SUPPLEMENTAL APPENDIX

NKTR-255, a novel polymer-conjugated rhIL-15 with potent antitumor efficacy

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Miyazaki T, *et al. J Immunother Cancer* 2021; 9:e002024. doi: 10.1136/jitc-2020-002024

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SUPPLEMENTAL METHODS

Reagents

rhIL-15 was expressed in E. coli. A gene encoding the mature form of IL-15 (residues 49-162, UnitProt P40933) with an additional N-terminal methionine was designed using E. coli-optimized codons, rhIL-15/IL-15Rα; Human IL-15Rα sushi comprises the extracellular sushi domain of IL-15Rα plus a natural hinge domain. The IL-15Rα sushi protein was expressed and purified to homogeneity (2.0 mg rhIL-15 mixed with 3.7 mg IL-15R sushi; the molar ratio between rhIL-15 and human IL-15Rα sushi is 2.5). After complex formation, rhIL-15 N72D/IL-15Rα Fc was purified by two-step affinity and ion-exchange chromatography and ligated into two separate pcDNA-based plasmids (Thermo Fisher). ExpiCHO cells (Thermo Fisher) were transiently transfected with these plasmids encoding each of the two molecules, grown for a week, and the supernatant harvested and filtered. The rhIL-15 N72D:IL-15Rα Fc was purified through a Protein A Column (GE Healthcare). rhIL-15/mIL-15Rα Fc (rhIL-15 and mouse IL-15Rα/Fc fusion protein complex) was prepared by incubating rhIL-15 (Nektar Therapeutics) and mouse IL-15Rα Fc (R&D systems) in a 1:4.5 ratio at 37°C for 30 min.

Flow cytometry

In mouse flow cytometry studies, fluorescent antibodies against CD3, CD4, CD8, CD11b, CD27, CD45, CD107a, CD122, pSTAT5 and pAKT were from BD Biosciences (San Jose, CA); antibodies against CD25, CD44, CD62L, FoxP3 and Ki67 were from eBioscience (San Diego, CA); antibodies against NKp46 and GzmB were from BioLegend (San Diego, CA). In human flow cytometry studies, fluorescent antibodies

against CD3, CD4, CD8, CD56, CD69, CD107a, CD122 and CD132 were from BioLegend (San Diego, CA); antibodies against pSTAT5 and HLA-DR were from BD Biosciences (San Jose, CA); antibodies against pAKT and pERK were from Cell Signaling Technology (Danvers, MA); antibodies against IL-15 and biotinylated IL-15Rα and associated secondary antibodies were from R&D Systems (Minneapolis, MN).

IL-15 receptor binding by surface plasmon resonance

The surface of a Biacore CM5 sensor chip (GE Healthcare, Marlborough, MA) was activated using a 1:1 mixture of N-Hydroxysuccinimide 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide) (NHS: EDC) to generate active NHS ester. Goat anti-Human Fc antibody (Thermo Fisher, Waltham, MA) was covalently attached to the surface by injecting it for 3 minutes in 10 mM sodium acetate (pH 4) at 50 µg/mL which resulted in approximately 5000 Response Units (RU) of antibody being immobilized to the surface. Remaining NHS ester was quenched with 1 M ethanolamine. At the initiation of each injection cycle, IL-15-Rα-Fc or IL-2-Rβ-Fc (at 2 µg/mL each; Timaru, New Zealand) was captured onto the activated sensor chip channel by a 3-minute injection step in 1X running buffer (HBS-EP buffer with 0.1 mg/mL BSA). rhIL-15, NKTR-255 and rhIL-15/IL-15Rα were diluted in 1X running buffer, and the starting concentrations were 30 nM (rhIL-15, NKTR-255 and rhIL-15/IL-15Rα) for IL-15Rα and 100 nM (rhIL-15 and rhIL-15/IL-15Rα) or 300 nM (NKTR-255) for IL-2Rβ, from which a series of 3-fold dilutions were injected onto a sensor chip coated with IL-15Rα and IL-2-Rβ.

Daudi Burkitt lymphoma xenograft model bone marrow preparation

Femurs were collected and bone marrow was flushed with 5ml of cold PBS. Bone marrows from both femurs per animal were pooled and washed with 1ml PBS. After centrifugation bone marrow cells were resuspended in 2ml cold PBS. Resuspended bone marrow samples were gently agitated into single cell suspensions and diluted 1:10 for counting. 2 million live cells were plated and red blood cell lysis, subsequent wash and resuspension in PBS were carried out to prepare samples for antibody staining.

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SUPPLEMENTAL TABLES

Supplemental Table 1. Mouse IL-15Rα affinity of rhIL-15 and NKTR-255.

