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Engineered Interleukin-2 Antagonists for the Inhibition of Regulatory T cells

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Abstract

The immunosuppressive effects of $CD4^+$ $CD25^{high}$ regulatory T cells interfere with anti-tumor immune responses in cancer patients. Here, we present a novel class of engineered human Interleukin (IL)-2 analogues that antagonize the IL-2 receptor, for inhibiting regulatory T cell suppression. These antagonists have been engineered for high affinity to the α subunit of the IL-2 receptor and very low affinity to either the β or γ subunit, resulting in a signaling-deficient IL-2 analogue that sequesters the IL-2 receptor α subunit from wild type IL-2. Two variants, "V91R" and "Q126T" with residue substitutions that disrupt the β and γ subunit binding interfaces, respectively, have been characterized in both a T cell line and in human primary regulatory T cells. These mutants retain their high affinity binding to IL-2 receptor α subunit, but do not activate STAT5 phosphorylation or stimulate T cell growth. The two mutants competitively antagonize wild-type IL-2 signaling through the IL-2 receptor with similar efficacy, with inhibition constants of 183 pM for V91R and 216 pM for Q126T. Here, we present a novel approach to CD25-mediated Treg inhibition, with the use of an engineered human IL-2 analogue that antagonizes the IL-2 receptor.

Keywords

Interleukin-2; regulatory T cells; antagonist engineering; cytokine engineering

Introduction

$CD4^+$C $D25^{high}$ regulatory T cells (Tregs) suppress immune responses[1,2] and have been found in the circulation of patients with various cancers[3–6] as well as near tumor sites,[5–6]

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correlating with reduced survival in some cancers.[6–8] Tregs dampen anti-tumor immune responses by inhibiting the effector functions of tumor-specific $CD4^+$ and $CD8^+$ T cells.[6, 9–11] As a result, the inhibition of Treg suppression of an anti-tumor immune response is one approach to cancer therapy currently being studied.

While the expression of the nuclear transcription factor Foxp3 appears to be the most specific marker for Tregs,[12–15] surface receptors for targeting Tregs include glucocorticoid-induced tumor necrosis factor receptor, cytotoxic T-lymphocyte antigen 4, and folate receptor 4.[16–18] We chose to target CD25, the Interleukin (IL)-2 receptor α subunit (IL-2Rα), which is constitutively expressed at high levels on $CD4^+CD25^{high}$ Tregs. The CD25 marker has been utilized successfully for Treg depletion. For example, anti-CD25 monoclonal antibodies and the diphtheria toxin / Interleukin -2 fusion protein, DAB_{389}-IL-2 (also known as denileukin difitox, or ONTAK) have been used to deplete Tregs alone or in conjunction with a cancer vaccine to induce effective anti-tumor immune responses in mice and humans.[19–23] However, these remain sub-optimal for several reasons. For example, the diphtheria toxin portion of DAB_{389}-IL-2 exhibits both toxicity and immunogenicity.[24] Anti-CD25 antibodies can cause elimination of Tregs by complement or by antibody dependent cell-mediated cytotoxicity, thereby disturbing T cell homeostasis.[25] While the inhibition of Treg function is desirable, their outright elimination would potentially be more disruptive to normal immune regulation and prevention of autoimmunity. Treg functional inhibition may be preferable to depletion as a therapeutic strategy, because Treg inhibition maintains normal T cell numbers, whereas Treg depletion simply leads to replenishment with new, uninhibited Tregs.[26.]

Here, we present a novel approach to CD25-mediated Treg inhibition, with the use of an engineered human IL-2 analogue that antagonizes the IL-2 receptor. Instead of using CD25 merely as a surface marker to identify and target Tregs, our approach is to inhibit the downstream effects of IL-2 receptor signaling. IL-2 is essential to Treg biology; it is required for the expansion, maintenance and the suppressive function of Tregs.[27–30] An IL-2 antagonist that is also an IL-2 analogue could potentially minimize the chances for systemic toxicity and immunogenicity, providing inhibition of Treg function without their elimination.

