

Efficacy and Mechanism-of-Action of a Novel Superagonist Interleukin-15: Interleukin-15 Receptor α Su/Fc Fusion Complex in Syngeneic Murine Models of Multiple Myeloma

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Abstract

ALT-803, a complex of an interleukin (IL)-15 superagonist mutant and a dimeric IL-15 receptor α Su/Fc fusion protein, was found to exhibit significantly stronger *in vivo* biologic activity on NK and T cells than IL-15. In this study, we show that a single dose of ALT-803, but not IL-15 alone, eliminated well-established 5T33P and MOPC-315P myeloma cells in the bone marrow of tumor-bearing mice. ALT-803 treatment also significantly prolonged survival of myeloma-bearing mice and provided resistance to rechallenge with the same tumor cells through a CD8⁺ T-cell-dependent mechanism. ALT-803 treatment stimulated CD8⁺ T cells to secrete large amounts of IFN- γ and promoted rapid expansion of CD8⁺CD44^{high} memory T cells *in vivo*. These memory CD8⁺ T cells exhibited ALT-803-mediated upregulation of NKG2D (KLRK1) but not PD-1 (PDCD1) or CD25 (IL2RA) on their cell surfaces. ALT-803-activated CD8⁺ memory T cells also exhibited nonspecific cytotoxicity against myeloma and other tumor cells *in vitro*, whereas IFN- γ had no direct effect on myeloma cell growth. ALT-803 lost its antimyeloma activity in tumor-bearing IFN- γ knockout mice but retained the ability to promote CD8⁺CD44^{high} memory T-cell proliferation, indicating that ALT-803-mediated stimulation of CD8⁺CD44^{high} memory T cells is IFN- γ -independent. Thus, besides well-known IL-15 biologic functions in host immunity, this study shows that IL-15-based ALT-803 could activate CD8⁺CD44^{high} memory T cells to acquire a unique innate-like phenotype and secrete IFN- γ for nonspecific tumor cell killing. This unique immunomodulatory property of ALT-803 strongly supports its clinical development as a novel immunotherapeutic agent against cancer and viral infections. *Cancer Res*; 73(10); 3075–86. ©2013 AACR.

Introduction

Multiple myeloma is a plasma cell malignancy, accounting for more than 1% of neoplastic diseases and 14% of all hematologic cancers (1). Multiple myeloma tumor cells are susceptible to immune cell recognition and elimination, as shown by the potentially curative graft-versus-myeloma activity observed in some patients following allogeneic hematopoietic stem cell transplantation and donor lymphocyte infusion therapies (2). However, these approaches are limited by transplantation-related mortality ranging from 30% to 50% and disease relapse in a majority of patients. Immunomodulatory chemotherapies, such as lenalidomide, are also thought to provide therapeutic benefit via mechan-

isms due in part to stimulation of T-cell and/or natural killer (NK) cell activity against myeloma cells (3). Although survival of patients with multiple myeloma has improved significantly by the use of these novel agents, multiple myeloma remains incurable due to the persistence of minimal residual disease (4, 5). Thus, novel modalities are needed to complement or improve the current treatment options for multiple myeloma.

Interleukin (IL)-15 is a critical cytokine for the development, proliferation, and activation of effector NK cells and CD8⁺ memory T cells (6, 7). IL-15 binds to the IL-15 receptor α (IL-15R α) and is presented in *trans* to the IL-2/IL-15 receptor β -common γ chain (IL-15R $\beta\gamma_c$) complex on effector cells. IL-15 and IL-2 share binding to the IL-15R $\beta\gamma_c$ and signal through STAT3 and STAT5 pathways. However, unlike IL-2, IL-15 does not support maintenance of CD4⁺CD25⁺FoxP3⁺ regulatory T (Treg) cells or induce cell death of activated CD8⁺ T cells (6); effects that may have limited the therapeutic activity of IL-2 against multiple myeloma (8). In addition, IL-15 is the only cytokine known to provide antiapoptotic signaling to effector CD8⁺ T cells (9). IL-15, either administered alone or as a complex with the IL-15R α , exhibits potent antitumor activities against well-established solid tumors in experimental animal models and, thus, has been identified as one of the most

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

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doi: 10.1158/0008-5472.CAN-12-2357

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promising immunotherapeutic drugs that could potentially cure cancer (10–17). However, there have been no reports showing efficacy of IL-15 against hematologic tumors.

To facilitate clinical development of an IL-15–based cancer therapeutic, we previously identified a novel IL-15 mutant with increased biologic activity compared with IL-15 (18). The pharmacokinetics and biologic activity of this IL-15 superagonist (IL-15N72D) was further improved by the creation of IL-15N72D:IL-15R α Su/Fc fusion complex (ALT-803), such that the superagonist complex has at least 25 times the activity of the native cytokine *in vivo* (19). Thus, we hypothesized that ALT-803 could potentially provide durable, immune cell-mediated antitumor efficacy. We evaluated this hypothesis by using 2 multiple myeloma models in syngeneic immunocompetent mice. The study also revealed that ALT-803 uses a novel mechanism of action against myeloma.

Materials and Methods

Mice and tumor cell lines

C57BL/6NHsd and BALB/c mice (5–6 week old females, Harlan Laboratories) and IFN- γ knockout (KO) [B6.129S7-Iflngtm1Ts/J] and perforin KO [C57BL/6-Prfl1tm1Sdz/J] mice (5–6 week old females, The Jackson Laboratory) were housed in the animal facilities at Altor BioScience. All animal studies were conducted according to NIH animal care guidelines under Institutional Animal Care and Use Committee-approved protocols.

The murine 5T33 multiple myeloma cell line (20) was kindly provided by Dr. Ulrich von Andrian, (Harvard Medical School, Boston, MA). The murine MOPC-315 myeloma cell line was purchased from American Type Culture Collection (ATCC). Tumor cell sublines, 5T33P and MOPC-315P, were developed by passage of the parental myeloma cells in C57BL/6NHsd and BALB/c mice, respectively. All cells were routinely cultured in Iscove's Modified Dulbecco's Medium (HyClone) supplemented with 10% Fetal Bovine Serum (HyClone) at 37°C with 5% CO₂ and harvested for animal injection at 80% to 90% confluency.

Tumor models

Following intravenous injection with 1×10^7 5T33P cells/mouse, 100% of C57BL/6NHsd mice developed tumor-induced hind-leg paralysis between 20 and 30 days. Similar tumor take rates were observed in BALB/c mice following intravenous injection of 1×10^7 MOPC-315P cells/mouse. Tumor-bearing mice were monitored daily for hind-leg paralysis, signs of overt disease progression, and mortality.

ALT-803 (IL-15N72D:IL-15R α Su/Fc) was generated as described previously (19). Recombinant human IL-15 (21) was kindly provided by Dr. Jason Yovandich (National Cancer Institute, Frederick, MD). ALT-803 at 0.2 mg/kg/dose (or as indicated), IL-15 at 0.056 mg/kg/dose (IL-15 molar equivalent dose of 0.2 mg/kg ALT-803) or PBS as a control was administered intravenously via the lateral tail vein to tumor-bearing mice. Levels of bone marrow (BM) myeloma cells and hind leg paralysis or survival were assessed as study end points.

Flow cytometry and ELISA analysis

To quantitate levels of murine lymphocyte subsets, BM, spleen, lymph node and blood were collected separately from

each mouse, cells were prepared and stained with fluor-labeled antibodies specific to CD4, CD8a, CD8b, CD11c, CD19, CD25, CD40, CD44, CD80, I-A(b), IFN- γ , IgG2b, IgA, NK1.1, NKG2D, NKp46, and/or PD-1, and appropriate isotype controls (eBiosciences, BD Biosciences, and Biolegend) as indicated in figure legends. Cell staining was analyzed on a FACVerse (BD Biosciences). The sorting of NKG2D^{neg}CD25^{neg}CD8⁺CD44^{high} T cells was conducted with FACS Aria and analyzed with Diva software (BD Biosciences).

Levels of 5T33P and MOPC-315P cells in BM preparations and IFN- γ in splenocytes were assessed by intracellular staining with antibodies specific to IgG2b, IgA and IFN- γ , respectively.

IFN- γ levels in mouse serum were quantitated by ELISA using anti-IFN- γ Ab (AN-18) capture and biotinylated anti-IFN- γ Ab (R4-6A2) detection following the manufacturer's instruction (Biolegend).

