

# Increased Affinity of Interleukin-2 Ligand to the Interleukin-2 $\alpha$ -Receptor Leads to Increased Ligand Persistence and Cell Growth

*Ian Driver*

*Department of Chemical Engineering; Project Advisor: Dr. Douglas A. Lauffenberger*

## Abstract

The therapeutic use of the interleukin-2 (IL-2) to stimulate T-cell proliferation has been exploited for the treatment of metastatic renal cell carcinoma and melanoma.<sup>1,2</sup> Current IL-2 based therapies are difficult due to the rapid clearance of intravenously administered IL-2 from the bloodstream. We have created mutant forms of IL-2 that demonstrate increased affinity for the IL-2 alpha-receptor subunit (IL-2R $\alpha$ ). Using a pulsed bioassay designed to closely approximate the clearance of IL-2 from the bloodstream, we show that the improved affinity of IL-2 for IL-2R $\alpha$  results in increased persistence of IL-2 on the cell surface and higher proliferation in a human T-cell line (KIT-225). We also compare our improved IL-2 mutants to IL-15, which has a high affinity interaction with its unique alpha-receptor subunit (IL-15R $\alpha$ ). Our best IL-2 mutant, 2-4, shows similar growth and binding to cells pulsed with the same concentration of IL-15. The mutant 2-4 we created represents a version of IL-2 with a nearly 100-fold increase in its affinity for IL-2R $\alpha$ , represented by an increase in Kd from approximately 30nM to 180 pM. This increase is comparable to the affinity of IL-15 (Kd 60-95 pM) for IL-15R $\alpha$ . The cell surface persistence of surface-bound cytokine (IL-2 or IL-15) was found to directly correlate with cell growth. Our findings demonstrate the power of protein engineering techniques to create therapeutically valuable proteins with tailored biophysical properties. These proteins also aid in the understanding of the biology of the system, as demonstrated by the comparisons between high affinity IL-2 mutants and IL-15.

## Introduction

The human immune system and inflammatory response are regulated by a large group of proteins called cytokines. Cytokines control growth of hematopoietic cells by binding to surface receptors. Most hematopoietic growth receptors bind their ligands with a high affinity, and consequently, optimal stimulation occurs at low concentrations. Despite being optimal for stimulation of growth, however, low concentrations of cytokines are rapidly cleared from the blood stream and degraded; therefore, the therapeutic use of cytokines is limited.

Interleukin-2 (IL-2) is the primary cytokine responsible for proliferative stimulation of activated T-cells. The therapeutic use of IL-2 has been demonstrated in the treatment of metastatic renal carcinoma and melanoma.<sup>1,2</sup> The main problem with current IL-2 therapies is the narrow therapeutic window. Low concentrations (1 pM or below) of IL-2 have little or no effect on T-cell stimulation and concentrations above 100 pM lead to stimulation of NK cells and undesired inflammatory activity.<sup>3,4</sup> The narrow range of concentrations over which useful T-cell stimulation can be accomplished is difficult to maintain due to the rapid systemic clearance of IL-2 from the bloodstream by the kidneys.

The activity of IL-2 in T-cells is mediated by the IL-2 receptor, a complex composed of three subunits: p55 (IL-2 $\alpha$ ), p75 (IL-2 $\beta$ ), and p64 (IL-2 $\gamma$ ). The IL-2 $\alpha$  subunit is unique to IL-2 where the IL-2 $\beta$  and IL-2 $\gamma$  subunits are expressed by NK cells.<sup>5</sup> It has been theorized that improving IL-2 affinity for the IL-2 $\alpha$  subunit could increase T-cell stimulation while decreasing NK activity.<sup>6</sup> The IL-2  $\beta$  and  $\gamma$  receptor subunits along with a different unique  $\alpha$ , are used by the IL-15 ligand. The IL-15 ligand has about a hundred-fold higher affinity towards its alpha-receptor than IL-2 towards its  $\alpha$ -receptor. The IL-15 system and its high affinity interaction serve as a comparative model for the increased affinity of IL-2 mutants.

Two rounds of IL-2 mutants were created by yeast surface display-directed evolution as previously described.<sup>6</sup> In both cases, high improvements in affinity (20-100 fold) resulted in increased persistence in the surface of T-cells and 2-6 fold increase in proliferation as compared to wild-type (WT) IL-2. In order to mimic actual IL-2 injection, T-cells were pulsed with various concentrations of mutant or WT IL-2 for 30 minutes and then washed with IL-2-free media. It appears that increased affinity of IL-2 to the IL-2 $\alpha$  subunit results in persistent signaling of T-cells and increased growth. The increased affinity of IL-2 towards its receptor creates a lasting local reservoir of IL-2 ligand. Instead of falling off the cell surface and being rapidly cleared, it remains tightly bound, effectively acting as a reservoir of IL-2.