To construct competitive IL-2 antagonists, we started with 2-4 IL-2, an IL-2 analogue that was originally designed for more potent T cell signaling responses and a better therapeutic index with selective targeting for $CD25^+$ T cells over $CD25^-$ natural killer cells. 2-4 IL-2 was engineered for increased binding affinity to the IL-2 receptor α subunit using directed evolution and yeast surface display.[31] For antagonists, we constructed and characterized two variants of 2-4 IL-2, one with a valine to arginine substitution at position 91, and the second with a glutamine to threonine at position 126, both on the 2-4 IL-2 background. These mutants, termed V91R and Q126T, are designed to disrupt binding to the IL-2 receptor β (IL-2Rβ) and IL-2 receptor γ (IL-2Rγ) subunits, respectively. They exhibit antagonistic properties without stimulating IL-2 receptor signaling in both the human T cell line Kit225 and human primary $CD4^+Foxp3^+$ Tregs *ex vivo*. These analogues could prove useful as pharmacological Treg inhibitors in the context of cancer immunotherapy. In addition, to the best of our knowledge, this is the first report of engineering a cytokine analogue with high

affinity to a non-signaling receptor subunit, and low affinity to the signaling receptor subunits to create effective cytokine antagonists. Therefore, this approach of engineering signaling deficient cytokine analogues that bind tightly and sequester the receptor from the wild type cytokine represents a novel paradigm that can be more broadly applied to antagonist engineering, such as for other γ common receptor cytokines, or for inflammatory cytokines such as IL-1, IL-6, or IL-12.

Materials and Methods

Preparation of IL-2 mutant proteins

Single point mutations were introduced to the 2-4 IL-2 coding sequence using the Quikchange kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. IL-2 mutants were expressed in yeast with an N-terminal FLAG tag and a C-terminal c-myc tag as previously described.[31] The supernatant was concentrated and buffer exchanged with PBS using a 10kDa MWCO ultrafiltration unit (Millipore, Billerica, MA). The retentate was purified using an anti-FLAG M2 agarose affinity gel (Sigma-Aldrich, St. Louis, MO), followed by size exclusion chromatography with a Superdex 75 column (GE Healthcare, Piscataway, NJ). Elution fractions that contained only monomeric protein were pooled and the protein concentration was determined using the Micro BCA Protein Assay kit (Pierce, Rockford, IL).

Tissue Culture

To characterize the IL-2 mutants, the human IL-2 dependent T cell line Kit225, which constitutively expresses all three subunits of the IL-2 receptor, was used. The cells were cultured in a humidified atmosphere in 5% CO_2 in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 40 pM IL-2, 2 mM L-glutamine, 2 mg/mL sodium bicarbonate, 50 U/mL penicillin, 50 μg/mL streptomycin and 50 μg/mL gentamicin.

Determination of IL-2Rα binding affinities of IL-2 mutants

The equilibrium binding affinities of V91R and Q126T binding to IL-2Rα were evaluated using a modification of a previously described protocol.[31] Kit225 cells were incubated in phosphate buffered saline (PBS) + 0.1% bovine serum albumin (BSA) at 37°C for 30 minutes, at 8×10^5 cells/mL with varying IL-2 mutant concentrations. At low IL-2 mutant concentrations, the total volume was increased to maintain an excess number of IL-2 mutant molecules over the number of IL-2Rα. After incubation, cells were kept on ice and stained with mouse anti-FLAG monoclonal antibody M2 (Sigma Aldrich, St. Louis, MO), followed by an Alexa Fluor 488 conjugated goat anti-mouse antibody (Invitrogen, Carlsbad, CA), to detect cell-surface bound protein. The mean single-cell fluorescence was measured, and the dissociation constant (K_D) and the 95% confidence interval were determined as previously described,[31] using the following equation: $F_{obs}=cL_0/(K_D+L_0)$, where F_{obs} is the background-corrected mean fluorescence, L_0 is the initial concentration of the protein being measured, and c is the proportionality constant. The K_D of IL-2 C125S, which is equivalent to aldesleukin (Proleukin, Novartis, Basel, Switzerland) and is referred to as "wild type IL-2," was also measured as a control.

Analysis of STAT5 phosphorylation

For all STAT5 phosphorylation assays, Kit225 cells were starved of IL-2 for 36 hours. Kit225 cells were incubated per at 10^6cells/mL culture medium at 37°C with IL-2 mutants for 30 minutes (for agonism studies), or with IL-2 mutants and 3 pM wild type IL-2 for 15 minutes (for antagonism studies). The cells were fixed and permeabilized using the method optimized by Krutzik et al.[32] The cells were stained with anti-pSTAT5 antibody clone 47 conjugated with Alexa Fluor 488 (BD Biosciences, San Jose, CA) and the mean single-cell fluorescence was measured. For antagonism studies, the half-maximal inhibitory concentration (IC_{50}) and the 95% confidence interval were determined using the following equation $F_{obs}=c/(1-L_0/(IC_{50}+L_0))$, where all variables are defined above. A global fit nonlinear regression was performed for each protein, using a global IC_{50} value and the proportionality constant from each of two separate experiments.

