Interleukin-2 Engineering for Improved Therapeutic Effectiveness

by

Balaji Madhav Rao

B. Chem. Engg., UICT (formerly UDCT), Mumbai, India, 1999
MSCEP, Massachusetts Institute of Technology, 2001

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Signature of Author _____
Department of Chemical Engineering
September 2004

Certified by _____
K. Dane Wittrup
J. R. Mares Professor of Chemical Engineering and Bioengineering
Thesis Supervisor

Certified by _____
Douglas A. Lauffenburger
Professor of Bioengineering and Chemical Engineering
Thesis Supervisor

Accepted by _____
Daniel Blankschtein
Professor of Chemical Engineering
Chairman, Committee for Graduate Students

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ABSTRACT

Interleukin-2 (IL-2) is an immunomodulatory cytokine that is clinically relevant for the treatment of metastatic renal cell carcinoma and melanoma. The primary objective of the research presented in this thesis was to generate IL-2 mutants with potentially improved therapeutic effectiveness. Based on qualitative considerations and simple mathematical modeling, we hypothesized that IL-2 mutants with increased affinity for the alpha subunit of the IL-2 receptor (IL-2Rα) would have increased potency for proliferation of activated T cells and hence potentially improved therapeutic value. Yeast surface display and directed evolution were used to generate a class of IL-2 mutants with enhanced IL-2Rα affinity. In a novel pulsed bioassay designed to approximate the rapid systemic clearance pharmacokinetics of IL-2, these mutants exhibit significantly increased potency for T cell proliferation, thus validating our hypothesis. Our results underscore the critical nature of the choice of appropriate bioassays to evaluate engineered proteins and other drugs. Conventional bioassays not only fail to reveal the increased potency resulting from enhanced IL-2Rα affinity (false negatives), but also suggest improved potency for a mutant without enhanced activity in the pulsed bioassay (false positive).

Cell-surface IL-2Rα acts as a ligand reservoir for the IL-2 mutants, leading to increased cell-surface persistence of the IL-2 mutants with increased IL-2Rα affinity and consequently increased integrated growth signal. This is analogous to the prolonged persistence of IL-15 on cell surface IL-15Rα reservoirs. IL-2 and IL-15 signal through the IL-2Rβ and IL-2Rγ subunits while each have a private non-signaling alpha receptor subunit. IL-15 has a high affinity (K_d ~ 10pM) for its private alpha receptor subunit, unlike wild-type IL-2 (K_d ~ 10 nM). IL-2 mutants with picomolar affinity for IL-2Rα stimulate T cell growth responses quantitatively equivalent to those mediated by IL-15. Our results suggest that the contrasting effects of IL-2 and IL-15 on T cells *in vivo* are largely due to the 1,000-fold different affinities of wild-type IL-2 and IL-15 for their respective private alpha receptor subunits.

Thesis Supervisor: K. Dane Wittrup
Title: J. R. Mares Professor of Chemical Engineering and Bioengineering

Thesis Supervisor: Douglas A. Lauffenburger
Title: Professor of Bioengineering and Chemical Engineering

To my parents

with love and humility

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Chapter 1: INTRODUCTION AND BACKGROUND

Cytokines are low molecular weight proteins that regulate the complex interactions between cells in the immune systems. Cytokines bind to receptors on the cell surface of target cells and mediate diverse biological responses such as proliferation, differentiation, cell motility or death. Interleukin-2 (IL-2), originally identified as a T cell growth factor (Gillis et al., 1978), is one of the most widely studied cytokines.

IL-2 is a variably glycosylated 15-18 kDa protein consisting of 133 amino acids that exerts a variety of immunoregulatory effects via specific cell surface receptors on different cell types (Probst et al., 1995). These include activation and expansion of lymphocyte subsets, including T helper cells, cytotoxic and suppressor cells, B cells, natural killer (NK) cells, as well as cytotoxic macrophages. Specifically, the proliferation of activated T cells and CD56 bright NK cells caused by IL-2 (Fehniger et al., 2002) has been exploited in the treatment of metastatic renal cell carcinoma and melanoma (Atkins et al., 1999; Fyfe et al., 1995). The goal of the research presented in this thesis is to generate IL-2 mutants with potentially improved therapeutic effectiveness.

1.1 The IL-2 Receptor

The biological activity of IL-2 is mediated through a multi-subunit IL-2 receptor complex (IL-2R), previously reviewed in (Nelson and Willerford, 1998). The IL-2 receptor is composed of three cell-surface subunits: p55 (IL-2Rα), p75 (IL-2Rβ) and p64 (IL-2Rγ), which span the cell membrane. IL-2Rα (also known as Tac antigen or CD125) is a ~ 55kDa protein with a 219 amino acid extracellular domain, a transmembrane domain of 19 amino acid residues and a short cytoplasmic domain with only thirteen residues.

IL-2Rα is highly homologous to the alpha chain of the IL-15 receptor. IL-2Rβ (also known as CD122) is a ~75 kDa protein with an extracellular domain having 214 residues, a 25 residue transmembrane domain and a large cytoplasmic domain of 286 residues. IL-2Rβ exhibits homology with several other receptors of the hematopoetic receptor superfamily. The β chain of the IL-2 receptor is shared with IL-15. IL-2Rγ (CD132) is a ~64 kDa protein having an extracellular domain with 232 residues, a transmembrane domain with 29 residues and 86 residues in the cytoplasmic domain. IL-2Rγ is also called the γ_c receptor (γ-common) as it is shared with IL-4, IL-7, IL-9 and IL-15.

The expression of the IL-2 receptor subunits on different cell types has been previously reviewed (Nelson and Willerford, 1998). It must be noted that conflicting data exists in the literature concerning the expression the three IL-2R subunits on peripheral blood mononuclear cells. This is possibly due to the sensitivity of receptor expression to the conditions of the blood sample analyzed (David et al., 1998). We restrict our discussion here to cell types that mediate the beneficial and deleterious effects in IL-2 based therapy. The expression of the α subunit is upregulated by T cells upon antigen activation, and is found in ~ 100-fold excess over IL-2Rβ and IL-2Rγ on the surface of activated T cell populations (Lowenthal and Greene, 1987). IL-2Rα is also expressed on a subset of NK cells that have high levels of expression of the CD56 antigen (CD56 bright NK cells). Both CD56 bright and CD56 dim (with low levels of CD56 expression) NK cells express IL-2Rβ and IL-2Rγ (Fehniger et al., 2002).

1.2 IL-2 interactions with receptor subunits

IL-2 binds the IL-2Rαβγ trimeric complex with high affinity (K_d ~ 10 pM) (Nelson and Willerford, 1998). IL-2 interacts directly with each subunit in the high affinity (αβγ) complex. IL-2 binds IL-2Rα with low affinity (K_d ~ 10 nM) and IL-2Rβ with very low affinity (K_d ~ 100 nM). IL-2Rγ, on the other hand, binds IL-2 with barely measurable binding affinity. Surface plasmon resonance studies with the soluble extracellular domain of IL-2Rγ indicate the K_d of this interaction is in the micromolar range (Liparoto et al., 2002). IL-2Rα and IL-2Rβ, when co-expressed, form a pseudo-high affinity complex capable of binding IL-2, (K_d ~ 30 pM). IL-2Rβ and IL-2Rγ can associate in a ligand-dependent fashion to form the IL-2Rβγ complex that binds IL-2 with intermediate affinity (K_d ~ 1 nM). Thus, the binding of IL-2 to its receptor subunits is modular and cooperative. Fluorescence resonance energy transfer (FRET) studies on a T cell line indicate that the IL-2 receptor subunits are co-localized on the cell surface (Damjanovich et al., 1997). The most likely mechanism for the binding of IL-2 to its receptor subunits involves the initial binding of IL-2 to pre-associated IL-2Rα and IL-2Rβ and subsequent recruitment of IL-2Rγ to the complex (Nelson and Willerford, 1998).

1.3 IL-2 mediated signaling

IL-2 mediated signal transduction through the IL-2 receptor complex has been previously reviewed (Gaffen, 2001; Nelson and Willerford, 1998). IL-2Rβ and IL-2Rγ associate in a ligand-dependent fashion and form the signaling complex (Nakamura et al., 1994). IL-2Rα displays no ability to transduce intracellular signals. This is most likely due to the

short cytoplasmic region of IL-2Rα. However, IL-2Rα on one cell can present IL-2 to IL-2Rβγ on an adjacent cell and enhance IL-2 signaling (Eicher and Waldmann, 1998).

Heterodimerization of IL-2Rβ and IL-2Rγ activates the Jak3 tyrosine kinase associated with IL-2Rγ. Jak3 is responsible for the initial phosphorylation of IL-2Rβ, initiating the recruitment of several signal-transducing molecules to the cytoplasmic tail of IL-2Rβ, such as Jak1, STAT5 and STAT3, the Shc-adaptor protein, Syk and $p56^{lck}$. These events result in the activation of the Jak/STAT, phosphatidylinositol 3-kinase and *ras/raf*/mitogen-activated protein kinase pathways (Yu et al., 2000). IL-2 signaling is linked to the increased expression of the proto-oncogenes *c-fos/c-jun* and *c-myc* and the anti-apoptotic gene *bcl-2*. IL-2 is also implicated in Activation Induced Cell Death of activated T cells through the Fas-FasL apoptotic pathway (Gaffen, 2001; Nelson and Willerford, 1998). The IL-2Rγ signals are essential for cell survival. This is because IL-2Rγ is crucial for the Jak3 phosphorylation of IL-2Rβ and hence the subsequent events that follow this. Decreased IL-2Rγ due to receptor downregulation leads to lowered expression of the *bcl-2* gene. This drives the T cells to apoptosis *in vivo* (Li et al., 2001).

1.4 IL-2R mediated trafficking

T-cell lines degrade exogenous IL-2 with a half-life ($t_{1/2}$) of 60-80 minutes, following addition of saturating amounts of ligand (Fujii et al., 1986). This is due to receptor-mediated internalization and degradation of IL-2. IL-2Rα alone cannot induce internalization, but IL-2/IL-2Rβγ and the IL-2/IL-2Rαβγ complexes are endocytosed with a $t_{1/2}$ of 10-15 minutes (Chang et al., 1996; Gullberg, 1987). The internalization of

the IL-2/IL-2R complex occurs through a mechanism that is likely to be distinct from endocytosis mediated by clathrin-coated structures (Subtil et al., 1994). Within ten minutes of internalization, IL-2 remains bound to α, β and γ in early endosomes. The components of IL-2-IL-2R complex undergo differential sorting wherein IL-2Rα is recycled to the cell surface, while IL-2 associated with IL-2Rβγ is routed to the lysosome and degraded (Fallon and Lauffenburger, 2000). The sorting of IL-2Rβγ to the lysosome is mediated by an alpha helical signal on the cytoplasmic domain of IL-2Rβ (Subtil et al., 1997). In the absence of receptor synthesis, the half-life of IL-2Rβ and IL-2Rγ is ~ 1 hr, while IL-2Rα has a half-life of ~ 48 hrs (Hemar et al., 1995). Thus IL-2/IL-2R trafficking results in degradation of IL-2, IL-2Rβ and IL-2Rγ and downregulation of the high affinity IL-2R.

Different motifs on the cytoplasmic and transmembrane domains of IL-2Rβ may act as weak signals that additively mediate internalization (Subtil and Dautry-Varsat, 1998). Mutational studies on the IL-2R subunits also indicate an important role for IL-2Rγ for internalization of IL-2. Rapid IL-2 internalization has been observed for T cells expressing IL-2Rα and IL-2Rγ (Morelon and Dautry-Varsat, 1998). There is evidence to suggest that there exist distinct cytoplasmic regions of IL-2Rγ function during endocytosis, one for ligand-independent constitutive endocytosis of IL-2Rγ and another for IL-2Rγ dependent IL-2 induced endocytosis (Yu et al., 2000). The IL-2R system exhibits modularity in signaling and trafficking events, in addition to IL-2 binding. Signaling impaired mutants of IL-2Rγ that can still contribute to receptor-mediated internalization have been identified. A mutant form of IL-2Rγ, which exhibits impaired

endocytosis, despite normal IL-2 induced signaling, has also been identified (Yu et al., 2000).

1.5 IL-2 based therapy

IL-2 based therapies exploit the proliferation of antigen-activated T cells and CD56 bright NK cells caused by IL-2 (Fehniger et al., 2002), for treatment of metastatic renal cell carcinoma and melanoma (Atkins et al., 1999; Fyfe et al., 1995). Low doses of IL-2 have been shown to enhance immune function in HIV positive individuals (Jacobson et al., 1996). However, a narrow therapeutic window has hampered IL-2 therapies: undesirable inflammatory responses are activated at IL-2 concentrations above 100 pM through stimulation of CD56 dim NK cells (Fehniger et al., 2002) while stimulation of T cells is not achieved below 1 pM. When administered intravenously, IL-2 is rapidly cleared from the body. IL-2 serum concentrations are in the nanomolar range initially, and fall rapidly with a double exponential clearance rate with half-lives of 12.9 and 85 minutes respectively (Konrad et al., 1990). Thus it is difficult to maintain the therapeutically effective serum concentration range (1 – 100 pM) over a sustained period of time. This narrow therapeutic window of effective concentration coupled with rapid systemic clearance adversely affects IL-2 therapy. An improved IL-2 variant with an enhanced therapeutic window would greatly impact IL-2 based therapies.

1.6 Thesis Overview

The primary objective of this thesis was to generate IL-2 mutants with potentially improved therapeutic value. Hypothesis-driven directed evolution was used towards this end. A directed evolution approach involves the generation of protein variants with

desired properties through multiple rounds of mutation and selection. A hypothesis linking the effect of altered binding affinity of the IL-2/ IL-2 receptor interaction on the cellular and systemic biological effects mediated by IL-2 was formulated based on existing data in literature. IL-2 mutants with suitably altered receptor binding affinities were generated by directed evolution and found to have improved biological properties in carefully designed bioassays. Our approach is distinct from a directed evolution approach that seeks to directly find protein variants with improved biological property without linking biophysical properties of the protein to biological activity. Examples of this direct approach include directed evolution to improve the potency of IL-12 to cause T cell proliferation (Leong et al., 2003) and engineering human interferon gamma to obtain variants with improved antiviral activity (Chang et al., 1999).

1.6.1 Hypothesis generation

Activated T cells and CD56 bright NK cells, which express IL-2Rα, mediate the beneficial effects of IL-2 while CD56 dim NK cells that do not express IL-2Rα are involved in the deleterious effects of IL-2 (Fehniger et al., 2002). On the basis of mathematical modeling and qualitative considerations detailed in subsequent chapters, we hypothesized that IL-2 mutants with increased IL-2Rα affinity would have potentially increased therapeutic effectiveness.

1.6.2 Molecular bioengineering by directed evolution

Display technologies such as phage display (Parmley and Smith, 1988) and yeast surface display (Boder and Wittrup, 2000), are powerful tools that link genotype to phenotype can be used for screening large libraries of protein variants for altered binding properties.

Interleukin-2 mutants with desired physical properties were generated using yeast surface display and directed evolution. The protein of interest is expressed or "displayed" as a fusion to a yeast cell surface protein Aga2p (Boder and Wittrup, 1997), as shown in Figure 1-1. Immunofluorescent labeling of epitope tags can be used to detect the presence of protein on the yeast cell surface. The surface displayed protein can bind to a soluble binding partner, in this case soluble IL-2Rα.

The procedure used to select IL-2 mutants with increased affinity for IL-2Ra is illustrated in Figure 1-2. A library of yeast displayed IL-2 mutants is generated. Each yeast cell expresses ~50000 copies of a single IL-2 mutant on the cell surface. Cells are labeled with fluorescently tagged soluble IL-2Rα. Yeast cells displaying mutants with improved binding to IL-2Rα are labeled to a greater extent than cells displaying wild-type IL-2. Quantitative screening by flow cytometry is used to isolate these yeast displayed IL-2 mutants with desired binding properties (Boder and Wittrup, 2000). After few rounds of sorting, a pool of yeast-displayed IL-2 mutants with increased affinity for IL-2Rα is obtained. DNA from the individual clones is isolated and sequenced to establish the identity of these high IL-2Rα binding IL-2 variants.

Variants with enhanced receptor binding affinities have been isolated for human growth hormone (Lowman et al., 1991), interleukin-6 (Toniatti et al., 1996) and ciliary neutrotrophic growth factor (Saggio et al., 1995), using phage display. IL-2 has been functionally displayed on phage (Buchli et al., 1997), but improved mutants have not previously been engineered by phage display.

1.6.3 Validation of hypothesis

The engineered IL-2 mutants were expressed solubly and improved binding to IL-2Rα was verified in a physiological context – on T cells expressing IL-2Rα. Carefully designed bioassays that mimic bolus pharmacokinetics were used to evaluate biological response of T cells to the engineered IL-2 mutants. In these assays, the mutants exhibit significantly higher activity than wild-type IL-2, indicating potentially improved therapeutic value. The IL-2 mutants also enable us to formulate a quantitative relationship between receptor-ligand molecular interactions and biological activity and understand better the differences between IL-2 and IL-15.

1.6.4 Thesis layout

Chapter 2 describes the generation of the IL-2 mutants with increased affinity for IL-2Rαand preliminary evaluation of the biological activity of these mutants. In Chapter 3, the design of novel bioassays that mimic bolus pharmacokinetics and the significantly improved activity of IL-2 mutants in these bioassays are discussed. This chapter also describes the mechanism leading to improved activity of the IL-2 mutants. Chapter 4 describes the further construction of a series of high IL-2Rα IL-2 mutants that are quantitatively equivalent to IL-15 in terms of T cell growth response generated. This chapter discusses the quantitative relationship between binding affinity and biological activity for IL-2 and IL-15 and the reasons for the vastly different biological responses mediated by IL-2 and IL-15. The final chapter discusses the conclusions of this study, perspectives on the broader applicability of the approach taken and suggested future work.

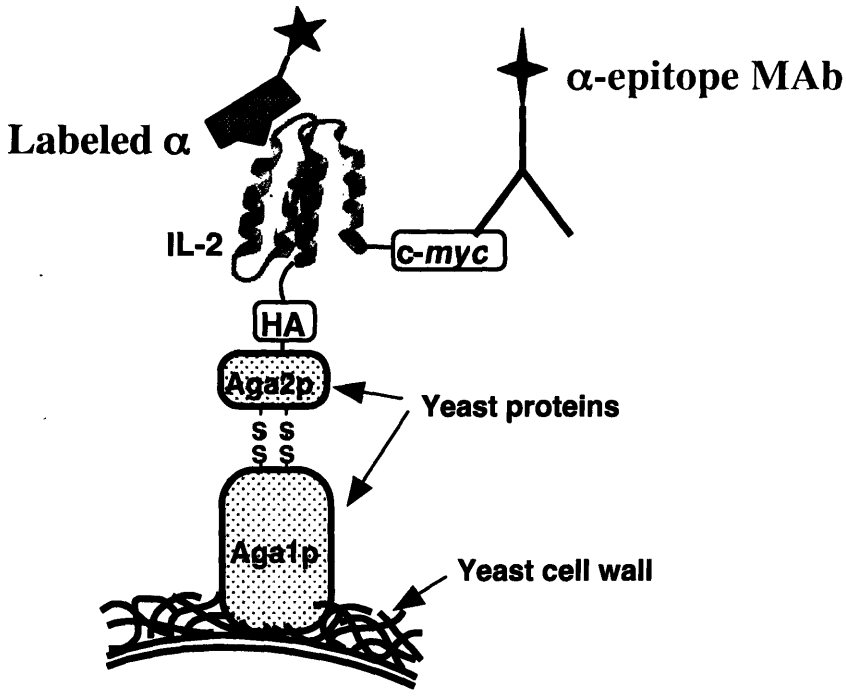

Figure 1-1: Yeast Surface Display of IL-2
IL-2 is expressed as a fusion to the yeast cell surface Aga2 protein.