## Materials and Methods

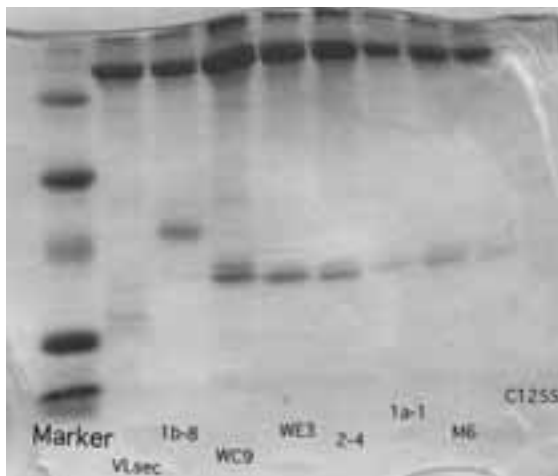
### Yeast surface display of IL-2 and library construction

The IL-2 mutants were constructed by Balaji Rao, with the same procedure that was used to create the previous generation mutant M6.<sup>6</sup>

### Purification of IL-2 variants

The mutant IL-2 proteins were secreted in yeast shake-flask<sup>6</sup> cultures. The supernatant was concentrated and purified using a FLAG immunoaffinity column (Sigma). The protein content of each elution was measured on a Cary 50 Bio UV-visible spectrophotometer (Varian). The four elutions containing the highest protein content were pooled. The pooled fractions were then run on a 15% acrylamide gel and stained with Coomassie Blue (0.25 g Coomassie Blue R-250, 100 mL ethanol, 100 mL water) overnight (Figure 1). In order to further purify the IL-2 proteins, they were separated by size exclusion chromatography on a Sephadex column on a Fast Performance Liquid Chromatography (FPLC) system (Pharmacia Biotech, UK). The FPLC system was set to a sensitivity of 0.0005, and 500 $\mu$ L of each protein was loaded into the loop. The eluent was collected in 1mL fractions. The fractions containing the peak protein, at the correct molecular weight, were pooled (Figure 2). The pooled fractions were loaded on a 15% acrylamide gel,

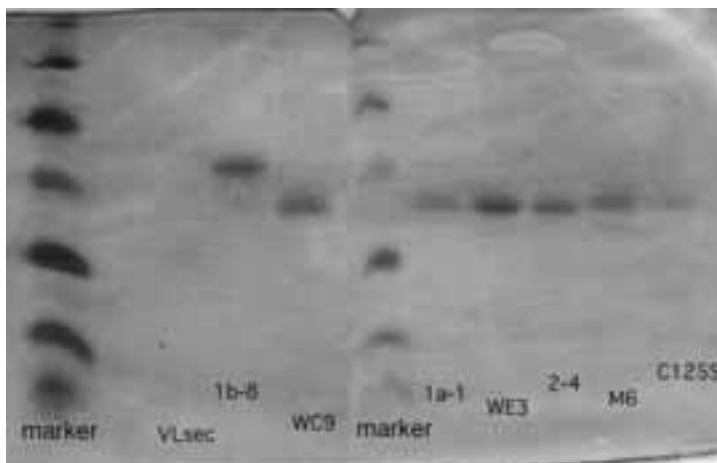
and protein purity was verified (Figure 3). The pooled fractions were then filtered using 0.22  $\mu$ m centrifuge tube filters (Costar). Protein concentration was measured using the Micro BCA Protein Assay (Pierce, IL), according to the manufacturer's protocol.



