NKTR-214, an Engineered Cytokine with Biased IL2 Receptor Binding, Increased Tumor Exposure, and Marked Efficacy in Mouse Tumor Models


Abstract

**Purpose:** Aldesleukin, recombinant human IL2, is an effective immunotherapy for metastatic melanoma and renal cancer, with durable responses in approximately 10% of patients; however, severe side effects limit maximal dosing and thus the number of patients able to receive treatment and potential cure. NKTR-214 is a prodrug of conjugated IL2, retaining the same amino acid sequence as aldesleukin. The IL2 core is conjugated to 6 releasable polyethylene glycol (PEG) chains. In vivo, the PEG chains slowly release to generate active IL2 conjugates.

**Experimental Design:** We evaluated the bioactivity and receptor binding of NKTR-214 and its active IL2 conjugates in vitro; the tumor immunology, tumor pharmacokinetics, and efficacy of NKTR-214 as a single agent and in combination with anti-CTLA-4 antibody in murine tumor models. Tolerability was evaluated in non-human primates.

**Results:** In a murine melanoma tumor model, the ratio of tumor-killing CD8+ T cells to Foxp3+ regulatory T cells was greater than 400 for NKTR-214 compared with 18 for aldesleukin, supporting preferential activation of the IL2 receptor beta over IL2 receptor alpha, due to the location of PEG molecules. NKTR-214 provides a 500-fold greater exposure of the tumor to conjugated IL2 compared with aldesleukin. NKTR-214 showed efficacy as a single agent and provided durable immunity that was resistant to tumor rechallenge in combination with anti-CTLA-4 antibody. NKTR-214 was well tolerated in non-human primates.

**Conclusions:** These data support further evaluation of NKTR-214 in humans for a variety of tumor types, adding to the repertoire of potent and potentially curative cancer immunotherapies.

Introduction

Immunotherapy offers potential for durable responses in a growing list of tumor types (1). Aldesleukin, recombinant human IL2, was the first cancer immunotherapy, and one of the first recombinant proteins, approved by the FDA in 1992. High-dose IL2 (HD-IL2) administration results in severe hypotension and vascular leak syndrome, which has relegated its use to specialized care centers. With use of appropriate supportive care, aldesleukin has demonstrated complete cancer regression in about 10% of patients treated for metastatic melanoma and renal cancer (2–4). Approximately 70% of patients with complete responses have been cured, maintaining complete regression for more than 25 years after initial treatment (2–6). Unfortunately, the side-effect profile restricts optimal IL2 dosing, limiting the number of patients who might achieve durable responses.

IL2 stimulates immune cell proliferation and activation through a receptor-signaling complex containing alpha (IL2Ra, CD25), beta (IL2Rβ, CD122), and common gamma chain receptors (γc, CD132; refs. 7, 8), but the effects are pleiotropic and contextual. At high doses, IL2 binds to heterodimeric IL2Rβγ receptor leading to desired expansion of tumor killing CD8+ memory effector T (CD8 T) cells (9). IL2 also binds to its heterotrimeric receptor IL2Rαβγ with greater affinity, which expands immunosuppressive CD4+CD25+ regulatory T cells (Tregs) expressing high constitutive levels of IL2Ra. Expansion of Tregs represents an undesirable effect of IL2 for cancer immunotherapy (7–13).

We hypothesized IL2 conjugation with polyethylene glycol (PEG) could be used to mask the region of IL2 that interacts with the IL2Ra subunit responsible for activating immunosuppressive Tregs, biasing activity towards tumor killing CD8+ T cells. Pegylation could alter the immunostimulatory profile of aldesleukin without mutation of its amino acid sequence while creating an inactive prodrug, mitigating rapid systemic immune activation, and improving tolerability (14).

We report the development of NKTR-214, a biologic prodrug consisting of IL2 bound by 6 releasable polyethylene glycol (PEG) chains. We evaluated receptor bias, bioactivity, tumor immunology, pharmacokinetics, pharmacodynamics, and tolerability of NKTR-214. Antitumor effects of NKTR-214 were compared with aldesleukin as a single agent and in combination with an...
Translational Relevance

Recent attention in immunotherapy for treatment of cancer has focused on the successes of checkpoint inhibitor antibodies. However, administration of the recombinant cytokine IL2 aldesleukin produces durable responses lasting up to two decades in approximately 10% of patients treated for metastatic melanoma and renal cancer. IL2 is an activator and suppressor of the immune system and is associated with severe side effects such as hypotension and vascular leak syndrome, limiting widespread use. Recent studies have shown that durable responses may increase with combined immunotherapies. We evaluated NKTR-214, a polymer conjugate of IL2 that tips the balance in favor of immune activation over suppression in the tumor, with improved efficacy and tolerability compared with aldesleukin. Our data provide the rationale for further evaluation of NKTR-214 in clinical studies as both a single agent and in combination with other immunotherapies including checkpoint inhibitor antibodies.