In vivo depletion of mouse NK1.1⁺ cells and CD8⁺ T cells

For *in vivo* depletion of NK1.1⁺ cells and CD8⁺ T cells, mice were injected intraperitoneally (i.p.) with 200 μ g/dose anti-NK1.1 (PK136, ATCC) and/or 500 μ g/dose anti-CD8 (53-6.72, ATCC) Abs. Control mice received PBS (0.2 mL). In pilot studies, the efficiency of NK1.1⁺ cell and CD8⁺ T-cell depletion was monitored by flow cytometry following staining of PBMCs and BM cells with appropriate antibodies.

T-cell labeling and adoptive transfer

CD3⁺ enriched cells (prepared with Mouse CD3⁺ T Cell Enrichment Column, R&D System), CD8⁺ enriched T cells [positive, CD8a (Ly-2) MicroBeads, mouse, Miltenyi Biotech] or sorted NKG2D^{neg}CD25^{neg}CD8⁺CD44^{high} memory T cells from spleens and lymph nodes of donor C57BL/6NHsd or IFN- γ KO B6 mice were labeled with Celltrace Violet (Invitrogen) at 1.5 μ mol/L/ 1×10^6 cells/mL, and then 1 to 1.5×10^6 violet-labeled cells were adoptively transferred into syngeneic C57BL/6NHsd or IFN- γ KO B6 recipients on study day 0 (SD0). On SD2, mice were treated (intravenously) with the following test articles: 0.02 mg/kg ALT-803, 0.2 mg/kg ALT-803 or PBS. On SD6, spleens were harvested and splenocytes were analyzed for proliferation of donor cells (violet label) or staining with antibodies specific to CD25, PD-1, CD44, CD8a, and NKG2D.

In vitro cytotoxicity assay

Tumor target cells (i.e., 5T33P, A20) were labeled with PKH67 (Sigma-Aldrich) according to the manufacturer's instructions. CD8⁺ T-cell-enriched spleen cells from normal, IFN- γ KO, and perforin KO B6 mice were isolated (untouched, CD8a⁺ T Cell Isolation Kit II, mouse, Miltenyi Biotech). Effector populations were produced by culturing prepared cells (2×10^7) in RPMI-1640 complete media containing ALT-803 (200 ng/mL) for 72 hours. Resulting effector cells were harvested, washed twice, and replated into 24-well plates with PKH67-labeled tumor target cells (E:T ratio; 10:1) in media containing varying doses of ALT-803. After incubation for 20 to 24 hours at 37°C with 5% CO₂, target cell killing was assessed by analysis of propidium iodide (PI) staining of PKH67-labeled tumor cells on a BD FACScan cytometer.

Data analysis

Data are expressed as the mean \pm SE. Survival data was analyzed using the log-rank test and Kaplan–Meier method. Comparisons of continuous variables were done using Student *t* tests or ANOVA (2-tailed; GraphPad Prism Version 4.03). *P* values of less than or equal to 0.05 are considered significant.

Results

Efficacy of ALT-803 in murine myeloma models

To conduct efficacy studies in hematologic tumor models, we derived highly tumorigenic myeloma lines 5T33P and MOPC-315P from the well-characterized 5T33 and MOPC-315 parental lines, respectively, and found that these cells could populate the BM and cause paralysis following intravenous inoculation of syngeneic mice. Tumor development in 5T33P-bearing C57BL/6NHsd mice and MOPC-315P-bearing BALB/c mice was assessed by staining myeloma cells in isolated BM cell preparations for intracellular 5T33P-specific

IgG2b and MOPC-315P-specific IgA paraproteins. In C57BL/6NHsd mice, IgG2b paraprotein-positive myeloma cell levels increased to more than 20% of the total BM cells by 21 days after 5T33P tumor cell inoculation (Supplementary Fig. S1). A single intravenous treatment of ALT-803 (0.2 mg/kg) had a marked effect on 5T33P cells in the BM of mice with well-established tumors (15 days after tumor implantation), providing more than 90% reduction in BM IgG2b⁺ myeloma cells 4 days after treatment compared with controls (0.8% versus 11.0%, $P \leq 0.02$; Fig. 1A). However, a molar equivalent dose of IL-15 was much less effective and only reduced BM 5T33P cells by 53% compared with PBS-treated mice ($P \geq 0.31$). Dose–response studies indicated that a single dose of ALT-803 at as low as 0.05 mg/kg was capable of reducing 90% of the BM 5T33P myeloma cells (Supplementary Fig. S2A). Similar studies in BALB/c mice bearing well-established MOPC-315P tumors confirmed that treatment with ALT-803, but not IL-15, resulted in a significant decrease in

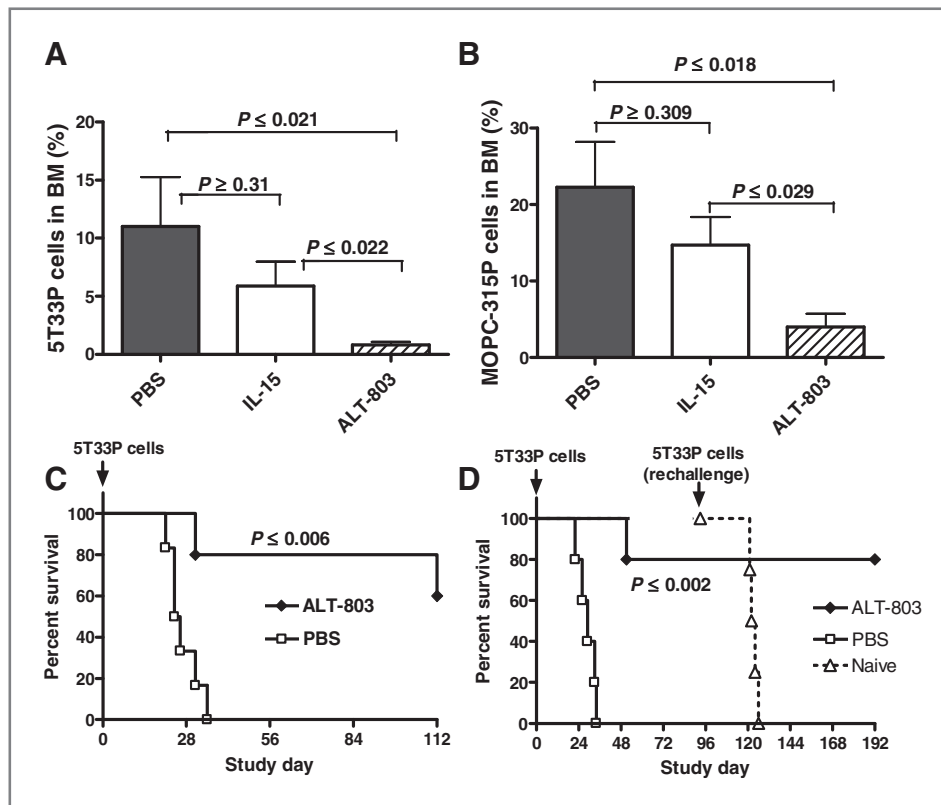


Figure 1. Antitumor effects of ALT-803 in murine myeloma models. A and B, effect of ALT-803 or IL-15 on myeloma cells in BM of 5T33P or MOPC-315P bearing mice. Female mice (5 mice/group) were injected intravenously with 5T33P (A) or MOPC-315P (B) myeloma cells (1×10^7 /mouse) on day 0. ALT-803 (0.2 mg/kg), IL-15 (0.056 mg/kg, an IL-15 molar equivalent dose to 0.2 mg/kg ALT-803), or PBS (dose volume equivalent) was then administered as a single intravenous injection on day 15 (5T33P) or 14 (MOPC-315P). BM cells were collected 4 days after study drug treatments. The cells were then stained with phycoerythrin (PE)-conjugated rat anti-mouse IgG2b or IgA Ab to evaluate the percentage of 5T33P or MOPC-315P cells, respectively, in BM. The plotted values represent the mean \pm SE; *P* values are presented. C, ALT-803 treatment prolonged survival of the examined mice bearing murine myeloma cells. Female C57BL/6NHsd mice ($n = 5$ /group) were injected intravenously with murine 5T33P myeloma cells (1×10^7 cells/mouse) on day 0. A single-dose of ALT-803 was administered i.v. at 0.2 mg/kg on day 4. Control mice were treated with PBS on day 4. Survival (or morbidity due to hind-leg paralysis) was monitored as a study end point. D, ALT-803 treatment prolonged survival of C57BL/6NHsd mice following subsequent rechallenge with 5T33P myeloma cells. 5T33P tumor-bearing mice ($n = 5$) were treated with ALT-803 on days 1 and 7 or with PBS as in Fig. 1C. ALT-803 treated mice that survived ($n = 4$) were rechallenged with 5T33P cells (1×10^7) on day 93. Five treatment-naïve mice were also administered 5T33P cells (1×10^7) on day 93 as a control for tumor development.