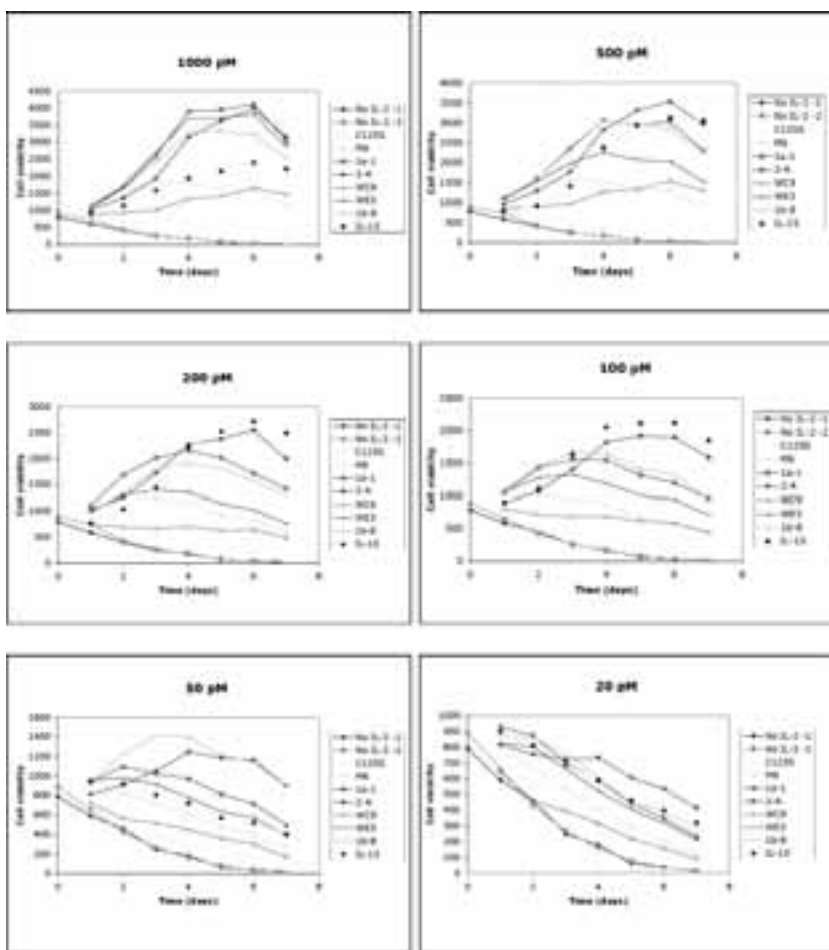
**Figure 1. Protein Fractions Before FPLC.** The IL-2 proteins appear at about 16.5 kb. 4 $\mu$ l of the protein was loaded at a 1:5 dilution. VLsec is a control consisting of the variable light chain fragment of the Huntington's protein.



**Figure 2. FPLC Traces.** For all proteins 500  $\mu$ L was loaded, and the FPLC system was run at a sensitivity of .0005. The trace in A is the standard curve. The trace in B is for mutant 2-4. The final peak in B is the peak containing IL-2 protein. All of the traces for the IL-2 mutants displayed similar curves to that of 2-4.



**Figure 3. Protein Fractions After FPLC.** The IL-2 proteins appear at about 16.5 kb. 4 $\mu$ l of the protein was loaded at a 1:5 dilution. Extraneous proteins no longer appear.



**Figure 4. KIT-225 IL-15R Proliferation.** Cell proliferation was measured over a range of concentrations (1000pM-20pM) for cells incubated in IL-2 (C125S, M6, 1a-1, 2-4, WC9, WE3, and 1b-8), IL-15 or no IL-2 media. Proliferation was measured using the Cell-Titer Glo™ Assay (Promega).

#### KIT-225 cell proliferation assay

KIT-225 is an IL-2 dependent human T-cell line that expresses about 3000-7000 IL-2 $\alpha\beta\gamma$  and 200,000-300,000 IL-2R $\alpha$ .<sup>7,8</sup> The KIT-225 cell line expressing IL-15 R $\alpha$  on its surface (KIT-225 IL-15R) was a gift from Sigrid Dubois from the NIH Center for Cancer Research.<sup>9</sup> The KIT-225 IL-15R cells were cultured in RPMI 1640 (Gibco) supplemented with 40 pM IL-2 (C125A), 10% FBS (Gibco), 200 mM L-glutamine (BD Bioscience), and 1mM G418 (Gibco).

KIT-225 IL-15R cells were cultured in media containing no IL-2 for one to three days. The starved cells at a concentration of 100,000 cells/ml, as measured on a Coulter Counter (Beckman Coulter), were split into nine 3mL cultures. Cells were incubated in 100pM IL-2 of each variant (C125S, M6, 1a-1, 2-4, WC9, WE3, 1b-8) and controls (IL-15 (R&D Systems) and no IL-2). After 30 minutes, the cell cultures were centrifuged (1000 rpm IEC HN-SII Centrifuge), washed in clean media, centrifuged again, and re-suspended in clean media.

Cell proliferation was measured using Celltiter-Glo™ Assay solution (Promega, WI), according to the manufacturer's protocol.