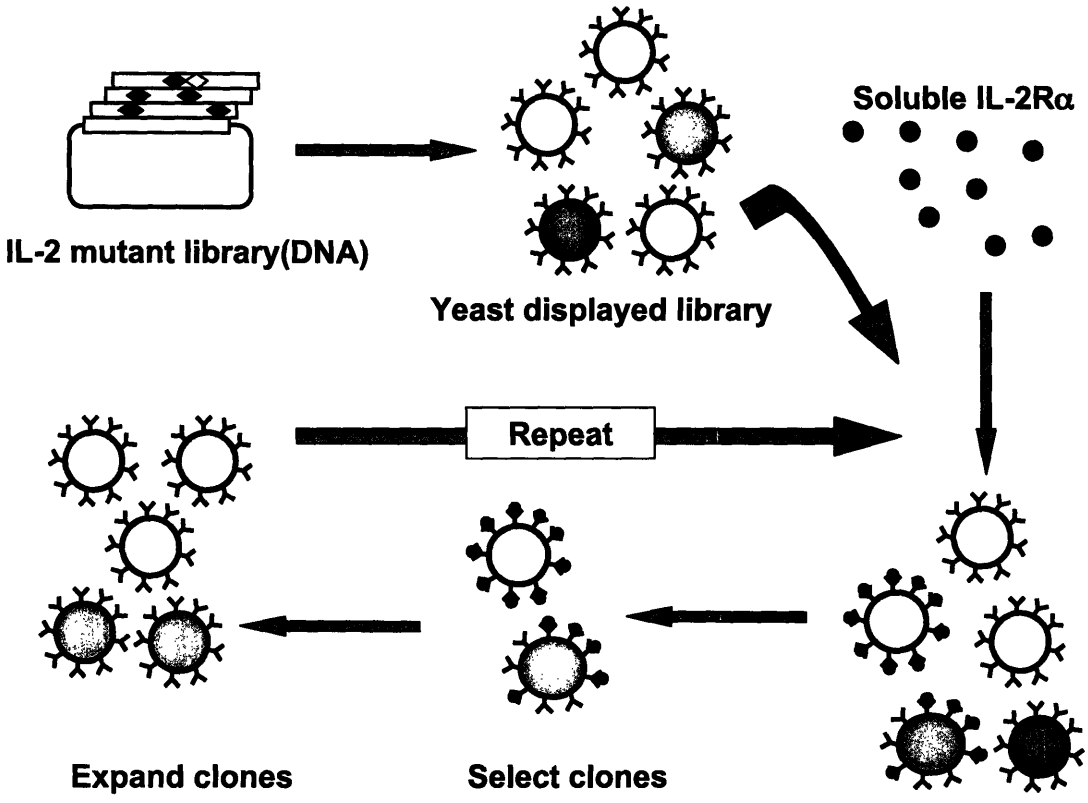

Figure 1-2: Directed evolution scheme to select high IL-2Rα affinity IL-2 mutants

Chapter 2: INTERLEUKIN-2 MUTANTS WITH ENHANCED α-RECEPTOR SUBUNIT BINDING AFFINITY

Stimulation of T-cells by IL-2 has been exploited for treatment of metastatic renal carcinoma and melanoma. However, a narrow therapeutic window delimited by negligible stimulation of T-cells at low picomolar concentrations and undesirable stimulation of NK cells at nanomolar concentrations hampers IL-2 based therapies. We hypothesized that increasing the affinity of IL-2 for IL-2Rα may create a class of IL-2 mutants with increased biological potency as compared to wild-type IL-2. Towards this end, we have screened libraries of mutated IL-2 displayed on the surface of yeast, and isolated mutants with a 15-30-fold improved affinity for the IL-2Rα subunit. These mutants do not exhibit appreciably altered bioactivity at 0.5-5 pM in steady-state bioassays, concentrations well below the IL-2Rα equilibrium binding constant for both the mutant and wild-type IL-2. A mutant was serendipitously identified that exhibited somewhat improved potency, perhaps via altered endocytic trafficking mechanisms described previously

2.1 Introduction

Interleukin-2 (IL-2) (Theze et al., 1996) is a 133-amino-acid cytokine that induces proliferation of antigen-activated T-cells and stimulation of NK cells. The proliferation of T-cells stimulated by IL-2 has been exploited for treatment of metastatic renal carcinoma and melanoma (Atkins et al., 1999; Fyfe et al., 1995). However, a narrow therapeutic window has hampered IL-2 therapies: undesirable inflammatory responses are activated

at IL-2 concentrations above 100 pM through stimulation of NK cells (Jacobson et al., 1996; Smith, 1993) while stimulation of T cells is not achieved below 1 pM. Given the very rapid systemic clearance of IL-2 (an initial clearance phase with a half-life of 12.9 min followed by a slower phase with a half-life of 85 min, (Konrad et al., 1990)), it is difficult to maintain therapeutic concentrations of IL-2 (1-100 pM) for a sustained period.

The biological activity of IL-2 in activated T cells is mediated through a multi-subunit IL-2 receptor complex (IL-2R) consisting of three cell-surface subunits: p55 (IL-2Rα), p75 (IL-2Rβ) and p64 (IL-2Rγ), which span the cell membrane (Nelson and Willerford, 1998). NK cells in general express only the IL-2Rβ and IL-2Rγ subunits (Voss et al., 1992), so enhanced affinity for IL-2Rα might be expected to increase the specificity of IL-2 for activated T cells relative to NK cells. Manipulation of the binding affinities to these receptor subunits might be used to alter the biological response to IL-2 and potentially create an improved therapeutic. Screening of over 2600 IL-2 variants created by combinatorial cassette mutagenesis has led to the isolation of an IL-2 variant (L18M, L19S) with increased potency (Berndt et al., 1994). Site-directed mutagenesis was also utilized to isolate IL-2 variants causing reduced stimulation of NK cells via reduced binding to IL-2Rβ and IL-2Rγ (Shanafelt et al., 2000).

Display technologies such as phage display (Parmley and Smith, 1988) and yeast surface display (Boder and Wittrup, 2000), are powerful tools that can be used for screening large libraries of protein variants for altered binding properties. Variants with enhanced receptor binding affinities have been isolated for human growth hormone (Lowman et al., 1991), interleukin-6 (Toniatti et al., 1996) and ciliary neutrotrophic growth factor (Saggio et al., 1995), using phage display. IL-2 has been functionally displayed on phage (Buchli

et al., 1997), but improved mutants have not previously been engineered by phage display.

Here we present IL-2 engineering by directed evolution with yeast surface display, to generate mutants with increased affinity for IL-2Rα. This is the first reported affinity maturation of IL-2 for a receptor subunit. T-cell response to IL-2 depends on the number of IL-2R occupied by IL-2 via: 1) the concentration of IL-2; 2) the number of IL-2R molecules on the cell surface; and 3) the number of IL-2R occupied by IL-2, i.e. the affinity of binding interaction between IL-2 and IL-2R (Smith, 1995). Increasing the affinity of IL-2 for IL-2Rα at the cell surface will increase receptor occupancy within a limited range of IL-2 concentration, as well as raise the number of IL-2 molecules localized at the cell surface. The IL-2-IL-2R complex is internalized upon ligand binding and the different components undergo differential sorting (Hemar et al., 1995). IL-2Rα is recycled to the cell surface, while IL-2 associated with the IL-2-IL-2Rβγ complex is routed to the lysosome and degraded. Increasing the affinity of IL-2 for IL-2Rα may shift trafficking of internalized IL-2 towards recycling, causing decreased degradation of IL-2 and hence favorably affect T-cell response (Fallon et al., 2000). Further, IL-2-IL-2Rα on one cell can augment IL-2 signaling on another cell (Eicher and Waldmann, 1998). IL-15, which exhibits picomolar binding affinity for its private IL-2Rα subunit, also performs such juxtacrine signaling (Dubois et al., 2002). Thus, it is conceivable that increasing the affinity of IL-2 for IL-2Rα may create a class of IL-2 mutants with increased biological potency as compared to wild-type IL-2. However, in the steady-state bioassays reported here, a 15-30-fold increase in IL-2Rα binding affinity does not contribute to improved IL-2 potency.

2.2 Materials and methods

2.2.1 Yeast surface display of IL-2

The IL-2 gene was subcloned into the pCT302 backbone at *NheI* and *BamHI* restriction sites. A serine was introduced at position 125 by site directed mutagenesis to obtain what will be termed "wild-type" C125S IL-2 (equivalent to Proleukin™). This vector is termed pCTIL-2.

IL-2 was expressed as an Aga2p protein fusion in *Saccharomyces cerevisiae* EBY100 transformed with vector pCT-IL-2, by induction in medium containing galactose (Boder and Wittrup, 1997). A haemagglutinin (HA) epitope tag is expressed N-terminal to IL-2, while a c-myc epitope tag is attached to the C-terminus of Aga2p-IL-2 fusion. The HA epitope tag can be detected using immunofluorescent staining using a mouse monoclonal antibody (mAb) 12CA5 (Roche Molecular Biochemicals) along with a goat anti-mouse antibody conjugated with Fluorescein Isothiocyanate (FITC). The c-myc epitope tag can be detected using a mouse monoclonal antibody (mAb) 9e10 (Covance) and a goat anti-mouse antibody conjugated with FITC. Detection of the c-myc epitope tag at the C-terminus of the Aga2p-IL-2 fusion is indicative of display of the full length IL-2 fusion on the yeast cell surface. Yeast cells were labeled with MAb 9e10 as described (Boder and Wittrup, 2000), to detect the presence of IL-2 fusions on the yeast cell surface

A soluble ectodomain of IL-2Rα (Wu et al., 1999), expressed in insect cell culture, was purified and biotinylated. Yeast cells were labeled with biotinylated soluble IL-2Rα as described (Boder and Wittrup, 2000), Labeling with soluble IL-2Rα is indicative of the

IL-2 fusion on the yeast surface being functional. Yeast displaying an irrelevant single chain antibody (scFv), D1.3, was used as a negative control.

2.2.2 Construction and screening of IL-2 library

The wild-type IL-2 coding sequence was subjected to random mutagenesis by error-prone polymerase chain reaction (PCR). The error rate was controlled by varying cycles of PCR amplification in the presence of nucleotide analogs 8-oxodGTP and dPTP (Zaccolo and Gherardi, 1999; Zaccolo et al., 1996). The PCR product obtained was further amplified by PCR without the nucleotide analogs. The final PCR product was transformed into yeast along with linearized pCT-IL-2. Homologous recombination in vivo in yeast between the 5' and 3' flanking 50 base pairs of the PCR product with the gapped plasmid resulted in a library of approximately $5x10^6$ IL-2 variants (Raymond et al., 1999).

Detailed protocols for screening yeast polypeptide libraries have been described (Boder and Wittrup, 2000). Yeast cells from the IL-2 library were labeled with biotinylated soluble IL-2Rα at a concentration of 0.2-0.8 nM and saturating concentration of mAb 12CA5 against the HA epitope tag, at 37°C, for 30 min- 1hr. Labeling with an antibody against one of the epitope tags is necessary to normalize for the number of IL-2 fusions on the yeast surface. The cells were washed, labeled with streptavidin conjugated with R-phycoerythrin (PE) (Pharmingen) and a goat anti-mouse antibody conjugated with FITC. The cells were then sorted on the Cytomation Moflo (first two sorts) or the Beckton Dickinson FACStar flow cytometer to isolate clones with improved binding to soluble IL-2Rα, relative to wild-type IL-2. Four rounds of sorting by flow cytometry, were carried out, with regrowth and reinduction of surface expression between each sort, After the fourth sort, DNA from twenty individual clones was extracted using the

Zymoprep kit (Zymo Research corporation). The DNA was amplified by transforming into XL-1 Blue cells (Stratagene). Sequences of the IL-2 mutants were determined by DNA sequencing.

IL-2 mutants isolated by flow cytometry were subcloned into secretion vectors, and secreted in yeast shake flask cultures, with an N-terminal FLAG epitope tag and a C-terminal c-myc epitope tag. The mutants were purified by FLAG immunoaffinity chromatography (Sigma). Quantification of IL-2 concentration was performed using quantitative western blotting, with a FLAG-BAP protein standard (Sigma) and mutant M6 as standards. The stock protein concentrations obtained were 11.7±1.2 μM for wild-type C125S (six measurements) IL-2, 20.7±1.4 μM for M6 (four measurements), 25.3±6.1 μM for M1 (four measurements) and 3.3±0.6 μM for C1 (eight measurements).

2.2.3 KIT-225 cell proliferation assay

KIT-225 is a human IL-2 dependent T-cell line, expressing roughly 3000-7000IL-2Rαβγ and 200000-300000 IL-2Ra (Arima et al., 1992; Hori et al., 1987). KIT-225 cells were cultured in RPMI 1640 supplemented with 20 pM IL-2, 10% FBS, 200 mM L-glutamine, 50 units/mL penicillin and 50 μg/mL gentamycin.

KIT-225 cells were cultured in medium without IL-2 for six days. The cell culture medium was changed after three days. On the sixth day, the cells were transferred into medium containing wild-type IL-2 or IL-2 mutants at different concentrations at 10^5 cells/mL. Cell culture aliquots were taken at different times and the viable cell density was determined using the Cell-titer Glo™ (Promega) assay.

2.2.4 Binding of IL-2 mutants to KIT-225 & YT2C2

KIT-225 cells were incubated (10^6 cells in 100 μL) with soluble IL-2 or mutants at 37 °C for 30 minutes, at pH 7.4. Cells were washed with ice-cold PBS, pH 7.4, containing 0.1% BSA and labeled with a biotinylated antibody against the FLAG epitope followed by streptavidin-phycoerythrin on ice. Cells were washed again and mean single cell fluorescence was determined using an EPICS-XL flow cytometer.

YT-2C2 is a human NK cell line expressing approximately 20000 IL-2Rβγ (Teshigawara et al., 1987) . YT-2C2 cells were cultured in the same medium as KIT-225 cells, without IL-2. YT-2C2 cells were incubated (10^6 cells in 100 μL) with the IL-2 mutants on ice for 30 minutes, at pH 7.4. Cells were washed with ice-cold PBS, (pH 7.4, 0.1% BSA) and labeled with a biotinylated antibody against the FLAG epitope followed by streptavidin-phycoerythrin on ice. Cells were washed again and mean single cell fluorescence was determined using an EPICS-XL flow cytometer. The equilibrium dissociation constants were determined using a global fit. 66% confidence intervals were calculated by as described (Lakowicz, 1999).

2.3 Results

2.3.1 Functional expression of IL-2 on the surface of yeast

Although IL-2 has been displayed on bacteriophage previously (Buchli et al., 1997), directed evolution using phage display, to obtain IL-2 mutants with improved binding for the IL-2R subunits has not been reported. IL-2 was expressed on the surface of yeast cells, on the assumption that expression in a eukaryotic system would produce a higher fraction of correctly folded protein. IL-2 was expressed as a fusion to the Aga2p agglutinin subunit, on the surface of yeast (Boder and Wittrup, 1997). Expression of the Aga2p-IL-2 fusion on the surface of yeast was measured by immunofluorescent labeling of the c-myc epitope tag attached to the C-terminus of the Aga2p-IL-2 fusion (Figure 2-1A). IL-2 displayed on the surface of yeast binds specifically to the soluble ectodomain of IL-2Rα (Figure 2-1B), while negative control yeast displaying an irrelevant scFv, D1.3, do not (Figure 2-1D). Presence of the c-myc tag indicates that the full-length IL-2 fusion is displayed on the yeast cell surface. Figure 2-1C shows immunofluorescent labeling of the c-myc tag on negative control yeast, displaying D1.3, indicating the presence of D1.3 fusions on the yeast cell surface.

2.3.2 Screening of IL-2 library for clones with improved binding to IL-2Rα

A yeast-displayed library of IL-2 mutants with a diversity of $5x10^6$ clones was constructed by error-prone PCR. This library was screened through four rounds of sorting by flow cytometry, with regrowth and reinduction of surface expression between each sort, to isolate clones with improved binding to soluble IL-2Rα. The ensemble of clones after four rounds of sorting shows improved binding relative to wild-type IL-2 at 0.4 nM

soluble IL-2Rα, normalized to the number of IL-2 fusions on the yeast surface by labeling with mAb 12CA5 (Figure 2-2).

Twenty mutants were sequenced (Table 2-1), and seven distinct sequences were obtained from the twenty clones sequenced. The most frequently occurring mutations (V69A and Q74P) cluster in a region predicted to be at the IL-2/IL-2Rα interface, by a homology model of IL-2 binding to its receptor subunits (Figure 2-3). Further, the mutant M6 has a mutation I128T, which is close to the predicted IL-2/IL-2β and IL-2/IL-2Rγ interface (Bamborough et al., 1994; Berman et al., 2000).

2.3.3 Binding of IL-2 mutants to KIT-225 cells expressing a large excess of IL-2Rα

The IL-2 mutants isolated by yeast surface display were tested in soluble form for tighter binding to IL-2Rα in its physiologically relevant context on the surface of KIT-225 cells. Three different mutants were tested - M6 (V69A, Q74P, I128T), M1 (V69A, Q74P) and C1 (I128T). We chose to test mutant M6 (and mutants derived from M6) due to the observation that M6, and not the other six mutants, exhibited slightly improved biological potency in preliminary KIT-225 cell proliferation assays (data not shown), described subsequently. M1 represents the most frequently occurring two mutations. We hypothesized, on the basis of the homology model of IL-2 binding to its receptor subunits, that the subset of mutations in M6 represented by M1 would be sufficient for increased binding affinity for IL-2Rα. C1 represents the mutation predicted to be close to the IL-2/IL-2β and IL-2/IL-2Rγ interface.

Figure 2-4 shows representative data for binding of M6, M1, C1 and wild-type (C125S) IL-2 to KIT-225 cells, at 37 °C. M6 and M1 have similar binding to KIT-225 cells, while

C1 exhibits similar binding to wild-type (C125S) IL-2. Since the KIT-225 cells express a large excess of IL-2Rα over IL-2Rβ and IL-2Rγ, the binding data obtained corresponds to IL-2Rα binding. Thus, M6 and M1 have a higher binding affinity for IL-2Rα on the surface of KIT-225 cells, as compared to C1 and wild-type (C125S) IL-2. The fluorescence data, in Figure 2-4, for concentrations 0.01 - 400 nM, were used to obtain a gross estimate of the K_d for M6 and M1. An equation describing a simple one-step binding equilibrium was used to fit the data.

$$F_{obs} = \frac{C \cdot L_0}{K_d + L_0} \quad \ldots (1)$$

Where:

F_{obs} = observed fluorescence,

L_0 = initial ligand concentration and,

C = proportionality constant.

The K_d for M6 and M1 can be estimated to be 1-2 nM (1.1±0.08 nM for M6 and 1.7±0.4 nM for M1). This represents roughly a fifteen to thirty-fold minimum improvement in binding affinity, relative to a wild-type K_d value of 28 nM for C125A IL-2, a mutant with alanine at position 125 (Liparoto et al., 2002). The errors represent variation due to the error in estimating concentrations using quantitative western blotting. This calculation underestimates the binding affinity than the actual value (overestimates the K_d) due to the following systematic errors: 1) The cell density used in the binding assay represents severe ligand (IL-2) depleting conditions. For equation (1) used above, to hold true, the initial ligand concentration must be approximately equal to the free ligand concentration

in solution at equilibrium. This assumption breaks down at concentrations less than roughly 10 nM, for the experimental setup used, and the free ligand concentration is less than the initial ligand concentration. This leads to an overestimate of the K_d (i.e. an underestimate of binding affinity). 2) Internalization of ligand-bound receptors occurs at 37 °C. The internalization rate of ligand-bound receptors can be assumed to be proportional to the fraction of ligand-bound receptors, leading to an overestimate of the K_d (underestimate of binding affinity).

The equilibrium dissociation constant (K_d) for C1 and C125S cannot be estimated from this data, due to the rapid dissociation of IL-2Rα-bound IL-2 (Liparoto et al., 2002). The receptor-bound IL-2 dissociates during the several wash steps involved in the experiment. This leads to very low fluorescence signal, even at high concentrations for C1 and C125S. M6 and M1 were also assayed for binding at these high concentrations for consistency. Increase in fluorescence signal beyond 400 nM concentrations of M6 and M1 may be due to binding to IL-2Rβ and IL-2Rγ on KIT225 cells and non-specific binding at micromolar concentrations of M6 and M1. In summary, the data in Figure 2-4 provides only a crude estimate of K_d, but definitively demonstrates that M1 and M6 exhibit substantial, qualitative improvements in binding affinity on the T cell surface, relative to C125S and C1.

2.3.4 Binding of IL-2 mutants to YT-2C2 cells expressing IL-2Rβ and IL-2Rγ

The binding of M1, M6 and C1 to YT-2C2 cells expressing IL-2Rβ and IL-2Rγ was determined (Figure 2-5). A global fit was used to estimate the equilibrium dissociation constants (K_d). These values are given in Table 2-2. The K_d values are consistent with reported affinities for the binding of IL-2 to IL-2Rβ (Liparoto et al., 2002). M1 was

found to have a significantly lower binding affinity for IL-2Rβ than wild-type, M6 and C1. This is interesting in light of M1's mutation sites, predicted to be on the opposite side from IL-2's contacts with IL-2Rβ.

2.3.5 Proliferation of IL-2 dependent KIT-225 cells in response to IL-2 mutants

The proliferation of a T-cell line (KIT-225) in response to the IL-2 mutants was studied to evaluate the effect of increase in affinity of IL-2 for IL-2Rα on biological potency. At low concentrations (0.5 pM) and long times, C1 and M6 caused approximately 50-60% greater proliferation of IL-2 dependent KIT-225 cells in cell culture, as compared to wild-type (C125S) IL-2 and M1. The proliferation of KIT-225 cells in culture with the different mutants, at different initial concentrations, is shown in Figure 2-6. It was surprising to note that both M6 and C1 had slightly improved biological potency while M1, with comparable affinity as M6 to IL-2Rα, did not. The observed increase in affinity of IL-2 for IL-2Rα did not have appreciable effect on biological potency for mutant M1 in this steady-state assay, suggesting that such an increase in affinity for IL-2Rα alone is not responsible for the increased potency of M6.