Materials and Methods

Recombinant protein and antibodies

Aldesleukin was purchased from Myoderm and diluted in the recommended product formulation buffer for in vivo studies. NKTR-214 was manufactured by Nektar Therapeutics. ELISA study materials are described in Supplementary Materials and Methods.

For in vivo murine studies, aldesleukin and anti-CTLA-4 antibody were administered intraperitoneally; NKTR-214 was administered intravenously by tail-vein injection. For each animal, the concentration was adjusted to achieve the specified dose at an injection volume of 0.2 mL for aldesleukin, 0.1 mL for anti-CTLA-4 antibody, and 8 to 10 mL/kg for NKTR-214. NKTR-214 dosing was based on the IL2 equivalent content of the conjugate.

Animals

Animal studies were conducted under accreditation by the Association for Assessment and Accreditation of Laboratory Animal Care or the Canadian Council on Animal Care. All studies met the ethical and humane criteria for transportation, housing, and care established by United States NIH guidelines or Canadian animal care regulators. Female C57BL/6 NCrI mice aged 6 to 8 weeks were purchased from Charles River Laboratories. Male and female cynomolgus monkeys, aged 2 to 3 years, were obtained from Worldwide Primates Inc. Male naïve adult cynomolgus monkeys, aged 3 to 4 years, weighing 3.7 to 4.3 kg at treatment initiation, were obtained from Primus Bio-Resources Inc. Male and female C57BL/6 NCrI mice aged 6 to 8 weeks weighing 16 to 20 g were purchased from Charles River Laboratories. Male and female Balb/C NCrl mice aged 6 to 8 weeks weighing 15 to 20 g and female Balb/C NCrl mice aged 6 to 8 weeks weighing 16 to 20 g were purchased from Charles River Laboratories.

Cell lines

IL2-dependent CTLL-2 mouse T-lymphocyte cell line, B16F10 melanoma, EMT6 mammary, and CT26 colon carcinoma cell lines were obtained from ATCC. All cells were verified pathogen and mycoplasma (IDEXX BioResearch) free and authenticated by ATCC using Cytochrome C subunit I PCR assay.

Production and purification of anti-CTLA-4

UC10-4F10-11 hybridoma (15), which secretes hamster anti-mouse CTLA-4 antibody with mouse IgG2a isotype, was purchased from ATCC (HB-304). PHM-11 medium (Invitrogen) was used to culture the cell line under 5% CO2. The cell line was maintained between 1 x 10^6 and 1 x 10^6 cells/mL and anti-CTLA-4 was expressed and purified (16).

Degree of PEGylation for NKTR-214

A releasable conjugate of glycine was used as a reference standard. Reference standard or NKTR-214 was incubated at room temperature for 45 minutes with 37.5 mmol/L NaOH to release PEG from IL2 or glycine. The reaction was neutralized with an equal volume of 100 mmol/L HCl. Samples were analyzed by reverse phase high-performance liquid chromatography (HPLC) to quantify released protein, glycine, and PEG. PEG moles released from NKTR-214 were calculated using the standard curve from releasable PEG-glycine standard. Degree of PEGylation was calculated as ratio of PEG moles to protein moles, or ratio of area under the curve (AUC) of released PEG to AUC of IL2. Procedures for mapping PEGylation sites are included in the Supplementary Materials and Methods.

In vitro hydrolysis kinetics of NKTR-214

Hydrolysis of NKTR-214 was monitored in vitro in PBS at pH 7.4 at 37°C. At indicated time points, levels of released IL2 conjugates were measured using reverse phase HPLC with corresponding IL2 conjugate reference standards. Data were fit to a kinetic model using the Berkeley-Madonna software. The study was performed long enough to obtain the half-life of release.

Identification of the active released IL2 conjugates

Activity of NKTR-214 and its released IL2 conjugates was measured by activation of pSTAT5 in CTLL-2 cells using Sector Imager 2400 plate reader and assay reagents from Meso Scale Discovery, LLC as described in Supplementary Materials and Methods. Total and phosphorylation of STAT5 (pSTAT5) levels were detected using the MSD Phospho[Tyr694]/Total STAT5a,b Whole Cell Lysate Kit. Methods for biophysical characterization and affinity of NKTR-214 binding to IL2 receptor subunits are described in Supplementary Materials and Methods.

