Clinical Cancer Research

The IL-15-Based ALT-803 Complex Enhances FcγRIIIa-Triggered NK Cell Responses and *In Vivo* Clearance of B Cell Lymphomas

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Abstract

Purpose: Anti-CD20 monoclonal antibodies (mAb) are an important immunotherapy for B-cell lymphoma, and provide evidence that the immune system may be harnessed as an effective lymphoma treatment approach. ALT-803 is a superagonist IL-15 mutant and IL-15Rα–Fc fusion complex that activates the IL-15 receptor constitutively expressed on natural killer (NK) cells. We hypothesized that ALT-803 would enhance anti–CD20 mAb-directed NK-cell responses and antibody-dependent cellular cytotoxicity (ADCC).

Experimental Design: We tested this hypothesis by adding ALT-803 immunostimulation to anti-CD20 mAb triggering of NK cells *in vitro* and *in vivo*. Cell lines and primary human lymphoma cells were utilized as targets for primary human NK cells. Two complementary *in vivo* mouse models were used, which included human NK-cell xenografts in NOD/SCID-$\gamma_c^{-/-}$ mice.

Results: We demonstrate that short-term ALT-803 stimulation significantly increased degranulation, IFNγ production, and ADCC by human NK cells against B-cell lymphoma cell lines or primary follicular lymphoma cells. ALT-803 augmented cytotoxicity and the expression of granzyme B and perforin, providing one potential mechanism for this enhanced functionality. Moreover, in two distinct *in vivo* B-cell lymphoma models, the addition of ALT-803 to anti-CD20 mAb therapy resulted in significantly reduced tumor cell burden and increased survival. Long-term ALT-803 stimulation of human NK cells induced proliferation and NK-cell subset changes with preserved ADCC.

Conclusions: ALT-803 represents a novel immunostimulatory drug that enhances NK-cell antilymphoma responses *in vitro* and *in vivo*, thereby supporting the clinical investigation of ALT-803 plus anti-CD20 mAbs in patients with indolent B-cell lymphoma. *Clin Cancer Res; 22(3); 596–608. ©2015 AACR.*

Introduction

Indolent B-cell non-Hodgkin lymphomas (iNHL) represent the most common clinical group of NHLs (1), are typically considered incurable, and the optimal approach to iNHL therapy remains unresolved (2). Currently, immunotherapy with anti-CD20 monoclonal antibodies (mAb), alone or in combination with chemotherapy, is a standard therapy for patients with iNHL (2, 3). However, responses are heterogeneous with some remissions lasting for years, and others a few months. While chemotherapy remains a mainstay of modern iNHL therapy, much of the toxicity of current combination regimens, including bone marrow suppression and the potential risk of secondary malignancies, results from the chemotherapy component. Recently, clinical research efforts have explored promising combinations that eliminate chemotherapy, and instead rely on doublets of therapeutic mAbs (3), survival pathway inhibitors (4), and/or utilizing immunomodulatory drugs (5). The goal of such a treatment paradigm is long-term disease control with minimal side effects for patients, without a requirement for cytotoxic chemotherapy or radiotherapy.

Use of anti-CD20 mAbs represents an effective, well-tolerated passive immunotherapy approach for iNHL, which may rely on several mechanisms of action including antibody-dependent cellular cytotoxicity (ADCC) to eliminate lymphoma cells (6, 7). NK cells are one cellular mediator of ADCC, with FcγRIIIa (CD16) being a dominant cell surface activating receptor for triggering NK-cell antitumor responses (8). The contribution of FcγRIIIa to anti-CD20 mAb responses is supported by enhanced clinical activity in patients with genetic polymorphisms that confer a higher affinity FcγRIIIa binding (9, 10). Furthermore,

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Translational Relevance

Therapeutic monoclonal antibodies (mAb) are a standard immunotherapy treatment for patients with various types of malignancy, including lymphoma. One cellular effector for therapeutic mAb-induced responses are natural killer (NK) cells, which recognize mAb-opsonized targets via the activating receptor FcγRIIIa (CD16). The IL-15 receptor is a central cytokine signal for NK-cell enhanced functionality, and physiologically the IL-15Rβγ recognizes IL-15 transpresented from accessory cells via the high-affinity IL-15Rα. ALT-803 is a superagonist IL-15 mutant and IL-15Rα–Fc fusion complex that exhibits prolonged *in vivo* pharmacokinetics, and effectively transpresents IL-15 in the absence of accessory cells. Here we show that ALT-803 augments the *in vitro* and *in vivo* response of NK cells when directed by anti-CD20 mAbs, against lymphoma targets, *in vitro* and *in vivo*. These results provide the rationale for translating this immunotherapy combination of immunostimulation by ALT-803 and therapeutic mAbs into clinical trials for cancer patients.

studies have demonstrated *in vivo* NK-cell activation in the blood of patients treated with anti-CD20 mAbs (11, 12). Second-generation anti-CD20 mAbs have been engineered to enhance the interaction between the Fc region and the low-affinity FcγRIIIa expressed on NK cells, resulting in even more potent ADCC (6). Recently, a study has identified a correlation between killer cell immunoglobulin-like receptor (KIR) genotype and delayed progression in iNHL patients treated with mAb therapy, further implicating NK cells as an important effector for iNHL (13). We reasoned that novel treatment approaches for iNHL that increase NK-cell ADCC in concert with anti-CD20 mAbs may result in enhanced antitumor responses without incurring serious or long-term complications that may occur with cytotoxic chemotherapy drugs.

NK cells are innate lymphoid cells that comprise 5% to 20% of human blood lymphocytes, and constitutively express a number of cytokine receptors, thereby making them amenable to cytokine-based priming *in vivo* (8, 14). Stimulation through the shared IL2/15Rβγ_c receptor by the cytokine IL-15 has been shown to enhance NK-cell ADCC *in vitro* (15), including that directed by anti-CD20 mAbs (16). IL-15 impacts other functions, including increased cytotoxic effector molecule expression, enhanced proliferation and survival, increased motility, and costimulation of NK-cell–derived cytokines (e.g., IFNγ; refs. 14, 17–19). IL-15 is physiologically transpresented by its high-affinity IL-15Rα from the surface of accessory cells to the IL2/15Rβγ_c on NK cells, resulting in the activation of multiple intracellular signaling pathways, including Jak1/3 and STAT3/5, MAPK, PI3K-Akt, and mTOR kinase (19). Approaches that utilize soluble IL-15/IL-15Rα complexes exhibit enhanced *in vivo* effects compared with IL-15 alone (20–23). ALT-803 is an IL-15 superagonist complex comprised of an IL-15 mutein bound to the sushi domain of IL-15Rα fused to the Fc region of IgG1 (23–25). This results in accessory cell-independent transpresentation of IL-15, prolonged *in vivo* pharmacokinetics, increased *in vivo* biologic activity compared with IL-15, and enhanced CD8 T-cell responses in multiple myeloma mouse models (25). Thus, we hypothesized that ALT-803 would effectively prime NK-cell ADCC against B-cell NHL in concert with anti-CD20 therapeutic mAbs, resulting in an enhanced antilymphoma innate immune response. Here, we tested this hypothesis by defining the preclinical effects of ALT-803 on NK-cell responses directed by anti-CD20 mAbs against primary B-cell lymphoma and lymphoma cell lines *in vitro* and *in vivo*.

Materials and Methods

Reagents and mice

Anti-human mAbs used were as follows: BD Biosciences: CD16 (3G8), IFNγ (B27), CD69 (FN50), CD107a (H4N3); Beckman Coulter: CD56(N901), CD3(UCHT1), CD45(J.33); Invitrogen: granzyme B(GB12); and BioLegend: perforin (dG9), HLA-DR (L243). Endotoxin-free recombinant human (rh)IL-15 (CellGenix) and ALT-803 (Altor) were used for NK-cell stimulation. Clinical grade antibodies included rituximab (Genentech) and cetuximab (Bristol Myers Squibb). hOAT (humanized anti-tissue factor IgG1 antibody, Altor) also served as a control. NOD-SCID-IL2Rγ$^{-/-}$ (NSG) mice were obtained from the Jackson Laboratory. Fox Chase SCID (C.B-17/IcrHsd-Prkdc-scid) mice were obtained from Harlan Laboratories. All mouse experiments were performed under a Washington University ASC protocol (NSG model) or Altor BioScience IACUC (SCID model) approved protocols.