BM myeloma cells compared with controls ($P \leq 0.02$, ALT-803 vs. PBS; $P \geq 0.31$, IL-15 vs. PBS; Fig. 1B). No toxicity was observed following treatment, indicating that ALT-803 administration and its antitumor effects, which resulted in the rapid killing of a large number of myeloma cells over a short duration, were well tolerated by mice.

ALT-803 effects on mouse survival were also evaluated in these myeloma models. 5T33P-bearing C57BL/6NHsd mice treated with a single 0.2 mg/kg dose of ALT-803 showed significantly increased survival when compared with PBS-treated mice, which all exhibited hind-leg paralysis (survival end point) between 21 and 35 days after tumor cell injection with a median survival time (MST) of 25 days ($P \leq 0.006$; Fig. 1C). Two or 3 weekly doses of ALT-803 also provided a significant survival benefit in this model ($P \leq 0.002$, ALT-803 vs. PBS; Fig. 1D) and in BALB/c mice bearing MOPC-315P tumors (Supplementary Fig. S2B).

Because ALT-803 treatment was capable of essentially curing mice bearing 5T33P myeloma, we evaluated whether these mice retain immunologic memory against the tumor cells. As shown in Fig. 1D, C57BL/6NHsd mice that survived initial 5T33P inoculation due to ALT-803 treatment were not affected by 5T33P cells rechallenge 3 months later, even in the absence of additional ALT-803 administration. These mice continued to survive over 190 days from the initial tumor cell inoculation. In contrast, all of the treatment-naïve mice administered with 5T33P cells on the same study day subsequently exhibited paralysis with a MST of 29 days after tumor cell injection. Together, these results show that a short course of ALT-803 treatment has significantly greater antitumor activity against established BM myeloma cells than IL-15 treatment, resulting in prolonged survival of myeloma-tumor bearing mice. ALT-803 was also capable of inducing long-lasting protective immunologic memory against subsequent tumor cell rechallenge.

CD8⁺ T cells mediate efficacy of ALT-803 against myeloma cells

Because ALT-803 treatment effectively eliminated myeloma cells *in vivo*, we tested whether ALT-803 had a direct effect on the viability and proliferation of 5T33P and MOPC-315P cells *in vitro*. Neither a decrease in cell numbers nor an increase in apoptotic cells was observed following incubation of tumor cells with ALT-803 even at high concentrations (Supplementary Fig. S3). Thus, ALT-803 antitumor activity *in vivo* is likely due to activation of immune responses rather than direct killing of tumor cells.

ALT-803 treatment is capable of significantly increasing the number of NK and T cells *in vivo* (19). To determine if these immune cells were responsible for ALT-803-mediated anti-myeloma efficacy, antibody immunodepletion of CD8⁺ T cells and NK1.1⁺ cells was conducted in tumor-bearing mice before ALT-803 treatment. Effective depletion of these immune cell subsets could be achieved by intraperitoneal administration of anti-CD8 and/or anti-NK1.1 antibodies starting with injections 48 and 24 hours before tumor inoculation and continued weekly after tumor inoculation (data not shown). When ALT-803 efficacy was examined in 5T33P-bearing mice, it was

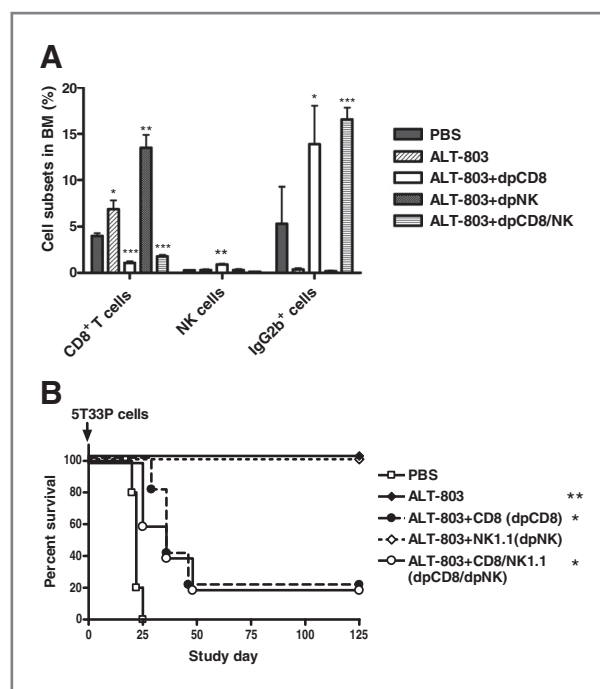


Figure 2. Immune cell effects on the antimyeloma activity of ALT-803. A, female C57BL/6NHsd mice ($n = 5/\text{group}$) were depleted of CD8⁺ T cells (dpCD8), NK1.1⁺ cells (dpNK), or both (dpCD8/NK) by intraperitoneal treatment with anti-CD8 and/or anti-NK1.1 antibodies on days -2 and -1 . Mice were then injected with 5T33P myeloma cells (1×10^7) on day 0 and treated with anti-CD8 and/or anti-NK1.1 antibodies on days 7 and 14. The mice were treated with ALT-803 (0.2 mg/kg) on day 14. Undepleted 5T33P-bearing mice receiving PBS served as controls. Four days after ALT-803 treatment, BM cells were isolated and stained with FITC-anti-CD8b, PE-anti-NKp46 and FITC-anti-IgG2b Abs, and analyzed by flow cytometry. The percentage of CD8⁺ T cells, NKp46⁺ NK cells, and IgG2b⁺ 5T33P myeloma cells in BM are shown. Bars represent the mean \pm SE. B, female C57BL/6NHsd mice ($n = 5/\text{group}$) were depleted of CD8⁺ T cells (dpCD8), NK1.1⁺ cells (dpNK), or both (dpCD8/NK) by treatment with anti-CD8 and/or anti-NK1.1 antibodies on days -2 and -1 as described in A. Mice were then injected with 5T33P myeloma cells (1×10^7) on day 0 and treated with anti-CD8 and/or anti-NK1.1 antibodies on day 4 and then weekly for 7 weeks. ALT-803 was administered intravenously at 0.2 mg/kg on days 4 and 11. Mice receiving 5T33P myeloma cells (1×10^7) on day 0 and PBS on days 4 and 11 were used as control. For comparison of ALT-803 vs. PBS or ALT-803+Ab depletion vs. ALT-803, *, $P \leq 0.05$; **, $P \leq 0.01$; and ***, $P \leq 0.001$.

found that CD8⁺ T-cell depletion alone or in combination with NK1.1⁺ cell depletion, but not NK1.1⁺ cell depletion alone, eliminated the antitumor effects of ALT-803 on BM 5T33P myeloma cells (Fig. 2A). Consistent with these results, antitumor activity correlated with ALT-803-mediated increases in BM CD8⁺ T-cell and not NK cell levels (Fig. 2A). We also conducted immune cell depletion studies in 5T33P-bearing C57BL/6NHsd mice treated with ALT-803 using survival as the efficacy end point. As described above, ALT-803 treatment effectively cured myeloma-bearing mice that otherwise developed paralysis within 28 days (Fig. 2B). Depletion of NK1.1⁺ cells had no effect on the antitumor activity of ALT-803, whereas depletion of CD8⁺ T cells or both CD8⁺ T cells and NK1.1⁺ cells significantly reduced the ALT-803-mediated survival benefit to 5T33P-bearing mice ($P < 0.013$). These

results support our conclusion that CD8⁺ T cells, not NK1.1⁺ cells, play a major role in ALT-803-mediated activity against 5T33P cells in C57BL/6NHsd mice.

ALT-803 induces CD8⁺CD44^{high} memory T cells to expand, upregulate innate receptors, and exhibit nonspecific cytotoxic activity

We have previously shown that a single dose of ALT-803 at 0.2 mg/kg dose level, but not IL-15, could significantly increase the CD8⁺ T cells and NK cells in naïve mice (19). As shown in Fig. 3A, we found that a single dose of ALT-803 (0.2 mg/kg) administered to either normal or 5T33P-bearing C57BL/6NHsd mice resulted in a similar 3 fold increase in the percentage of CD8⁺CD44^{high} memory T cells. This is consistent with observations by others that certain cytokines, such as IL-12, IL-18, IFN- γ , or IL-15, can promote proliferation of CD8⁺CD44^{high} T cells, but not the naïve CD8⁺ T cells, *in vivo* (22–24).