#### IL-2 Surface Persistence Assay

The KIT-225 IL-15 cells from the proliferation assay were also used in the persistence assay. After the cells were incubated with IL-2 and re-suspended in clean media 100,000 cells from each group were labeled with primary and secondary antibodies. The primary antibody for all of the IL-2 pulsed cells was 1:200  $\alpha$ -cmv chicken polyclonal antibody (Molecular Probes) in PBS + 0.1% BSA for 30 minutes and the secondary was 1:100 Goat-anti-Chicken conjugated to Alexafluor (Molecular Probes) in PBS + 0.1% BSA for 10 minutes. The cells incubated in IL-15 were labeled with 1:100 biotinylated  $\alpha$ -human IL-15 antibody (R&D Systems) in PBS + 0.1% BSA and then in 1:100 Streptavidin-PE (Molecular Probes) in PBS + 0.1% BSA. All cells were washed in 1 mL PBS + 0.1% BSA and centrifuged. Cells were then re-suspended in 400  $\mu$ L PBS + 0.1% BSA and analyzed in a Coulter EPICS XL Flow Cytometer (Beckmann Coulter).

## Results

#### Purification of IL-2 Variants

After the secreted proteins were initially separated on a FLAG immunoaffinity column, protein containing fractions were pooled and run on a 15% acrylamide gel and stained with Coomassie Blue (Figure 1).

The IL-2 variants were observed at the correct molecular weight but a significant amount of other protein was also observed. In order to isolate the IL-2 protein from extraneous proteins, the pooled fractions were loaded into a FPLC system. The traces from the FPLC (Figure 2) were used to pool fractions containing the IL-2 protein.

The peak corresponding to the IL-2 protein was verified by running the fractions on a 15% acrylamide gel and checking the molecular weight (Figure 3). Figure 3 shows a solid band around 16.5 kb, where the protein should be, and almost no other protein. The concentrations of the various proteins, as measured by the BCA assay were repeated in triplicate. The concentrations and standard deviations are reported in Table 1.

**Table 1. Protein Concentrations.** Using the BCA assay (Pierce), protein concentration was measured. From the gel in Figure 3, it can be assumed that the protein concentration is nearly the IL-2 concentration.

Protein	Concentrations ( $\mu$ g/mL)	Std Dev ( $\mu$ g/mL)
C125S	107	10
M6	159	15
1a-1	98	10
2-4.	147	13
WC9	238	9
WE3	273	16
1b-8	301	20
Vlsec	89	16

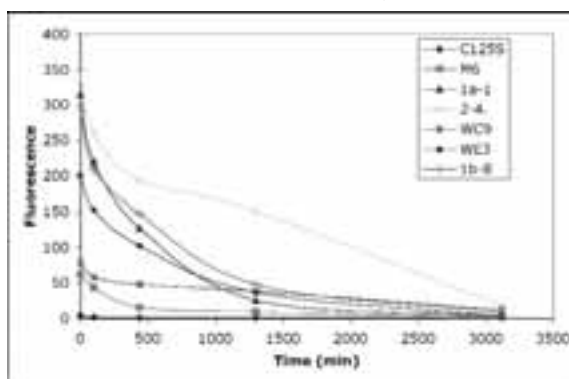
#### KIT-225 Cell Proliferation

Cell proliferation was measured every day for up to ten days using the Cell-Titer Glo™ Assay. The time zero cell-viability was measured in two different samples without IL-2 cells immediately after the incubation

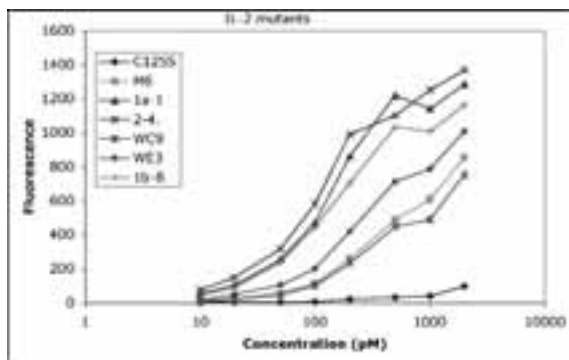
period. Since all of the cells were taken from the same population of cells, it can be assumed that the no IL-2 time zero point is a representative starting point for all of the cell populations.

The pulse of IL-2 roughly models renal clearance. When IL-2 is administered *in vivo*, it is rapidly cleared from the body, with half-lives of 12.9 and 85 minutes.<sup>10</sup> By exposing the cell populations to IL-2 for 30 minutes and then washing them, we approximate *in vitro* systemic clearance of IL-2. When the viability of the pulsed cells over time was measured, cells pulsed with mutants 2-4 and 1a-1 showed significantly higher viability than cells exposed to wild-type IL-2 (C125S). Mutant 2-4 over the range of concentrations showed similar viability to that of IL-15 exposed cells (Figure 4).