Cell lines

K562 cells (ATCC, CCL-243) and Raji cells (ATCC, CCL-86) were obtained from ATCC in 2008, viably cryopreserved and stored in LN2, thawed for use in these studies, and maintained for no more than 2 months at a time in continuous culture as described (26). Prior to our studies, the K562 cells were authenticated by confirming cell growth morphology (lymphoblast), growth characteristics, and functionally as NK-cell–sensitive targets in 2014 and 2015. Raji cells were authenticated by confirming cell growth morphology (lymphoblast), growth characteristics, phenotype of uniform expression of human CD20, and functionally as anti–CD20 mAb-opsonized targets for ADCC in 2014 and 2015. Daudi cells (ATCC, CCL-213) were obtained from ATCC in 2004, viably cryopreserved, and stored in LN2. Prior to use in these studies, the Daudi cells were authenticated in 2014 and 2015 by confirming cell growth morphology (lymphoblast), growth characteristics, phenotype of uniform expression of human CD20 by flow cytometry, and functionally as anti–CD20 mAb-opsonized targets for ADCC. Cells were cultured in R10: RPMI1640 supplemented with L-glutamine, HEPES, NEAA, sodium pyruvate, and Pen/Strep/Glutamine containing 10% FBS (Hyclone or Sigma Aldrich). Cells were washed in PBS (Hyclone) prior to mouse injections.

NK-cell purification and cell culture

Human normal donor PBMCs were obtained anonymously from platelet-apheresis donors or from OneBlood (26). NK cells were purified using RosetteSep (StemCell Technologies) or Ficoll centrifugation and CD56$^+$CD16$^+$ NK cell isolation kit (Miltenyi). Cells were cultured at 3 to 5 × 10^6 cells/mL in HAB10: RPMI1640 supplemented with L-glutamine, HEPES, NEAA, and Pen/Strep containing 10% human AB serum (Sigma-Aldrich). Cells were preactivated for 18 to 20 hours using rhIL-15 or ALT-803 as indicated. Cells were washed in HAB10 before functional assays were performed. For some experiments, cells were cultured for 48 hours in R10.

Functional and proliferation assays

NK-cell functional and proliferation assays were performed as described (26, 27). In experiments using rituximab-opsonized targets, cells were preincubated with mAbs for 30 minutes, washed, and used at the indicated E:T ratios.

Flow-based killing assays

Cytotoxicity assays were performed as described (27, 28). Data were acquired on a Gallios flow cytometer (Beckman Coulter) and analyzed using Kaluza (Beckman Coulter) software. For the ADCC assays with Raji, target cells were preincubated with rituximab 10 mcg/mL or cetuximab 10 mcg/mL as a control in HAB10, washed, cocultured with human NK cells at the indicated E:T ratios. For the ADCC assays with Daudi, target cells were labeled with CellTrace Violet (Invitrogen) and incubated for 2 days with the indicated effectors at the indicated E:T ratios. Daudi viability was assessed by detecting PI using flow cytometry.

Patient samples

Primary lymph node lymphoma cells were collected under the Institutional Review Board–approved Washington University Lymphoma Banking Protocol (2011–08251) after informed consent. A single-cell suspension was generated by mechanical disruption, and mononuclear cells isolated by Ficoll centrifugation. Cryopreserved single-cell suspensions were thawed (>95% viable) and used immediately as targets in ADCC assays.

Human NK-cell NSG xenografts with Raji-luciferase cells

Raji cells expressing a luciferase–eGFP fusion were generated by spinfection of a Luc-eGFP-cassette–containing U3 retrovirus, followed by flow sorting of stable GFP-positive cells, as described (29). The NSG-Raji model was chosen to examine the impact of ALT-803 administration on human NK-cell anti-CD20 mAb-directed clearance of human lymphoma cells *in vivo*. NSG mice were irradiated (250 cGy) 24 hours prior to i.v. injection with 1×10^6 Raji-luciferase cells. After 3 days, human NK cells were injected (4×10^6/mouse, retro-orbital). Concurrently, 10 mg/kg rituximab and ALT-803 were injected i.v. Where indicated, ALT-803 or PBS (control) was administered i.v. twice weekly. Tumor burden was assessed by bioluminescence imaging (BLI). For BLI, mice were injected i.p. with 150 mcg/g D-luciferin (Biosynth) in PBS, anesthetized, and imaged with a CCD camera (IVIS 100; PerkinElmer); exposure time 1 to 60 seconds, binning 16, field of view 12, f/stop 1, open filter. Both dorsal and ventral whole body BLI images were taken and quantified as photon flux (29). Mice were also followed for survival, and euthanized when any unacceptable morbidity developed.

SCID mice with Daudi models

This second model was chosen to examine the impact of ALT-803 administration on mouse NK-cell anti-CD20 mAb-directed clearance of human lymphoma cells *in vivo*. SCID mice were injected i.v. with 1×10^7 Daudi cells, and at day 15 randomized into the indicated treatment groups. Mice were injected with rituximab (10 mg/kg) and ALT-803 or controls on days 15 and 18. On day 22, the percentage of HLA-DR$^+$ Daudi cells in femur bone marrow was determined using flow cytometry. For survival experiments, hind limb paralysis was used as the endpoint.

Statistical analysis

Statistical comparisons were performed using Student *t* test, Kruskal–Wallis test, ANOVA, and Kaplan–Meier analysis (Log–rank, Mantel–Cox) as appropriate (Prism v5, GraphPad Software). For all testing, significance levels required $P < 0.05$.

Results

ALT-803 stimulation augments human NK-cell cytotoxicity and granzyme B and perforin expression

To determine whether initial findings that ALT-803 augmented murine NK-cell function (24, 25) is applicable to humans, we first defined the impact of short-term stimulation with ALT-803 on human NK-cell cytotoxicity against the MHC class I low, NK-cell–sensitive target K562. Purified (>95% CD56$^+$CD3$^-$) human NK cells were stimulated with varying equimolar concentrations of ALT-803 or rhIL-15 for 20 hours, washed, and used as effector cells in a 4-hour cytotoxicity assay against K562 targets. ALT-803 and rhIL-15 enhanced human NK-cell killing in a comparable, dose-dependent fashion (Fig. 1A and B). We next investigated the impact of short-term ALT-803 stimulation on key effector proteins that mediate NK-cell killing. ALT-803 induced a dose-dependent increase in the expression of perforin and granzyme B by CD56^{dim} human NK cells (Fig. 1C). In addition, NK-cell stimulation was also evident via induction of the early activation marker CD69 (Fig. 1D and E). These data demonstrate that ALT-803, comparably with rhIL-15, enhances the cytotoxic potential and activation state of human NK cells *in vitro*.

ALT-803 augments NK-cell ADCC directed by an anti-CD20 mAb *in vitro*

ADCC represents a specialized NK-cell attack against mAb-coated target cells, and depends primarily on triggering via FcγRIIIa. We next tested the ability of short-term ALT-803 stimulation to enhance ADCC against the CD20$^+$ B-cell lymphoma cell lines Daudi and Raji *in vitro*. Coincubation of Daudi targets with human PBMC plus an anti-CD20 mAb (rituximab) for 2 days resulted in increased Daudi cell death, compared with control antibody (Fig. 2A, 0 nmol/L ALT-803). This anti-CD20 mAb-directed ADCC was significantly augmented by ALT-803. In additional experiments, NK cells were purified from PBMC (>90% CD56$^+$CD3$^-$) and utilized as effectors, revealing that NK-cell killing was dose dependently increased by ALT-803 (Fig. 2B). Analysis of the non–NK-cell fraction of PBMC as effectors demonstrated no Daudi cell killing, consistent with NK cells being the primary mediator of ADCC in PBMC in these experiments (data not shown). In separate experiments, purified human NK cells were stimulated for 20 hours with ALT-803 or rhIL-15, and used as effectors in ADCC assays against Raji targets. Summary results demonstrated that ALT-803 increased rituximab-directed ADCC against Raji cells, similar to equimolar concentrations of rhIL-15 (Fig. 2C). At the highest concentrations of ALT-803 or rhIL-15 (1 nmol/L), NK cells were able to kill Raji cells in the presence of control mAb, indicating that Raji cell resistance to NK cells is also overcome by maximal stimulation via the IL-15Rβγ$_c$.