A recent study also showed that certain immunotherapies promote antigen-nonspecific expansion of memory CD8⁺ T cells with innate-type cell receptors (25). Unlike the memory CD8⁺ T cells stimulated by antigen-dependent TCR signaling that upregulate PD-1 and CD25 cell surface molecules, the immunotherapy-mediated expanded memory CD8⁺ T cells express NKG2D, granzyme B, and possess broadly antigen-nonspecific lytic capability. Interestingly, we found that the splenic memory CD8⁺ T cells expanded *in vivo* by ALT-803 treatment also expressed NKG2D and not CD25 or PD-1 (Fig. 3B). To examine ALT-803-mediated changes in this cell population, we isolated CD3⁺ enriched cells from spleens and lymph nodes of C57BL/6NHsd mice and labeled them with Celltrace Violet, and then adoptively transferred these cells into syngeneic recipients. Two days after transfer, the mice were treated with PBS or ALT-803 (0.02 mg/kg or 0.2 mg/kg) and the phenotype and proliferation of the adoptively transferred cells were examined 4 days later. As shown in Fig. 3C, ALT-803 treatment resulted in a significant, dose-dependent increase in proliferation of donor CD8⁺CD44^{high} T cells isolated from spleens of recipient mice, whereas donor memory CD8⁺ T cells did not proliferate in PBS-treated mice. In the expanded memory CD8⁺ T-cell population from 0.2 mg/kg ALT-803-treated mice, more than 90% expressed NKG2D with increased positive staining in cells that underwent multiple rounds of proliferation. To rule out the possibility that this is due to an enormous expansion of a small population of NKG2D⁺ cells following ALT-803 treatment, we conducted similar adoptive transfer studies with sorted NKG2D^{neg}CD25^{neg}CD8⁺CD44^{high} T cells labeled with Celltrace Violet. Treatment of recipient mice with 0.2 mg/kg ALT-803 caused an increase in NKG2D⁺ memory CD8⁺ T cells from 0% to 13% (Fig. 3D; see gating strategy in Supplementary Fig. S4). Thus, ALT-803 treatment not only induced the proliferation of the memory CD8⁺ T cells but also upregulated the NKG2D receptor on their surface. Donor memory CD8⁺ T-cell expressing CD25 also proliferated following ALT-803 treatment but the percentage of these cells (~4%) was the same in ALT-803- and PBS-treated mice, consistent with the findings in 5T33P tumor-bearing mice.

To assess whether the induced CD8⁺ T-cell responses were associated with changes in antigen presentation potential *in vivo*, we administered ALT-803 (0.2 mg/kg), LPS (12.5 μ g/mouse), or poly IC (10 μ g/mouse) to normal and 5T33P-bearing C57BL/6NHsd mice and examined the upregulation of activation/maturation markers on BM dendritic cells (DC). We found that ALT-803, unlike poly IC or LPS, did not increase CD11c, MHC II (I-A^b), CD80 or CD40 levels on BM DCs (Fig. 4). Similar results were found for splenic DCs. Thus, the rapid expansion of CD8⁺CD44^{high} memory T-cell population stimulated by ALT-803 is unlikely a result of increased antigen-specific responses, consistent with the results of others showing antigen-independent activation of innate-type memory T cells following immunotherapy or microbial or viral infection (26–28).

The cytotoxic activity of ALT-803-treated immune cells was also examined *in vitro*. CD8⁺CD44^{high} T cells increased 5-fold in splenocytes and 3-fold in CD8⁺-enriched splenic T cells from normal C57BL/6NHsd mice following a 3-day incubation with 0.2 μ g/mL ALT-803. Similar to the findings *in vivo*, upregulation of NKG2D but not CD25 or PD-1 was observed on memory CD8⁺ T cells following ALT-803 incubation (Fig. 5A). The ALT-803-stimulated splenocytes and CD8⁺-enriched splenic T cells exhibited elevated cytolytic activity against 5T33P cells (Fig. 5B) as well as A20 lymphoma cell lines (Fig. 5C). Killing of 5T33P cells was further enhanced by inclusion of ALT-803 during the cytotoxicity assay, suggesting a continued activation of immune cell antitumor activity by ALT-803. Interestingly, 5T33P myeloma-targeted cytotoxicity of ALT-803-stimulated CD8⁺ enriched splenocytes was not affected by inclusion of an NKG2D-blocking antibody, whereas this antibody reduced 5T33P killing by whole splenocyte cultures (Fig. 5B). These results suggest that *in vitro* cytotoxicity of the NK cells in the whole splenocyte cultures are dependent on NKG2D, whereas that of CD8⁺ T cells does not require NKG2D but may be mediated through other innate-like activating receptors induced by ALT-803. The cytotoxicity of CD8⁺ T cells was partially dependent on perforin expression as CD8⁺ T cells obtained from perforin KO mice showed reduced 5T33P cell killing in this assay (Fig. 5D).

Overall, these studies indicate that ALT-803 potently induces CD8⁺CD44^{high} T cells and upregulates innate-cell receptor NKG2D without the requirement of antigen-specific stimulation. Also, this type of ALT-803-stimulated CD8⁺ memory T cells exhibit cytotoxic activity against myeloma and other tumor cells.

Serum IFN- γ is elevated by ALT-803 treatment in a CD8⁺ T-cell-dependent manner and is required for ALT-803-mediated efficacy

In addition to stimulating immune cells, we found that a single dose of ALT-803 to C57BL/6NHsd mice could significantly increase serum IFN- γ levels (Fig. 6A). Immune-depletion studies were then carried out to identify the immune cell types responsible for IFN- γ production after ALT-803 treatment. As shown in Fig. 6A, depletion of CD8⁺ T cells, but not NK1.1⁺ cells, eliminated most of the high-level expression of serum IFN- γ , indicating that CD8⁺ T cells were the dominant source of ALT-803-induced IFN- γ . To further determine whether