Tumor exposure of NKTR-214 compared with aldesleukin

C57BL/6 mice were implanted subcutaneously into the right flank with B16F10 melanoma cells (1 x 10^6 per animal). Seven days after implantation, when tumors measured 200 mm³, animals were administered NKTR-214 (2 mg/kg × 1) or aldesleukin (3 mg/kg daily × 5). Tumors were harvested (n = 4 per observation time), homogenized in ice-cold PBS containing protease inhibitor (Roche) and 0.25% acetic acid, and centrifuged to obtain supernatant. To quantify NKTR-214 levels in tumor tissue, PEG was released from IL2 by incubating the supernatant in a pH 9 buffer at 37°C overnight. IL2 was measured by sandwich ELISA specific for human IL2. To calculate AUC, data were fit with Phoenix WinNonLin using a noncompartmental model. AUC after aldesleukin was estimated on the basis of day 1 AUC multiplied by 5.
Immune cell phenotyping

Mice bearing B16F10 melanoma tumors (as described above) were administered NKTR-214 (2 mg/kg × 1), aldesleukin (3 mg/kg daily × 5), or vehicle (daily × 5). Tumors were collected 5, 7, and 10 days following initial dose and were within one SD of mean tumor volume for each group. Following mechanical and enzymatic digestion, phenotypic assessment of immune cell subtypes in tumor microenvironment was determined by flow cytometry. Immunohistochemical analysis of T-cell density was performed on frozen tumor sections 7 days after administration (Supplemental Materials and Methods).

Activity of NKTR-214 in murine tumor models

All studies were conducted in therapeutic mode with dosing initiated in the presence of established tumors with volume in excess of 100 mm³. Tumor measurements and body weights were recorded 3 times per week. For all studies, mean tumor volume for each group was graphically represented until >20% of the original cohort was removed due to tumor volume constraints. For the single-agent study, mice bearing B16F10 melanoma tumors were randomized 7 days after implantation and administered NKTR-214 2 mg/kg every 9 days for 3 doses (n = 9 mice), aldesleukin 3 mg/kg twice daily for 5 days (2 cycles, n = 9 mice), or vehicle for 5 doses (n = 12 mice). For EMT6 mammary (n = 10 per group) and CT26 colon carcinoma (n = 12 per group) combination efficacy studies, 2 × 10⁶ cells were implanted into the right flank of BALB/c mice. Seven days after implantation, mice were randomized and administered NKTR-214 0.8 mg/kg every 9 days for 3 doses, anti–CTLA-4 100 µg twice weekly through day 18, vehicle control, or anti–CTLA-4 and NKTR-214 given in combination. Two groups implanted with EMT6 were administered either aldesleukin 0.5 mg/kg daily for 5 days (2 cycles) or aldesleukin combination with anti–CTLA-4.

For the tumor rechallenge study, Balb/c mice were implanted subcutaneously in the right flank with 2 × 10⁶ EMT6 mammary carcinoma cells. Seven days after inoculation, mice were randomized and administered antibody control (n = 10 mice) or a combination of anti–CTLA-4 and NKTR-214 (n = 80 mice). Forty-nine days after dosing initiation, tumor-free animals exhibiting complete response to combined immunotherapy were enrolled into two groups receiving either rechallenge of 2 × 10⁶ EMT6 or unrelated CT26 colon carcinoma cells. An additional cohort of age-appropriate naïve animals was challenged with EMT6 cells (n = 10 mice). After 109 days, animals that rejected the EMT6 rechallenge were inoculated with 2 × 10⁶ EMT6 cells (n = 14 mice). Age-appropriate naïve animals (n = 5 mice) were similarly challenged; all groups were followed with no additional treatment.

Evaluation of immune markers and potential hypotension and vascular leak syndrome in non-human primates

For evaluation of immune markers, cynomolgus monkeys (n = 3/sex/dose) received a single intravenous bolus dose into the saphenous vein of NKTR-214 0.01, 0.03, and 0.1 mg/kg. Blood samples were collected to evaluate effects of NKTR-214 on lymphocyte and soluble CD25 counts as described in Supplementary Materials and Methods.

Arterial blood pressure was used to monitor potential hypotension and vascular leak syndrome. Male monkeys (n = 4) were surgically implanted with telemetry transmitters (TL11M2-D70-PCT; Data Sciences International). Each received NKTR-214 as a slow intravenous bolus every 14 days for 4 doses (0, 0.01, 0.03, and 0.1 mg/kg) according to Latin-square study design. Continuous telemetry recordings were collected from each conscious unrestricted monkey for 0 to 24 hours (time for peak NKTR-214 concentration), 30 to 54 hours (time for peak active 2-PEG-IL2 and 1-PEG-IL2 concentration), and 80 to 104 hours (peak immunological and clinical signs observed) after each dose. Arterial blood pressure was collected every 15 minutes over each telemetry-recording period.