ALT-803 primes NK-cell ADCC and cytotoxicity against primary follicular lymphoma cells isolated from patient lymph nodes

Purified normal donor NK cells were stimulated with ALT-803 for 20 hours, and used as effector cells in killing assays against primary follicular lymphoma target cells. The specific death of light-chain restricted CD19$^+$ clonal B cells were identified by CFSE labeling and CD19 surface staining. A representative donor is shown in Fig. 2D, demonstrating ALT-803 enhancement of both

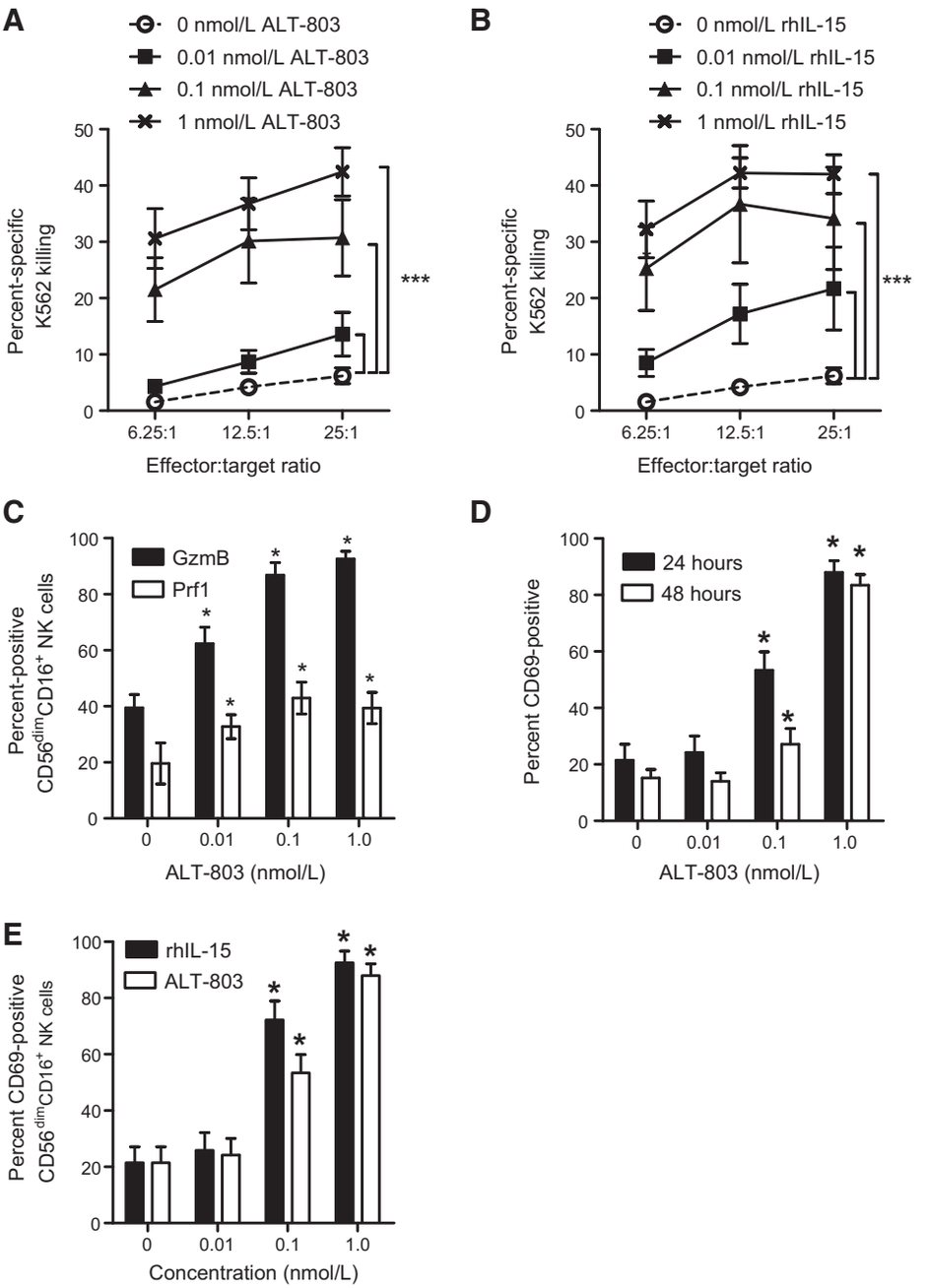

Figure 1.
ALT-803 enhances human NK-cell cytotoxicity and increases cytotoxic effector molecule expression. Purified human NK cells (>95% $CD56^+CD3^-$) were stimulated with the indicated concentrations of ALT-803 (A) or rhIL-15 (B) for 20 hours and used as effectors in a standard flow-based cytotoxicity assay against K562 target cells. Mean ± SEM of specific K562 killing is shown at the indicated effector:target cell ratios. As the ALT-803 and rhIL-15 experiments were performed simultaneously the same no cytokine control conditions are used for A and B. Specific killing is the amount of 7AAD-positive target cells present subtracted by the spontaneous (no effector) cell death (routinely <5%). C and D, purified NK cells were cultured with the indicated concentrations of ALT-803. Following stimulation, cells were harvested, and $CD56^{dim}CD16^+$ NK cells were analyzed for intracellular perforin (Prf1) and granzyme B (GzmB) protein (C, 24 hours), or cell surface CD69 expression (D). Equimolar concentrations of rhIL-15 and ALT-803 were compared for induction of cell surface CD69 expression after 24 hours on $CD56^{dim}$ NK cells (E). As the ALT-803 and rhIL-15 experiments were performed simultaneously, the same no cytokine control conditions are used for ALT-803 and rhIL-15 in E. Results are mean percentage positive ± SEM for $N = 5$ normal donors. *, $P < 0.05$; ***, $P < 0.001$.

ADCC (rituximab) and NK cytotoxicity (control mAb) against these primary lymphoma target cells. This finding was reproducible between multiple lymphoma samples (Supplementary Table S1) and NK-cell donors (Fig. 2E). Thus, ALT-803 also enhances primary human NK-cell killing of primary lymphoma cells *in vitro*.

ALT-803 enhances NK-cell IFNγ production and degranulation triggered by anti–CD20 mAb-coated tumor cells

To further define the impact of ALT-803 on NK-cell responses to mAb-opsonized target cells, we also examined NK-cell IFNγ production and degranulation. Purified NK cells were stimulated for 20 hours with ALT-803, washed, and then cocultured for 6 hours with Raji target cells that were preincubated with rituximab or control mAb. ALT-803 prestimulation resulted in a dose-dependent enhancement of both IFNγ production and degranulation (CD107a surface expression) in response to rituximab-coated Raji cells (Fig. 3A). In addition, ALT-803 enhanced IFNγ production and degranulation to a modest degree in the control–mAb conditions, especially at its maximal concentration. The mature $CD57^+$ subset of $CD56^{dim}$ NK cells has been reported to exhibit enhanced FcγRIIIa-triggered IFNγ