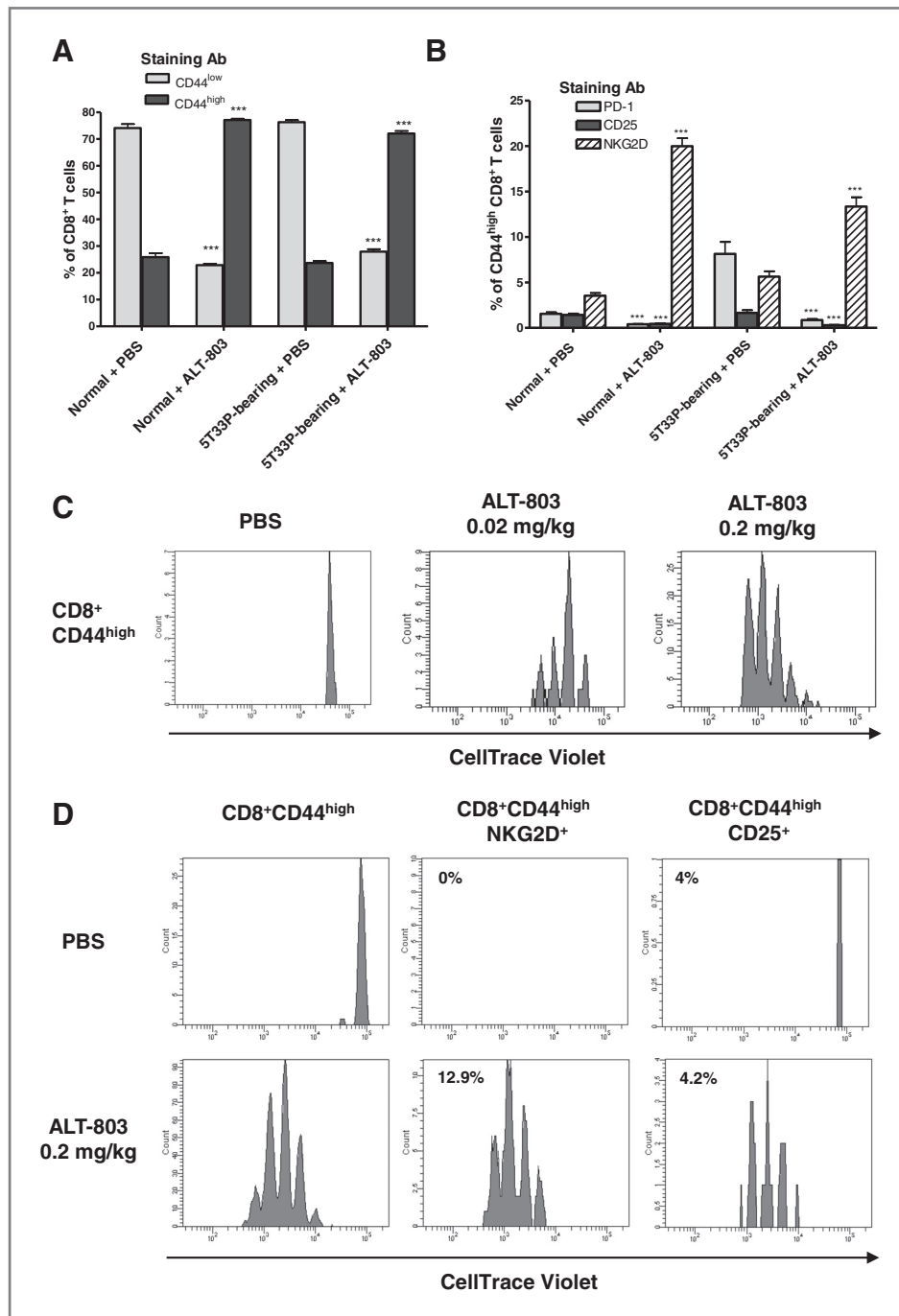
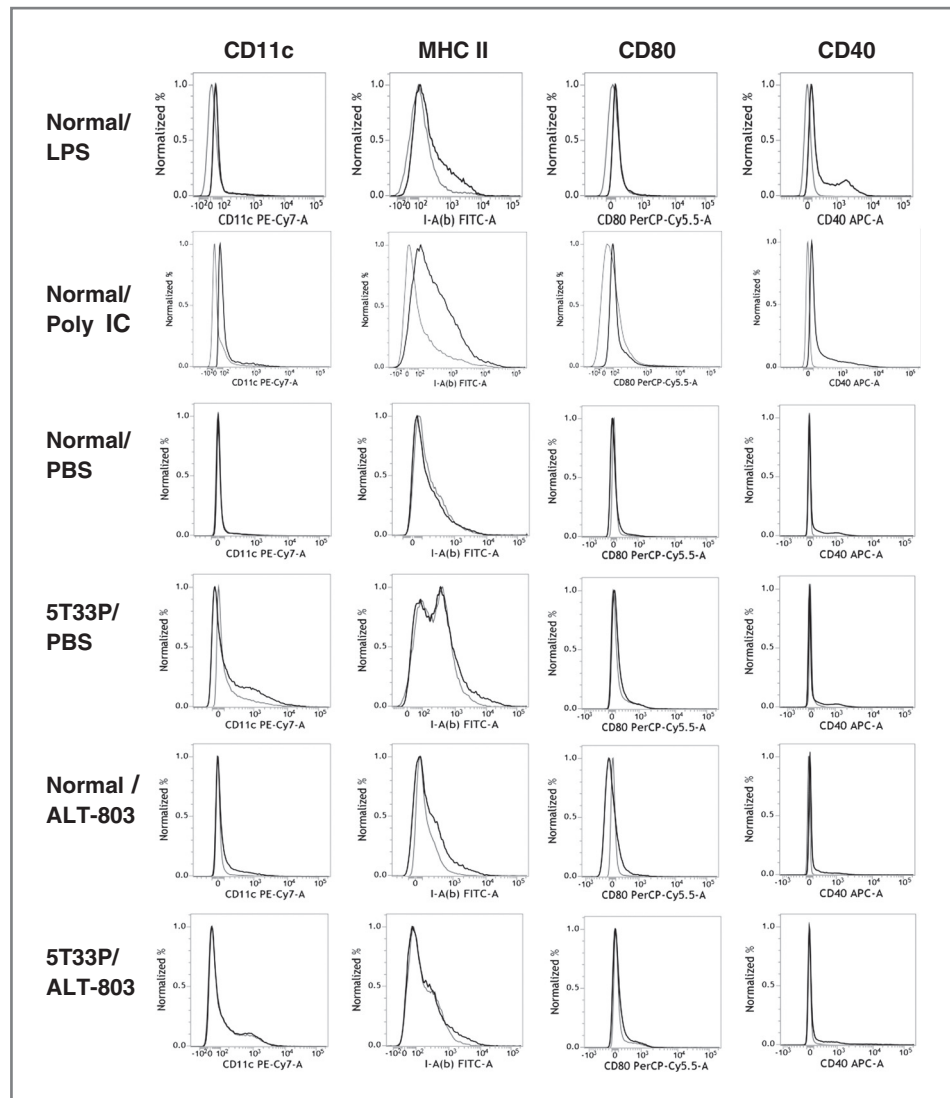


Figure 3. ALT-803 induces CD8⁺CD44^{high} memory T cell proliferation and upregulation of NKG2D. **A** and **B**, female C57BL/6NHsd mice (5–6 weeks old, 6 mice/group) were untreated (normal) or injected intravenously with 5T33P myeloma cells (1×10^7 /mouse; 5T33P-bearing) on day 0. ALT-803 (0.2 mg/kg) or PBS (dose volume equivalent) was administered intravenously on day 14. Four days after treatment, mouse splenocytes were isolated and stained with Abs specific to CD44 (PE-Cy7), NKG2D (APC), PD-1 (FITC), CD25 (PE), and CD8 (PerCP-Cy5.5). Stained cells were analyzed by flow cytometry. The percentage of CD44^{low} and CD44^{high} in CD8⁺ T cells (**A**) and percentage of PD-1-, CD25- and NKG2D-positive cells in CD8⁺CD44^{high} memory T-cell population (**B**) are shown. For comparison of ALT-803 vs. PBS treatment effects in normal or 5T33P-bearing mice, $***, P < 0.001$. **C**, CD3⁺ enriched cells from spleens of donor C57BL/6NHsd mice were labeled with Celltrace Violet and then adoptively transferred (1.5×10^6 cells/mouse) into syngeneic recipients (3 mice/group) on study day 0 (SD0). On SD2, mice were treated (i.v.) with 0.02 mg/kg of ALT803, 0.2 mg/kg of ALT-803 or PBS (dose volume equivalent). On SD6, spleens were harvested and analyzed individually by flow cytometry for donor cells (violet label) and positive staining with antibodies specific to CD44 (PE-Cy7), NKG2D (APC), PD-1 (FITC), CD25 (PE), and CD8 (PerCP-Cy5.5). Histograms show proliferation of violet-labeled CD8⁺CD44^{high} memory T-cell population. **D**, NKG2D^{neg}CD25^{neg}CD8⁺CD44^{high} memory T cells from spleens and lymph nodes of donor C57BL/6NHsd mice were sorted with BD FACS Aria (Supplemental Fig. S4) and labeled with Celltrace Violet. Donor cells (1×10^6 cells/mouse) were then adoptively transferred into syngeneic recipients (3 mice/group) on SD0. On SD2, mice were treated (i.v.) 0.2 mg/kg ALT-803 or PBS (dose volume equivalent). On SD6, spleens were harvested and analyzed by flow cytometry as described in Fig. 3C. Histograms show proliferation of violet-labeled CD8⁺CD44^{high} memory T-cell population and CD8⁺CD44^{high}NKG2D⁺ and CD8⁺CD44^{high}CD25⁺ subpopulations. The value indicates the percentage of NKG2D⁺ or CD25⁺ cells in the donor CD8⁺CD44^{high} memory T-cell population.

Figure 4. ALT-803 did not increase expression maturation markers on BM dendritic cells. Female C57BL/6NHsd mice (5–6 weeks-old, 3 mice/group) were untreated (normal) or injected intravenously with 5T33P myeloma cells (1×10^7 /mouse; 5T33P-bearing) on day 0. ALT-803 (0.2 mg/kg) or PBS (dose volume equivalent) was administered intravenously on day 14. Four days after treatment, BM cells were isolated, pooled, and stained with antibodies specific to CD11c (PE-Cy7), MHC II [I-A(b)] (FITC), CD80 (PerCP-Cy5.5), and CD40 (APC), then analyzed by flow cytometry. Mice treated intraperitoneally either with 12.5 μ g of LPS (*E. coli* 055:B5, Sigma-Aldrich) and sacrificed 12 hours later or with 10 μ g of Poly IC (InvivoGene) and sacrificed 24 hours later served as positive controls. Histograms show staining with positive Abs (black line) or isotype controls (gray line).



CD8⁺CD44^{high} memory or CD8⁺CD44^{low} naïve T cells were the primary producers of IFN- γ after ALT-803 treatment, we analyzed IFN- γ production of splenic CD8⁺ T cells from ALT-803-treated mice. Intracellular IFN- γ was detectable as early as 12 hours after ALT-803 treatment in the CD8⁺CD44^{high} memory T-cell population and the percentage of IFN- γ -producing memory T cells continued to remain elevated for at least 48 hours after ALT-803 treatment (Fig. 6B). Significant ALT-803-mediated induction of intracellular IFN- γ was not observed in CD8⁺CD44^{low} naïve T cells. Thus, ALT-803 activates CD8⁺CD44^{high} memory T cells to proliferate and secrete IFN- γ via an antigen-independent pathway.