Inflammatory cytokines TNFα and IFNγ were analyzed in monkeys at 1, 3, 6, 12, 24, 48, 72, and 168 hours after a single 0.1 mg/kg dose of NKTR-214 by flow cytometry using a Cytoometric Bead Array for Non-Human Primate (NHP) Th1/Th2 kit (BD Biosciences). One milliliter of blood was collected via saphenous vein and resulting serum samples were diluted 1 in 10 in tubes containing a mixture of beads coated with capture antibodies specific to IFNγ and TNFα. Bound cytokines were detected using phycoerythrin-conjugated detection antibodies. The IFNγ and TNFα concentrations were interpolated using a 5-parameter logistic nonlinear regression equation, established from the human Th1/Th2 cytokine standard curves ranging from 20 to 5,000 pg/mL for IFNγ and of 10 to 5,000 pg/mL for TNFα.

Statistical analyses

For identification of active released IL2 conjugates, EC₅₀ values for each compound were determined from sigmoidal dose–response curves using GraphPad Prism 5.01. In mouse efficacy studies, results are shown as mean ± SEM. Tumor growth inhibition was calculated as percent reduction in volume for treated groups relative to vehicle. The significance between experimental and vehicle groups was compared using ANOVA, with the Tukey post test. Differences between NKTR-214 and aldesleukin were analyzed using unpaired Student t test. P values < 0.05 were considered significant. Kaplan–Meier survival curves represent percent of tumors in each group with a volume less than their 4-fold of initial day 0 value (tumor volume quadrupling time, TVQT). Statistical significance was calculated utilizing GraphPad Prism v6.04.

Results

Design and characterization of NKTR-214

NKTR-214 is a prodrug consisting of IL2 conjugated to 6 releasable PEG chains. The amino acid sequence of the IL2 is the same as aldesleukin. The PEG reagent and conjugation reaction were optimized to facilitate localization on IL2 at lysine residues of NKTR-214 followed by hydrolytic removal of PEG on the interface. Below), the last remaining PEG is distributed at the IL2/IL2R interface (Fig. 1). Enzymatic digestion of NKTR-214 is hydrolyzed to its most active 1-PEG-IL2 state (described below), the last remaining PEG is distributed at the IL2/IL2R interface.

Because of the high degree of PEylation initially, NKTR-214 is an inactive prodrug and serves as "reservoir" revealing active conjugated IL2 after slow release of PEG chains in vivo (Fig 2A). The prodrug effect is designed to mitigate rapid systemic immune
activation such as what might occur with HD-IL2 regimen, while allowing sustained concentrations of active conjugated IL2 in the tumor. In vitro hydrolysis studies demonstrate PEG release occurs sequentially, with a half-life of 20 hours for each step in the release, supporting the lack of a “burst” IL2 release effect (Supplementary Fig. S1). Unconjugated IL2 is cleared rapidly in vivo with a half-life of 13 minutes in mice and 1.4 hours in humans (18, 19). Therefore, slow release of PEG relative to fast clearance of IL2 provides minimal exposure to free IL2 in vivo.

Identification of the active IL2 conjugates released from NKTR-214

To assess bioactivity of NKTR-214 and identify active conjugated IL2 species formed after PEG release, we evaluated levels of pSTAT5 in murine T cells (Fig. 2B) after treatment in culture. As anticipated, NKTR-214 was not active with an EC_{50} > 4,000 ng/mL, consistent with the prodrug design. Unconjugated aldesleukin had an EC_{50} of 0.4 ng/mL, similar to reported values (18). In vitro, PEG chains were released hydrolytically from NKTR-214, forming a mixture of conjugated IL2 species ranging from 5-PEG-IL2 to 1-PEG-IL2, which were each purified. 1-PEG-IL2 and 2-PEG-IL2 species were identified as the most active IL2 conjugates with EC_{50} values of 2 and 20 ng/mL, respectively. Positional isomer analysis of synthesized 1-PEG-IL2 indicated two positional isomers (of the six potential K sites described above), which were identically bioactive in the pSTAT assay. The 3-PEG-IL2 was found to be inactive with EC_{50} > 1,300 ng/mL. Therefore, species containing ≥ 3-PEGs are inactive; those with < 3-PEGs are active.

Biophysical affinity characterization of NKTR-214 binding to IL2 receptor subunits indicates PEG chains impact affinity to IL2Rα to a greater extent than IL2Rβ compared with IL2 (Supplementary Table S1; Supplementary Fig. S2).