2.4 Discussion

We hypothesized that increasing the affinity of IL-2 for its alpha-receptor subunit would create an IL-2 mutant with improved biological potency, for reasons described earlier (Introduction). To this end, IL-2 mutants with improved affinity for IL-2Rα were selected from a yeast-displayed randomly mutated library. The mutants obtained were tested for proliferation of a T-cell line (KIT-225). The concentrations at which the KIT-225 proliferation assays were carried out lie in the picomolar range (0.5 – 5 pM), however the equilibrium dissociation constants for the IL-2 mutants selected lie in the nanomolar range. One of the predicted mechanisms for IL-2 mutants with increased IL-2Rα binding affinity to have increased biological potency, is an increased concentration of IL-2 localized at the cell surface, by binding to IL-2Rα. Under the steady-state conditions of the bioactivity assay, increase in occupancy of IL-2Rα would not be significantly different for the mutants as compared to wild-type IL-2. The T-cell response to the IL-2 mutants is therefore not detectably different from wild-type IL-2. A greater increase in occupancy of IL-Rα would conceivably lead to an increase in potency, through increase in the local concentration of IL-2, at the cell surface. We hypothesize that a greater difference in T-cell response, in these assays, may be observed for mutants with IL-2Rα affinity in the picomolar range. However, on the basis of our results, we can conclude that a fifteen to thirty-fold increase in affinity for IL-2Rα does not result in a corresponding increase in biological potency, in proliferation assays at picomolar concentrations, as described. The quantitative relationship between such steady-state, low-concentration assays and the pharmacological situation in vivo is not clear however, given the rapid renal clearance of parenterally administered IL-2.

The mutations responsible for the higher affinity for IL-2Rα (V69A, Q74P) cause a decrease in affinity for IL-2Rβ. One of the reasons for the decreased biological activity of M1 relative to M6 may be this decrease in affinity for IL-2Rβ. We could not analyze the effect of the selected mutations on the binding affinity for IL-2Rγ due to the extremely weak affinity of interaction between IL-2 and IL-2Rγ (Liparoto et al., 2002).

M6 and C1 exhibit slightly improved biological potency relative to wild-type, in the T-cell proliferation assays described. C1 has no appreciable change in affinity for IL-2Rα and IL-2Rβ, as compared to wild-type, while M1, with increased affinity for IL-2Rα, has slightly decreased biological potency. Also, as explained earlier, there would not be a significant increase in IL-2Rα receptor occupancy under the particular conditions of the T-cell proliferation assays. These observations suggest that the increased potency of M6 in the T-cell proliferation assays is through a mechanism unrelated to the increase in affinity for IL-2Rα. Previous studies have investigated the increased biological potency of 2D1, a mutant of IL-2. 2D1 internalized by receptor-mediated endocytosis is recycled to a greater extent than wild-type IL-2, leading to decreased depletion of 2D1 in cell culture and hence improved biological potency (Fallon et al., 2000). Increased recycling of internalized M6 and C1 could be a potential mechanism for increased biological potency of M6 and C1, relative to wild-type IL-2.

Our results establish the proof of concept of a strategy to isolate IL-2 mutants with tailored binding characteristics and characterize T-cell response to these mutants. The YT-2C2 cell-binding assay provides a convenient preliminary test to check and ensure that the mutants selected do not have their affinities for IL-2Rβ greatly weakened. The IL-2 mutants did not show increased potency in T-cell proliferation assays at low

picomolar concentrations. Conversely, none of the seven isolated mutants showed loss of biological potency as compared to wild-type IL-2, in preliminary assays (data not shown). This work lays the foundation for the generation and characterization of IL-2 mutants with further improved affinities for IL-2Rα, sufficient to drive greater receptor occupancy in the 0.1-10 pM concentration range. In addition, bioassays designed to better mimic the transient nature of IL-2 exposure in vivo may highlight the altered properties of these mutants.

Table 2-1: Mutations in IL-2 clones with greater affinity for IL-2Ra compared to C125S

Seven distinct sequences were obtained out of twenty clones sequenced.

	Mutants	M16	M13	M12	M9	M5	M30	M6
	Isolates	1	3	7	4	1	1	3
Position	WT aa							
1	*A*	T						
11	*Q*						R	
46	*M*	L						
48	*K*						E	
49	*K*	R						
61	*E*	D						
64	*K*			R				
68	*E*						D	
69	*V*	A	A	A	A	A		A
71	*N*						T	
74	*Q*			P	P	P		P
79	*H*	R						
90	*N*						H	
101	*T*				A	A		
103	*F*						S	
114	*I*						V	
128	*I*							T
133	*T*					N		

Table 2-2: Binding affinities of IL-2 mutants for IL-2Rβ on YT-2C2 cells

	Kd (nM)	66% confidence intervals
WT (C125S)	94	70-135
C1	132	110-161
M6	210	149-331
M1	480	388-630

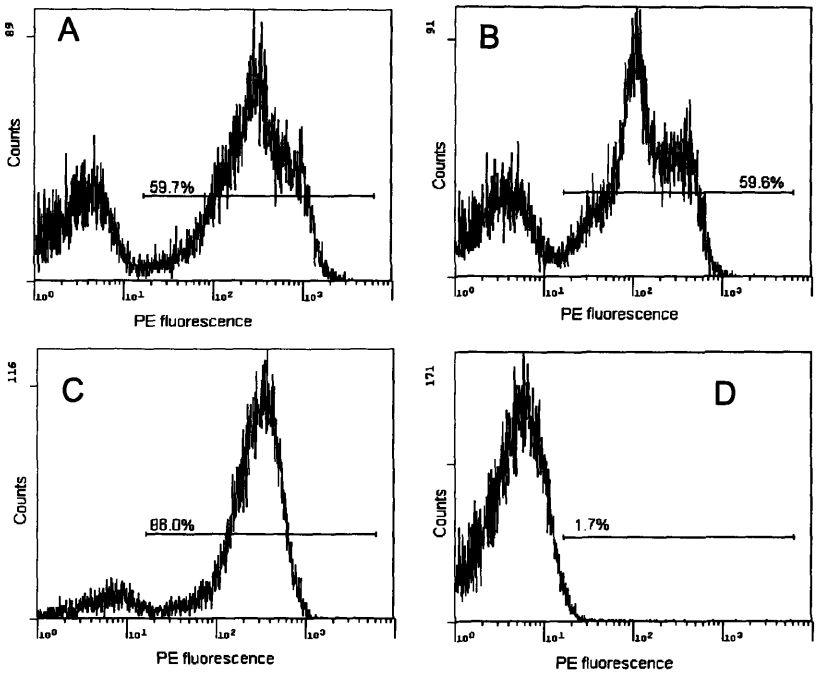

Figure 2-1: IL-2 is functionally displayed on the surface of yeast.

(A) Labeling of IL-2 displaying yeast with saturating concentration of anti-c-myc antibody (9e10), (B) Labeling of IL-2 displaying yeast with 52 nM soluble IL-2R (C) Labeling of D1.3 (an irrelevant negative control scFv) displaying yeast with saturating concentration of anti-c-myc antibody (9e10), (D) Labeling of D1.3 displaying yeast with 52 nM soluble IL-2Rα.

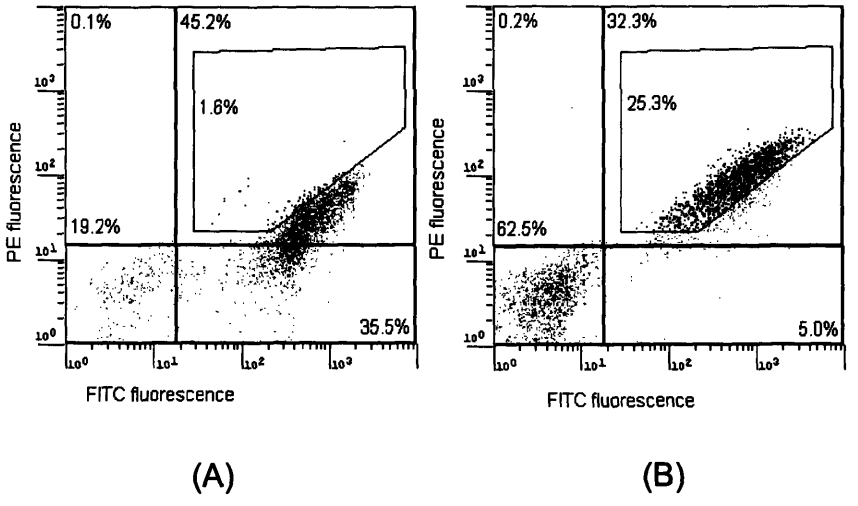

Figure 2-2: Ensemble of clones with better binding for IL-2Rα compared to wild-type C125S

Labeling with saturating concentration of anti-HA antibody(12CA5) and 0.4 nM IL-2Rα ($2x10^6$ cells, 100 μL volume) at 37 °C. (A) Wild-type (C125S) (B) Population isolated after four rounds of sorting.

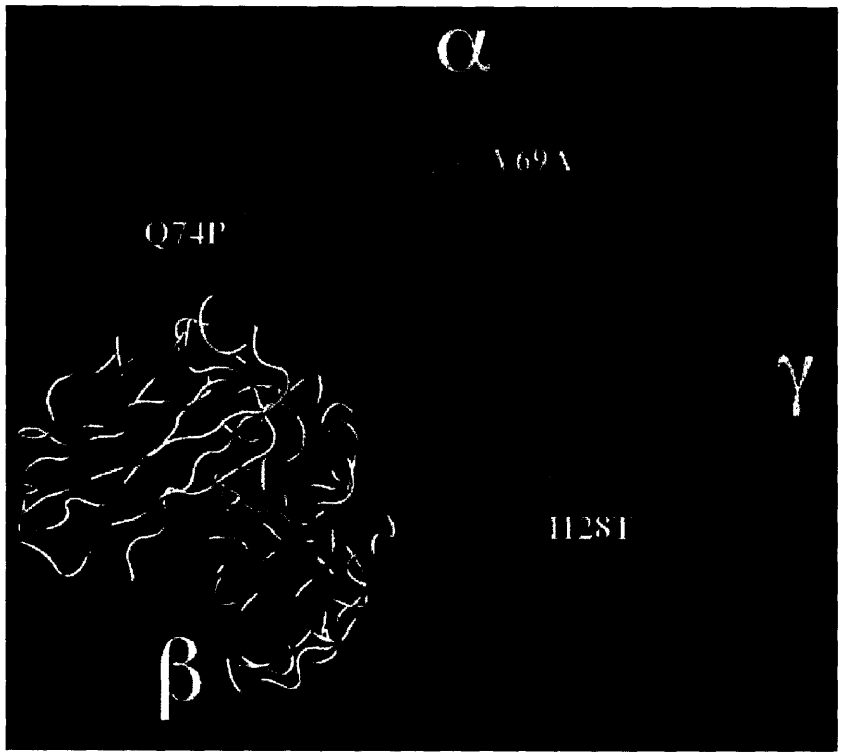

Figure 2-3: Locations of mutations on a model of IL-2/receptor complex

IL-2 is shown in red, IL-2Rα subunit in blue, IL-2Rβ subunit in white, IL-2Rγ subunit in grey. The residues where mutations were encountered, in improved IL-2 mutants, are marked – Q74 (orange), V69 (brown) and I128 (green).

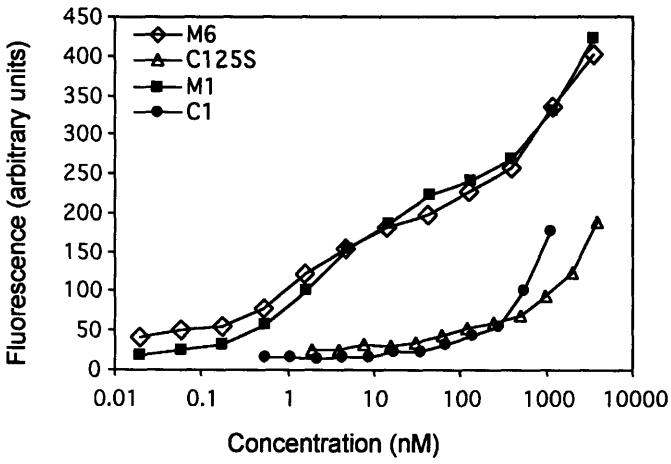

Figure 2-4: Binding of solubly expressed IL-2 mutants to KIT-225 cells expressing large excess of IL-2Rα, at 37 °C

Data shown is representative data from at least two experiments, for each mutant or wild-type IL-2; the binding curves look similar at 4 °C (data not shown)

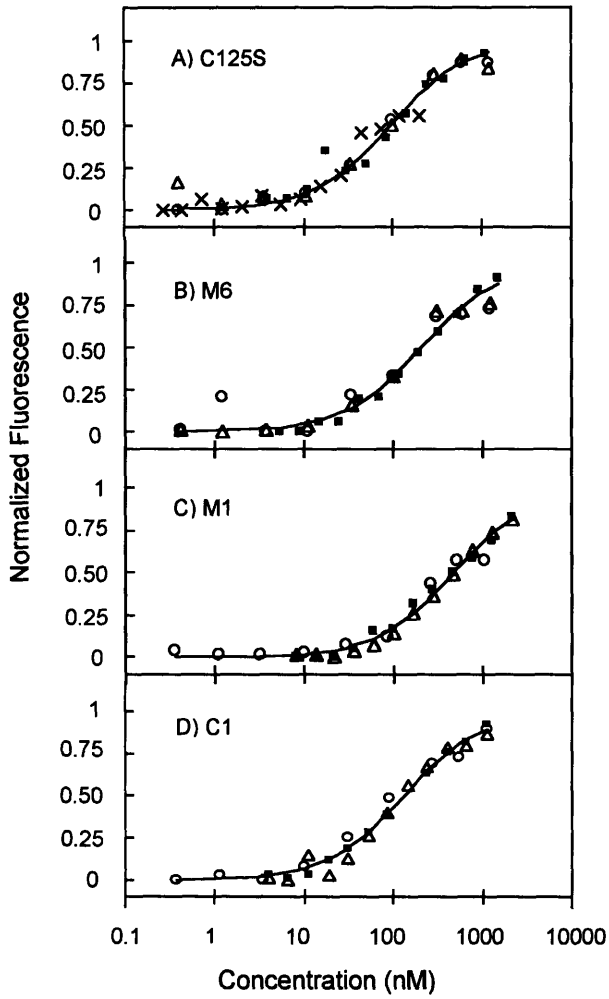

Figure 2-5: Binding of solubly expressed IL-2 mutants to YT-2C2 cells expressing IL-2Rβ and IL-2Rγ

Different symbols denote different data sets.

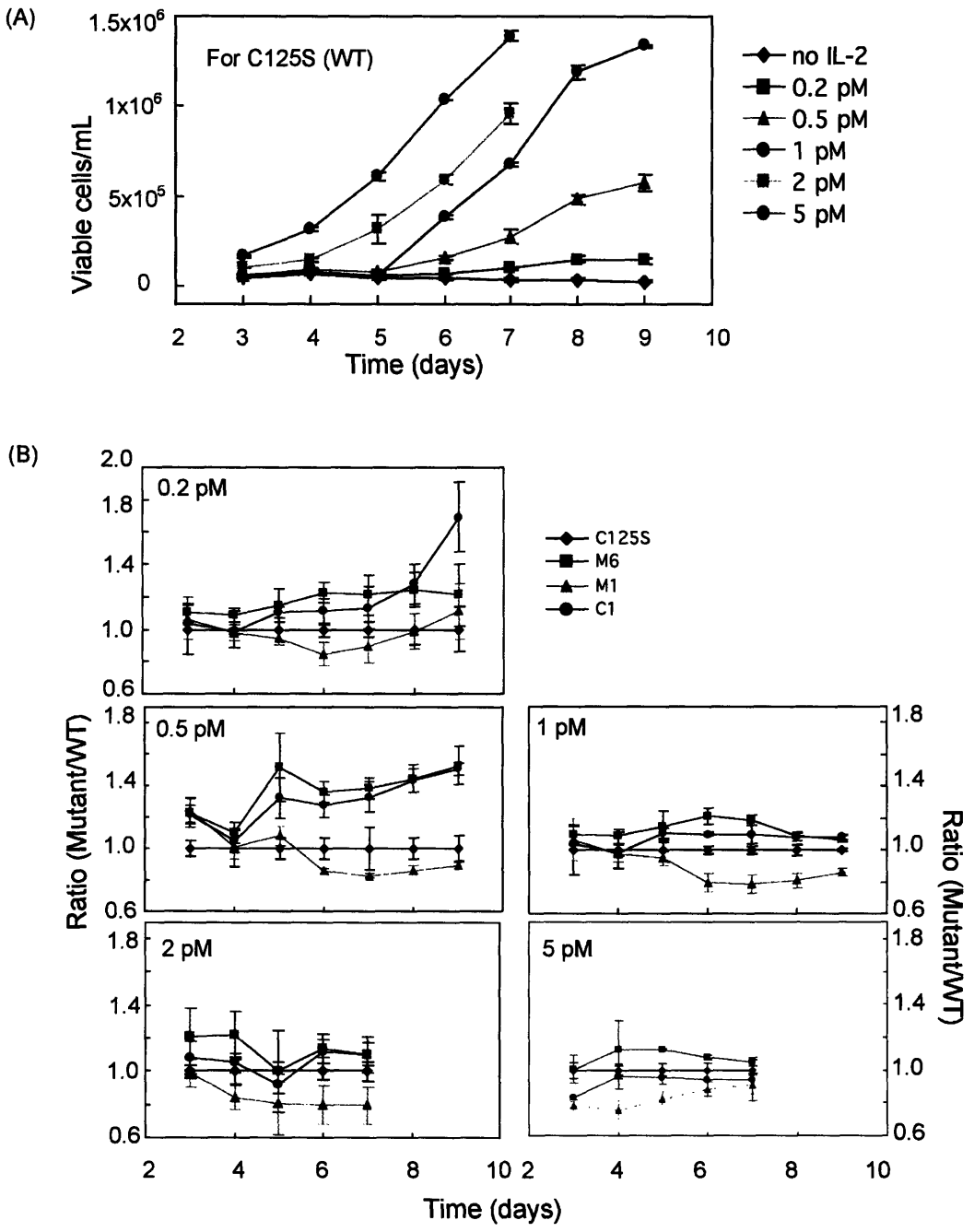

Figure 2-6: Proliferation of IL-2 dependent KIT-225 cells in response to wild-type (C125S) IL-2 and IL-2 mutants

(A) Number of viable KIT-225 cells in culture with time, at different concentrations of wild-type (C125S) IL-2. Error bars indicate the standard deviation for three separate cultures.

(B) Ratio of number of viable cells in culture with the mutants to number of cells in culture with wild-type (C125S) IL-2. The concentrations are indicated on the plots. Error bars indicate the standard deviation for three separate cultures.

Chapter 3: IL-2 VARIANTS ENGINEERED FOR INCREASED IL-2Rα AFFINITY EXHIBIT INCREASED POTENCY ARISING FROM A CELL SURFACE LIGAND RESERVOIR EFFECT

Proliferation of activated T cells and CD56 bright NK cells (Fehniger et al., 2002) caused by interleukin-2 (IL-2) has been exploited in IL-2-based therapies for the treatment of metastatic renal cell carcinoma and melanoma (Atkins et al., 1999; Fyfe et al., 1995). Here we demonstrate the potentially improved therapeutic value of IL-2 variants engineered to gain 15-30-fold increased affinity for the IL-2 alpha-receptor subunit (IL-2Rα). A novel pulsed bioassay was used to more closely approximate the rapid systemic clearance pharmacokinetics of cytokines such as IL-2, compared to conventional static bioassays. In this assay, mutants with increased affinity for IL-2Rα exhibit significantly increased activity for T-cell proliferation, whereas static bioassays not only fail to reveal the increased activity resulting from enhanced IL-2Rα affinity (false negatives), but also suggest improved activity for another mutant without enhanced activity in the pulsed assay (false positive). Our studies on the mechanism leading to increased activity of IL-2 mutants with increased IL-2Rα affinity suggest that cell-surface IL-2Rα acts as a ligand reservoir for the IL-2 mutants. This leads to increased cell-surface persistence of the IL-2 mutants with increased IL-2Rα affinity in cell-surface ligand reservoirs and consequently increased integrated growth signal. Furthermore, a mathematical model predicts increased persistence of cell-surface bound IL-2 in vivo for enhanced IL-2Rα-binding IL-2 mutants, suggesting potentially improved therapeutic value of allowing cellular capture of ligands in persistent cell-surface reservoirs. Finally,

our findings emphasize the critical choice of appropriate bioassays to evaluate engineered proteins and other drugs.