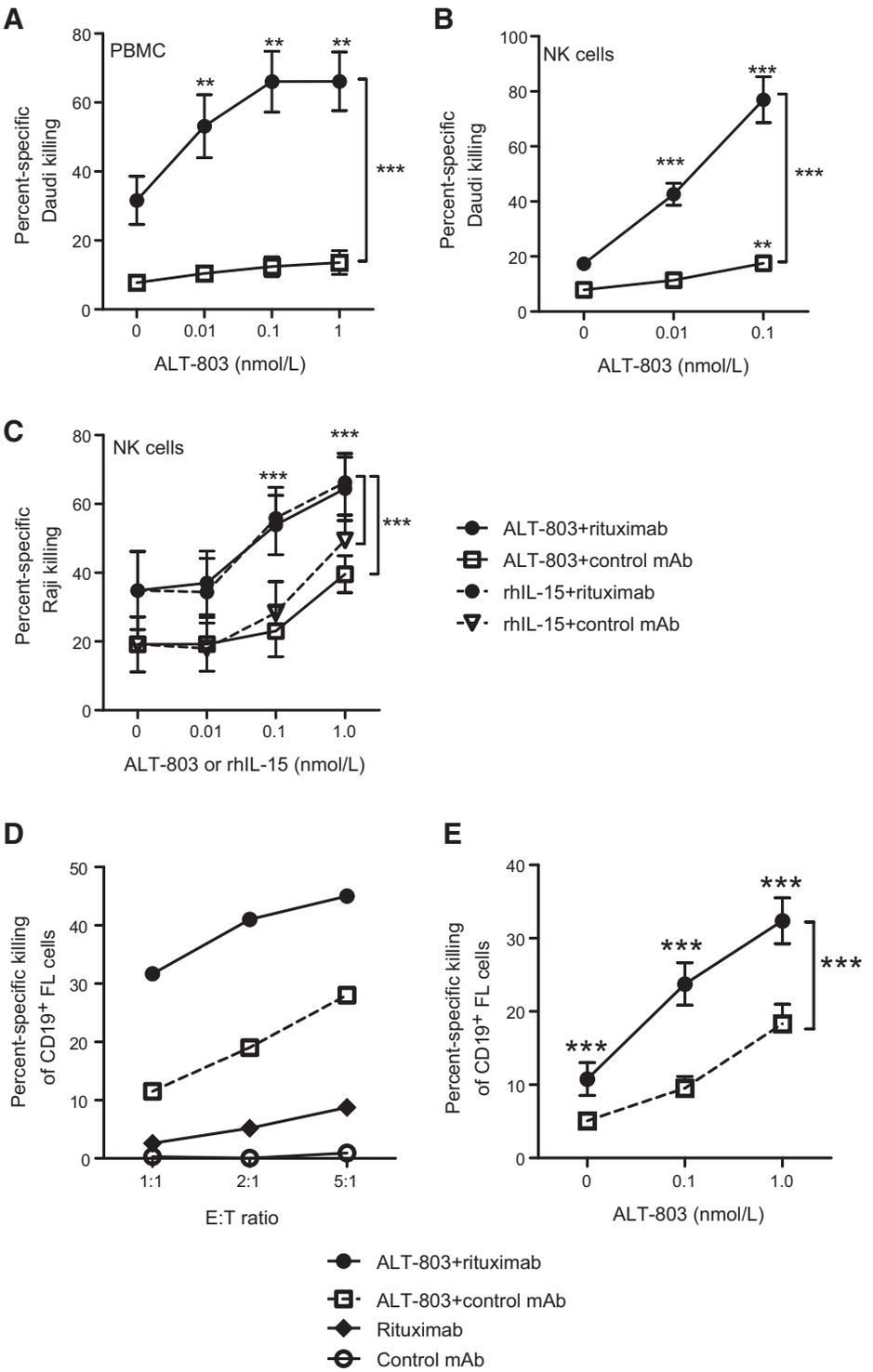

Figure 2.
ALT-803 augments human NK-cell ADCC directed by anti-CD20 mAbs against B lymphoma cell lines and primary human follicular lymphoma cells. A, human PBMC were used as effector cells and CellTrace violet-labeled Daudi cells were the targets (E:T ratio 2:1) with ALT-803 doses and antibody conditions (rituximab or control) present as indicated. After 2 days, Daudi cell viability was determined by flow cytometry (PI) with the mean ± SEM of Daudi cell death shown ($N = 8$ donors). B, in separate experiments, NK cells were purified from PBMC (>90% $CD56^+$ $CD3^-$) and used in Daudi target cell ADCC assays (E:T ratio 1:1). There were significant differences in mean ± SEM ($N = 4$ donors) between rituximab and control, and ALT-803 concentrations, as indicated. C, in separate experiments, purified human NK cells (>95% $CD56^+CD3^-$) were stimulated with the indicated concentration of ALT-803 or rhIL-15 for 20 hours, and then used as effectors in a flow-based cytotoxicity assay against Raji B-cell lymphoma target cells. Mean ± SEM of specific Raji cell killing is shown with an E:T ratio of 2.5:1 with the indicated ALT-803 or rhIL-15 concentrations and rituximab or control mAb present ($N = 4$ normal donors). There were significant differences between rituximab and control, and ALT-803 or rhIL-15 concentrations, as indicated. There were no significant differences between equimolar concentrations of ALT-803 and rhIL-15. D, Representative normal donor NK cells in a 4-hour flow-based ADCC assay demonstrating killing of $CD19^+$ primary lymphoma cells at the indicated E:T ratios after 20 hours of stimulation with 1 nmol/L ALT-803 and 30-minute lymphoma cell prelabeling with rituximab or control mAbs. Effector cells were purified (>95% $CD56^+CD3^-$) NK cells. E, summary data of mean ± SEM (E:T 2:1, $N = 5$ lymphoma sample targets, $N = 15$ normal NK-cell donors, $N = 5$ independent experiments) showing the dose-dependent increase in mean ADCC enhanced by ALT-803. There was a significantly increased ADCC response with increasing ALT-803 concentration and the mAb treatment group. **, $P < 0.01$; ***, $P < 0.001$.

production and redirected killing responses (30). We therefore evaluated these NK-cell functional readouts, stratified by CD57 expression, following ALT-803 stimulation. We unexpectedly observed no impact of CD57 expression on NK-cell IFNγ responses to FcγRIIIa-triggering via rituximab-coated Raji cells, while degranulation was significantly (albeit modestly) greater in the $CD57^-$ less mature subset (Fig. 3B). Collectively, these data reveal that ALT-803 enhances multiple NK-cell effector responses, including degranulation, cytotoxicity, ADCC, and IFNγ production triggered by FcγRIIIa.

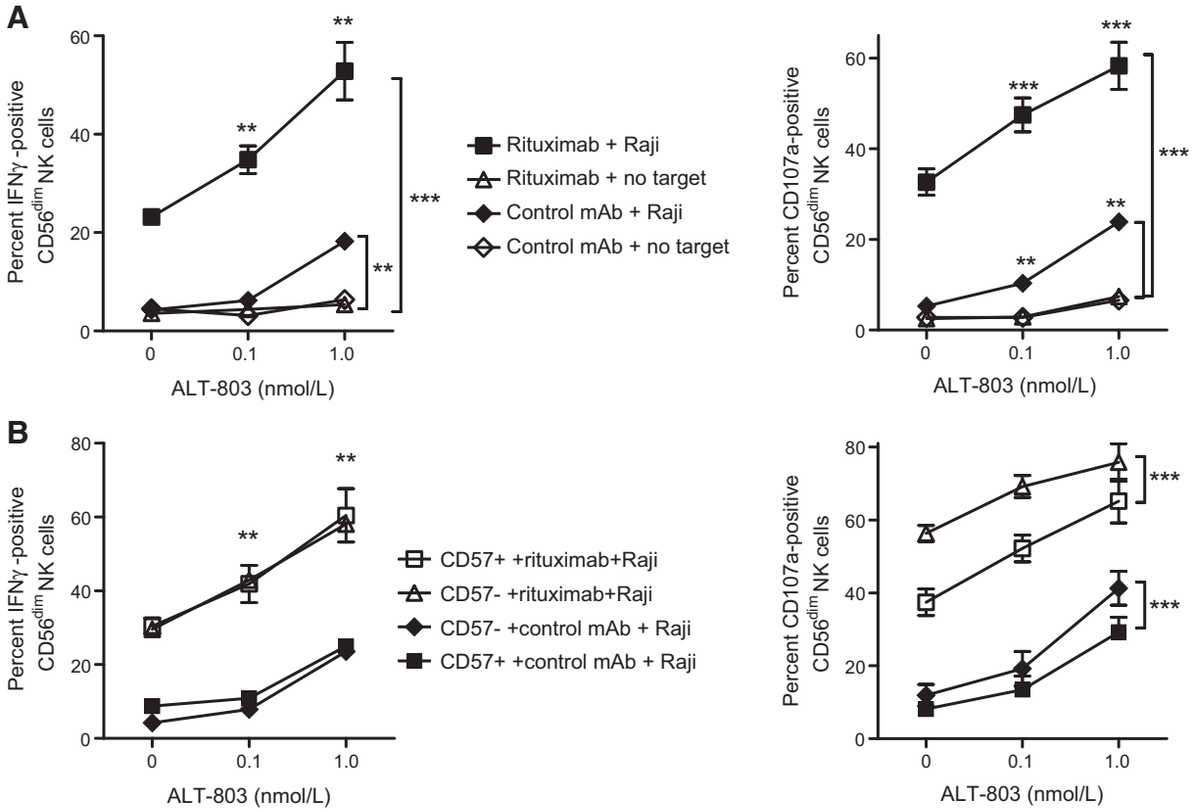

Figure 3.

