ALKS 4230, a novel engineered IL-2 fusion protein with an improved cellular selectivity profile for cancer immunotherapy

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Supplemental Methods

Crystallization and structural determination. Crystals were grown at room temperature by hanging drop vapor diffusion. An equal volume of protein was mixed with crystallization buffer, 25 % (w/v) PEG 3350, 0.2 M MgCl2, 0.1 M Hepes 7.5. Large crystals appeared within 48-72 hours and were frozen in a cryoprotectant consisting of mother liquor with 10 % glycerol. Out of sixteen crystals tested, only one diffracted X-rays to better than 4.0 Å. To assess the effect of the cryosolvent on crystal order, several capillary-mounted crystals were also tested for diffraction quality, but none diffracted X-rays appreciably. A complete dataset to 3.4 Å resolution was collected at 100 K at Advanced Light Source beamline 4.2.2, on an RDM 8M CMOS detector in shutterless mode.

Data were integrated and scaled in XDS, indexing in space group $P3_221$, a=b= 86.8 Å, c= 119.6 Å. The structure was solved using molecular replacement and confirmed using several available models of the IL-2 / receptor complex, as well as structures of IL-2 alone. There was strong F_o-F_c difference density corresponding to sections which were designed in ALKS 4230 to be ***contiguous*** in primary sequence, which in the IL-2 / receptor complex are ***noncontiguous*** but are close in three-dimensional space, indicative of the correct solution.

Refinement was performed in Phenix. Due to the moderate resolution of the data, a conservative refinement protocol was employed to minimize model bias, with grouped

B-factor, rigid body, and positional refinement using tightly restrained stereochemical parameters. Translation-libration-screw tensors were applied in later stages of refinement, with noticeable improvement in R_{free} and R_{cryst}. No solvent molecules were added due to the resolution of the structure. Structural analyses were performed using MOE™ and PyMOL (v 1.7.6).

***In vitro* immune cell stimulation.** Mouse splenocytes were isolated following a standard red blood cell (RBC) lysis procedure. The final cell sample was counted and resuspended in RPMI media. These cells were serum starved for 60 minutes, washed twice and resuspended in HBSS, and plated in the presence of decreasing concentrations of ALKS 4230, rhIL-2, or unstimulated, for approximately 30 minutes in a 37°C, 5% CO_2 environment. Leukocytes obtained from fresh human blood after standard RBC lysis were counted, resuspended in X-VIVO 10 media, plated, and incubated in the presence of decreasing concentrations of ALKS 4230, rhIL-2 or unstimulated, for approximately 30 minutes in a 37°C, 5% CO2 environment. For both mouse and human cell assays, following stimulation, cells were fixed, washed, and Fc-blocked. After surface marker staining, cells were washed and permeabilized for subsequent intracellular staining steps before analysis by flow cytometry.

To characterize the activity of ALKS 4230 and rhIL-2, two human cell lines with differential expression of IL-2Rα/CD25 were used: the HH cell line, an IL-2-responsive human $CD4^+$ T cell line derived from a human patient with cutaneous T cell lymphoma (ATCC® CRL-2105™). HH cells lack expression of IL-2Rα, but still express a functional intermediate-affinity IL-2 receptor complex; the NK-92 cell line is IL-2-dependent, originally isolated from a patient with non-Hodgkins

lymphoma (ATCC® CRL-2407™). Cells were treated with ALKS 4230 or rhIL-2 and intracellular pSTAT5 levels were quantified after directly fixing, permeabilizing, and staining intact cells with a fluorochrome-conjugated antibody specific for pSTAT5.

Isolation, expansion, and stimulation of human PBMCs. Peripheral whole blood samples were previously collected from human donors by apheresis and then separated into lymphocyte and monocyte fractions either by cold agglutination, elutriation, or by positive selection (CliniMACs with CD14 beads) and then preserved by cryogenesis. Lymphocytes and monocytes for each donor were thawed rapidly, washed, and counted by trypan-blue exclusion. Cells were resuspended in complete media and mixed at a 4:1 lymphocyte-to-monocyte ratio so that all samples had similar cell proportions which were representative of the ratio normally found in peripheral blood mononuclear cell (PBMC) fractions. These mixed samples are referred to as PBMCs in the text.

On Day 0, 1.5 x 10^7 PBMCs were put in culture at 7x10^5 cells/ml in complete media plus additional 3% human AB serum, 0.2 µg/ml α-CD3 antibody, and either 0.5 nM ALKS 4230 or 0.5 nM rhIL-2, and incubated at 37˚C in the presence of 5% CO_2. On Day 5, cells were harvested, washed, and resuspended at 7x10^5 cells/ml in fresh media with all components except for exclusion of α-CD3 antibody. On Days 7 and 9, cell cultures were harvested, and the cells were washed and reseeded in fresh media with fresh components as on Day 5. On Day 12, cell cultures with varying treatments were harvested, washed and cells were stained as described below.

Cells were washed, and labeled with live-dead viability reagent, then washed and incubated with a Fc receptor-blocking reagent and stained with fluorescently-conjugated

antibody panels specific for the markers outlined in Supplementary Table S3. Cells were then washed and fixed in 0.1% formalin and stored at 4°C until acquisition. Flow cytometric acquisition was performed on a MACSQuant-10 and cell populations, were identified and quantified using FlowJo software (v.X.0.7).