NKTR-214 provides 500-fold increased tumor exposure relative to aldesleukin

To assess tumor exposure, NKTR-214 or aldesleukin levels were measured in mice bearing B16F10 melanoma tumors. As shown in Fig. 3, tumor aldesleukin levels rapidly reached C_{max} and then rapidly declined, leading to <4 ng/g concentrations 24 hours after each dose and a daily AUC of 0.09 ± 0.02 μg/hour/g. In contrast, NKTR-214 was detectable in tumors for up to 8 days after a single dose achieving an AUC of 30 ± 6.9 μg/hour/g. On the basis of AUC, a single dose of NKTR-214 led to a 67-fold higher exposure compared with 5 daily doses of aldesleukin, even though 7.5 fewer IL2 equivalents were dosed using NKTR-214 (3 mg/kg daily × 5 = 15 mg/kg vs. 2 mg/kg). Thus, normalizing exposure on the basis of IL2 equivalents, NKTR-214 achieved a 500-fold increased exposure relative to aldesleukin. The active conjugated IL2 form of NKTR-214 (2-PEG-IL2 and 1-PEG-IL2 together) was also quantified and remained detectable in tumor for up to 5 days yielding an AUC of 23 ± 4.4 μg/g. Hence, exposure to active conjugated IL2 was 50-times higher compared with aldesleukin, translating to 380 times increased exposure relative to an equivalent dose of aldesleukin. The tumor exposure of NKTR-214 allows dosing once every 9 days in mice compared with twice daily for two 5-day cycles for aldesleukin.

Immune cell phenotyping: NKTR-214 favors proliferation and infiltration of memory CD8 T cells over Tregs in the tumor

The in vitro binding and activation profiles described above suggest PEGylation interfered with the interaction between IL2 and IL2Rα relative to aldesleukin. We therefore tested whether these effects bias the profile of immune cell subtypes in vivo. The number of CD8 T and Treg cells in the tumor following administration of either NKTR-214 or IL2 is a key measure of whether pleiotropic effects of IL2 have been shifted due to conjugation of IL2 to PEG at the IL2/IL2Rα interface. To address the question, mice bearing subcutaneous B16F10 mouse melanoma tumors were treated with a single dose of NKTR-214 or 5 doses of aldesleukin, and immune cells in the tumor microenvironment were quantified by flow cytometry. In tumors of aldesleukin-treated mice, total and memory CD8 cells were increased as a percentage of tumor-infiltrating lymphocytes; however, these effects were transient, reaching significance relative to vehicle on day 5 (Fig. 4A). In contrast, significant (P < 0.05) and sustained total and memory CD8 T-cell stimulation was achieved following a single NKTR-214 administration, with superior percentages of memory CD8 (day 7) and total CD8 (days 7 and 10) relative to aldesleukin. Both NKTR-214 and aldesleukin treatment resulted in increased activated natural killer (NK) cells 5 and 7 days after treatment initiation, though this effect was diminished by day 10. CD4 cell percentages of tumor-infiltrating lymphocytes were diminished following NKTR-214 treatment relative to vehicle on day 5. On day 10, NKTR-214 resulted in fewer CD4 cell percentages compared with vehicle and aldesleukin. The CD4 cell population was further analyzed for the FoxP3+ subset, which defines the Treg population. NKTR-214 administration reduced percentage of Tregs at every time point, consistent with reduced access to the IL2Rα subunit arising from the PEG chains. In
contrast, Treg reduction with aldesleukin was modest achieving significance on day 5. The increase of CD8 T cells and reduction of Tregs led to a marked elevation of the CD8/Treg ratio in the tumor by day 7. The ratio of CD8/Treg for NKTR-214, aldesleukin, and vehicle was 449, 18, and 4, respectively.

While these results provide quantification of immune cells in the tumor microenvironment, the spatial relationship between T cells and tumor cells cannot be visualized. Therefore, immunohistochemical staining was performed (Fig. 4B), with representative images showing CD8 T cells are not only increased in number but are interspersed with tumor cells.

These results indicate NKTR-214 is capable of inducing more robust in vivo memory effector CD8 T-cell response, than seen with aldesleukin, without a commensurate stimulation of Tregs in tumor, consistent with in vitro IL2Rβ-biased binding profile of active IL2 conjugates derived from NKTR-214. These in vivo effects arise although 7.5-fold fewer IL2 equivalents were administered for NKTR-214 compared with aldesleukin.

NKTR-214 provides superior single-agent efficacy compared with aldesleukin in an aggressive mouse melanoma model

Given the markedly increased ratio of CD8 T cells/Tregs in the tumor microenvironment following NKTR-214 treatment, we compared the efficacy of NKTR-214 to aldesleukin in the B16F10 murine melanoma model. NKTR-214 and aldesleukin led to significant tumor growth inhibition at day 9 compared with vehicle, 80% and 91%, respectively (Fig. 5A). In animals who received vehicle, the tumors rapidly grew to the endpoint of 1,500 mm³ 8 days after treatment, leading to median tumor growth delay, defined as time to reach 4 times the initial volume or TVQT of 5 days. Aldesleukin significantly increased the TVQT to 10 days compared with vehicle. For animals treated with NKTR-214, the TVQT was increased 16.7 days providing significant tumor growth inhibition compared with aldesleukin, despite 10-fold fewer IL2 equivalents and reduced dosing frequency (Fig. 5B). Although no significant body weight loss was recorded, mice in the aldesleukin group became hypothermic and exhibited shivering behavior, unlike the NKTR-214–treated group where mild weakness was observed.