For analysis of human whole blood, phosphorylation state analysis was performed using BD Phosflow technology according to the manufacturer's instructions (BD Biosciences, San Jose, CA), and as previously described.[33] All human blood samples were obtained with informed consent and according to the Institutional Ethics Review Board Protocols. All blood samples were collected in sterile 10 mL lithium heparin Monoject tubes. For each condition and time point, 4 mL fresh, *ex vivo* blood from healthy control donors were used. Blood samples were incubated with IL-2 or with a cocktail of IL-2 and IL-2 antagonists in 50 mL polypropylene Falcon conical tubes for 30 min in a 37°C water bath. Fixation of cells and preservation of phosphorylation status was obtained by adding pre-warmed BD Lyse/Fix buffer and incubation in a 37°C water bath. Permeabilization of cells was performed by incubation of cells in BD Perm Buffer III on ice for 30 min. Cells were subsequently washed twice with 2% FBS/PBS and stained using BD Staining Buffer (all reagents from BD Bioscience, San Jose, CA). Cells were stained using APC mouse anti-human CD4 (clone RPA-T4) (BD Bioscience, San Jose, CA), PE anti-human Foxp3 (clone 206D) (Biolegend, US) and Alexa Fluor-488 mouse anti-human pSTAT5 (pY694; clone 47) (BD Bioscience, San Jose, CA).

Kit225 Cell Proliferation Assays

Kit225 cells were starved of IL-2 for 36 hours. Then, 4×10^5 cells were incubated in 3 mL culture medium at 37°C with IL-2 mutants, either in the absence (for agonism studies), or presence (for antagonism studies) of 25 pM wild type IL-2. At each time point, the live cell number in 100μL culture medium was determined in triplicate using the CellTiter-Glo assay (Promega, Madison, WI) and a Cary Eclipse luminometer (Varian, Palo Alto, CA) according to the manufacturer's instructions.

Results

Design of IL-2 Mutant Antagonists

IL-2 analogue antagonists were designed using the following criteria: 1) high binding affinity to IL-2Rα, the IL-2 specific capture subunit, and 2) low predicted binding affinity to IL-2Rβ or IL-2Rγ, the two subunits responsible for receptor signaling. The high binding affinity to IL-2Rα leads to preferential IL-2Rα binding of the IL-2 analogue over wild type

IL-2, while the low binding affinity to IL-2Rβ or IL-2Rγ would prevent the IL-2 analogue from activating the IL-2 receptor signal itself. We achieved the first design criterion by using a previously engineered mutant of human IL-2, 2-4 IL-2, as a starting point for our IL-2 analogue. 2-4 IL-2 is an IL-2 analogue previously developed in our lab using directed evolution and yeast surface display to have high binding affinity to IL-2Rα.[31] The K_D of 2-4 IL-2 binding to IL-2Rα is ~200 pM whereas the K_D of wild type IL-2 binding to IL-2Rα is ~30 nM. 2-4 IL-2 persists on the surface of cells expressing IL-2Rα for days, significantly longer than the cell surface persistence of wild type IL-2.[31]

For the second design criterion, we used recently published crystal structures of wild type IL-2 bound to the three IL-2 receptor subunits[34, 35] to identify candidate residues likely to make energetically important interactions with the IL-2Rβ or IL-2Rγ subunits. Assuming that these interactions are preserved in 2-4 IL-2 binding to IL-2Rβ and IL-2Rγ, we disrupted binding of 2-4 IL-2 to IL-2Rβ or IL-2Rγ by introducing amino acid substitutions at these locations. Five mutants, each with a single residue substitution on the 2-4 IL-2 background were generated in small scale pilot studies (Table I). Several of these point mutations have been reported in the literature on a wild type IL-2 background to disrupt biological activity[36] or more explicitly, to disrupt IL-2 receptor subunit binding affinity.[37–40] Of the five mutants generated, V91R and Q126T, which contain single residue substitutions at the binding interfaces with IL-2Rβ and IL-2Rγ, respectively, were secreted in yeast most efficiently and were characterized further (Fig. 1). On wild type IL-2, a valine at position 91 is in the center of the IL-2 / IL-2Rβ interface and makes van der Waals interactions with IL-2Rβ.[35] Therefore, a charged amino acid substitution, such as arginine, at position 91 (V91R) was hypothesized to disrupt binding to IL-2Rβ. As for IL-2Rγ binding, previous reports have shown the importance of Q126 for biological activity;[40, 41] the crystal structures used also identified Q126 as the most important IL-2 residue that interacts with IL-2Rγ.[34, 35] Cassell and coworkers performed an extensive study of the activity of wild type IL-2 mutants on T cells with each of the 20 amino acids in the 126 position, and showed that threonine yielded the lowest activity.[36] We assumed that this was due to abrogated IL-2Rγ binding and was the basis for introducing a threonine substitution at position 126 (Q126T) on the 2-4 IL-2 background.