To determine whether induced IFN- γ plays a role in the antimyeloma activity of ALT-803, treatment effects on survival were evaluated in IFN- γ KO B6 mice bearing 5T33P cells. Similar to the findings in myeloma-bearing C57BL/6NHsd mice following CD8⁺ T-cell depletion, ALT-803 treatment provided little or no protection from mortality to IFN- γ KO mice after 5T33P cell inoculation, indicating IFN- γ is required

for ALT-803 efficacy (Fig. 6C). However, IFN- γ had no direct effect on 5T33P cell growth or apoptosis *in vitro* (Supplementary Fig. S3), consistent with previous reports (29). These results support a mechanism where ALT-803 activates IFN- γ production and cytotoxic activity of CD8⁺ memory T cells and together these responses promote rapid elimination of myeloma cells and prolonged survival of tumor-bearing mice.

To assess whether IFN- γ is needed for ALT-803-mediated effects on CD8⁺ memory T-cell responses, adoptive cell transfer studies were conducted using donor Celltrace Violet-labeled CD8⁺ T cells from IFN- γ KO mice transferred into IFN- γ KO and wild-type recipient mice. As shown in Fig. 7, ALT-803 treatment of IFN- γ KO or wild-type recipients induced comparable CD8⁺CD44^{high} memory T-cell proliferation and upregulation of NKG2D of the adoptively transferred cells. This indicates that ALT-803-induced CD8⁺CD44^{high} memory T cell responses were IFN- γ independent. Interestingly, we also found that that CD8⁺ T cells isolated from IFN- γ KO mice exhibited less ALT-803-stimulated *in vitro* cytotoxic activity

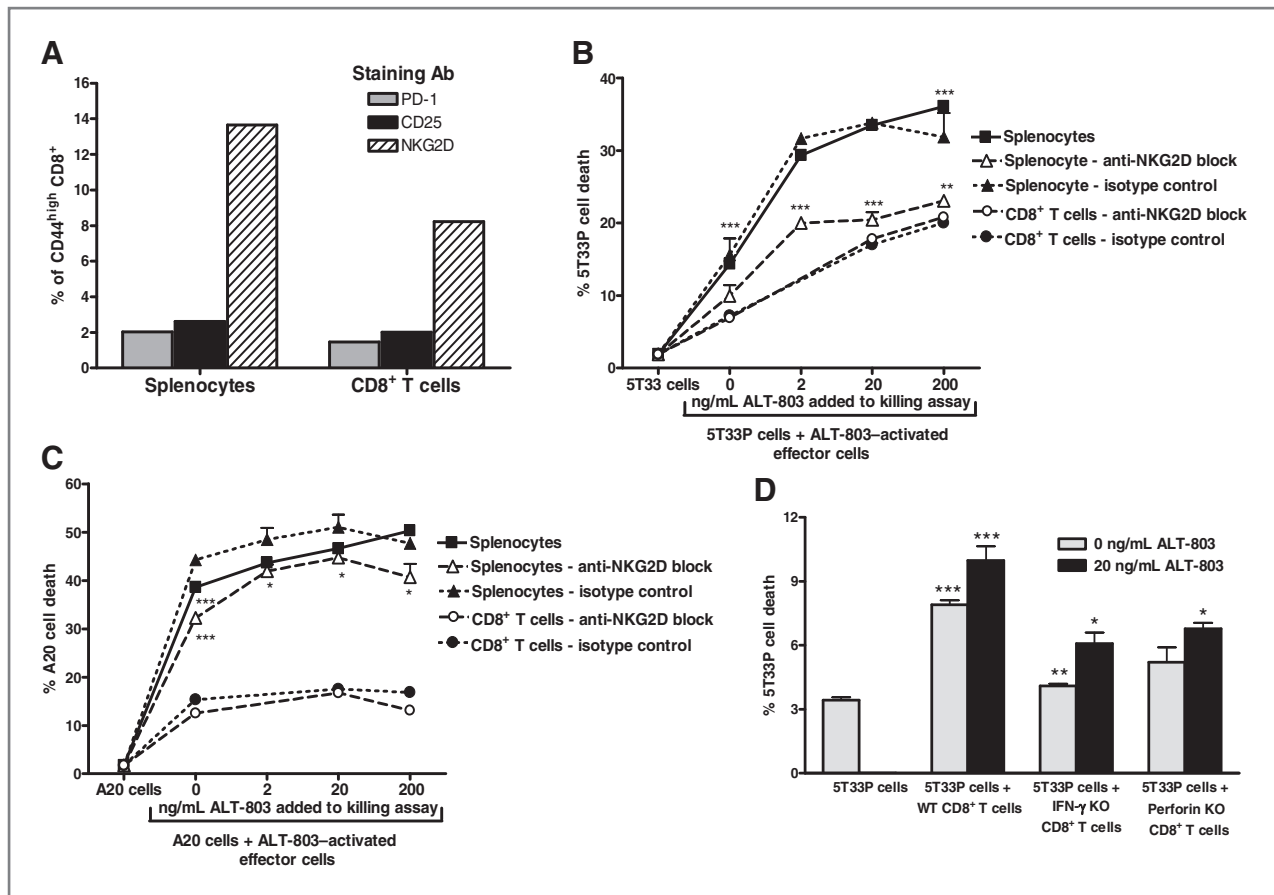


Figure 5. *In vitro* cytotoxic activity of ALT-803-treated immune cells. A, unfractionated or CD8⁺ T-cell-enriched splenocytes (untouched) from normal C57BL/6NHsd mice (pool of 3/group) were cultured with 200 ng/mL of ALT-803 for 72 hours. Cells were then harvested, stained with Abs specific to CD44 (PE-Cy7), NKG2D (APC), PD-1 (FITC), CD25 (PE), and CD8 (PerCP-Cy5.5), and analyzed by flow cytometry for expansion of CD8⁺CD44^{high} memory T-cell populations. B and C, unfractionated or CD8⁺ T-cell-enriched splenocytes were activated as described in A, then washed thoroughly and replated in duplicate wells (1 × 10⁶ cells/well) containing 0, 2, 20, or 200 ng/mL ALT-803. NKG2D blocking antibody (10 μg/mL) or isotype control antibody (10 μg/mL) was added to appropriate wells as indicated. PHK-67 labeled 5T33P (1 × 10⁵ cells/well; B) or A20 tumor cells (1 × 10⁵ cells/well; C) were added (E:T ratio = 10:1) and incubated for 24 hours. Target cell killing of the individual cultures was assessed by analysis of PI staining of PHK-67 labeled tumor cells on a BD FACScan. The level of PI staining in cultured PHK-67 labeled 5T33P or A20 cells alone served as a background control. The results are representative of two independent studies. For comparison of target cells + activated splenocytes versus target cells alone or target cells + activated splenocytes + 200 ng/mL ALT-803 versus target cells + activated splenocytes; ***, *P* ≤ 0.001. For comparison of the effects of anti-NKG2D antibody versus isotype control; *, *P* ≤ 0.05; **, *P* ≤ 0.01; and ***, *P* ≤ 0.001. D, *in vitro* 5T33P killing assay of CD8⁺ T cell enriched spleen cells from normal, IFN-γ KO B6, and perforin KO B6 mice (pool of 3/group). As described above, enriched CD8⁺ T cells (2 × 10⁷) were incubated with ALT-803 (0.2 μg/mL) for 72 hours and then replated into triplicated wells (3 × 10⁶ cells/well) without or with ALT-803 (20 ng/mL). PHK-67-labeled 5T33P tumor cells (3 × 10⁵ cells/well) were added as target cells (E:T ratio = 10:1). After incubation for 20 hours, target cell killing was assessed as described above. The percentage of PI-positive 5T33P cells is shown. For comparison of target cells + effector cells versus target cells alone or target cells + KO effector cells versus target cells + WT effector cells under the same culture conditions; *, *P* ≤ 0.05; **, *P* ≤ 0.01; and ***, *P* ≤ 0.001.

against 5T33P cells than was observed in CD8⁺ T cells from normal C57BL/6NHsd mice (Fig. 5D). Together, these results suggest that while IFN-γ is not required for ALT-803-mediated activation and expansion of CD8⁺ memory T cells, it still plays a role in augmenting the cytotoxicity of these cells against tumors via an as yet undetermined mechanism.