NKTR-214 is synergistic with an anti–CTLA-4 antibody

Checkpoint inhibitor antibody anti–CTLA-4 antagonizes the inhibitory interaction between CTLA-4 and its ligand B7 on antigen-presenting cells (20). To test the concept of T-cell activation using dual mechanisms of checkpoint blockade and direct cytokine stimulation, NKTR-214 and murine anti–CTLA-4 were evaluated in two syngeneic tumor models. No significant effect on tumor growth was seen when the EMT6 murine breast tumor model was treated with the EMT6 murine breast tumor model.
model was treated with single-agent NKTR-214, aldesleukin, or anti–CTLA-4, Fig. 6A. When aldesleukin was combined with anti–CTLA-4, tumor growth inhibition of 83% was achieved on day 18; however, the combination of NKTR-214 and CTLA-4 proved more efficacious leading to tumor regression of −3.8%. After treatment ended on day 21, both groups were followed for long-term tumor growth delay. At the end of study (day 100), the aldesleukin combination group maintained 40% tumor-free animals, with a mean TVQT of 32.5 days, which was significant compared with vehicle (TVQT 10.8 days). On the other hand, the TVQT of the NKTR-214 and anti–CTLA-4 group could not be determined as 70% of the animals were tumor-free at day 100; 78 days after the last dose of NKTR-214 was given. Although the CT26 murine colon tumor model also exhibits aggressive growth, single-agent NKTR-214 and anti–CTLA-4 each produced significant, if moderate, tumor growth inhibition of 51% and 60%, respectively, at day 11 compared with vehicle (Fig. 6B). Neither single agent led to significant mean TVQT changes, with 10% of animals remaining tumor free at day 100. However, the combination of these agents proved synergistic, with 89% tumor growth inhibition at day 11 and 67% tumor-free animals at day 100. For all treatment groups, the combinations were well tolerated with no body weight loss and no other obvious clinical signs. These data indicate synergy between NKTR-214 and checkpoint blockade, with each using a complementary mechanism of immune activation. Data demonstrating the antitumor immunity stimulated by this combination requires innate and adaptive immune responses are shown in Supplementary Fig. S3.

NKTR-214 combined with anti–CTLA-4 induces durable and specific antitumor immunity

The combination studies described above suggest durability of response; animals remain tumor-free for an extended period after their last therapeutic dose. To more fully explore the durability and specificity of antitumor activity induced by NKTR-214 combined with anti–CTLA-4 the EMT6 model, mice with complete responses to therapy (n = 30) were rechallenged with a second EMT6 tumor implant or nonrelated CT26 implant (n = 10) and monitored without additional therapy (Fig. 6C and D). Tumor-free animals proved resistant to EMT6 rechallenge with 70% of the mice remaining tumor-free, while CT26 tumor implants proceeded to grow. The tumor-free mice from the EMT6 tumor rechallenge were challenged a third time with EMT6, and followed without additional treatment. After 60 days, 100% of these animals remained tumor-free. The resistance to tumor growth strongly suggests antitumor immunity induced by this combination therapy is tumor-specific and long-lasting. Antibody depletion experiments (Supplementary Materials and Methods and Supplementary Fig. S3) demonstrated CD8 T and NK cells were required for protection from rechallenge.

NKTR-214 does not produce hypotension or vascular leak syndrome in non-human primates at the maximum tolerated dose

To proceed with clinical testing of NKTR-214 in humans, safety was evaluated in cynomolgus monkeys. To establish the cynomolgus monkey as a suitable toxicology species, human and monkey peripheral blood mononuclear cells from three donors each were exposed to human IL2 and active 1-PEG-IL2 from NKTR-214. The resulting IL2R activation measured by pSTAT5 signaling was similar between monkey and human primary T cells (Supplementary Table S2). The maximum tolerated dose (MTD) was 0.1 mg/kg. Soluble CD25 and total lymphocyte counts were used as pharmacodynamic markers of immune stimulation (21). Both biomarkers increased in a dose-responsive manner above baseline producing 24-fold and 4-fold increases in soluble CD25 and lymphocytes respectively at MTD (Supplementary Fig. S4A). As vascular leak syndrome is a known toxicity of HD-IL2 that is detected through hypotension, cardiovascular safety (ECG and blood pressure) was monitored in cynomolgus monkeys using telemetry. No hypotension or ECG abnormalities were observed at MTD for 104 hours after dose administration (Supplementary Fig. S4B). Lack of pulmonary edema was further confirmed by histopathology (data not shown) with no evidence of lung infiltrates. Studies of inflammatory cytokines TNFα and IFNγ showed no detectable levels, suggesting lack of toxicity-associated cytokine release, despite robust immune activation (22). Together with the murine antitumor efficacy, these results support the improved safety and tolerability of NKTR-214.