IL-2 Analogue Binding Affinity to the IL-2 Receptor α Subunit

The first design criterion for the IL-2 antagonists was to maintain high binding affinity to IL-2Rα in order for IL-2Rα to preferentially bind the antagonist over wild type IL-2. Therefore, the first step in characterizing the IL-2 analogues was to measure their IL-2Rα binding affinity using Kit225, a human T cell line that is dependent on IL-2 for growth. Kit225 constitutively expresses all three subunits of the IL-2 receptor, with IL-2Rα in ~10 fold excess. The surface labeling of Kit225 is thus dominated by binding to IL-2Rα, and the IL-2Rα binding affinity can be measured using cell surface titrations on Kit225.

The binding domains to each of the three IL-2 receptor subunits are on distinct areas of the surface of IL-2.[34, 35] Therefore, single residue substitutions at the IL-2Rβ or IL-2Rγ interfaces on the 2-4 IL-2background were estimated to have little or no effect on the binding affinity to IL-2Rα. Indeed, the measured IL-2Rα binding affinities of V91R and

Q126T are similar to that of 2-4 IL-2, indicating that the introduction of each of the two point mutations did not disrupt high affinity binding to IL-2Rα (Fig. 2). On the other hand, a Q126T/V91R double mutant that we sought to explore had significantly lower IL-2Rα binding affinity (K_D = 2 nM, data not shown), presumably due to some synergistic destabilization by the two mutations that reduces the IL-2Rα binding affinity by disrupting the protein's conformation. Although this was an unexpected result, this example underscores the importance of ensuring that the protein's conformation and its IL-2Rα affinity is preserved when introducing other residue substitutions for other antagonists designed in this manner.

Disruption of Binding to the IL-2 Receptor β and γ Subunits

The second design criterion for the IL-2 antagonists was the disruption of binding affinity to the IL-2Rβ and IL-2Rγ subunits, so that the IL-2 mutants themselves would not agonize the IL-2 receptor. The binding affinities of wild type IL-2 to IL-2Rβ or to IL-2Rγ alone are relatively low, with K_D values of approximately 0.5 mM and 0.7 mM, respectively.[42] The affinities of the IL-2 analogues with disrupted binding interactions to IL-2Rβ or IL-2Rγ would likely be too low to be measured reliably. Therefore, instead of directly measuring those binding affinities, the inability of the IL-2 analogues to agonize the IL-2 receptor at both an early and late signaling event was measured.

The Jak/STAT pathway is activated by the IL-2 receptor in both non-regulatory T cells and regulatory T cells,[43] and thus phosphorylated STAT5 (pSTAT5) was used as an early marker of IL-2 receptor activation. During initial testing, STAT5 phosphorylation in Kit225 was found to be extremely sensitive to wild type IL-2, with a measured half-maximal effective concentration (EC_{50}) of approximately 2 pM wild type IL-2 (Fig. 3A). However, the pSTAT5 profiles of cells treated with 100 nM V91R or Q126T are indistinguishable from those of untreated cells (Fig. 3B), thus indicating that the V91R and Q126T mutations severely inhibit the IL-2 mutants' ability to activate the IL-2 receptor.

Because the Kit225 cell line is dependent on exogenous IL-2 for growth, Kit225 cell proliferation was used as a late signaling event in measuring IL-2 receptor activation. The half maximal effective concentration for wild type IL-2 induced cell growth has been reported to be ~10 pM,[44] consistent with our results (data not shown). Similar to the pSTAT5 analysis, there was minimal to no Kit225 proliferation stimulated by V91R and Q126T at concentrations as high as 100 nM (Fig. 3C). At 100 nM, V91R did induce a very slight residual amount of cell growth, but this was still significantly less than the growth induced by 25 pM IL-2, signifying an over 4000-fold reduction in cell proliferative activity on a molar basis.

Antagonism by the IL-2 Analogues

Next, the ability of the IL-2 mutants to antagonize the IL-2 receptor was studied using the same early and late signaling events measured in the agonism studies. In pSTAT5 assays in Kit225, both V91R and Q126T antagonized the IL-2 receptor with equal efficacy and IC_{50} values of ~500 pM (Fig. 4A). In Kit225 cell proliferation assays, V91R and Q126T effectively antagonized IL-2 receptor as well (Fig. 4B). V91R and Q126T were also tested

for antagonism of STAT5 phosphorylation in primary human Tregs *ex vivo*. As shown in Figure 4C and Figure 1, Supplemental Digital Content 1, http://links.lww.com/JIT/A10, these antagonists potently interfere with wild-type IL-2 signaling.