Discussion

IL-15 and IL-15Rα are coexpressed and form a protein complex in antigen-presenting cells for *trans*-presentation to T and NK cells (6). Studies have shown that soluble IL-15:IL-15Rα complexes exhibit a 50-fold better immune stimulatory activity *in vivo* than IL-15 alone (10) and potent efficacy against solid and metastatic tumors in various mouse models (11–13);

however, its activity against hematologic tumors has not been reported. In this study, we describe the antimyeloma activity and mechanism of action of ALT-803, a protein complex consisting of an IL-15 superagonist mutant associated with a dimeric IL-15RαSu/Fc fusion protein (18, 19). We found that a single dose of ALT-803 was much more effective than IL-15 at reducing the levels of well-established murine 5T33P and MOPC-315P myeloma cells in the BM of tumor-bearing immunocompetent mice. ALT-803 was also found to prolong survival of 5T33P and MOPC-315P tumor-bearing mice and effectively cured a majority of the mice of tumors. Moreover, 5T33P-bearing mice cured by prior ALT-803 treatment were protected against subsequent 5T33P rechallenge, indicating that ALT-803 mediated the induction of long-lasting antimyeloma

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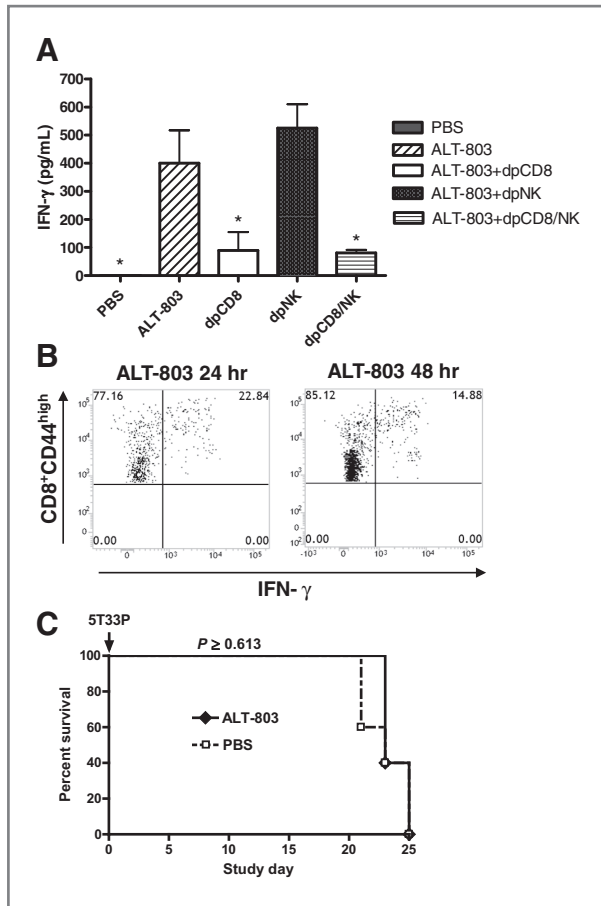


Figure 6. CD8⁺ T-cell production of IFN-γ plays a role in ALT-803-mediated efficacy. **A**, ALT-803 induced high levels of serum IFN-γ via CD8⁺ T cells. C57BL/6NHsd mice (*n* = 5) received 3 doses of anti-CD8 Ab (dpCD8), anti-NK1.1 Ab (dpNK), or both Abs (dpCD8/NK) intraperitoneally on days -2, -1, and 7. On day 8, a single intravenous dose of ALT-803 (0.2 mg/kg) was administered and 2 days later (day 10) serum IFN-γ levels were examined. Bars represent the mean ± SE. *, *P* ≤ 0.05, compared to ALT-803 response. **B**, C57BL/6NHsd mice (*n* = 3) were administered a single intravenous dose of ALT-803 (0.2 mg/kg) on day 1 or day 2 respectively. On day 3, isolated splenocytes were stained with Abs to CD44 (PE-Cy7), and CD8 (PerCP-Cy5.5), and then intracellularly stained with FITC-anti-IFN-γ antibody. Dot plots show the percentage of IFN-γ-producing CD8⁺CD44^{high} memory T cells. **C**, IFN-γ is required for ALT-803 antimyeloma activity. Female IFN-γ KO B6 mice (*n* = 5/group) were injected intravenous with 5T33P myeloma cells (1 × 10⁷ cells/mouse) on day 0. ALT-803 (0.2 mg/kg) or PBS was administered intravenously on days 4 and 11. Survival (or morbidity due to hind-leg paralysis) was monitored as a study end point.

immune memory responses. These results are consistent with our previous report that ALT-803 exhibited significantly better activity compared with IL-15 in stimulating NK cell and CD8⁺ T-cell responses *in vivo* (19). This enhanced immunostimulatory activity is likely the result of a combination of the increased *in vivo* half-life of ALT-803 compared with IL-15 (25 hours vs. <40 minutes) and the dimeric nature of the cytokine domain in the complex increasing its binding avidity to IL-15Rβγ_c (19). It is also possible that the Fc domain of the complex enables *trans*-presentation of the cytokine to IL-15Rβγ_c receptor-bearing NK and T cells via binding to the

Fc-γ receptors (FcγR) on the surface of dendritic cells, macrophages, NK cells, and other cell types (11). We have recently created an FcγR-binding deficient derivative of ALT-803 for studies to further evaluate the contribution of the Fc-γ domain to ALT-803-mediated immune stimulation.

Previous studies have shown that IL-15 and IL-15:IL-15Rα complexes can stimulate antitumor activity via either effector NK cells or T cells, showing the remarkable capacity of IL-15 to induce different effector cell responses against diverse tumor types and tumor microenvironments (11–14, 16). In the 5T33P myeloma model reported here, we found that treatment with ALT-803 resulted in an increase in CD8⁺ T-cell levels in the BM of tumor-bearing mice that correlated with the complex's

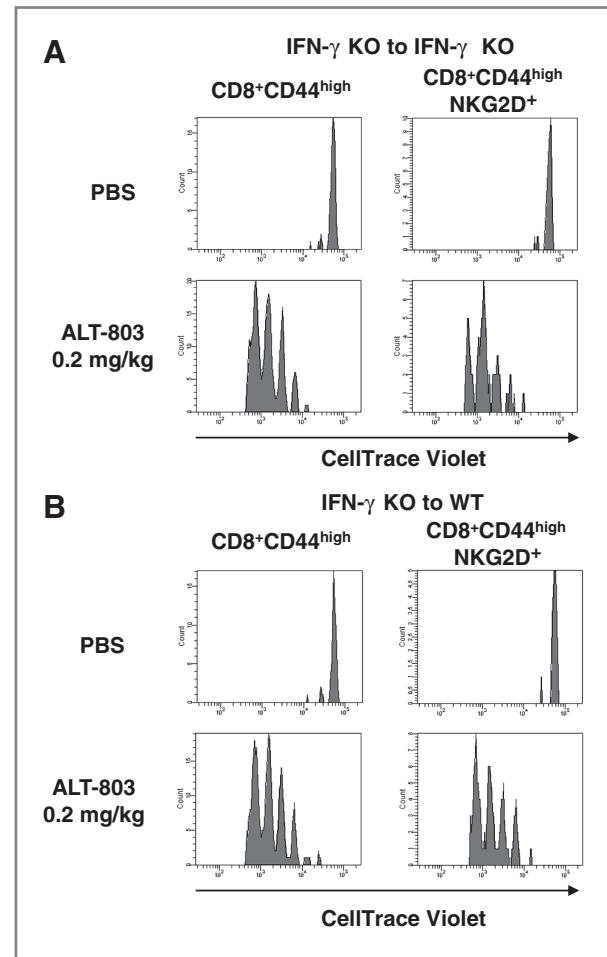


Figure 7. ALT-803 induction of CD8⁺CD44^{high} memory T-cell responses was not dependent on IFN-γ. **A** and **B**, enriched CD8⁺ T cells (positive selection) from splenocytes and lymph nodes of IFN-γ KO B6 mice (6-week-old) were labeled with CellTrace Violet and adoptively transferred (1.5 × 10⁶ cell/mouse) into IFN-γ KO B6 recipients (KO, *n* = 5; **A**) or wild-type C57BL/6NHsd recipients (WT, *n* = 5; **B**) on day 0 (SD0). On SD2, 3 KO and 3 WT mice were treated with 0.2 mg/kg ALT-803 (*i.v.*) and the remaining 2 KO and 2 WT mice received PBS (*i.v.*) as controls. On SD6, spleens were harvested and analyzed individually by flow cytometry for donor cells (violet label) and positive staining with antibodies specific to CD44 (PE-Cy7), NKG2D (APC), and CD8 (PerCP-Cy5.5). Histograms show proliferation of violet-labeled CD8⁺CD44^{high} and CD8⁺CD44^{high}NKG2D⁺ memory T-cell population.