ALT-803 enhances human NK-cell IFNγ production and degranulation (CD107a) responses directed by an anti-CD20 mAb. A, purified human NK cells (>95% $CD56^+$ $CD3^-$) were stimulated with ALT-803 for 20 hours and then triggered by coincubation with Raji tumor targets (5:1 E:T ratio) in the presence of rituximab or control mAb as indicated. IFNγ production (left) and degranulation (CD107a, right) were determined using standard 6-hour flow cytometry assay. The combined mean ± SEM percentage IFNγ-positive (left) or CD107a-positive (right) NK cells is shown with data from 3 normal donors. There was a significantly increased response due to ALT-803 concentration and coincubation condition as indicated. B, CD57 was used to stratify $CD56^{dim}CD16^{bright}$ NK cells into terminally matured ($CD57^+$) and less mature ($CD57^-$) subsets. While the IFNγ production stimulated by FcγRIIIa engagement or tumor targets was identical in ALT-803 primed human NK cells, there was significantly increased degranulation in less mature $CD57^-$ NK cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

ALT-803 augments rituximab-directed clearance of Daudi lymphoma cells in SCID mice

Prior studies with ALT-803 have demonstrated its capacity to expand murine NK cells in mice (24, 25). Here, 1×10^7 Daudi lymphoma cells were engrafted into SCID mice on day 0, and mice were treated with vehicle (PBS), rituximab (R), ALT-803, or ALT-803 plus rituximab in two doses on day 15 and day 18 post-Daudi cell challenge (Fig. 4A). First, the Daudi cell burden in the bone marrow of mice was examined at day 22 after challenge (Fig. 4B and C). As shown in Fig. 4B, there was a significant enhancement of rituximab-mediated Daudi cell clearance in the bone marrow at all doses of ALT-803 tested. These results were confirmed in an additional experiment, albeit with lower overall Daudi cell engraftment (Fig. 4C). To further assess the control of Daudi cells *in vivo*, groups of SCID mice were treated as in Fig. 4A and followed for survival. There was significantly enhanced survival of SCID mice treated with ALT-803 plus rituximab, compared with rituximab alone, or other conditions (Fig. 4D). In separate experiments, the ability of rhIL-15 and ALT-803 were compared for their ability to augment rituximab-directed antilymphoma responses (Fig. 4E) Equivalent doses and schedule of ALT-803 plus rituximab demonstrated significantly reduced Daudi cell burden compared with rhIL-15 plus rituximab. NK-cell numbers and percentages in the bone marrow of mice treated with ALT-803 and/or rituximab in Fig. 4B and C are shown in Fig. 4F and G. Overall, there was no biologically significant alteration in bone marrow NK-cell numbers or percentages with ALT-803 treatment in two doses in this model, suggesting that enhanced tumor clearance is due to NK-cell activation, as opposed to increased numbers. NK-cell analyses in the spleen of the mice are provided in Supplementary Fig. S1. This analysis was performed at day 22 at the time of Daudi cell examination, and only provides information as to the NK-cell modulation at this single time point. These findings demonstrate that ALT-803 enhances anti-CD20-directed control of Daudi B cell lymphoma *in vivo*.

ALT-803 enhances rituximab-directed control of Raji lymphoma cells in a human NK-cell NSG xenograft model

We next investigated the ability of ALT-803 administration to enhance human NK-cell clearance of lymphoma cell lines *in vivo* in conjunction with rituximab in an immunodeficient NSG mouse xenograft system. NSG mice were injected with Raji cells expressing luciferase, and groups of mice were treated with human NK cells and rituximab or control mAb on day 3, followed by ALT-

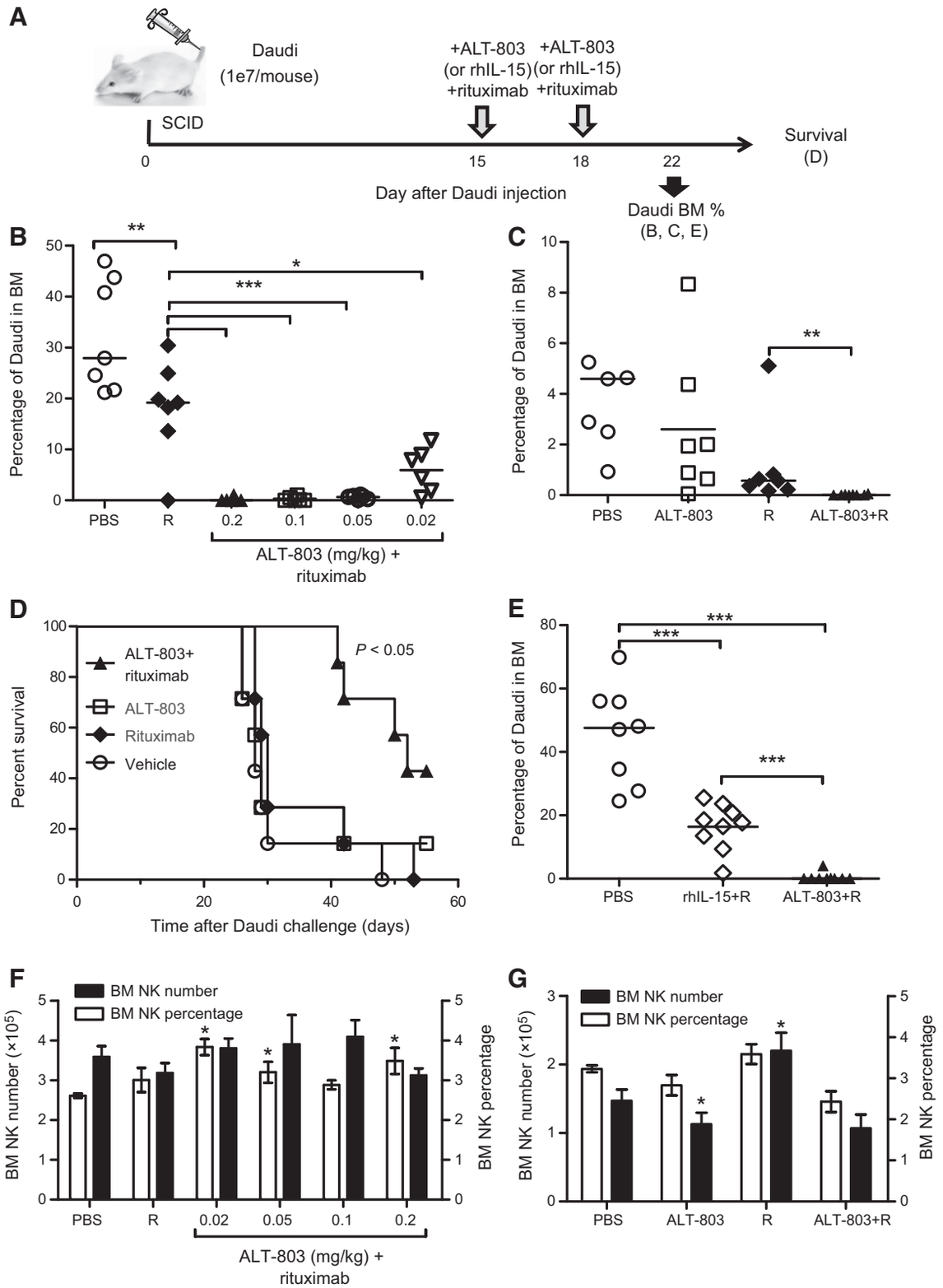

Figure 4.

ALT-803 enhanced rituximab-directed protection from a lethal Daudi lymphoma challenge *in vivo*. A, schema for experimental model. SCID mice were injected (i.v.) on day 0 with 1×10^7 Daudi cells. On days 15 and 18 mice were treated i.v. with PBS (vehicle), rituximab (10 mg/kg), and/or ALT-803 (as indicated for B, 0.2 mg/kg for C and D). Two readouts were utilized: the percentage of Daudi cells (human HLA-DR$^+$) in the bone marrow (BM) at day 22 postinjection (B and C) and survival (D). (*Continued on the following page.*)

803 administration twice weekly (0.05 mg/kg; Fig. 5A). Raji cell burden was monitored with BLI. Mice not receiving rituximab, including those treated with PBS, human NK cells, or human NK cells plus ALT-803, exhibited rapid tumor growth (Fig. 5B). Notably, although mice treated with human NK cells and rituximab exhibited tumor control for 2 weeks, there was significantly lower tumor cell burden measured by BLI in human NK cell plus ALT-803 plus rituximab-treated mice (Fig. 5B). In a separate experiment, the reduced Raji cell burden in the human NK cell plus ALT-803 plus rituximab group, compared with human NK cells plus rituximab group, was confirmed (Fig. 5C). In this NSG model of human NK-cell engraftment and ALT-803 therapy, we verified that human NK cells were present in the blood, spleen, and bone marrow of NSG mice at day 12 in both ALT-803 and control groups (data not shown). Human NK-cell–engrafted mice treated with ALT-803 plus rituximab had significantly prolonged survival compared with rituximab alone in this model (Fig. 5D). Finally, when equivalent doses and schedules of rhIL-15 and ALT-803 were compared in combination with rituximab, ALT-803 plus rituximab exhibited significantly improved Raji lymphoma cell control *in vivo* (Fig. 5E). In our NSG model, there exists the possibility that murine macrophages contribute to antitumor responses following anti-CD20 mAb treatment, which would require macrophage-depleting experiments to definitively address.