3.1 Introduction

Interleukin-2 is a potent immunomodulatory cytokine that acts on various immune cell types. IL-2 based therapies exploit the proliferation of antigen-activated T cells and CD56 bright NK cells caused by IL-2 (Fehniger et al., 2002), for treatment of metastatic renal cell carcinoma and melanoma (Atkins et al., 1999; Fyfe et al., 1995). When administered intravenously, IL-2 is rapidly cleared from the body. IL-2 serum concentrations are in the nanomolar range initially, and fall rapidly with a double exponential clearance rate with half-lives of 12.9 and 85 minutes respectively (Konrad et al., 1990). Thus it is difficult to maintain the therapeutically effective serum concentration range (1 – 100 pM) over a sustained period of time. This narrow therapeutic window of effective concentration coupled with rapid systemic clearance adversely affects IL-2 therapy.

The biological activity of IL-2 is mediated through the interaction of IL-2 with its multi-subunit receptor (Nelson and Willerford, 1998). The IL-2 receptor system consists of the alpha (IL-2Rα), beta (IL-2Rβ) and gamma (IL-2Rγ) receptor subunits. IL-2Rα is not involved in intracellular signaling, while IL-2Rβ and IL-2Rγ are necessary and sufficient to mediate intracellular signaling. IL-2 binds with a very high affinity (Kd = 10^{-11} M) to the trimeric IL-2Rαβγ complex, an intermediate affinity to IL-2Rβγ (Kd = 10^{-9} M) and low affinity to the IL-2Rα subunit (Kd = 10^{-8} M). Antigen-activated T cells and CD56 bright NK cells, which mediate the therapeutically relevant effects of IL-2, express all three receptor subunits and respond to picomolar concentrations of IL-2. However, at

nanomolar IL-2 concentrations, activation of the IL-2Rβγ on CD56 dim NK cells leads to toxicity (Fehniger et al., 2002).

We hypothesized that increasing affinity of IL-2 for IL-2Rα would be a useful strategy to construct IL-2 variants with potentially improved therapeutic properties. IL-2Rα is overexpressed on the surface of activated T cells (Smith, 1989; Theze et al., 1996) and has a long half-life (~48 hrs) on the cell surface (Hemar and Dautry-Varsat, 1990). An IL-2 mutant with increased affinity for IL-2Rα may bind to the surface of activated T cells for a longer time and hence remain in circulation, captured on T cells, for much longer than wild-type IL-2. Thus IL-2Rα on activated T cells may act as a reservoir for IL-2 in circulation, leading to a prolonged persistence of IL-2 signaling. This should enable reduced dosage and consequently lower toxicity. Yeast surface display and directed evolution have been previously used to generate IL-2 mutants with increased affinity for IL-2Rα (Rao et al., 2003). In this paper, we describe the bioassay evaluation of such mutants in a novel pulsed bioassay assay that more closely approximates the systemic clearance of IL-2 than conventional static bioassays. In this assay, a T cell line shows significantly greater proliferation in response to IL-2 mutants with higher affinity for IL-2Rα than wild-type IL-2 or a mutant with wild-type affinity for IL-2Rα. This result not only demonstrates the increased activity of IL-2 mutants with increased IL-2Rα affinity, but also emphasizes the pitfalls of false negatives and false positives arising from an inappropriate choice of assay for evaluating the engineered IL-2 variants. Conventional static bioassays at low picomolar concentrations suggested that increased affinity of IL-2 for IL-2Rα did not exhibit a significant effect on the activity of the IL-2 mutants relative to wild-type IL-2 (Rao et al., 2003). Also, a mutant with no change in

IL-2Rα affinity was implicated to have higher activity in the static assay, while the pulsed bioassay shows no change in activity for this mutant.

We also investigated the mechanism conferring increased activity to IL-2 mutants with increased affinity for IL-2Rα. Our results suggest that IL-2Rα on the T cell surface acts as a ligand reservoir for IL-2 and mediates this increased activity for the IL-2 mutants. Furthermore, a mathematical model predicts longer persistence of the IL-2 mutants with increased affinity for IL-2Rα on the surface of T cells in circulation, when administered as an intravenous (i.v.) bolus. This could conceivably lead to prolonged signaling from the IL-2 mutants with increased affinity for IL-2Rα, even at low dosages, suggesting potentially improved therapeutic value for these IL-2 mutants.

3.2 Materials and Methods

3.2.1 IL-2 mutants

For clarity, the term "Interleukin-2" will be used throughout to refer to wild-type Interleukin-2 and the Interleukin-2 mutants M6, M1, and C1. The wild-type IL-2 considered has a serine at position 125 (C125S – equivalent to Proleukin™). Yeast surface display and directed evolution were used to generate IL-2 mutants with increased binding affinity for IL-2Rα (Rao et al., 2003). We considered three mutants - M6 (V69A, Q74P, I128T), M1 (V69A, Q74P), and C1 (I128T). M6 and M1 have a fifteen-thirty fold increased affinity for IL-2Rα as compared to wild-type IL-2. Mutant C1 has wild-type affinity for IL-2Rα. The rationale behind the choice of these mutants for analysis has been previously detailed (Rao et al., 2003).

Wild-type IL-2 and the IL-2 mutants were expressed solubly in a yeast expression system, with an N-terminal FLAG epitope tag and a C-terminal c-myc epitope tag, as previously described (Rao et al., 2003).

3.2.2 Conventional Static Bioassay

Proliferation of an IL-2 dependent cell line was used as a read-out to evaluate the activity of the IL-2 mutants generated. KIT-225 is a human IL-2 dependent T-cell line, expressing roughly 3000-7000IL-2Rαβγ and 200000-300000 IL-2Rα (Arima et al., 1992; Hori et al., 1987). A frozen stock of KIT-225 cells was created using cells cultured in a humidified atmosphere with 5% CO_2, at 37°C, in RPMI 1640 supplemented with 1nM IL-2, 10% FBS, 200 mM L-glutamine, 50 units/mL penicillin and 50 μg/mL gentamycin. Subsequently, frozen aliquots were revived and cultured in medium containing 40 pM

IL-2. Prior to the bioassay, KIT-225 cells were cultured in medium without IL-2 for one day. Cells were then resuspended in medium containing wild-type IL-2 or IL-2 mutants at different concentrations at 10^5 cells/mL. Cell culture aliquots were taken at different times and the viable cell density was determined using the Cell-titer Glo™ (Promega) assay.

3.2.3 Pulse Bioassay

The pulse bioassay, where cells are exposed to IL-2 for a short period of time, was designed as the simplest approximation for systemic clearance of IL-2. KIT225 cells were starved in IL-2 free medium for one day. Cells were resuspended at 10^5 cells/mL in medium containing wild-type IL-2, mutants or a negative control (no IL-2). After 30 minutes of incubation in a humidified atmosphere with 5% CO_2, at 37°C, the cells were centrifuged (8 minutes at 4°C) and the medium containing IL-2 was removed. Cells were washed with medium without IL-2, at room temperature, and centrifuged again. The supernatant was discarded and the cells were resuspended in medium without IL-2. The cells were transferred to an incubator at 37°C containing a humidified atmosphere with 5% CO_2. Cell culture aliquots were taken at different times and the viable cell density was determined using the Cell-titer Glo™ (Promega) assay.

3.2.4 Persistence of IL-2 on surface of KIT225 cells

The cell-surface associated IL-2 during the course of the pulse assay was determined using flow cytometry. The protocol followed was exactly the same as described for the pulse assay. KIT225 cells were pulsed with M6 or wild-type IL-2, at a concentration of 2 nM. At different time points after the final re-suspension step in medium without IL-2,

cell culture aliquots were taken and centrifuged. Cells were resuspended in 100 μL ice-cold PBS, pH 7.4, containing 0.1% BSA and a biotinylated antibody against the FLAG epitope (Sigma). This was followed by incubation with streptavidin-phycoerythrin on ice (Molecular Probes). Cells were centrifuged, the supernatant discarded, and resuspended in ice-cold PBS, pH 7.4, containing 0.1% BSA. The mean single cell fluorescence was determined using an EPICS-XL flow cytometer. The cell-surface bound IL-2 may dissociate and re-bind after the final re-suspension step. To study this effect, soluble human IL-2Rα (R&D Biosystems) at a concentration of 1 nM was used as a capture reagent for any dissociated IL-2. This concentration represents a considerable excess of soluble IL-2Rα molecules over the total cell-surface bound IL-2Rα in the culture.

3.2.5 Mathematical model

A simple mathematical model was developed to describe the effect of increased affinity of IL-2 mutants for the IL-2Rα subunit, on persistence of IL-2 on the cell surface, in the context of systemic clearance of IL-2. The physical processes considered are

1) Systemic clearance of IL-2 from the body
2) Interaction of IL-2 with IL-2Rα on the cell surface
3) Endocytosis and degradation of IL-2Rα complexes

The differential equations governing the processes described are as follows:

$$\frac{d}{dt}[IL-2] = -A \cdot k_1 \cdot [IL-2] \cdot e^{-k_1 t} - B \cdot k_2 \cdot [IL-2] \cdot e^{-k_2 t} + (-k_{on} \cdot [IL-2] \cdot ([\alpha]_0 - [IL-2 \cdot \alpha]) + k_{off} \cdot [IL-2 \cdot \alpha] - k_{ec} \cdot [IL-2 \cdot \alpha]) \cdot n/N_{av} \quad \ldots (1)$$

$$\frac{d}{dt}[IL-2\cdot\alpha] = k_{on}\cdot[IL-2]\cdot[\alpha]_0 - [IL-2\cdot\alpha]\cdot(k_{on}\cdot[IL-2] + k_{off} + k_{ec}) \quad \ldots (2)$$

The terms used and the parameter values chosen are described in Table 3-1. The differential equations were solved using MATLAB.

The total number of IL-2Rα on the T-cell surface is assumed to remain constant. The number of activated T cells in circulation is assumed to be 10% of the total T cells in circulation and is an overestimate. This estimate is based on the CD25+ T cells in circulation. The number of activated T cells is used to calculate molar concentrations of cell-surface associated IL-2. Greater numbers of activated T cells would lead to an increased contribution of depletion of IL-2 through endocytosis of IL-2-IL-2Rα complexes. Thus we choose the number of activated T cells as 10% of the total number of T cells in circulation, as a conservative estimate.

Endocytic degradation through IL-2Rαβγ is not considered. This simplification arises primarily because there are far fewer (~100 fold lesser) IL-2Rαβγ than IL-2Rα, on the T-cell surface. The endocytic sink due to the IL-2-IL-2Rαβγ complexes under conditions of maximal endocytic degradation was calculated and found to be negligible at times less than 400 minutes. The details of this calculation are as follows:

All IL-2Rβ and IL-2Rγ subunits are assumed to be associated with the IL-2Rα subunit. All IL-2Rαβγ trimers are assumed to be associated with IL-2. Maximal endocytic degradation of IL-2-IL-2Rαβγ complexes will occur under these conditions. At steady state the endocytic rate should equal the rate of synthesis of IL-2Rαβγ. The rate of synthesis of IL-2Rαβγ can be estimated as ($V_R + k_{syn}C$) (Fallon and Lauffenburger, 2000)

Where

V_R is the constitutive rate of IL-2Rabg synthesis and is 11/min

k_{syn} is the induced rate of IL-2Rabg synthesis and is 0.0011/min

C is the total number of IL-2-IL-2Rαβγ complexes. A value of 3000 is used as the maximum estimate of IL-2-IL-2Rαβγ complexes

The maximal endocytic degradation rate is thus estimated as 14/min/cell. Considering 10^8 cells/liter, this translates to a decrease of 2 femtomolar/min, in serum and cell surface IL-2 concentration. After 400 min, this corresponds to a decrease in serum concentration of IL-2 by 0.8 pM. At this time, the serum concentration of IL-2 is ~ 10 pM. Thus the decrease in serum concentration of IL-2 due to endocytic degradation is negligible relative to systemic clearance of IL-2. Also, at times less than 400 min, inclusion of the endocytic degradation term in the model does not significantly alter the cell surface IL-2 concentration for wild-type IL-2 or the IL-2 mutants. Beyond this time, the maximal endocytic rate considered affects the cell surface IL-2 concentration for wild-type IL-2 and the IL-2 mutants. Incidentally, the recommended dosing regimen for Proleukin™ (wild-type IL-2) involves a 15 min. intravenous infusion every 8 hours (480 min).

3.3 Results

3.3.1 A pulse assay approximates renal clearance

We earlier reported the generation of mutants with increased affinity for IL-2Rα (Rao et al., 2003). Mutants M6 and M1 exhibit increased affinity for IL-2Rα while mutant C1 has wild-type IL-2Rα affinity. In static bioassays, in the 0.5-5 pM range of concentration, M6 and C1 were found to have slightly increased activity relative to wild-type IL-2. However, these concentrations represent severely ligand-depleting conditions since the total number of IL-2Rα present on cell surfaces in the culture is greater than the number of molecules of IL-2. To fully evaluate these mutants, we performed these assays under a wider range of concentrations. Figure 3-1A shows the viable cell density, in response to varying concentrations of wild-type IL-2 or the IL-2 mutants, assayed at 60 hours after IL-2 addition. Consistent with previous results, we find that M6 and C1 have increased activity relative to wild-type IL-2. M6 has an increased affinity for IL-2Rα, while C1 has wild-type affinity. Thus, the observed slight increase in activity of M6 and C1 in this bioassay is not attributable to increased affinity for IL-2Rα.

When administered as an i.v. bolus, IL-2 is rapidly cleared from the body, with half-lives of 12.9 and 85 minutes (Konrad et al., 1990). Because the static assay does not reflect this rapid clearance that occurs under physiological conditions, we designed a pulse assay to crudely approximate systemic clearance. KIT225 cells were exposed to IL-2 for a period of 30 minutes, then washed and resuspended in IL-2-free media, and the viable cell density measured as a function of time. When exposed to 1 nM concentration of cytokine for 30 minutes in the pulse assay, M6 and M1 showed significantly improved activity as

compared to wild-type IL-2. C1 and wild-type IL-2 showed similar activity. This is shown in Figure 3-1B.

We assayed the effect of varying the concentration of cytokine in the pulse assay on the viable cell density. Figure 3-1C is a snapshot of viable cell density as a function of pulse concentration, at 60 hrs after the cytokine pulse, while Figure 3-2 shows the effect viable cell density in response to varying concentrations of wild-type IL-2 or the IL-2 mutants, as a function of time. M6 and M1 show significantly higher activity than wild-type IL-2 and C1 over a broad pulse concentration range. Also, the level of proliferation obtained using less than 100 pM pulse of mutants with higher affinity for IL-2Rα (M6 and M1) cannot be achieved by using any concentration of C125S or C1. It is interesting to note that C1 and M6 show similar activity in a static assay (Figure 3-1A). A conventional static bioassay would implicate C1 as a more active IL-2 variant than wild-type IL-2 and M1. However C1 is likely to be ineffective in a physiological context, as suggested by a pulse assay that more closely approximates systemic clearance. Conversely, M1 does not show significantly improved activity in the static assay, but has similar activity as M6 in the pulse assay. Thus, inappropriate assays can clearly lead to both false negatives and false positives, demonstrating that the kinetic details of bioassay design are critical for effective evaluation of protein variants with potentially improved therapeutic properties.

3.3.2 IL-2Rα acts as a ligand-reservoir in a pulse assay

We hypothesized that M6 and M1 exhibit increased activity in a pulse bioassay due to increased persistence on the surface of KIT225 cells, with IL-2Rα acting as a reservoir for IL-2 and promoting proliferation. To test this hypothesis, we used flow cytometry to probe the cell-surface associated IL-2 as a function of time. As shown in figure 3-3A,

surface-associated wild-type IL-2 is negligible after the wash steps in the pulse bioassay. However, even after 6 hours, mutant M6 persists on the cell surface. The closed symbols in Figure 3-2B show the kinetics of persistence of M6 on the surface of KIT225 cells. Thus, the overexpressed IL-2Rα acts as a reservoir for IL-2 and mediates prolonged signaling.

After the final wash step, when the cells are resuspended in IL-2-free medium, the cell-surface bound IL-2 may dissociate and re-bind subsequently. We used soluble IL-2Rα as a reagent to capture any dissociated IL-2, to detect any re-binding occurring in the pulse assay. The cell surface associated M6 was probed using flow cytometry. As shown by the open symbols in Figure 3-3B, there is greater decrease in cell-surface bound IL-2, in the presence of the soluble IL-2Rα, indicating a certain amount of re-binding of M6 in the pulse assay. The key observation, however, is that M6 persists on the cell surface even in the presence of a capture agent IL-2Rα. In presence of soluble IL-2Rα, a rapid initial decrease in cell-surface IL-2 followed by a slower rate of decrease is observed. One explanation for these heterogeneous kinetics may be the presence of complexes with both IL-2Rα and IL-2Rαβ species on the cell surface. This aspect of the mechanism is currently under investigation. It should be pointed out that re-binding and equilibration could also occur in the physiological context of systemic clearance, as is evident from the mathematical model described below.

3.3.3 A mathematical model predicts potentially improved therapeutic value

IL-2Rα is overexpressed on the surface of antigen-activated T cells (Smith, 1989; Theze et al., 1996) and has a long half-life on the cell surface (Hemar and Dautry-Varsat, 1990). Conceivably, an IL-2 variant that bound to IL-2Rα with a slow dissociation rate

would remain in circulation, bound to IL-2Rα-overexpressing cells for a significantly longer period than wild-type IL-2, even as serum IL-2 undergoes rapid systemic clearance. We evaluated the effect of changing the dissociation rate of binding of IL-2 to IL-2Rα in the context of systemic clearance of IL-2 with a mathematical model. The model predicts significantly increased levels of cell-surface associated IL-2 and significantly increased persistence of cell-surface bound IL-2, with as little as a ten-fold decrease in the off-rate. Thus the model quantitatively confirms the concept of a cell surface ligand-reservoir effect mediated by IL-2Rα. Prolonged elevated cell-surface levels of IL-2 would lead to persistent signaling. A significantly reduced dose of the IL-2 mutants should therefore be sufficient to achieve the wild-type response, leading to reduced toxicity and hence potentially improved therapeutic value. The greatly increased activity of M6 and M1 in pulse assays is proof of concept for the improved therapeutic potential of the class of IL-2 mutants with increased affinity for IL-2Rα.

The predicted free serum concentration levels of IL-2 are not significantly different for the mutants with decreased dissociation rate of binding of IL-2 to IL-2Rα. However the local concentration of IL-2 at the cell surface is very different for these mutants, relative to wild-type IL-2. This is a conceptually distinct strategy compared to conjugation of polyethylene glycol (PEGylation) to proteins, to increase their serum half-life (Harris and Chess, 2003). PEGylation causes an increased serum concentration of cytokine, but not necessarily increased cell-surface bound cytokine levels, as these are governed by the affinity of the cytokine-receptor binding interaction. Furthermore, PEG-ylated IL-2 remains available for interaction with NK cells in the circulation, with

attendant side effects. By contrast, mutant IL-2 such as M1 or M6 are sequestered to the surfaces of those cells specifically targeted for stimulation.

3.4 Discussion

We have demonstrated here that IL-2 mutants with enhanced IL-2Rα binding affinity have significantly increased activity for proliferation of activated T cells overexpressing IL-2Rα, through a cell-surface ligand-reservoir effect. A novel pulsed bioassay was utilized to approximate the systemic clearance pharmacokinetics of IL-2. The IL-2 mutants exhibit increased activity in these assays, but not in conventional static bioassays. Thus our results emphasize the critical nature of the choice of appropriate bioassays to evaluate engineered protein variants.

We investigated the mechanism conferring increased activity to the IL-2 mutants with enhanced IL-2Rα affinity. Our findings suggest that the overexpressed IL-2Rα on the T cell surface acts as a ligand reservoir for the IL-2 mutants. This leads to increased cell-surface persistence of the IL-2 mutants and hence increased integrated growth signal. Our mathematical model predicts increased persistence of the IL-2 mutants in vivo, even at low dosage concentrations. This suggests potential therapeutic value for the IL-2 mutants. Also the lowered dosage concentration would help in the reducing the undesirable inflammatory responses associated with the existing dosage concentration of IL-2. The half-life of IL-2Rα on the antigen-activated T-cell surface is on the order of 48 hours (Hemar and Dautry-Varsat, 1990). In the pulse assay, the half-life of M6, an IL-2 mutant with 15-30 fold enhanced IL-2Rα affinity, on the surface of KIT225 cells is on the order of 4 hours. This suggests that further substantial improvements in activity may be

obtained by further decreasing the dissociation rate of interaction between IL-2 and IL-2Rα.