ability to reduce BM 5T33P-cell burden. However, systemic depletion of CD8⁺ T cells, but not NK1.1⁺ cells, was shown to largely eliminate the antitumor activity of ALT-803 on BM myeloma cells and the treatment-related survival benefit in 5T33P-bearing mice. This indicates that CD8⁺ T cells, but not NK1.1⁺ cells, play a pivotal role in ALT-803 antimyeloma activity. This finding is perplexing since we found that a single intravenous treatment of ALT-803 (0.2 mg/kg) had a marked effect on 5T33P cells in the BM of mice with well-established tumors, providing more than 90% reduction in BM IgG2b⁺ myeloma cells 4 days after treatment. Such a robust and rapid onset of immune responses is generally believed to only be associated with the innate immune system. In addition, we further found that a single dose of ALT-803 was capable of inducing high serum levels of IFN- γ and promoting the proliferation of CD8⁺ T cells in non-tumor bearing mice shortly after treatment. The source of serum IFN- γ was largely from CD8⁺CD44^{high} T cells, not NK1.1⁺ cells, based on our immune-depletion analysis. Therefore, we questioned whether the activation of CD8⁺ T cells and subsequent antitumor activity mediated by ALT-803 was antigen dependent. To address this, we examined whether ALT-803 induced dendritic cell activation/maturation. Our finding that ALT-803 treatment did not upregulate CD11c, CD80, MHC-II, and CD40 in BM and splenic DCs from either tumor- or non-tumor-bearing mice suggested that ALT-803 did not promote antigen presentation at the initial phase of the immune response. Thus, it seems unlikely that antigen-dependent clonal expansion of naïve CD8⁺ T cells immediately after ALT-803 treatment is responsible for the potent antimyeloma activity observed in mice bearing established 5T33P and MOPC-315P tumors.

The proliferation of memory-phenotype (CD44^{high}) CD8⁺ T cells, but not naïve CD8⁺ T cells, can be induced *in vivo* by the cytokines IL-12, IL-18, and IFN- γ , most likely via production of IL-15, or directly by IL-15 (22–24). A recent study also showed that cytokine-mediated stimulation could promote antigen-nonspecific expansion of memory CD8⁺ T cells with a unique phenotype (25). Unlike TCR signaling that upregulates PD-1 and CD25 surface markers on memory CD8⁺ T cells, treatment with IL-2 in combination with anti-CD40 antibody resulted in expansion of memory CD8⁺ T cells that express NKG2D, granzyme B, and possess broad lytic capabilities. These cells have been suggested to be responsible for the dramatic antitumor effects of this therapy in animal models (26–28). Herein, using the adoptive-cell transfer approach, we showed that ALT-803 alone could also induce CD8⁺CD44^{high} memory T cells, but not naïve T cells, to acquire innate cell receptors, such as NKG2D, without inducing PD-1, *in vitro* and *in vivo*. ALT-803 seems to act by both inducing CD8⁺ memory T-cell proliferation and upregulating NKG2D expression rather than preferentially expanding preexisting CD8⁺CD44^{high} memory T cells carrying this receptor. *In vitro*, the ALT-803-activated CD8⁺CD44^{high} memory T cells exhibited antigen-nonspecific and potent antitumor activity against 5T33P myeloma. Because of the presence of the large numbers of the CD8⁺CD44^{high} memory T cells after ALT-803 treatment with an innate-like phenotype and their high antitumor activity, it is conceivable that these cells represented the main effector

cells responsible for mounting robust and rapid immune responses against myeloma in the initial phase after ALT-803 infusion.

A single dose of ALT-803 was capable of inducing high serum levels of IFN- γ in mice. This activity seemed to be different from that in previous studies in which monotherapy with IL-15 or single-chain IL-15:IL-15R α complexes was shown to induce mouse immune cell proliferation, but not to affect serum IFN- γ levels (30, 31). IL-15 has been reported to elevate IFN- γ levels *in vivo* when coadministered with IL-12, IL-18, or other immunostimulatory molecules via a cytokine feedback cascade involving NK cells and macrophages (7). In contrast, we show that the effect of ALT-803 on serum IFN- γ levels was largely dependent on CD8⁺CD44^{high} memory T cells and not NK1.1⁺ cells. It has been found that treatment of mice with IL-15:IL-15R α /Fc complexes similar to ALT-803 can cause naïve CD8⁺ T cells to expand and acquire an activated phenotype that includes the ability to secrete IFN- γ and mediate antigen-specific cytolytic function (10). These responses were dependent on MHC class I molecules, TCR avidity and were enhanced in the presence of peptide antigen (32), suggesting that IL-15:IL-15R α /Fc complexes increase the sensitivity and responsiveness of naïve CD8⁺ T cells to endogenous antigen presentation. In contrast, ALT-803 has the unique feature of inducing high levels of serum IFN- γ by activating CD8⁺ memory T cells in an antigen-independent fashion *in vivo*. Although IFN- γ has no direct effects on growth or induction of apoptosis of 5T33P tumor cells *in vitro* as shown in this study, the loss of treatment-mediated antimyeloma activity in the IFN- γ KO mice bearing 5T33 tumors shows the pivotal role of IFN- γ in the therapeutic potency of ALT-803. The effect of IFN- γ on ALT-803 antitumor activity is apparently via an indirect mechanism as ALT-803 did not lose its ability to induce IFN- γ -deficient CD8⁺CD44^{high} memory T cells in IFN- γ KO mice.

IFN- γ is a remarkable cytokine that orchestrates a diverse array of cellular programs through transcriptional regulation of immunologically relevant genes (33). IFN- γ skews the immune response toward a T-helper (T_H) 1 phenotype by inducing T-bet, a critical transcription factor of T_H1 cells, which directly induces many T_H1 cell-related genes, but indirectly suppresses the T_H2 cell-related genes (34). IFN- γ also orchestrates the trafficking of specific immune cells to sites of inflammation (e.g., tumor sites) through upregulating expression of adhesion molecules (e.g., ICAM-1, VCAM-1) and chemokines (e.g., IP-10, MCP-1, MIG-1 α / β , RANTES; refs. 35–42). Thus, the loss of IFN- γ could lead to the loss of the T_H1 cell-type antitumor environment and the inability to upregulate the necessary chemokine receptors and/or adhesion molecules on the ALT-803-activated CD8⁺CD44^{high} T cells for trafficking to the tumor site. In addition, IFN- γ is a potent activator of macrophages that kill pathogens and tumor cells by producing reactive oxygen species and reactive nitrogen intermediates via induction of NADPH oxidase system and iNOS (43–45). IFN- γ is also known to repolarize the stage M2 tumor-promoting tumor-associated macrophages (TAM) to M1 tumor-destroying macrophages at the tumor sites, which in turn could mount an effective immune response against tumors (46, 47).

Thus, IFN- γ secreted by ALT-803-activated memory T cells could significantly contribute to the antitumor potency of ALT-803 by directly activating macrophages to enhance their tumor-killing activities or to repolarize the TAMs for tumor destruction.

In summary, we reveal the novel mechanism of action of ALT-803, an IL-15 superagonist complex, against multiple myeloma that acts mainly through its stimulation of CD8⁺ CD44^{high} memory T cells to expand, acquire an innate-type phenotype and secrete IFN- γ independent of antigen requirement resulting in enhancement of host survival. These findings suggest a novel therapeutic strategy of exploiting the innate-cell function of adoptive immune cells. Although our results suggest clinical application for treatment of multiple myeloma, this IL-15-based approach may also be efficacious for other cancers and infectious diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Development of methodology: W. Xu, B. Liu, X. Zhu, M.P. Rubinstein

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Xu, B. Liu, X. Zhu, E.K. Jeng, M.P. Rubinstein, P.R. Rhode, H.C. Wong

Writing, review, and/or revision of the manuscript: W. Xu, M. Jones, B. Liu, X. Zhu, C.B. Johnson, W.D. Marcus, M.P. Rubinstein, P.R. Rhode, H.C. Wong

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. Xu, M. Jones, B. Liu, C.B. Johnson, K.-P. Han, P.R. Rhode

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Acknowledgments

The authors thank Dr. Ulrich von Andrian, Harvard Medical School, for providing the 5T33 myeloma cell line and Dr. Jason Yovandich, Biological Resources Branch, National Cancer Institute-Frederick, for providing recombinant human IL-15. The authors also thank Dr. Dean Taylor for his critical reading of the manuscript.

Grant Support

This study was supported by grants from the NIH (CA167925; H.C. Wong). 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 June 23, 2012; revised February 14, 2013; accepted February 25, 2013; published OnlineFirst May 3, 2013.

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