Discussion

This report describes the development of NKTR-214 for cancer therapy. To our knowledge, this is the first use of a prodruk design for biologic therapy using PEG, releasable PEG linkers, and site-directed PEG to alter the immunologic, pharmacokinetic, and biodistribution profile of the molecule (Figs. 3 and 4). The agent shows marked efficacy in aggressive murine tumors as a single agent and in combination with anti–CTLA-4 (Figs. 5 and 6). NKTR-214 is a highly *‘combinable cytokine’* dosed more like an antibody than a cytokine; anticipated dosing schedule in humans is once every 21 days. Yet NKTR-214 provides a mechanism of direct immune stimulation
The combination of NKTR-214 with murine anti–CTLA-4 results in tumor-free animals remaining tumor-free for up to 100 days and resistant to tumor rechallenge (Fig. 6). NKTR-214 is well tolerated in non-human primates, with no evidence of hypotension and vascular leak syndrome at its MTD (Supplementary Fig. S4B).

PEGylation technology has existed for decades leading to availability of multiple protein conjugates across diverse therapeutic areas including oncology (pegfilgrastim), infectious disease (peginterferon alpha-2b) and autoimmune disease (certolizumab pegol; ref. 23). In the past, the primary function of PEG was to improve half-life using standard stable conjugation chemistry (24–26). In this report, PEG is used to bias receptor selectivity and alter pharmacokinetics. No mutations were introduced into the molecule; therefore, the core of NKTR-214 is identical to the well-understood aldesleukin amino acid sequence. The presence of multiple PEG chains creates an inactive prodrug, which prevents rapid systemic immune activation upon administration. Use of releasable linkers allows PEG chains to slowly hydrolyze, continuously forming active conjugated IL2 bound by 2-PEGs or 1-PEG. PEGylation dramatically alters the pharmacokinetics of NKTR-214 compared with IL2, providing a 500-fold increase in AUC in the tumor compared with an IL2 equivalent dose. The location of PEG chains at the IL2/IL2Rα interface interferes with binding to high-affinity IL2Rα, while leaving binding to low-affinity IL2Rβ.
unperturbed (Supplementary Fig. S2). The bias observed in vitro changes the immune cell profile in vivo in the tumor microenvironment, leading to a maximal CD8/Tregs ratio of >400 for NKTR-214 compared with 18 for aldesleukin treatment (Fig. 4A). Overcoming immune tolerance in the tumor microenvironment may require tipping the balance in favor of CD8 T over Tregs in the tumor (10–13). Furthermore, CD8 T cells are extensively interspersed with tumor cells (Fig. 4B). Infiltration of CD8 T cells is relevant because previous immunotherapies such as tumor antigen–specific vaccines may have failed due to poor trafficking of these cells into the tumor (11). NK cell responses are also mobilized by NKTR-214 treatment; both innate and adaptive immune responses appear to play a role in antitumor immunity. Together, these results suggest NKTR-214 favors immune activation over suppression in the tumor, with a mechanism different from the original cytokine and complementary to checkpoint blockade antibodies.

Numerous approaches to improve the safety profile of aldesleukin have been reported; however, these were challenged by the absence of a defined mechanism for IL2 toxicity. One hypothesis implicated NK cells as the mechanism of toxicity; a mutein biased towards IL2Rα and against activation of NK cells was developed. However, this proved ineffective in the clinic with a lower MTD than aldesleukin (27, 28). Subsequently, other reports described modified forms of IL2 that have attempted to favor ILRβ binding, prolong exposure, or mitigate toxicity. These include mutation of the IL2 amino acid sequence, fusion to a targeting antibody, or use of IL2/anti-IL2 antibody complexes (29–31). These alterations, like every new agent, need to be evaluated clinically. Notably, NKTR-214 provides increased tumor exposure without assuming tumor target selectivity and without the complications of fusing an antibody to a cytokine. Unnatural protein fusion junctions pose greater risk for immunogenicity. Receptor bias shown with NKTR-214 is achieved using the clinically validated aldesleukin amino acid sequence and PEG, which is regarded as safe and nonimmunogenic.