It is interesting to note the striking similarity of cell surface retention of M6 with interleukin-15 (IL-15) (Dubois et al., 2002). IL-2 and IL-15 share the IL-2Rβ and IL-2Rγ subunits while each have their own private alpha receptor subunit (Fehniger et al., 2002). IL-15 has a high affinity for its private alpha receptor subunit, unlike wild-type IL-2. In assays where the cytokine is withdrawn from the medium, IL-15 persists on the cell surface for a long period of time, through association with IL-15Rα, and mediates prolonged signaling (Dubois et al., 2002). M6, with increased affinity for the private IL-2Rα subunit exhibits an increased persistence on the cell surface in similar assays. Thus, our results support a possible role for the high affinity IL-15Rα as a capture reagent to generate a ligand-reservoir, supporting previous work in this area.

The "cell surface reservoir" concept may be broadly applicable to numerous other cytokine receptor systems with multi-subunit receptors, to generate super-agonists or super-antagonists. Examples include interleukin-3 (IL-3), interleukin-5 (IL-5) and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF). IL-3, IL-5 and GM-CSF use private alpha receptor subunits and a common beta subunit that is implicated in most of the signaling associated with these cytokines (Guthridge et al., 1998). Cytokine variants with increased affinity for their private alpha subunits would conceivably increase persistence of these cytokines in circulation and hence lead to improved cytokine super-agonists (through persistent signaling) or super-antagonists (through persistent blocking of signaling). Thus cytokine variants with potentially improved therapeutic effectiveness may be generated by this approach. However, as stated earlier, it is very

important to use an appropriate in vitro bioassay to evaluate the activity of the cytokine variants generated.

Table 3-1: Description of terms used and parameter values chosen for the mathematical model

Term	Description	Value	Reference
[IL-2]	Serum concentration of IL-2 (molar units)	-	-
[IL-2•α]	IL-2 in complex with IL-2Rα (molar units)	-	-
$[\alpha]_0$	Total IL-2Rα on T cell surface	100,000/cell	
t	Time (min)	-	-
A	Magnitude of fast component of double exponential clearance	0.866	(Konrad et al., 1990)
k_1	Rate constant for fast component of double exponential clearance	0.0537 min^{-1}	(Konrad et al., 1990)
B	Magnitude of slow component of double exponential clearance	0.134	(Konrad et al., 1990)
k_2	Rate constant for slow component of double exponential clearance	0.00815 min^{-1}	(Konrad et al., 1990)
k_{on}	Association rate constant for IL-2-IL-2Rα interaction (wild-type)	6e8 M^{-1} min^{-1}	(Liparoto et al., 2002)

k_{off}	Dissociation rate constant for IL-2-IL-2Rα interaction (wild-type)	18 min^{-1}	(Liparoto et al., 2002)
k_{ec}	Rate constant for endocytosis and degradation of IL-2/IL-2Rα complex	0.00024 min^{-1}	(Hemar and Dautry-Varsat, 1990)
n	Total number of activated T cells in circulation	10^8 L^{-1}	(Hodge et al., 2000; Storek et al., 2000)
N_{av}	Avogadro number	6.023e23	

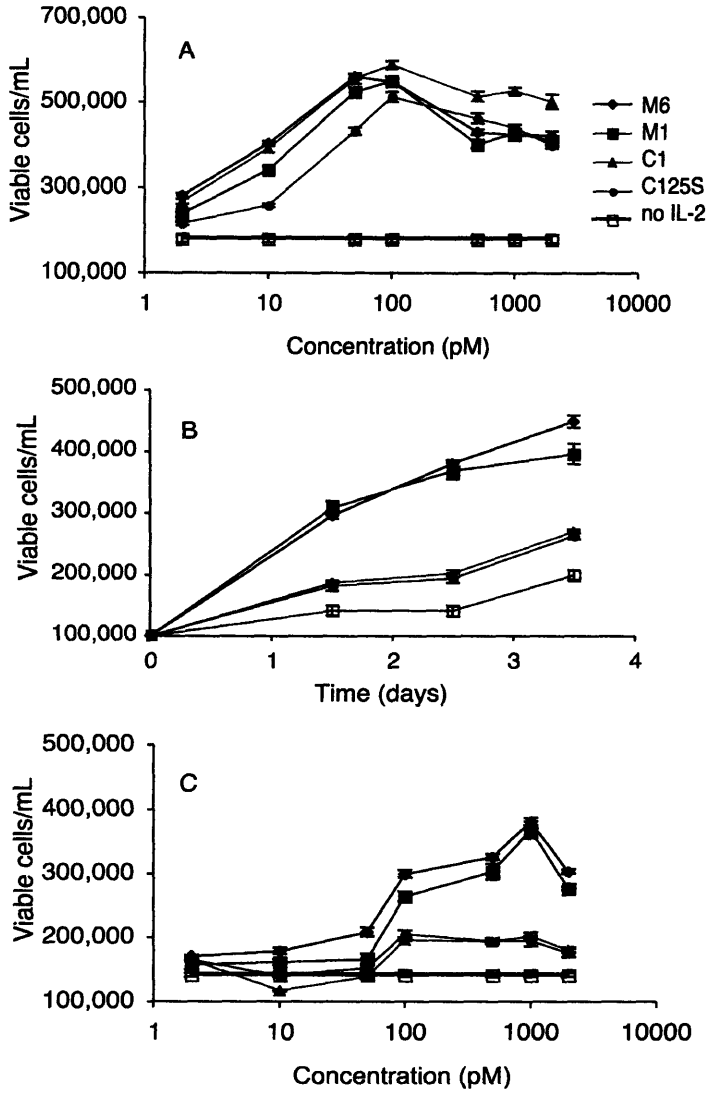

Figure 3-1: IL-2 mutants with higher affinity for IL-2Rα exhibit greatly increased activity in a pulse assay, but not in a conventional steady-state proliferation assay

Error bars indicate standard deviation of triplicate measurements. Data shown is representative of three separate experiments.

(A) Viable cell density as a function of IL-2 concentration, in a steady-state assay, after 60 hrs.

(B) Viable cell density as a function of time, in a pulse-assay with an initial pulse concentration of 1 nM IL-2.

(C) Viable cell density as a function of IL-2 concentration, in a pulse-assay, after 60 hrs.

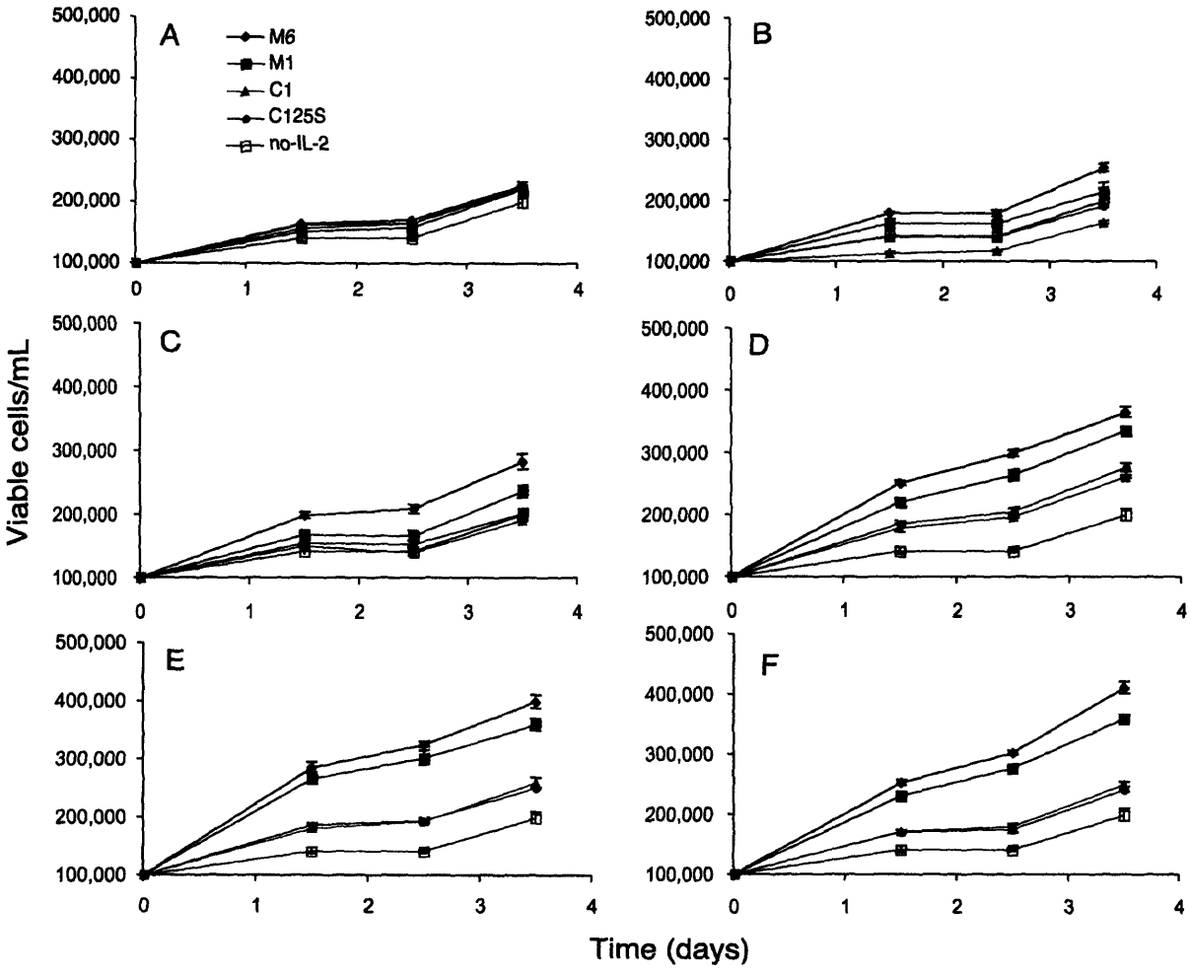

Figure 3-2: Viable cell density as a function of time, in a pulse assay, with varying initial pulse concentrations of IL-2.

The pulse concentrations are as follows: A – 2 pM, B – 10 pM, C – 50 pM, D – 100 pM, E – 500 pM, F – 2000 pM.

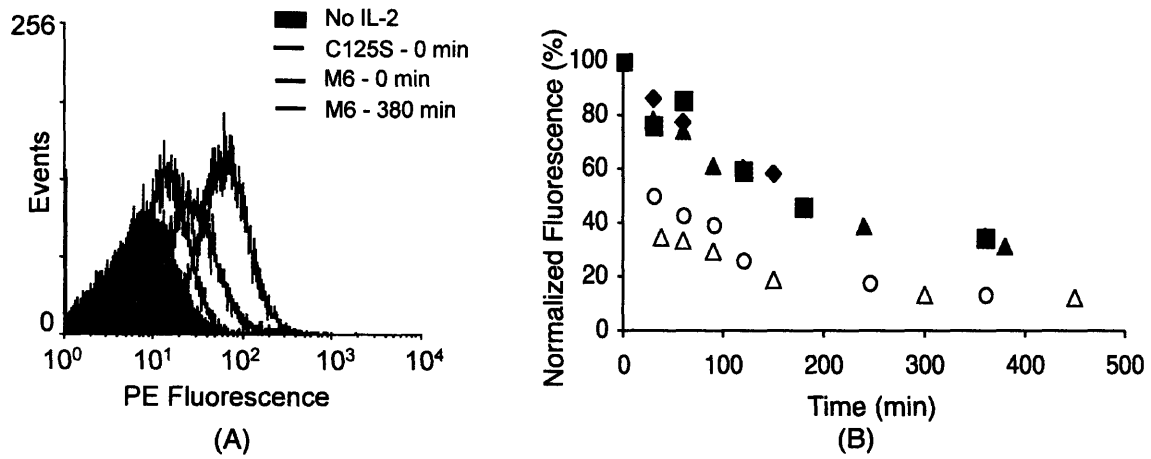

Figure 3-3: IL-2Rα acts as a ligand-reservoir and mediates prolonged persistence of M6 on the cell surface of KIT-225 cells relative to wild-type IL-2 (C125S)

PE fluorescence is a measure of cell-surface bound IL-2. Data shown is a representative set of histograms for three different experiments

Different colored closed symbols denote data from different experiments where no capture reagent is present. Different colored open symbols denote data from different experiments where soluble IL-2Rα was used as a capture reagent for dissociated M6.

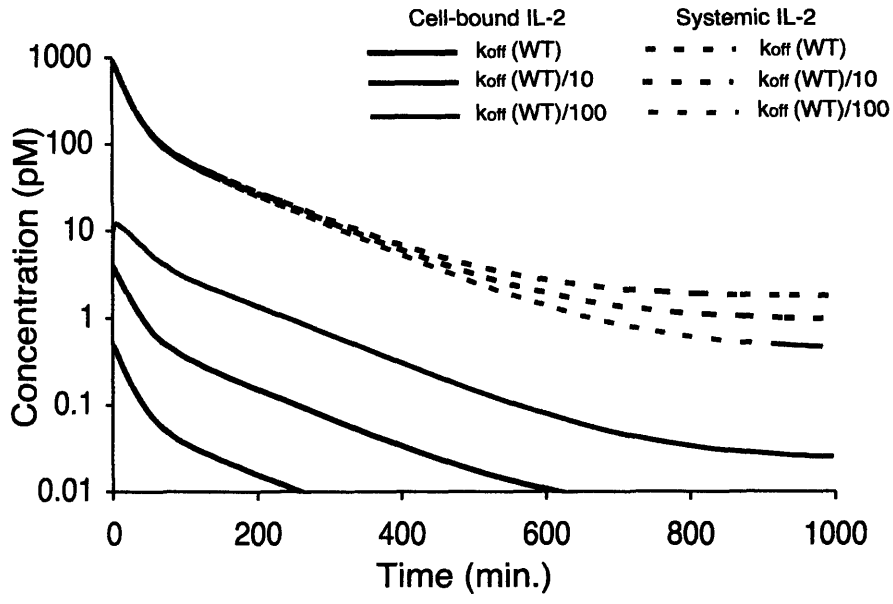

Figure 3-4: Model for persistence of IL-2 mutants with decreased off-rate for IL-2Rα on activated T cells, in blood circulation.

Decreased off-rate of IL-2 mutants for IL-2Rα results in greater levels and longer persistence of IL-2 on activated T cells in blood circulation.

Chapter 4: IL-2 MUTANTS WITH PICOMOLAR IL-2Rα BINDING AFFINITY STIMULATE GROWTH RESPONSES QUANTITATIVELY EQUIVALENT TO IL-15

Several alternative mechanisms have been proposed to explain the contrasting effects of IL-2 and IL-15 on T cell proliferation and death. We have used directed evolution to construct IL-2 mutants that bind IL-2Rα with affinities comparable to the IL-15/IL-15Rα interaction. These IL-2 mutants exhibit T cell growth response/receptor occupancy curves indistinguishable from that for IL-15, suggesting that much of the difference between the IL-2 and IL-15 effects arise simply from their 1,000-fold differing affinities for their private alpha receptor subunits. T cells proliferate for up to six days following a 30-minute incubation with these IL-2 mutants, which may lead to potential applications for cancer & viral immunotherapy.

4.1 Introduction

Interleukin-2 (IL-2) and Interleukin-15 (IL-15) can exert qualitatively differing effects on T cells (Fehniger et al., 2002; Waldmann, 2002; Waldmann et al., 2001) - for example, IL-2 promotes Activation Induced Cell Death (AICD) while IL-15 inhibits AICD. Transgenic mice lacking the IL-2 alpha receptor subunit (IL-2Rα) exhibit T cell expansion and autoimmune disease (Willerford et al., 1995), while IL-15 alpha receptor subunit (IL-15Rα) null transgenic mice have reduced numbers of $CD8^+$ T cells and other lymphocytes **(Lodolce et al., 1998)**. However, paradoxically, both IL-2 and IL-15 signal through the same IL-2Rβ and IL-2Rγ receptor subunits. The mechanisms leading to different biological effects of IL-2 and IL-15 are poorly understood. IL-2 and IL-15

mediate their biological response through a trimeric receptor consisting of shared beta and gamma subunits and a private alpha receptor subunit (Fehniger and Caligiuri, 2001; Nelson and Willerford, 1998). IL-15Rα is expressed in a broad range of cells and tissues, by contrast with IL-2Rα, whose overexpression is in general limited to activated mononuclear cells (Kobayashi et al., 2000). For cells simultaneously expressing the alpha receptor subunit for both IL-2 and IL-15, there are marked differences in the growth response to IL-2 and IL-15 (Dubois et al., 2002). Evidence from fluorescence resonant energy transfer (Damjanovich et al., 1997) and antibodies binding IL-2Rβ at different epitopes (Lehours et al., 2000) suggest that the IL-15/IL-2Rβ/IL-2Rγ complex differs topologically from IL-2/IL-2Rβ/IL-2Rγ, and consequently may trigger qualitatively different signals. It has also been proposed that the small cytoplasmic portion of IL-15Rα may be involved in signaling, by contrast with IL-2Rα (Bulanova et al., 2001). Further evidence for qualitative differences in signaling between IL-2 and IL-15 is that IL-15-mediated proliferation is inhibited to a greater extent than IL-2 by rapamycin inhibition of FKBP (Dubois et al., 2003).

We hypothesize that a major contributor to differences between IL-2 and IL-15 is simply altered cell-surface receptor occupancy, driven by differing alpha subunit binding affinities. Both IL-2 and IL-15 bind the IL-2Rβγ heterodimeric receptor with similar affinity (Kd ~ 1 nM) and can signal through IL-2/15Rβγ in the absence of the private alpha receptor subunit. However, they differ starkly in their binding to their private alpha receptor subunits. IL-15 has a very high binding affinity for IL-15Rα (Kd ~10 pM), while IL-2 binds with low affinity to IL-2Rα (Kd ~ 10 nM) (Fehniger and Caligiuri, 2001). This leads to prolonged persistence of IL-15 but not IL-2 on the surface of T cells, in *in*

vitro assays where cytokine is withdrawn from the medium (Dubois et al., 2002). Here we have generated IL-2 mutants with picomolar IL-2Rα binding affinity by directed evolution, and find that the T cell proliferative response to these mutant IL-2 species is essentially the same as responses to equivalent receptor occupancies for IL-15. These results support the hypothesis that IL-2Rβ/IL-2Rγ receptor occupancy is a primary determinant of growth responses for both IL-2 and IL-15.

4.2 Results and Discussion

IL-2 mutants with a range of affinities for IL-2Rα that approach the affinity of IL-15 for IL-15Rα (Table 4-1, Figure 4-1) were generated using yeast surface display and directed evolution (Rao et al., 2003). We compared the bioactivity of high IL-2Rα affinity IL-2 mutants with IL-15 in a T cell line (F15R-Kit) expressing both IL-2Rα and IL-15Rα subunits, in addition to IL-2Rβ and IL-2Rγ. Pulse bioassays, where cells are incubated in cytokine for only a short period of time, were used to mimic the transient cytokine exposure history typical of bolus pharmacokinetics (Rao et al., 2004). Our objective was to quantitatively analyze the relationships between receptor occupancy and proliferation response for IL-2, IL-15, and a series of IL-2 mutants bridging the three-order-of-magnitude gap between them in alpha receptor subunit binding affinity.

In pulse assays, IL-15 persists on the cell surface for over two days, while wild-type IL-2 has negligible persistence on the cell surface (Figure 4-2A), consistent with previous results (Dubois et al., 2002). IL-2 mutants with increased IL-2Rα affinity however, have greatly increased persistence on the cell surface relative to wild-type IL-2 (Figure 4-2B, C, D), since IL-2Rα acts as a ligand reservoir for high IL-2Rα affinity IL-2 mutants.

Artifactual binding of these IL-2 mutants to IL-15Rα was excluded by the observation that excess IL-15 does not diminish surface labeling by these mutants (data not shown). Increased initial private alpha receptor occupancy after cytokine withdrawal leads to increased cell surface persistence of cytokine over the course of several days (Figure 4-3). The increased persistence of high IL-2Rα affinity IL-2 mutants is likely a result of both decreased dissociation of the mutants from cell surface IL-2Rα and more efficient recycling of the mutants to the cell surface following internalization, since high IL-2Rα affinity IL-2 mutants also have improved binding at endosomal pH (data not shown) and conceivably recycle to a greater extent than wild-type IL-2 (Fallon et al., 2000). For a given initial receptor occupancy, IL-15 exhibits somewhat greater persistence than the IL-2 mutants (Figure 4-2, 4-3). This may be due to a significantly lesser sensitivity of the IL-15/IL-15Rα binding interaction to lowered pH relative to the binding of IL-2 mutants to IL-2Rα , and consequently more efficient endocytic recycling to the surface following internalization (Figure 4-4).