The impact of ALT-803 treatment on human NK cells transferred into NSG mice was also investigated (Supplementary Fig. S2). Nine days after transfer, ALT-803–treated mice demonstrated increased human NK-cell numbers in the blood and liver, equivalent numbers in the bone marrow, and reduced engraftment in the spleen, compared with control mice (Supplementary Fig. S2A and S2B). Moreover, ALT-803 treatment *in vivo* resulted in the expansion of a $CD56^{bright}CD16^{+}$ NK-cell effector population of NK cells. This was in contrast with the conventional $CD56^{bright}$ $CD16^{dim/-}$ and $CD56^{dim}CD16^{+}$ NK-cell subsets recovered from control mice (Supplementary Fig. S2C). Finally, $CD56^{bright}$ $CD16^{+}$ NK cells recovered from ALT-803 treated NSG mice showed evidence of extensive proliferation, with significantly greater dilution of CFSE compared with NK cells recovered from control mice. Collectively, these data suggest that human NK cells proliferate, expand, and mediate superior anti-lymphoma functionality with ALT-803 treatment *in vivo*.

Prolonged ALT-803 stimulation of NK cells induces proliferation, alteration in NK-cell subsets, and preserved ADCC

Prolonged ALT-803 stimulation may potentially alter the human NK-cell subset composition and hence the ability to mediate ADCC against lymphoma cells. Human NK cells expanded in NSG mice treated with ALT-803 after 9 days demonstrated a predominantly $CD56^{bright}CD16^{+}$ phenotype. To further explore the long-term effects of ALT-803 on human NK cells, we cultured purified NK cells (>95% $CD56^{+}CD3^{-}$) with varying concentrations of ALT-803 for 14 days and assessed NK-cell subsets and proliferation. Short-term (1 day) stimulation of human NK cells did not alter NK-cell subset composition, nor did it change FcγRIIIa expression (Fig. 6A). However, chronic ALT-803 stimulation induced the emergence of a $CD56^{bright}CD16^{+}$ NK-cell subset after 1 to 2 weeks (Fig. 6A). Notably, ALT-803 induced the proliferation of both $CD56^{bright}CD16^{-}$ and $CD56^{bright}CD16^{+}$ to a comparable degree, whereas $CD56^{dim}CD16^{+}$ NK cells exhibited less proliferation and subset contraction (Fig. 6B). Similar results were observed with equimolar concentrations of rhIL-15 (data not shown). To determine whether NK cell changes associated with prolonged ALT-803 stimulation altered ADCC potential, we tested the ability of normal donor NK cells to kill rituximab-coated Raji lymphoma cells after 1, 8, or 15 days of ALT-803 stimulation (1 nmol/L). To control for assay variation, this experiment was conducted using cryopreserved NK cells were thawed, stimulated for the indicated times, and then simultaneously assessed for ADCC function in the same assay. ADCC functionality was clearly present at all days assessed in this experiment (Fig. 6C). Thus, although prolonged ALT-803 selectively expands $CD56^{bright}CD16^{+}$ cells *in vitro* and *in vivo*, there was a retained, enhanced capacity for ADCC.

Discussion

IL-15 is an immunostimulatory cytokine currently in clinical development for the immunotherapy of several cancers (14, 17–19). The primary immune cell types activated by IL-15 are NK cells, $CD8^{+}$ T cells, and innate nonclassical T cells, resulting in proliferation, survival, and enhanced effector function (14, 17–19). Here, we defined the effects of ALT-803 (23–25), an IL-15 superagonist complex engineered to enhance receptor binding, transpresentation, and *in vivo* stability, on human NK cells. Short-term ALT-803 stimulation of primary human NK cells resulted in increased granzyme B and perforin protein expression and enhanced cytotoxicity against NK-cell–sensitive targets. We investigated whether ALT-803 potentiated human NK-cell responses triggered via FcγRIIIa by anti–CD20 mAb-opsonized lymphoma targets. Short-term ALT-803 activation enhanced degranulation, IFNγ production, and ADCC against rituximab-opsonized lymphoma cell lines. Importantly, ALT-803 also augmented anti–CD20 mAb-directed ADCC responses by human NK cells against primary human follicular lymphoma cells. Two different *in vivo* models with NK cells directed by rituximab against $CD20^{+}$ B-cell lymphoma lines demonstrated that combination treatment with ALT-803 plus rituximab significantly reduced lymphoma burden

(*Continued.*) B, the percentage of Daudi cells in the BM was significantly reduced in the ALT-803 plus rituximab group, compared with rituximab alone, as indicated. Results shown are the mean for $N = 6$–7 mice/group (bar) with individual mice shown as symbols. C, in a separate experiment from (B) Daudi-bearing SCID mice were treated with PBS (vehicle), ALT-803 (0.2 mg/kg), rituximab (10 mg/kg), or ALT-803 plus rituximab and analyzed at day 22 post-injection. In this experiment, the overall Daudi cell burden was lower in the BM, but confirmed a significant reduction in Daudi cells in the BM of ALT-803 plus rituximab-treated mice, compared with rituximab alone. D, groups ($N = 7$–8 mice/group) of Daudi-bearing SCID mice were treated as in (C) and followed for survival. Kaplan–Meier analysis is shown, demonstrating that ALT-803 plus rituximab significantly improves survival compared with the other treatment conditions. E, in separate experiments, groups ($N = 7$–8 mice/group) of Daudi-bearing SCID mice were treated as in A and B with PBS (vehicle), rituximab (R, 10 mg/kg), and equivalent amounts of ALT-803 (0.2 mg/kg) or rhIL-15 (0.055 mg/kg; all intravenously), and at day 22 BM was assessed for the percentage of Daudi cells. ALT-803 plus rituximab had significantly reduced Daudi cell burden compared with rituximab plus rhIL-15. F and G, the percentage and number of NK cells in the BM of mice treated in B and C are shown in F and G, respectively. Overall, there was not a substantial impact of ALT-803 on the absolute number of NK cells in the BM at day 22.
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

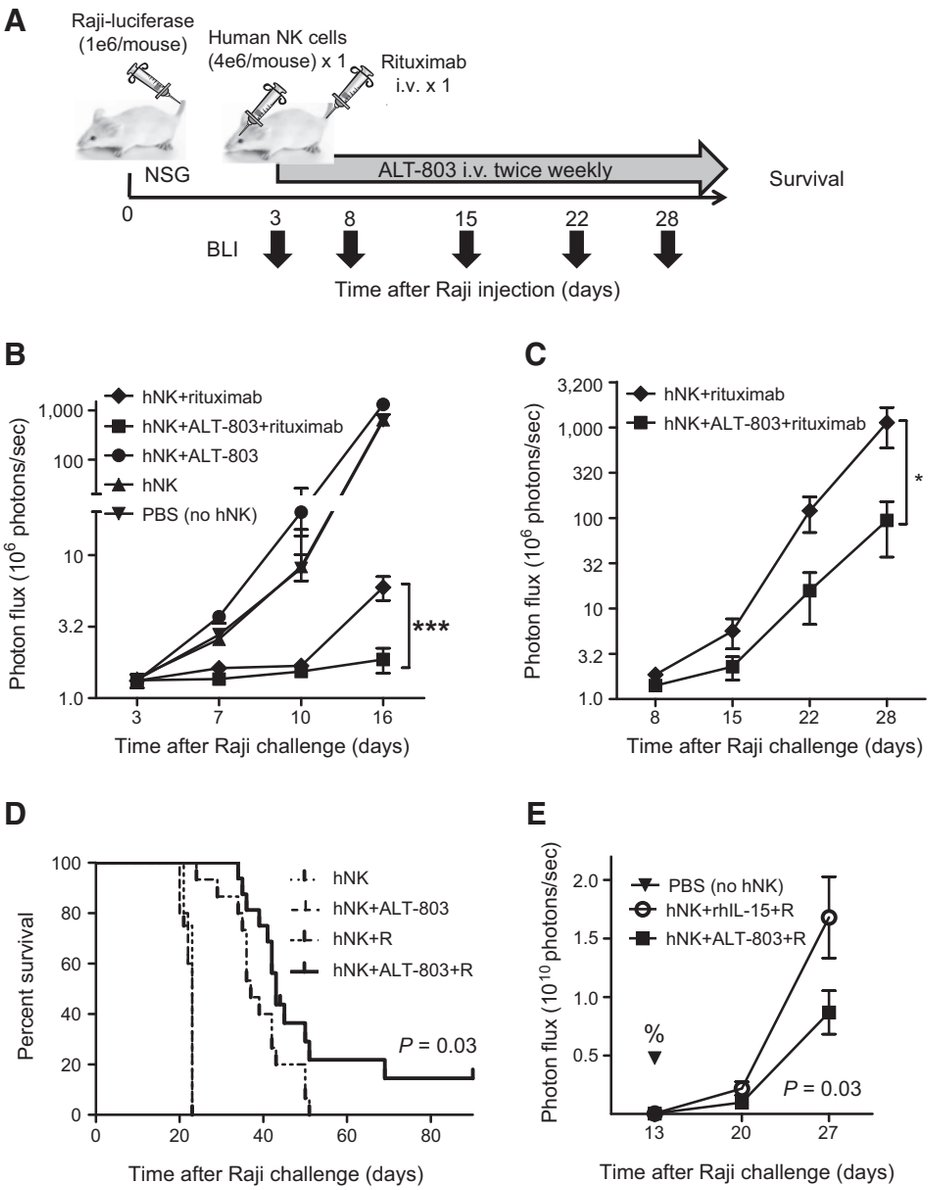

Figure 5.