Mouse IL-15Rα was captured on a Biacore surface plasmon resonance sensor chip and relative affinities were calculated from kinetic measurements. Each assay was performed in duplicate. K_D, dissociation constant (affinity); k_{off}, mean dissociation rate constant; k_{on}, mean association rate.

	IL-15Rα		
	k_{on} ($M^{-1}sec^{-1}$)	k_{off} (sec^{-1})	K_D (pM)
rhIL-15	$3.2 \pm 0.31 \times 10^{7}$	$6.7 \pm 0.69 \times 10^{-5}$	2.1 ± 0.42
NKTR-255	$1.4 \pm 0.073 \times 10^{6}$	$7.0 \pm 0.73 \times 10^{-5}$	48 ± 2.4

Supplemental Table 2. Signaling profiles of rhIL-15, NKTR-255, rhIL-15/IL-15Rα and rhIL-15 N72D/IL-15Rα Fc

Relative activation was assessed by the percent positivity of pSTAT5, pAKT or pERK in each population. Mean EC_{50} values were obtained from experiments with human whole blood from six healthy donors. EC_{50}, half maximal effective concentration; NK, natural killer; ND, not determined; SD, standard deviation.

	EC_{50} nM (mean ± SD)								
	NK cells			$CD8^+$ T cells			$CD4^+$ T cells		
	pSTAT5	pAKT	pERK	pSTAT5	pAKT	pERK	pSTAT5	pAKT	pERK
rhIL-15	0.025 ± 0.011	1.3 ± 1.3	0.76 ± 0.65	0.021 ± 0.0063	ND	1.6 ± 1.5	0.023 ± 0.0070	ND	ND
NKTR-255	0.15 ± 0.055	7.7 ± 4.0	4.4 ± 3.3	0.083 ± 0.025	ND	6.5 ± 9.4	0.084 ± 0.032	ND	ND
rhIL-15/ IL-15Ra	0.0047 ± 0.0039	0.12 ± 0.17	0.051 ± 0.052	0.018 ± 0.013	ND	0.19 ± 0.11	0.036 ± 0.022	ND	ND
rhIL-15 N72D/IL-15Ra Fc	0.0052 ± 0.0020	0.11 ± 0.13	0.052 ± 0.025	0.027 ± 0.014	ND	0.23 ± 0.072	0.053 ± 0.024	ND	ND

SUPPLEMENTAL FIGURES

Supplemental Figure 1. Surface expression profile of IL-15 receptor subunits in human whole blood

Representative flow cytometry histogram plots of surface IL-15Rα, IL-2Rβ and IL-2Rγ expression in NK, $CD8^+$ T and $CD4^+$ T cells from three independent experiments. Solid line antibody staining; dotted line: FMO control. FMO, fluorescence-minus-one; NK, natural killer.

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Supplemental Figure 2. rhIL-15 and NKTR-255 have similar leukocyte proliferation and activation properties in vitro compared with precomplexed rhIL-15/IL-15Rα cytokines

(A) Dose-response curves representing proliferation of NK, $CD8^+$ T and $CD4^+$ T cells and **(B)** CD69 surface expression in human PBMCs (n=3) stimulated in vitro overnight with rhIL-15, NKTR-255, rhIL-15/IL-15Rα or rhIL-15 N72D/IL-15Rα Fc. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 (Dunnett's multiple comparisons test versus vehicle). CFSE, carboxyfluorescein diacetate succinimidyl ester; NK, natural killer; PBMCs, peripheral blood mononuclear cells; SEM, standard error of the mean.

Supplemental Figure 3. NKTR-255 preferentially induces proliferation of all NK cell subpopulations and CD8⁺ T cell memory subpopulations

(A) Total NK cells and NK cell subpopulations, **(B)** total CD8⁺ T cells and CD8⁺ T subpopulations, and **(C)** total CD4⁺ T and CD4⁺ regulatory T cells after a single i.v. dose of vehicle control or NKTR-255 (0.01, 0.03, 0.1, or 0.3 mg/kg) in mice. NK, natural killer; SD, standard deviation; Tcm, central memory T cell; Tem, effector memory T cell; Treg, CD4⁺ regulatory T cell.

Supplemental Figure 4. NKTR-255 has superior antitumor activity compared with precomplexed rhIL-15 N72D/IL-15Rα Fc in a Daudi lymphoma model

Survival plot of Daudi Burkitt lymphoma xenograft model after the administration of a single dose of NKTR-255 (0.3 mg/kg, i.v.) or rhIL-15 N72D/IL-15Ra Fc (0.3 mg/kg, i.v.) or vehicle,on Day 4 after tumor inoculation. *p<0.05, log-rank test.

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