We compared the T cell growth response mediated by IL-15 and high IL-2Rα affinity IL-2 mutants in a pulse assay to explore the relationship between cell surface persistence and bioactivity. Consistent with previous observations (Dubois et al., 2002), IL-15 promotes the growth of T cells in a pulse assay, unlike wild-type IL-2 (Figure 4-5). An insignificant level of wild-type IL-2 is associated with IL-2Rα on the cell-surface following pulse exposure, whereas IL-15 persists in IL-15Rα ligand reservoirs on the cell surface for a prolonged period of time (Figure 4-2A). Mutants with increasing IL-2Rα binding affinity exhibit prolonged cell surface persistence (Figure 4-2B-D) and also stimulate increased growth of T cells (Figure 4-5). Picomolar concentrations of several

IL-2 mutants stimulate T cell growth for up to six days following a 30-minute pulse exposure, while even nanomolar pulse concentrations of wild-type IL-2 fail to produce any net proliferation.

The relationship between T cell growth response and initial receptor occupancy after cytokine withdrawal was quantitatively examined (Figure 4-6A,B). Maximum viable cell density attained and the integral of viable cell number over a ten-day period were used as metrics to quantify the family of growth response curves shown in Figure 4-5. The integral of cell viability as well as the maximum cell number correlate linearly with the number of interleukin molecules initially captured in cell surface receptor reservoirs (Figure 4-6A, B), demonstrating the existence of a single pharmacodynamic dose-response curve relating growth to initial receptor occupancy for IL-2, IL-15, and all of the six IL-2 mutants.

IL-2 induced T cell growth is consistent with a quantal signal transduction hypothesis (Smith, 1989), by which once the signal mediated through IL-2R$\alpha\beta\gamma$ exceeds a certain threshold, growth occurs at a specific growth rate independent of the magnitude of the growth signal. The concept of threshold signaling complex levels governing cell fate decisions has additionally found applicability in stem cell biology more broadly (Zandstra et al., 2000). For an IL-2 (or IL-15) dependent T cell line such as F15R-Kit, a similar threshold response also converts an actively growing T cell culture to a death phase once the IL-2R$\alpha\beta\gamma$ signal is depleted below a certain level. In order to compare the responses to different mutants and different pulse concentrations on a common basis, each growth curve was normalized by its maximum viable cell density (Figure 4-7) and then aligned on the time axis such that the peak cell number occur at an approximately common time

(Figure 4-8). All of the IL-2 wild-type and mutant growth curves from Figure 4-5 map by this procedure onto a single universal growth curve, indicating that the specific growth and death rates are indeed approximately constant (Figure 4-6C). Consequently, the amount of time spent in the growth phase is sufficient to completely characterize the growth curves in Figure 4-5. In other words, the growth curves in Figure 2 can be reconstructed from the universal growth curve (Figure 4-6C) once the initial time (time of cytokine withdrawal) is mapped on to the universal growth curve by aligning the time for transition from the growth phase to the death phase, presumed to occur upon depletion of IL-2Rαβγ signal below a threshold signal value. This time shift correlates strongly with initial receptor occupancy, with growth maintained for up to six days at the highest occupancies achieved with the IL-2 mutants (Figure 4-9). The growth kinetics of IL-15 are also described by a universal growth curve (Figure 4-6D), although with somewhat slower death rates than the IL-2 mutants, possibly due to prolonged signaling due to increased persistence of IL-15 relative to any of the IL-2 mutants (Figure 4-2A vs. 4-2B-D). The very similar universal growth curves for IL-2 and IL-15 (Figure 4-6C,D) further reinforce the functional equivalence of the pharmacodynamic proliferative response mediated by IL-15 and the high IL-2Rα affinity IL-2 mutants at equivalent receptor occupancies.

We establish here a quantitative relationship between receptor alpha subunit binding affinity and mitogenic activity for IL-2 and IL-15. Cell surface IL-15Rα, with high affinity for IL-15, acts as a capture reagent for IL-15 that retains IL-15 on the cell surface for a prolonged period of time and mediates growth of T cells expressing IL-15Rα (Dubois et al., 2002). IL-2Rα acts as a similar cell surface ligand reservoir for IL-2

mutants with increased affinity for IL-2Rα. Our results strongly suggest that a primary difference in biological activity between wild-type IL-2 and IL-15 results from their different affinities for their respective private alpha receptor subunit.

Lymphocyte homeostasis is maintained in vivo by a balance between signals from lymphotrophic survival factors and apoptotic death pathways (Khaled and Durum, 2002). Numerous cytokines with central roles in controlling the number of cells in the lymphoid compartment signal through the common gamma (γ_c) subunit of the IL-2R: IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, indicating a broad and central role for the growth and anti-apoptotic signal provided by IL-2Rγ_c. It has been hypothesized that differences in the effects of IL-4 and IL-7 relative to IL-2 and IL-15 are a result of altered receptor expression on different cell types, rather than qualitatively distinct intracellular signaling pathways (Marrack and Kappler, 2004), and our results are consistent with this view. The general approach of creating a cell-bound ligand reservoir might enable engineering of mutant cytokines with enhanced potency for GM-CSF, IL-3, and IL-5 (Guthridge et al., 1998) , since they also possess non-signaling alpha capture receptor subunits.

The IL-15-like behavior of high IL-2Rα affinity IL-2 mutants has potentially significant implications for IL-2 based cancer immunotherapy. IL-2 is FDA-approved for the treatment of metastatic renal cell carcinoma and melanoma (Atkins et al., 1999; Fyfe et al., 1995). At nanomolar concentrations, wild-type IL-2 mediates deleterious toxic effects, through IL-2Rβγ on CD56 dim NK cells (Fehniger et al., 2002). The increased potency of high IL-2Rα affinity IL-2 mutants relative to wild-type IL-2 should allow the use of reduced doses, with consequently lower toxicity. The use of IL-15 to replace IL-2 in cancer immunotherapy has been suggested due to the inhibitory effects of IL-15 on

AICD (Waldmann et al., 2001), however the broad tissue distribution of IL-15Rα might conceivably lead to detrimental side effects (Fehniger and Caligiuri, 2001). IL-2Rα is expressed by activated T cells, and so high IL-2Rα affinity IL-2 mutants should mediate a biological response selectively in these desired target cells. Thus the IL-2 mutants described here may combine the specificity of wild-type IL-2 with the desired immunotherapeutic effects of IL-15.

4.3 Materials and Methods

4.3.1 Generation of IL-2 mutants

Human IL-2 with a cysteine to serine mutation at position 125 (C125S) was considered as wild-type IL-2 (equivalent to Proleukin™). A previously described protocol (Rao et al., 2003) was used to express wild-type IL-2 as an Aga2p protein fusion in the EBY100 strain of yeast *Saccharomyces cerevesiae*. A library of approximately 10^7 yeast-displayed IL-2 mutants (termed "library A") was generated as previously described (Rao et al., 2003).

A soluble, biotinylated form of the ectodomain of human IL-2Rα was a kind gift of Dr. T. L. Ciardelli (Dartmouth Medical School, Hanover, NH). An equilibrium binding screen, as previously described (Rao et al., 2003), was used to isolate IL-2 mutants with increased binding affinity for soluble IL-2Rα. WC9 and WE3 are two of the clones with improved IL-2Rα binding affinity isolated after two rounds of sorting by flow cytometry.

After one round of sorting library A, DNA from the pool of mutants isolated was extracted using the Zymoprep kit (Zymo Research Corporation). The mutant IL-2 coding sequences in this pool of DNA were amplified by PCR (termed "reaction 1").

Concurrently, the mutant IL-2 coding sequences were subjected to random mutagenesis by error-prone PCR in the presence of nucleotide analogs 8-oxodGTP and dPTP to control mutagenesis rates, as previously described (Rao et al., 2003). The product obtained in this mutagenic PCR was amplified further by PCR (termed "reaction 2") in the absence of the nucleotide analogs. The products from the reactions 1 and 2 were mixed in a 1:1 ratio and DNA shuffling (Stemmer, 1994) was carried out on the combined pool of DNA. The final PCR product after DNA shuffling was used to generate a library of approximately $1.5x10^8$ yeast-displayed IL-2 mutants (termed "library B"), using the protocol described previously (Rao et al., 2003). IL-2 mutants with decreased dissociation rate of binding (off-rate) to soluble IL-2Rα were isolated from library B using a kinetic screen (Boder and Wittrup, 2000) with wild-type IL-2 (Chiron) as soluble competitor. 1a-1, 2-4 and 1b-8 are three of the clones with slower IL-2Rα binding off-rate isolated after four rounds of sorting by flow cytometry.

WC9, WE3, 1a-1, 2-4, 1b-8 and M6, a previously reported IL-2 mutant (Rao et al., 2004; Rao et al., 2003), were chosen in particular for detailed evaluation since they have a range of IL-2Rα binding affinities that bridge the three-order-of-magnitude gap in private alpha receptor subunit binding affinity between wild-type IL-2 and IL-15. Wild-type IL-2 and the IL-2 mutants were expressed solubly in yeast, with an N-terminal FLAG epitope tag and a C-terminal c-myc epitope tag. FLAG immunoaffinity chromatography (Sigma) followed by size exclusion chromatography on a Superdex™ 200 column (Pharmacia Biotech) was used to purify the proteins. The protein concentrations were measured using the Micro BCA Protein Assay (Pierce).

4.3.2 Tissue Culture

F15R-Kit cells were a kind gift of Dr. T. A. Waldmann (National Cancer Institute, NIH, MD). F15R-Kit is an IL-2 dependent human T cell line with stable overexpression of human IL-15Rα and IL-2Rα (Arima et al., 1992; Dubois et al., 2002; Hori et al., 1987). These cells also express IL-2Rβ and IL-2Rγ (less than 10,000 copies per cell), though at much lesser levels than IL-2Rα and IL-15Rα (~ 10^5-10^6 copies per cell). The IL-15Rα expressed in F15R-Kit cells has an extracellular N-terminal FLAG epitope tag. Cells were cultured in a humidified atmosphere with 5% CO_2, in RPMI 1640 supplemented with 0.5 nM IL-2, 10% FBS, 1 mM sodium pyruvate, 200 mM L-glutamine and 0.8 g/L geneticin. Prior to any assay, cells were cultured in medium without IL-2 for one day.

4.3.3 Quantifying alpha receptor number on cells

Cell surface IL-2Rα was detected using a mouse monoclonal antibody against human IL-2Rα conjugated with R-Phycoerythrin (PE) (Pharmingen). Cell surface IL-15Rα has an N-terminal FLAG epitope tag and was detected by immunofluorescent staining with a mouse monoclonal antibody M2 (Sigma) against the FLAG epitope along with a goat anti-mouse antibody conjugated with PE (Molecular Probes/Sigma). Cell surface bound IL-15 was detected using a mouse monoclonal antibody MAB247 (R&D Biosystems) against IL-15 along with a goat anti-mouse antibody conjugated with PE. Quantum Simply Cellular™ microbeads (Sigma) were used to quantify the total number of IL-2Rα, IL-15Rα or cell surface bound IL-15 molecules on F15R-Kit cells, using the manufacturer's protocol. Briefly, the microbeads and the cells were subjected to identical immunofluorescent staining using antibodies to detect IL-2Rα, IL-15Rα or cell surface bound IL-15. The median fluorescence values of distinct microbead populations that bind

to known, incremental amounts of mouse immunoglobulin were used to generate a standard curve. The median fluorescence value of the cells labeled to detect IL-2Rα, IL-15Rα or cell surface bound IL-15 was used with the appropriate standard curve to determine the number of cell surface alpha receptor or bound IL-15 in antigen binding capacity (ABC) units.

4.3.4 Binding Assays

F15R-Kit cells were labeled (~ 100,000 cells/mL) with varying concentrations of soluble wild-type IL-2, IL-2 mutants or IL-15 (R&D Biosystems) for 30 minutes at 37 °C and pH 7.4. It should be noted that because the cells are metabolically active under these conditions, endocytic trafficking could perturb the estimate of the binding constant K_d; however, this procedure provides a more accurate estimate for cell-surface interleukin levels in the proliferation experiments performed at 37°C than titrations on ice. The cells were subsequently labeled with a polyclonal anti-c-myc chicken antibody (Molecular Probes) followed by a goat-anti-chicken antibody conjugated with Alexa dye (Molecular Probes) on ice, to detect cell-surface-bound wild-type or mutant IL-2. Alternately, the cells were labeled with mouse monoclonal antibody MAB247 against IL-15 followed by a goat-anti-mouse antibody conjugated with PE on ice, to detect cell-surface-bound IL-15. The median single cell fluorescence was determined using an EPICS-XL flow cytometer. Due to the vast overexpression of IL-2Rα and IL-15Rα (~ 10^5-10^6 copies per cell) relative to IL-2Rβ and IL-2Rγ (less than 10,000 copies per cell) (Arima et al., 1992; Hori et al., 1987) on F15R-Kit cells, the fluorescent signal from cell-surface bound cytokine can be assumed to be entirely due to binding to the alpha receptor subunit.

4.3.5 Estimation of K_d

Equilibrium dissociation constants for binding of wild-type and mutant IL-2 and IL-15 to their respective private alpha receptor subunit were determined using the equation:

$$F_{obs} = \frac{c \cdot L_0}{K_d + L_0}$$

Where:

F_{obs} = Observed background-corrected median fluorescence

L_0 = Concentration of wild-type or mutant IL-2 or IL-15 used

c = proportionality constant

Data from two separate experiments were used to determine the K_d values for wild-type and mutant IL-2 and IL-15. For each protein, one global K_d value and a proportionality constant c for each experiment were used in a global data fit procedure. The maximum median fluorescence observed at saturating concentrations of wild-type or mutant IL-2, corresponding to saturation of all cell surface IL-2Rα, should be the same. Therefore, the value of c was constrained to be the same for wild-type IL-2 and the IL-2 mutants for each experiment. Even though the complete binding isotherm is not captured in the concentration range considered for wild-type IL-2 and some of the IL-2 mutants, the constraint on c allows a K_d value to be estimated for each IL-2 species. 66% confidence intervals were determined using the procedure described in (Lakowicz, 1999).

4.3.6 Persistence Assays

A pulse bioassay, as previously described (Rao et al., 2004), was carried out on F15R-Kit cells with a 100 pM pulse concentration of wild-type IL-2, IL-2 mutants or IL-15. Briefly, cells were exposed to a 30-minute pulse of cytokine and then washed with and resuspended in cytokine-free medium. At different time points after cytokine withdrawal, cell-surface associated ligand was determined using immunofluorescent staining (as described earlier) and flow cytometry.

4.3.7 Bioassays

A pulse bioassay is the simplest approximation for bolus pharmacokinetics. Pulse bioassays, as previously described (Rao et al., 2004), were carried out on F15R-Kit cells with varying pulse concentrations of wild-type IL-2, IL-2 mutants or IL-15. The viable cell density was determined over a ten-day period using the Cell-titer Glo™ (Promega) assay. IL-2Rα and IL-15Rα numbers on the cell surface were determined for the cells used in the pulse bioassay.

Table 1: IL-2 mutants with increased IL-2Rα binding affinity.

F15R-Kit cells expressing IL-2Rα and IL-15Rα were labeled with wild-type IL-2, IL-2 mutants or IL-15 for 30 minutes at 37°C and pH 7.4. Cell surface bound protein was measured using flow cytometry. Data from two different experiments (Figure 4-1) were used to estimate K_d values as described (materials and methods).

Protein	Mutations	Kd (pM)	66% Confidence Intervals
C125S (WT)	-	30030	ND
WC9	S4P, T10A, Q11R, V69A, Q74P, N88D, T133A	1585	950-2700
M6	V69A, Q74P, I128T	1215	740-2000
WE3	N30S, V69A, Q74P, I128T	778	400-1270
1b-8	K8R, Q13R, N26D, N30T, K35R, T37R, V69A, Q74P, I92T	409	230-690
1a-1	N30S, E68D, V69A, N71A, Q74P, S75P, K76R, N90H	254	150-420
2-4	N29S, Y31H, K35R, T37A, K48E, V69A, N71R, Q74P, N88D, I89V	180	110-300
IL-15	-	76	52-110

ND – not determined

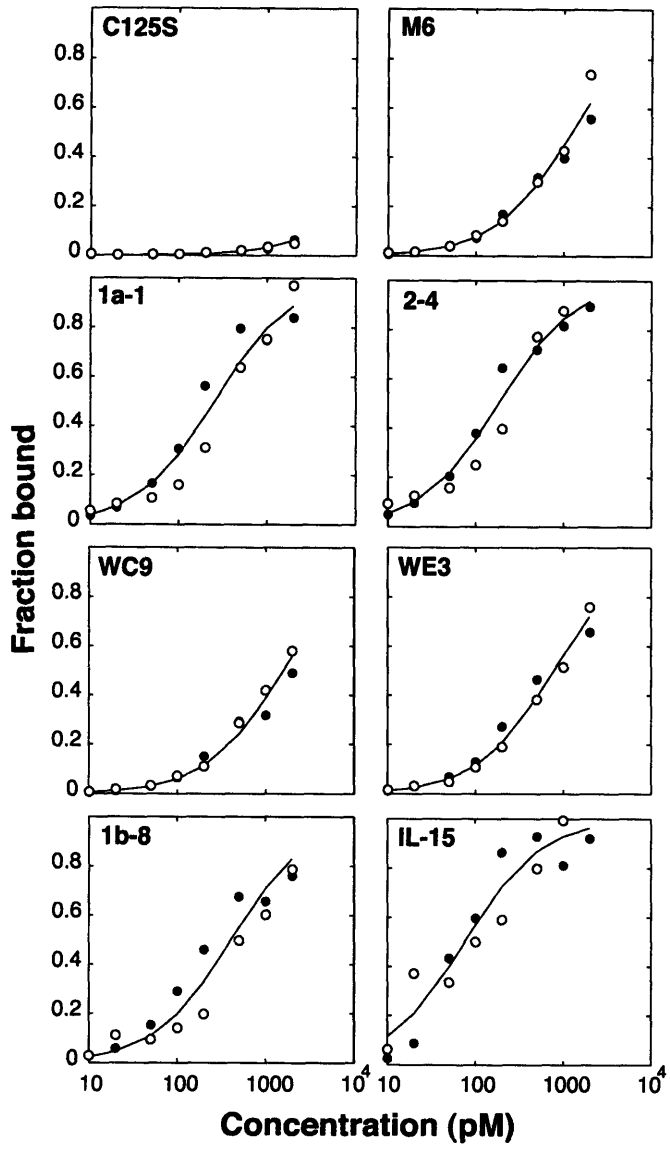

Figure 4-1: Binding of wild-type and mutant IL-2 to IL-2Rα and IL-15 and IL-15Rα
Different symbols denote different data sets. Solid lines denote the estimated bound fraction using the best-fit value of K_d. The K_d values estimated reasonably predict the fraction of alpha receptors bound at a given concentration of wild-type or mutant IL-2 or IL-15.

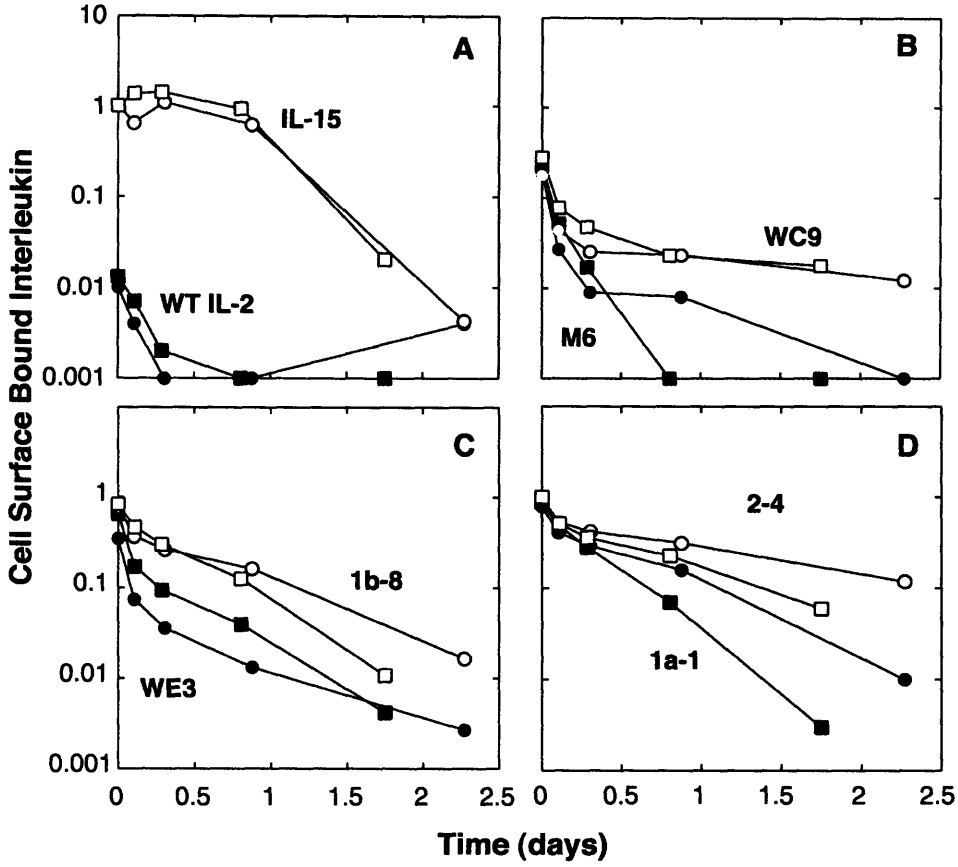

Figure 4-2: Cell surface persistence of wild-type IL-2, IL-2 mutants and IL-15
F15R-Kit cells (~100,000 cells/mL) expressing IL-2Rα and IL-15Rα were labeled with 100 pM wild-type IL-2, IL-2 mutants or IL-15 for 30 minutes at 37°C and pH 7.4. Cells were then washed with and resuspended in cytokine-free medium. Cell surface bound protein was measured using flow cytometry at different time points following cytokine withdrawal. The initial (time zero) value corresponds to the cell-surface bound ligand before the wash step. The median fluorescence value for 2-4 at time zero was used to normalize the data for wild-type IL-2 and IL-2 mutants for each data set. The median fluorescence value for IL-15 at time zero was used to normalize data for IL-15, for the data set denoted by open circles. For the IL-15 data set denoted by open squares, the K_d value for IL-15 (Table 4-1) and the experimentally determined total number of IL-15Rα were used to estimate the number of IL-15-bound IL-15Rα at time zero. The number of IL-15-bound IL-15Rα on the cell surface was directly determined for subsequent time points. The number of IL-15-bound IL-15Rα at time zero was used to normalize this data set. Normalized values less than 0.001 are plotted as 0.001.