ALT-803 enhanced rituximab-directed control of Raji lymphoma challenge by human NK-cell xenografts in NSG mice. A, schema of experimental model. Immunodeficient NSG mice were challenged with 1×10^6 Raji cells engineered to express luciferase. After 3 days, human NK cells (hNK) were transferred into the mice and groups were treated with PBS (no hNK control), ALT-803, rituximab, or ALT-803 plus rituximab. At the indicated time points post Raji challenge, mice were injected with D-luciferin, and evaluated for relative tumor burden *in vivo* using BLI. B, mice were treated as in A and imaged at days 3, 7, 10, and 16 post-Raji cell challenge with $N = 3$–5 mice/group. ALT-803 was administered at 0.05 mg/kg in this experiment. The mean ± SEM photon flux of separate ventral and dorsal whole body BLI measurements at each time point is shown for each group. There was a significantly decreased Raji cell burden in the hNK plus ALT-803 plus rituximab, compared with the hNK plus rituximab groups. C, in a separate experiment, groups of mice were treated with hNK plus rituximab ± ALT-803 (0.2 mg/kg), with the mean ± SEM photon flux of separate ventral and dorsal whole body BLI measurements shown. There was significantly decreased Raji cell burden in the hNK plus ALT-803 plus rituximab condition, compared with hNK plus rituximab. D, groups of mice were treated as in A and followed for survival, with an ALT-803 dose of 0.2 mg/kg. Kaplan–Meier analysis is shown, demonstrating that ALT-803 plus rituximab significantly improves survival compared with hNK plus rituximab (log-rank Mantel–Cox, $P = 0.03$). E, in separate experiments, groups of mice were engrafted with Raji-luciferase cells as in A and treated with PBS ($N = 4$, negative control) or hNK plus rituximab followed by rhIL-15 (0.055 mg/kg, $N = 8$) or ALT-803 (0.2 mg/kg, $N = 7$) twice weekly. The ALT-803 group had significantly reduced Raji tumor burden compared with rhIL-15 administered at equivalent doses and an identical schedule. % Mice from the PBS control group are not shown past day 13 due to death from Raji cells. *, $P < 0.05$; ***, $P < 0.001$.

and increased survival, compared with rituximab treatment alone. As expected, *in vivo* lymphoma burden in mice treated with ALT-803 plus rituximab was also significantly reduced, compared with an equivalent dose/schedule of rhIL-15 plus rituximab. Finally, long-term activation of human NK cells with ALT-803 resulted in extensive proliferation and altered NK-cell subset composition,

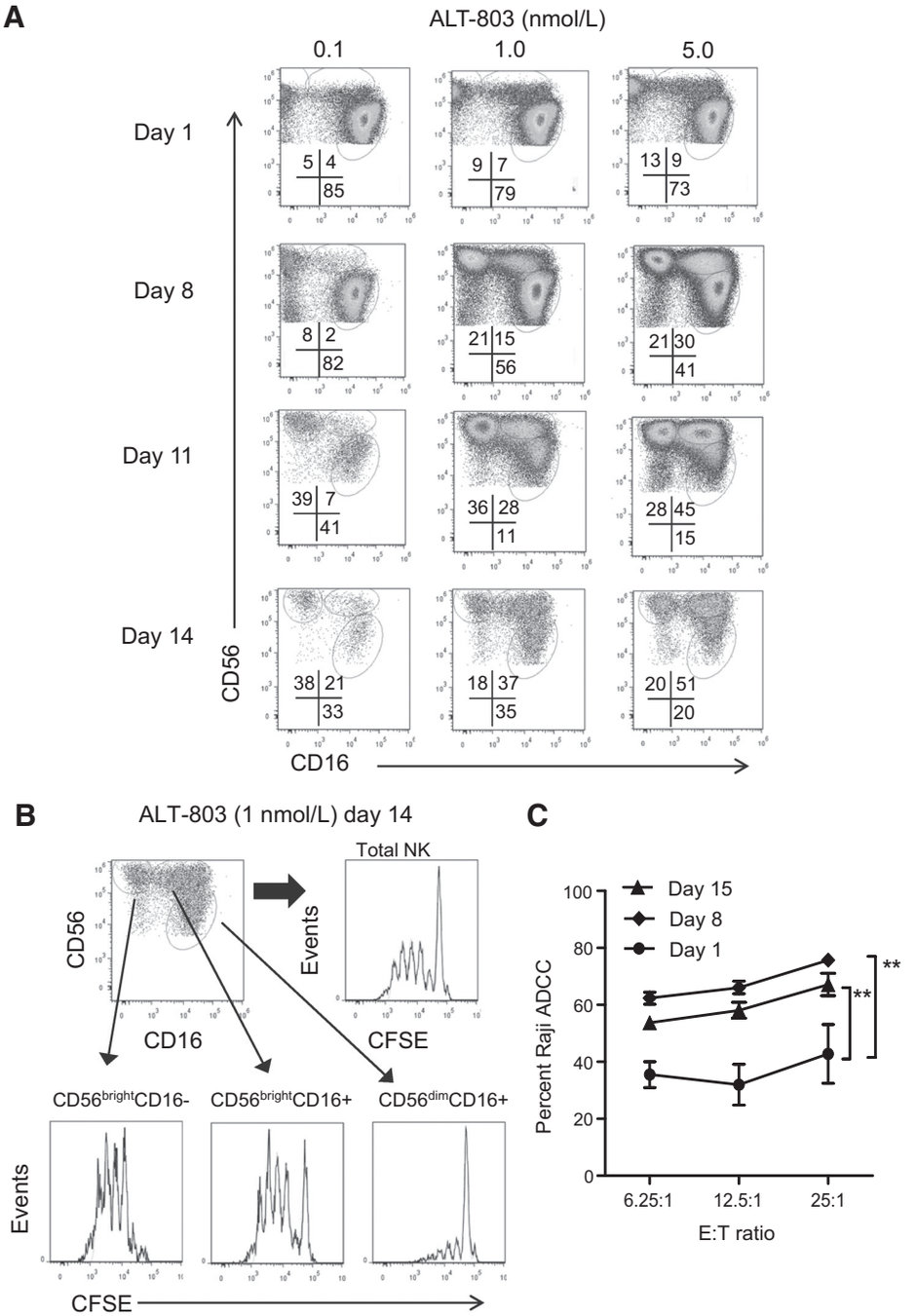

Figure 6.
Prolonged ALT-803 stimulation induces NK-cell proliferation and alters NK-cell subset composition while preserving ADCC. A, purified human NK cells (>95% $CD56^{+}CD3^{-}$) were labeled with CFSE and cultured for two weeks in HAB10 medium with the indicated concentration of ALT-803. At 1, 8, 11, and 14 days of stimulation, NK-cell subsets were analyzed by flow cytometry. A representative donor is shown that demonstrated minimal impact on the $CD56^{bright}CD16^{-}$, $CD56^{bright}CD16^{+}$, and $CD56^{dim}CD16^{+}$ NK cells subsets after 1 day of stimulation. However, after 8 to 14 days of culture, a subset of $CD56^{bright}CD16^{+}$ NK cells emerges as the dominant NK-cell subset. Results are representative of 3 normal donors. Numbers represent the percentages of the three NK-cell subsets as gated. B, analysis of proliferation (CFSE dilution) of NK-cell subsets after 14 days of ALT-803 stimulation demonstrated that both $CD56^{bright}CD16^{-}$ and $CD56^{bright}CD16^{+}$ NK cells have extensively proliferated, while minimal proliferation was evident in the now minor $CD56^{dim}CD16^{+}$ NK population. Results are representative of $N = 3$ normal donors. C, cryopreserved purified NK cells from the same donors were thawed and stimulated for the indicated length of time in ALT-803 (1 nmol/L) and used as effector cells in the same ADCC assay with rituximab-labeled Raji tumor target cells. ADCC was present after 1, 8, or 15 days of ALT-803 stimulation, suggesting that the human NK-cell subset molding that occurs with prolonged stimulation does not impede ADCC. Results are representative of 3 normal donors. **, $P < 0.01$.

but preserved their ADCC against lymphoma cell lines. Thus, ALT-803 represents an immunostimulatory drug that enhances the functionality of NK cells triggered by FcγRIIIa against lymphoma cells *in vitro* and *in vivo*.