### IL-2 Surface Persistence and Binding



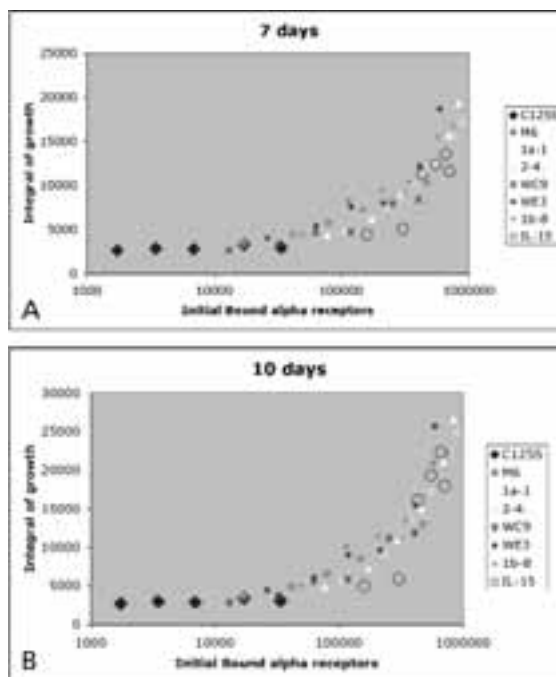
**Figure 5. IL-2 Surface Persistence.** IL-15R cells were exposed to 100pM of respective proteins for 30 minutes, then washed. The zero time point was taken immediately after the wash step.



**Figure 6. Time Zero Surface Binding.** KIT-225 IL15R cells were incubated with respective proteins at varying concentrations (10-200pM) and the initial surface binding was measured.

**Table 2. Approximate Kd values extrapolated from binding curves.** The data from time zero binding was fit to a curve for each mutant. By assuming that the top of each curve represented receptor saturation, Kd values could be extrapolated.

	Kd (pM)
C125S	30030
M6	1215
1a-1	254
2-4	180
WC9	1585
WE3	778
1b-8	409
IL-15	76



**Figure 7. Initial alpha-receptors bound vs. total growth.** The correlation between initial number of receptors bound and the integral of growth is nearly perfectly exponential. The one significant outlier is IL-15, which is not entirely unreasonable given that it is an entirely different protein than the IL-2 mutants. Each point for each mutant represents one concentration. Total growth was calculated using the either A) 7 day or B) 10 day growth curve.

### Discussion

We have demonstrated a strong correlation between the persistence of IL-2 ligand on the surface and the proliferative response of T-cells. Using a cell line that contains both IL-2 $\alpha$  and IL-15 $\alpha$  in addition to the shared IL-2R $\beta$  and IL-2R $\gamma$  on the surface, we have proven that our engineered IL-2 proteins have remarkably similar binding and growth characteristics to IL-15. Compared to wild-type IL-2, wild-type IL-15 shows increased affinity toward its private receptor and prolonged signaling.<sup>9</sup> We have created an IL-2 protein that maintains its specificity while increasing signaling.<sup>6</sup>

An IL-2 variant capable of specific stimulation of T-cells that also has the binding characteristics of IL-15 is therapeutically advantageous. Current IL-2 therapies have a narrow therapeutic window due to the weak binding and rapid clearance of the ligand from the blood stream. Our best IL-2 variants offer the ability to stimulate T-cells specifically with persistent signaling, such that the therapeutic window can be greatly increased.


Figure 6 demonstrates the direct correlation between persistence of ligand on the cell surface and cell growth. For both curves of the initial persistence with respect to total growth, all of the mutants and IL-15 fit the same exponential curve. Our directed evolution techniques can create a range of mutants with different affinities, and also affinity can be correlated with growth.

Our work indicates that it is possible to increase specificity, while creating a cytokine with a wide range

of binding and signaling properties. *In vivo* assays need to be performed to evaluate the benefit of high affinity cytokines. Proleukin® (Chiron Corporation) is the only FDA approved IL-2 therapy. Proleukin® therapy uses essentially wild-type IL-2 comparable to C125S. Even if former research finds that it is not desirable to induce very high levels of T-cell growth, current therapies could be carried out with lower doses and possibly lower side

effects using higher affinity mutants of IL-2. Our work indicates that it is possible to manipulate the binding properties of cytokines over a wide range, thus offering many potential new therapeutic advances.

### Acknowledgements

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