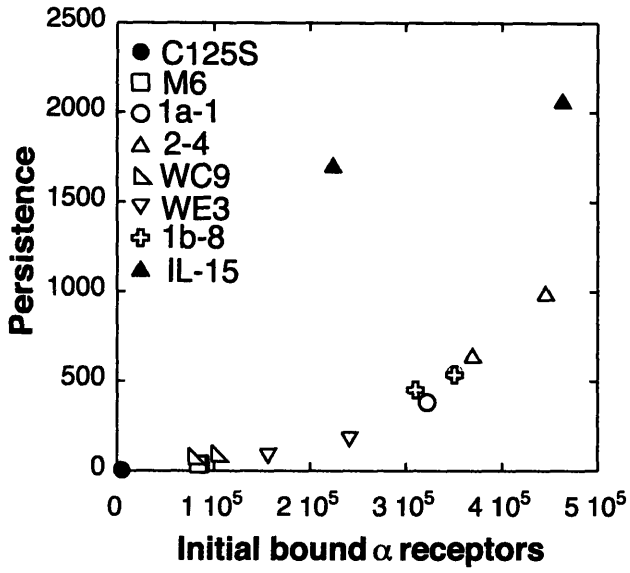

Figure 4-3: Persistence on cell surface correlates strongly with initial receptor occupancy

The area under the curve of a plot of cell-surface-bound interleukin versus time (Figure 4-2) serves as a quantitative measure of the exposure history of the cells to surface bound ligand i.e. persistence and was estimated using trapezoidal rule. The K_d values for 2-4 and IL-15 and total numbers of cell surface IL-2Rα or IL-15Rα were used to estimate the initial number of 2-4-bound IL-2Rα and IL-15-bound IL-15Rα. The product of normalized cell surface bound wild-type or mutant IL-2 (Figure 4-2) and the initial number of 2-4-bound IL-2Rα at time zero was used as an estimate of the number of wild-type or mutant IL-2-bound IL-2Rα on the cell surface.

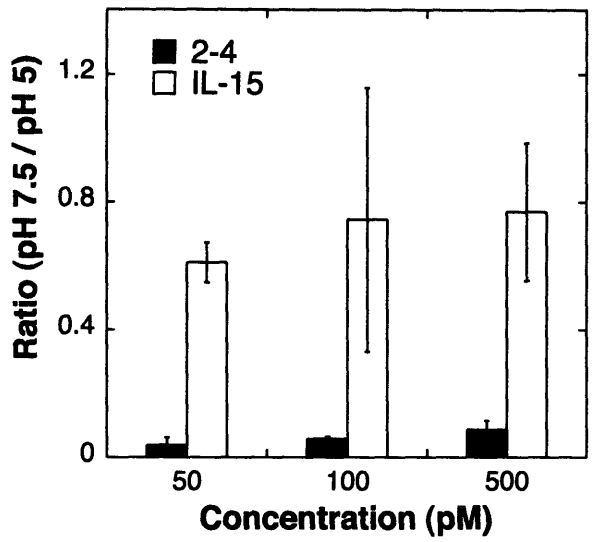

Figure 4-4: IL-15 binding to IL-15Rα is less sensitive to lowered pH than 2-4 binding to IL-2Rα

Binding of both 2-4 and IL-15 to their respective alpha receptor subunits is decreased at pH 5 (relative to pH 7.5). However, the degree of decrease in binding at pH 5 (relative to pH 7.5) is significantly greater for 2-4 than IL-15. The significantly lesser sensitivity of IL-15 binding to IL-15Rα at endosomal pH is consistent with IL-15 recycling to a much greater extent than the IL-2 mutants, leading to increased persistence for a given initial receptor occupancy. F15R-Kit cells were cultured in medium without IL-2 for 1-2 days prior to the assay. Cells were labeled with 50, 100 or 500 pM of 2-4 or IL-15, at pH 7.5 or pH 5, for 30 minutes at 37 °C. Single cell fluorescence was determined by flow cytometry. For each concentration, the ratio of background-corrected median fluorescence at pH 5 to the value at pH 7.5 was calculated. Error bars denote standard deviation of three separate experiments.

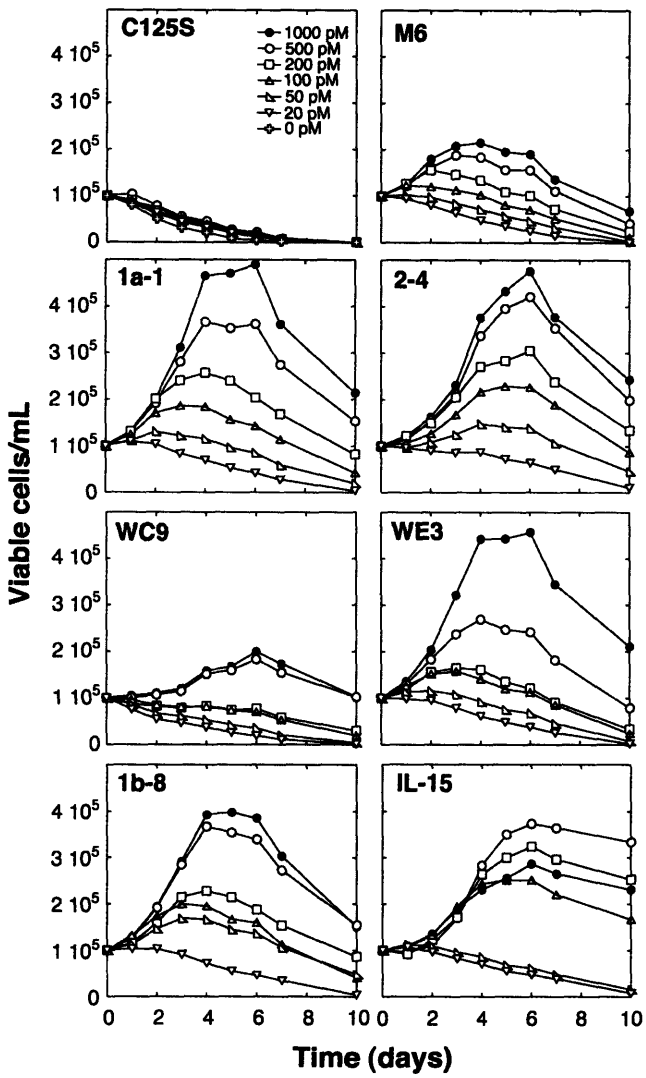

Figure 4-5: IL-2 mutants and IL-15, but not wild-type IL-2, stimulate T cell growth
F15R-Kit cells (~ 100,000 cells/mL) expressing IL-2Rα and IL-15Rα were incubated with a range of concentrations of wild-type IL-2, IL-2 mutants or IL-15 for 30 minutes at 37°C and pH 7.4. Cells were then washed with and resuspended in cytokine-free medium. Viable cell number over a ten-day period was determined using the Cell Titer-Glo™ (Promega) luminescence assay.

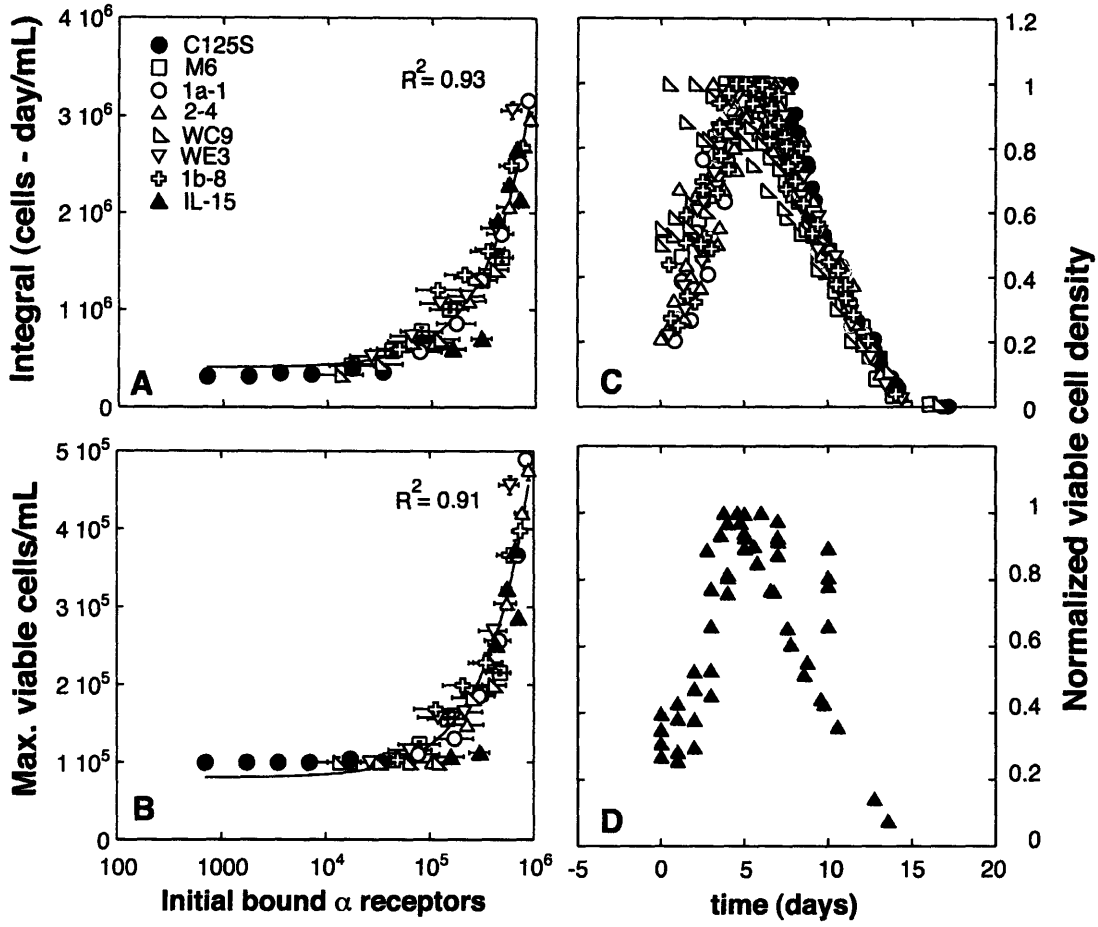

Figure 4-6: Pharmacodynamic response of IL-2 mutants and IL-15 is equivalent

(A and B) Growth response of 15R-Kit cells as quantified by the integral of viable cell density over a ten-day period and the maximum cell density linearly correlates with initial receptor occupancy for wild-type IL-2, IL-2 mutants and IL-15. Although the logarithm of surface interleukin is plotted to condense the three-log dynamic range, the relationship is well represented by linear regression with the correlation coefficient R^2 shown. The total numbers of IL-2Rαand IL-15Rα were determined experimentally. The initial receptor occupancy was estimated using K_d values and the total receptor number. X-error bars represent error in estimating initial receptor occupancy due to error in K_d value estimates. Y-error bars represent standard deviation of triplicate measurements. (C) The growth kinetics of wild-type IL-2 and all IL-2 mutants, for all concentrations, is described by one universal growth curve (D) IL-15 follows an essentially similar universal growth curve as IL-2

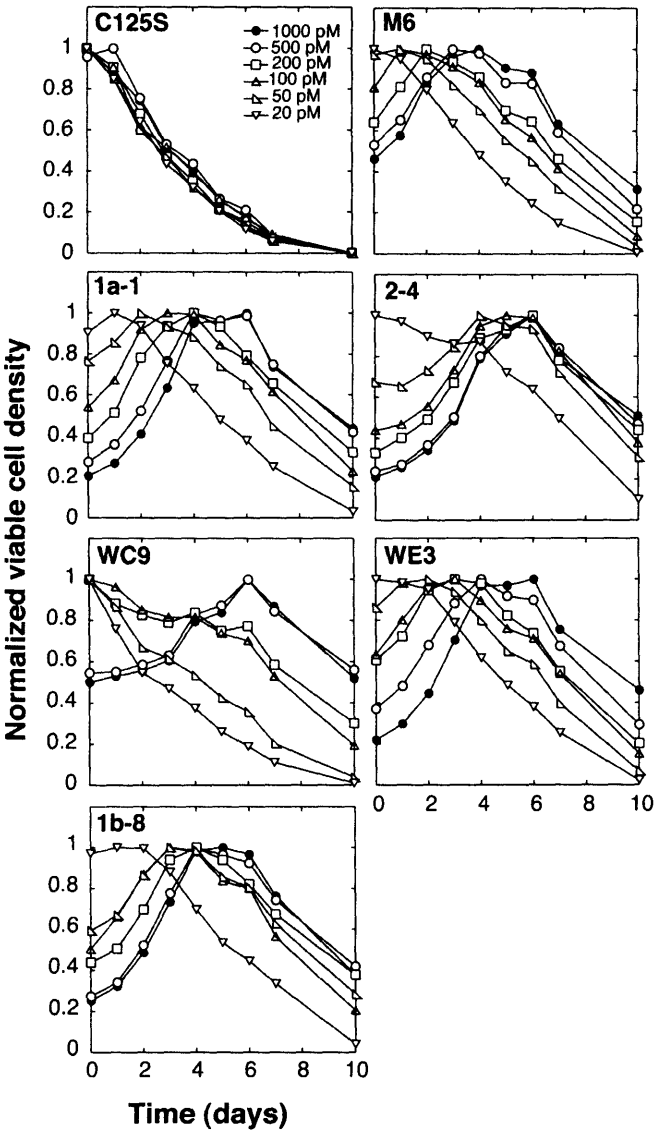

Figure 4-7: Normalized T cell growth responses

The growth curves (Figure 4-5) for wild-type IL-2 and IL-2 mutants were normalized by the maximum viable cell density for each concentration

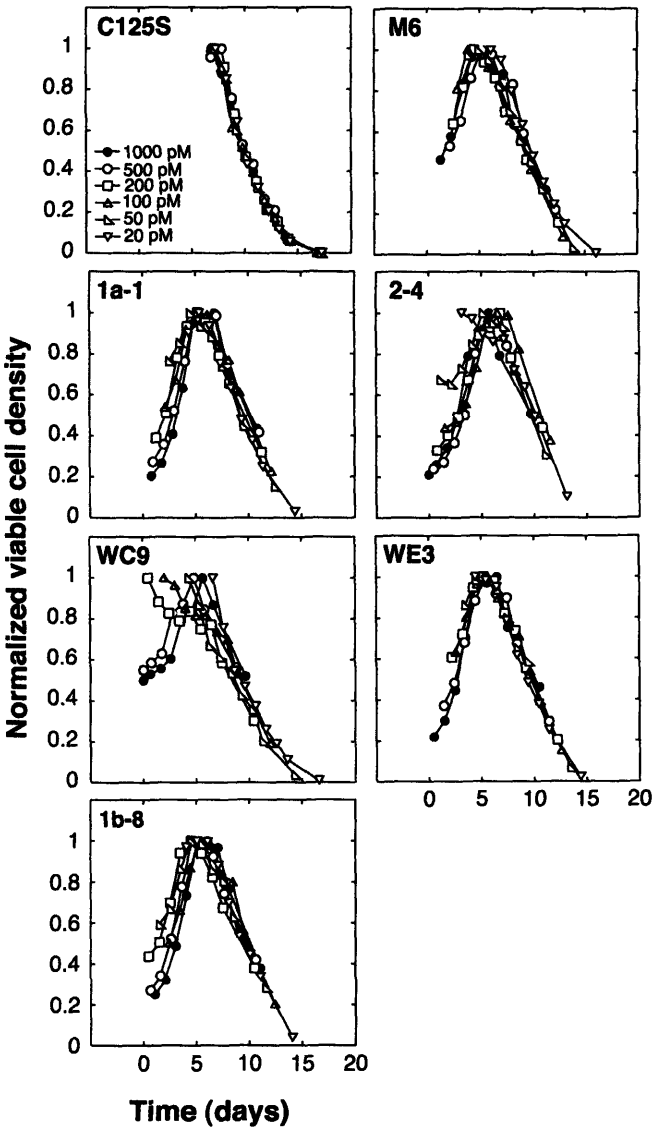

Figure 4-8: Normalized and time-shifted T cell growth responses

The normalized growth curves (Figure 4-7) were shifted on the time axis such that the viable cell density extrapolates to zero after at least 14 days. The value of 14 days was arbitrarily chosen because the viable cell density for T cell cultures treated with 1 nM 2-4 (highest concentration used for the IL-2 mutant with highest IL-2Rα binding affinity) extrapolates to zero after 14 days.

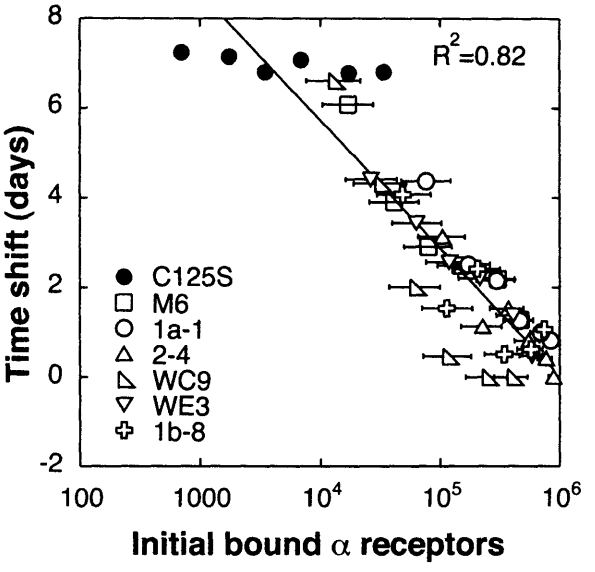

Figure 4-9: The magnitude of shift on time axis correlates strongly with initial receptor occupancy

Experimentally determined total IL-2Rα numbers and K_d values were used to determine the initial number of IL-2Rα bound with wild-type or mutant IL-2, following cytokine withdrawal. The X-error bars denote the error in estimating the initial bound IL-2Rα due to error in K_d estimates.

Chapter 5: CONCLUSIONS

The primary objective of the research presented in this thesis was to generate IL-2 mutants with potentially improved therapeutic value. We used existing data in the literature to formulate a strategy for engineering improved IL-2 variants. On the basis of qualitative considerations and mathematical modeling, we hypothesized that IL-2 variants with increased binding affinity for the alpha receptor subunit of IL-2 will have potentially improved therapeutic value. Yeast surface display and directed evolution were used to generate IL-2 mutants with increased IL-2Rα affinity. In novel pulsed bioassays designed to mimic bolus pharmacokinetics, these mutants exhibit significantly increased potency for T cell proliferation relative to wild-type IL-2. Conventional bioassays not only fail to reveal the increased potency resulting from enhanced IL-2Rα affinity (false negatives), but also suggest improved potency for a mutant without enhanced activity in the pulsed bioassay (false positive). Our results underscore the critical nature of the choice of appropriate bioassays to evaluate engineered proteins and other drugs.