The current standard-of-care treatments of iNHL rely primarily on nonspecific cytotoxic chemotherapy agents that affect rapidly dividing cells and damage DNA, thereby yielding clinical remissions, but with the cost of substantial potential toxicity (1, 2, 5). The effectiveness of anti-CD20 mAbs against lymphoma, including their significant improvement in long-term clinical outcomes for NHL patients, provides proof-of-principle that immune-based therapy has activity against NHL (31). This clinical activity has spurred interest in developing immunotherapy combinations that eliminate chemotherapy altogether, especially for iNHL. For example, doublets of therapeutic mAbs have been investigated in relapsed/refractory iNHL (32, 33). In addition, the immunomodulatory (IMiD) drug lenalidomide has been shown to enhance NK-cell ADCC (34), and when combined with rituximab has provided promising activity in patients with indolent (35, 36), or subsets of aggressive, B-cell NHL (37). Other cytokines (IL2, IL12,

IL18, IL21) and CpG ODNs have also been combined with therapeutic mAbs, with the aim of augmenting NK-cell responses directed by the disease-specific mAb (38–46). However, IL2 (unlike IL-15) has the capacity to selectively augment regulatory T cells (47), potentially explaining the limited efficacy of combining IL2 and therapeutic mAbs. Recent studies have indicated that blockade of immune inhibitory receptors (e.g., PD-1) also result in clinical responses against iNHL, likely via enhancing endogenous T and NK-cell antilymphoma responses (48). Finally, the reprogramming of T cells with chimeric antigen receptors has demonstrated remarkable preliminary efficacy in related B-cell malignancies (49), including preliminary reports in B-cell NHL (50). ALT-803 represents a novel approach to provide immunostimulation of selected antitumor immune effector cells that would likely complement standard therapeutic mAbs, and emerging immune checkpoint blockade, IMiDs, and CAR-T cell approaches. Our data suggest that the combination of ALT-803 and anti-CD20 mAbs may be a rational clinical approach based on ADCC potentiation, especially in iNHL where single-agent rituximab is a standard-of-care.

On the basis of the potential to enhance NK-cell proliferation, survival, natural killing, and ADCC (14, 17–19), several types of IL-15 receptor agonists are in preclinical or clinical development for cancer immunotherapy, including rhIL-15, ALT-803, unmodified IL-15/IL-15Rα complexes, and IL-15–IL-15Rα fusion proteins (18). Recently, the *in vivo* feasibility of NK-cell and $CD8^+$ T-cell modulation by rhIL-15 administration to patients with advanced cancer was reported in a first-in-human rhIL-15 clinical trial (51). This study reported on the safety of IL-15 administered as an i.v. bolus at 0.3, 1.0, or 3.0 mcg/kg for 12 consecutive days in cancer patients, which resulted in substantial NK-cell modulation. After a transient redistribution from the peripheral blood, hyperproliferation of NK cells was observed in the periphery evidenced by Ki67 expression, and numbers expanded 10- to 50-fold over several weeks, including $FcγRIIIa^+$ NK cells. The data presented here are consistent with IL-15/ALT-803 induction of NK-cell tissue redistribution, proliferation, and reveal that prolonged IL-15/ALT-803 exposure results in the expansion of a $CD56^{bright}$ $CD16^+$ NK-cell subset with preserved functionality. Future studies will focus on NK-cell trafficking and expansion differences induced by various formulations of IL-15, which may have clinical relevance. In addition, blood $CD8^+$ and γδ-T cells increased in number, with $CD8^+$ T cells demonstrating increased Ki67, CD38, and HLA-DR expression. Additional early-phase clinical trials with rhIL-15 are ongoing that include subcutaneous administration (NCT01727076) for solid tumor patients, and i.v. rhIL-15 in metastatic carcinoma (NCT01572493) or following NK-cell infusion for myeloid diseases (NCT01385423). ALT-803 administered weekly is also currently being tested in phase I dose-escalation studies in advanced solid tumors (NCT01946789), relapse following allogeneic stem cell transplantation (NCT01885897), relapsed/refractory multiple myeloma (NCT02099539), and non-muscle invasive bladder cancer in combination with BCG (NCT02138734). Thus, available safety and correlative biological data suggest that IL-15 receptor engaging drugs may be safely administered with substantial *in vivo* human NK-cell modulation. As our data indicate that such IL-15–based modulation may be combined with anti-CD20 mAbs to enhance the innate immune response against B-cell lymphomas, clinical investigation of ALT-803 plus anti-CD20 mAbs in patients with iNHL is underway (NCT02384954). The large number of ongoing studies with rhIL-15 and ALT-803 will expand our understanding of whether these two agents will exhibit comparable or distinct immune modulation based on pharmacokinetics or *in vivo* distribution and will assist in the design of future clinical studies.

There are a number of potential mechanisms whereby ALT-803 may enhance antitumor immune responses to lymphoma. In our experiments examining ALT-803 enhancement of human NK-cell function, we observed comparable effects of equimolar concentrations of rhIL-15 and ALT-803 *in vitro* when examining cytotoxicity, ADCC, FcγRIIIa-triggered degranulation and IFNγ production, and proliferation. These findings suggest that prior studies demonstrating that rhIL-15 augments direct NK-cell antitumor responses via a pleiotropic array of functional antitumor improvements (14, 17–19), will also apply to ALT-803. However, *in vivo* ALT-803-induced NK-cell activation has been demonstrated to be superior to rhIL-15 (25), as ALT-803 exhibits a greater serum half-life (24), and prolonged distribution to lymphoid organs compared with rhIL-15 (Wong HC and colleagues, In Press, Cancer Immunology Research). In addition, stimulation of NK cells by therapeutic mAbs has been linked to "exhaustion" after triggering via FcγRIIIa through depletion of the cytotoxic effector molecules perforin and granzyme B (52). Notably, signals through the $IL2/15Rβγ_c$ reversed the cytotoxic effector molecule depletion, and allowed for effective serial NK-cell killing. We therefore expect that ALT-803 stimulation may reverse FcγRIIIa-triggered "exhaustion", providing a more sustained NK-cell ADCC response when used in combination with therapeutic mAbs. While not directly examined in our study, IL-15 (18) and ALT-803 (25) have been shown to promote $CD8^+$ T-cell responses, resulting in enhanced T-cell–dependent antitumor immunity. As rituximab has been shown to induce an endogenous adaptive immune response in the setting of indolent NHL (53, 54), ALT-803 therapy may also potentiate such autologous $CD8^+$ T-cell antitumor immunity. This effect may be further enhanced by coengagement of NK cells via anti-CD20 mAbs, resulting in NK-cell–derived cytokine production and DC activation and maturation. Thus, provision of ALT-803 plus rituximab may have additional favorable antitumor effects by promoting endogenous tumor-specific memory $CD8^+$ T-cell responses to lymphoma as has been shown in models of multiple myeloma (25), in addition to directly augmenting NK-cell anti-CD20 triggered responses.

In summary, we report that the immunostimulatory agent ALT-803 augments NK-cell proliferation, cytotoxic capacity, FcγRIIIa-triggered degranulation, and IFNγ production, and ADCC against B-cell lymphoma. Furthermore, ALT-803 potentiated anti–CD20 mAb-directed tumor clearance of mice challenged with B-cell lymphoma lines *in vivo*. These preclinical results suggest that the combination of ALT-803 plus an anti-CD20 mAb merits testing in early-phase clinical trials in patients with iNHL in varying settings, including relapsed/refractory disease, high-risk iNHL disease in remission, or iNHL patients with evidence of minimal residual disease. Moreover, investigations of IL-15–based immunostimulation plus therapeutic mAbs in multiple cancer types are warranted.

Disclosure of Potential Conflicts of Interest

P.R. Rhode has ownership interest (including patents) in Altor BioScience Corporation. No potential conflicts of interest were disclosed by the other authors.

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