To assess the impact of receptor bias, prodruk design, and high tumor concentrations on in vivo efficacy, we chose the subcutaneous B16F10 mouse melanoma model, a tumor known to be aggressive and difficult to treat (32). NKTR-214 showed significantly improved efficacy in this model as a single agent compared with aldesleukin. The improvement was achieved despite using 10-fold fewer IL2 equivalents. While 20 total doses of aldesleukin were needed to achieve modest efficacy, only 3 doses of NKTR-214 were administered (Fig. 5). Interestingly anti–CTLA-4 was not active in the B16F10 melanoma model as a single agent (32, 33). The combination of NKTR-214 with anti–CTLA-4 proved synergistic in mouse efficacy models of breast and colon tumors, with 83% and 67% of animals remaining tumor-free up to 100 days, respectively (Fig. 6A and B). Tumor-free animals could be challenged twice with the same tumors, and up to 100% of these animals resisted tumor regrowth without further therapy (Fig. 6C). Recent clinical and preclinical studies combining immunotherapies such anti–CTLA-4 and anti–PD-1 indicate more patients may benefit when multiple mechanisms of immune activation are targeted (34–37). Our results suggest NKTR-214 is positioned to provide a complementary mechanism of immune activation in combination, using dosing schedules concurrent with antibody therapy.

NKTR-214 is administered in vivo as an inactive prodruk, potentially mitigating toxicities associated with rapid off-target systemic immune activation, which would occur upon administration of HD-IL2 or other biologically active cytokines. Ability to dose fewer IL2 equivalents at a significantly reduced frequency may also contribute to the improved tolerability observed with NKTR-214. In cynomolgus monkeys, NKTR-214 could be dosed at MTD without cardiovascular adverse effects, reflected in the absence of hypotension and vascular leak syndrome. Total lymphocyte and soluble CD25 levels were used as pharmacodynamic markers and indicate clear dose-dependent immune stimulation in monkeys, with no evidence of cytokine release syndrome. These results suggest unlike HD-IL2, NKTR-214 may be dosed safely in humans, potentially increasing the number of patients achieving durable responses.
Although it is difficult to directly translate efficacy in mouse tumor models to human cancers, it is encouraging that the IL2 pathway is already a validated cancer immunotherapy target with a history of durable benefit for some patients. The efficacy of combination therapy using checkpoint blockade antibodies appears to translate between mice and humans, increasing the probability that NKTR-214 will produce robust efficacy in patients with cancer (34, 37). Although much of the current focus in cancer immunotherapy is on immune checkpoint blockade using antibodies, cytokines will play an important role in providing complementary mechanisms of action, as single agents and in combination. The data presented in this report indicate the immunologic, pharmacokinetic, and pharmacologic properties of the endogenous cytokine IL2 were significantly altered by strategic polymer conjugation. As such, NKTR-214 is currently being evaluated in a Phase 1 clinical study in solid tumors.

Figure 6.
Combination of NKTR-214 with checkpoint inhibition and tumor rechallenge. A. EMT6 breast model: aldesleukin or NKTR-214 in combination with anti-CTLA-4 generated significant tumor growth inhibition (left; *, day 18) compared with vehicle. A higher percentage of tumor-free animals was achieved when NKTR-214 was combined with anti-CTLA-4 (right; †, 70% day 100); n = 12 mice per group. B. CT-26 colon model: anti-CTLA-4 and NKTR-214 as single agents generated significant growth inhibition (*), but the combination of both agents was synergistic (left; †, day 11) and lead to significant percentage of tumor-free animals (right; ††, 67% day 100); n = 12 mice per group. Tumor rechallenge data: mean tumor volumes (C) and individual animal measurements (D). Combination therapy induced complete responses in 44% of treated animals. Tumor-free responders were resistant to first EMT6 rechallenge, with 70% remaining tumor-free, but were susceptible to CT26 tumor growth. Complete responders from the first rechallenge group were rechallenged a second time with EMT6 implants, and 100% remained tumor-free without additional treatment. Symbols represent mean ± SEM. *P < 0.05 compared with vehicle (ANOVA, Tukey post test); †P < 0.05 compared with aldesleukin (unpaired Student t test).
Disclosure of Potential Conflicts of Interest

D. Sheng, T.K. Chang, and J. Huang have ownership interest (including patents) in Nektar Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors thank Dr. Mario Szolov, Yale University, for his careful reading and critique of this manuscript. The authors also thank Pia Charaweyewang, Nektar Therapeutics, for his technical contributions and Phillips Gilmore, Oncology Communications, Inc. for technical support with manuscript preparation.

Grant Support

This study was financially supported by Nektar Therapeutics. 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 July 14, 2015; revised October 21, 2015; accepted October 23, 2015; published online February 1, 2016.

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