Cell surface IL-2Rα acts as a ligand reservoir for the mutants with increased IL-2Rα affinity, leading to increased persistence of the mutants on the cell surface and subsequently increased growth signal. This is analogous to the prolonged persistence of IL-15, which binds with high affinity to IL-15Rα, on cell surface IL-15Rα reservoirs. We have shown that T cell growth responses mediated by the high affinity IL-2Rα mutants are quantitatively equivalent to those mediated by IL-15. Our results suggest that the contrasting effects of IL-2 and IL-15 on T cells in vivo is largely due to the 1,000-fold

different affinities of wild-type IL-2 and IL-15 for their respective private alpha receptor subunits. The major contributions of this thesis can be summarized as follows:

1) IL-2 mutants with potentially improved therapeutic value were generated.

2) The cell surface ligand reservoir mechanism leading to increased potency of the IL-2 mutants was elucidated.

3) A novel bioassay was designed to mimic bolus pharmacokinetics. Our results show that the choice of appropriate bioassays is critical for the evaluation of engineered proteins.

4) A quantitative relationship between binding affinity for the private alpha receptor subunit and T cell growth response that is valid for both IL-2 and IL-15 was established. The quantitative functional equivalence of high IL-2Rα affinity IL-2 mutants and IL-15 suggests that much of the difference between IL-2 and IL-15 stems from their vastly different affinities for their respective alpha receptor subunit.

The work described in this thesis lays the foundation for the development of IL-2 variant based improved IL-2 therapies. The next step involves the evaluation of the IL-2 mutants in proliferation and toxicity assays using human peripheral blood mononuclear cells (PBMC). Secretion of pro-inflammatory cytokines by CD56 dim NK cells in response to the IL-2 mutants can be used as a measure of toxicity. The IL-2 mutants were engineered for increased binding to human IL-2Rα. Increased affinity for IL-2Rα mediates increased persistence of the IL-2 mutants on cell surface IL-2Rα reservoirs and subsequently increased T cell growth response. These mutants do not exhibit improved binding to

murine IL-2Rα (data not shown) and conceivably will not lead to increased murine T cell proliferation. Therefore, *in vivo* tests on mice should be conducted on transgenic mice with human IL-2Rα. In general, prior to any animal testing, the increased binding of the IL-2 mutants to IL-2Rα expressed by the animal should be verified.

The IL-2 mutants can be used to better understand any differences in signaling mediated by IL-2 and IL-15. An interesting question would be if indeed distinct intracellular signaling pathways are involved in the signaling mediated by wild-type IL-2, the IL-2 mutants and IL-15.

The general approach of creating a cell-bound ligand reservoir, exploited in the case of IL-2, might also enable engineering of mutants with enhanced potency for GM-CSF, IL-3, and IL-5 (Guthridge et al., 1998) , since they also possess non-signaling alpha capture receptor subunits.

APPENDIX A: ADDITIONAL EXPERIMENTS

A.1 IL-2 mutants do not perceptibly bind to IL-15Rα

Protocol

KIT225 cells over-expressing IL-2Rα and IL-15Rα were labeled with the IL-2 mutants with and without saturating amounts of IL-15. A decrease of IL-2 binding signal in the presence of IL-15 would indicate binding of IL-2 mutants to IL-15Rα

Results

Experiment 1:

KIT225 cells were labeled with wild-type IL-2 (C125S) or IL-2 mutants at 1 nM concentration, with or without the presence of 2 nM IL-15. Cell surface bound IL-2 was detected.

As shown in Figure A-1, The binding of IL-2 to KIT225 cells is not perceptibly affected by the presence of saturating amounts of IL-15. This suggests that the IL-2 mutants do not perceptibly bind IL-15Rα.

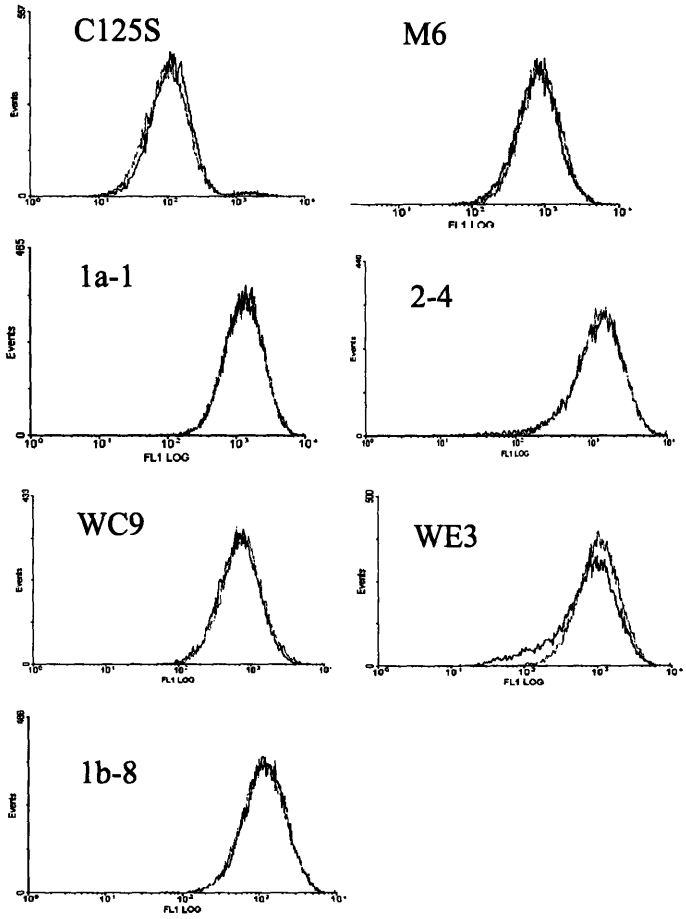

Figure A-1: Presence of IL-15 does not perceptibly affect binding of IL-2 to KIT225 cells Black curves denote binding of IL-2 (1 nM) to KIT225 cells in absence of IL-15
Red curves denote binding of IL-2 in the presence of 2 nM IL-15

Experiment 2:
At different concentrations of 2-4, there is no perceptible effect of the presence of saturating amounts of IL-15, on the binding of 2-4 to KIT225 cells (Figure A-2). This suggests that 2-4 does not perceptibly bind IL-15Rα. (Note that for the 500 pM the distributions are not the same and therefore cannot be compared accurately)

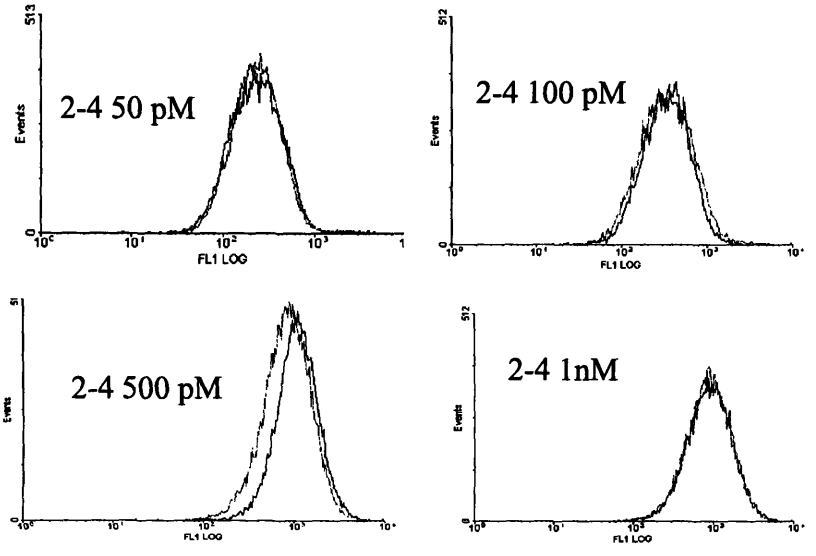

Figure A-2: Presence of IL-15 does not perceptibly affect binding of 2-4 to KIT225 cells at various concentrations
Black curves denote binding of 2-4 to KIT225 cells in absence of IL-15
Red curves denote binding of 2-4 in the presence of 2 nM IL-15

Experiment 3:
The IL-15Rα on KIT225 cells has an N-terminal FLAG tag. KIT225 cells were labeled with 2-4 (1nM) and an anti-FLAG antibody (M2) with and without saturating amounts of IL-15. 2-4 also has an N-terminal FLAG tag. Therefore, the M2 binding signal comes from M2 bound to 2-4 and M2 bound to IL-15Rα.
In the presence of IL-15, there is a decrease in M2 signal indicating that IL-15 blocks the binding of M2 to IL15Rα, at least partially. However, there is no perceptible decrease in the binding of 2-4 to KIT225 cells in the presence of IL-15. This suggests that 2-4 does not perceptibly bind IL-15Rα. This is shown in Figure A-3

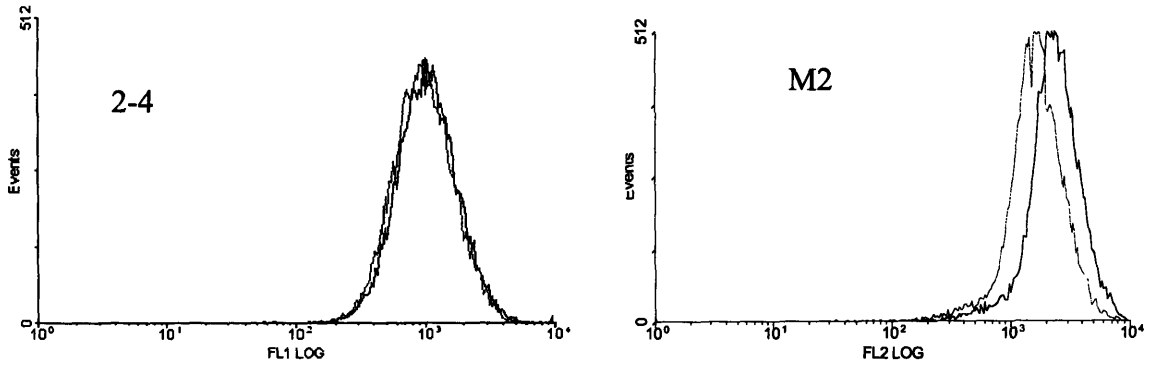

Figure A-3: Presence of IL-15 does not perceptibly affect binding of 2-4 to KIT225 cells but blocks M2 binding to IL-15Rα

Black curves denote binding of 2-4 (1 nM) and M2 to KIT225 cells in absence of IL-15
Red curves denote binding of 2-4 (1 nM) and M2 in the presence of 2 nM IL-15

Notes

The experimental system used is not the optimal system to prove that IL-2 does not bind IL-15Rα. IL-15Rα are roughly half in number relative to IL-2Ra. Thus the maximum decrease in signal in the presence of IL-15 is approximately 33%. On a logarithmic scale this is a fairly small change.

Nevertheless, at 1 nM (the maximum IL-2 concentration used in our bioassays) there is no perceptible change in signal (over noise which is typically 10%) as indicated by the overlay plots

A.2 Effect of acid strip on cell surface ligand reservoir

Protocol

KIT225 cells were subjected to an acid strip procedure as follows. Cells were washed once with ice-cold PBS and twice with acid strip solution (10 mM citrate, 0.14 M NaCl, 50 mg/mL BSA, pH 2.8).
Cells were subjected to an acid strip before incubation with 2-4 in the pulse assay or after incubation with 2-4. Cells subjected to an acid strip, but incubated in medium without IL-2 during the pulse assay were used as a negative control. Cell surface bound 2-4 after the acid strip was determined using flow cytometry. Only cells with the same forward scatter – side scatter profile as the control cells (without acid strip) were considered. The viable cell number after three days was measured using the Promega Cell Titer-Glo luminescence bioassay.

Results

- **Acid strip causes decrease in cell viability**
  Cells subjected to the acid strip procedure have less than 10% viability, relative to control cells.

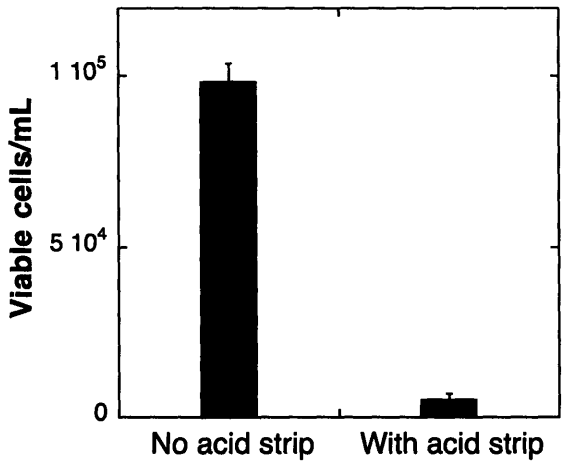

Figure A-4: Acid strip causes decrease in cell viability
Error bars indicate standard deviation of triplicate measurements.

- **Acid strip removes cell surface bound 2-4**
  The acid strip procedure completely removes cell surface bound 2-4, as shown in Figure A-5

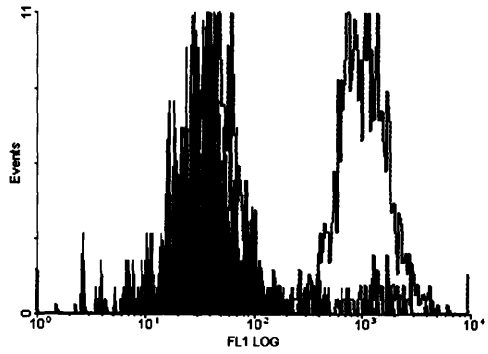

Figure A-5: Acid strip removes cell surface bound 2-4
The shaded green histogram is the negative control. Red histogram represents cells labeled with 2-4 after the acid strip. The blue histogram represents cells subjected to the acid strip after incubation with 2-4 in the pulse bioassay

- **Cell surface bound 2-4 leads to increased proliferation**
  Cells subjected to the acid strip procedure prior to incubation with 2-4 in the pulse assay show increased proliferation relative to a negative control. Cells where 2-4 is stripped from the cell surface after the pulse assay do not undergo proliferation. This indicates that the cell surface bound ligand mediates cell proliferation in the pulse bioassay (Figure A-6).

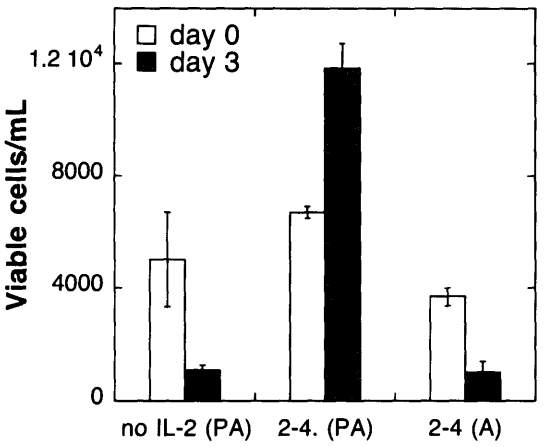

Figure A-6: Cell surface bound ligand mediates cell proliferation in the pulse assay
PA – acid strip prior to incubation with ligand in pulse assay
A - acid strip prior to incubation with ligand in pulse assay
Error bars indicate standard deviation of triplicate measurements

Notes:
Histograms in Figure A-6 were obtained using less than 1000 cells. This is because most of the cells were affected by the acid strip procedure used.

APPENDIX B: MATLAB CODES

Main program

```
clear all;

i = 1000; % initial concentration in pM

n = 1e8; % cells per liter

Nav = 6.023e23; % Avagadro number

koff1 = 18; % off rates in min-1

koff2 = 1.8;

koff3 = 0.18

options = [ ];

x0 = [i 0];

[t1,x] = ode23s(@odeset, [0 1000], x0, options,i,n,koff1);

ia1 = n/Nav*x(:,2)*1e12;

[t2,x] = ode23s(@odeset, [0 1000], x0, options,i,n,koff1);

ia2 = n/Nav*x(:,2)*1e12;

[t3,x] = ode23s(@odeset, [0 1000], x0, options,i,n,koff1);

ia3 = n/Nav*x(:,2)*1e12;
```

Function

```
function dx = odeset(t,x,i,n,koff1);

%define all parameters

kon1 = 6e8; % association rate M-1 min-1
```

```
kec1 = 0.00024; % endocytic rate min-1

a0  = 100000; % receptors/cell

k1 = 0.693/12.9; % min-1 alpha clearance from blood

k2 = 0.693/85; % min-1 beta clearance from blood

Nav = 6.023e23; % Avagadro no.

% x1 = IL-2

% x2 = IL-2-a

% i = initial IL-2 concentration (pM)

dx = zeros(2,1);

dx(1)=-k1*(0.866*i*exp(-k1*t))-(0.134*i*exp(-k2*t))*k2 - kon1*x(1)*(a0-x(2))*n/Nav
+ koff1*x(2)*n/Nav*1e12 - kec1*x(2)*n/Nav*1e12;
%
dx(2)=kon1*x(1)*1e-12*a0 - x(2)*(kon1*x(1)*1e-12+koff1+kec1);
%
```

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CURRICULUM VITAE

Balaji M. Rao

Education

9/99 – 8/04 Massachusetts Institute of Technology, Dept. of Chemical Engineering
- Master of Science (S.M.) in Chemical Engineering Practice, June 2001
- Ph D. in Chemical Engineering, September 2004
Minor: Biology
- *GPA:* 5.0/5.0

7/95 – 5/99 University Department of Chemical Technology (UDCT), University of Mumbai
- Bachelor of Chemical Engineering (B. Chem. Engg.), May 1999
- Gold Medalist at the B. Chem. Engg. Examination (First in a class of 65)

Research Experience

1/00 – 8/04 Graduate Research Assistant, Dept. of Chemical Engineering, Massachusetts Institute of Technology
Thesis title: Interleukin-2 Engineering for Improved Therapeutic Effectiveness
Thesis Advisors: Prof. K. D. Wittrup and Prof. D. A. Lauffenburger

5/97 – 6/97 Worked on a research project, "Design and Optimization of a continuous flow immobilized amylase bioreactor for the hydrolysis of starch", at Bhabha Atomic Research Center (BARC), Mumbai, India

Teaching Experience

1/02 – 5/02 Graduate Teaching Assistant, Dept. of Chemical Engineering, Massachusetts Institute of Technology
Course details: Undergraduate course in Separations
Course Instructor: Dr. C. M. Mohr

6/03 –8/04 Mentor for the Undergraduate Research Opportunity Program (UROP)

Industrial Experience

6/00 – 8/00 MIT Practice School

- Worked on a project involving the capacity increase of two petrochemical plants at GE Plastics, Mt. Vernon, IN (June 2000)
- Worked on a project interfacing Chemical Engineering and Information Technology, to improve quality control in a polymer production and processing facility at GE Plastics, Mt. Vernon, IN (July 2000)
- Worked on a project involving the study of the various aspects of clog-formation during injection of a controlled release drug delivery system at Alkermes Inc, Cambridge, MA (August 2000)

1/99 – 4/99 Worked on the project, "Design of a plant to manufacture 1500 TPA Methyl Benzoate", at UDCT, Mumbai, India.

5/98 – 6/98 Worked as a summer intern in Cumene-Phenol and Phthalic Anhydride plants and completed a project on purification of AMS (Alpha Methyl Styrene), at Herdillia Chemicals Ltd., Mumbai, India.

Honors and Awards

- Merck Best Student Poster Award at the Biochemical Engineering (XIII) 2003 Conference (July 2003)
- MIT Presidential Fellowship for graduate study, September 1999 – June 2000
- G. P. Kane Gold Medal for standing first at the B. Chem. Engg. Examination, May 1999
- Undergraduate design project nominated for the P. C. Ray award, a national level competition organized by the Indian Institute of Chemical Engineers, May 1999

Publications

Rao, B. M., Girvin, A. T., Ciardelli, T., Lauffenburger, D. A., and Wittrup, K. D. "Interleukin-2 mutants with increased α-subunit binding affinity", Protein Engineering 2003 Dec; 16(12): 1081-1087.

Rao, B. M., Driver, I., Lauffenburger, D. A., and Wittrup, K. D. "IL-2 variants engineered for increased IL-2Rα affinity exhibit increased potency arising from a cell surface ligand reservoir effect" Molecular Pharmacology 2004 (In Press)

Rao, B. M., Driver, I., Lauffenburger, D. A., and Wittrup, K. D. "IL-2 mutants with picomolar IL-2Rα binding affinity stimulate growth responses quantitatively equivalent to IL-15" (Submitted)

Rao, B.M., Lauffenburger, D. A., and Wittrup, K. D. "Engineering protein therapeutics within a systems-level computational context" (Submitted)

Cochran, J. R., **Rao, B. M.**, Kim, Y., and Wittrup, K. D. "Directed evolution selects mutations biased towards homologous substitutions at variable sites" (In preparation)

Patents

Rao, B. M., Lauffenburger, D. A. and Wittrup, K. D. "Mutant Interleukin-2 Polypeptides" (US and PCT Applications filed)

Posters

Balaji M. Rao, Ian Driver, Douglas A. Lauffenburger and K. Dane Wittrup. "Interleukin-2 Engineering for Improved Therapeutic Effectiveness". Poster presented at the Biochemical Engineering (XIII) 2003 Conference, July 2003 *(This poster won the Merck Best Student Poster Award)*