For a competitive antagonist, the Cheng-Prusoff relationship[45] describes the relationship between the half-maximal inhibitory concentration of antagonist, IC_{50}, and the inhibition constant, K_I. It is given by: $IC_{50}=K_I(1+[A]/EC_{50})$, where [A]=wild type IL-2 agonist concentration, and the EC_{50} is the half-maximal effective concentration of wild type IL-2 agonist in the absence of antagonist. Based on the measured IC_{50} values and the wild type IL-2 concentration used in Figure 4A, the corresponding K_I values are 183 pM for V91R and 216 pM for Q126T. These values are consistent with the binding affinity measured (Fig. 2). They are also consistent when repeating the pSTAT5 antagonism assay with a different wild type IL-2 agonist concentration (See Figure 2, Supplemental Digital Content 2, http://links.lww.com/JIT/A11). Because IC_{50} values are dependent on assay conditions, such as wild type IL-2 agonist concentration, the measured IC_{50} values are not directly applicable to other *in vitro* or *in vivo* assays. Instead, a better indicator of the antagonists' effectiveness and potency is the assay-independent K_i which are sub-nanomolar for both antagonists and indicate relatively potent antagonists. V91R and Q126T also potently antagonize wild type IL-2 in human primary Tregs. However, a similar analysis based on the Cheng-Prusoff equation for a consistency check cannot be performed, due to a lack of an EC_{50} value for the assay. The donor variability in IL-2 sensitivity as well as limitations in the amount of blood taken from a single donor make it difficult to measure both an IL-2 agonist dose response (for EC_{50} determination) and antagonist inhibition curves (for IC_{50} determination) for use in calculating K_I values in primary Tregs.

Discussion

Protein engineering techniques were used to customize the binding affinities of IL-2 to each of its receptor subunits, resulting in a novel class of IL-2 analogues with high affinity to IL-2Rα and low affinity to either IL-2Rβ or IL-2Rγ. These mutants antagonize wild type IL-2 in both a T cell line and in primary Tregs *ex vivo*. By targeting the downstream effects of the IL-2 receptor rather than simply using CD25 as a cellular marker to deliver cytotoxic payloads, these IL-2 antagonists provide a novel mode of pharmacological Treg inhibition potentially of use for cancer immunotherapy.

The IL-2 variants created by substituting on 2-4 IL-2 either a valine to arginine at position 91 or a glutamine to threonine at position 126 retain high affinity IL-2Rα binding, minimally activate the IL-2 receptor, and antagonize the IL-2 receptor. The assumption that the wild type IL-2 / IL-2 receptor crystal structure could be used to rationally design mutants of 2-4 IL-2 appears to hold up well. In fact, it was surprising that single residue substitutions at V91R or Q126T were so effective at disrupting binding to IL-2Rβ and IL-2Rγ, respectively, and created such signaling deficient analogues of 2-4 IL-2. Of course, effective antagonists are not limited to these substitutions on the 2-4 IL-2 background. Residue substitutions, such as those listed in Table I, or other unidentified substitutions at the IL-2Rβ or IL-2Rγ binding interfaces, or combinations of these, could also potentially yield potent antagonists on the 2-4 IL-2 background.

The potency of antagonism of both mutants tested were nearly identical in this study. However, if additional residue substitutions are explored, a 2-4 IL-2 mutant with disrupted IL-2Rγ affinity may be preferable to one with disrupted IL-2Rβ affinity. Unbound IL-2Rα and IL-2Rβ have been shown to pre-associate on the cell surface[46–48] and an IL-2 mutant with high IL-2Rα affinity may still bind preformed IL-2Rα/IL-2Rβ complexes, even with lowered IL2-Rβ affinity.[49] Once bound to IL-2Rα/IL-2Rβ complexes, the mutant might bind IL-2Rγ due to undisrupted IL-2Rγ binding affinity, and undesirably create a signaling complex. We speculate this may be the cause of the low levels of agonism by V91R. Second, if a mutant with disrupted IL-2Rβ binding affinity only binds IL-2Rα, this leaves IL-2Rβ and IL-2Rγ available for signaling by wild type IL-2, although signaling in the absence of IL-2Rα is much less efficient. A 2-4 IL-2 analogue with disrupted binding to both IL-2Rβ and IL-2Rγ is another potential design to be explored, but such a double mutant may not sequester preformed IL-2Rα/IL-2Rβ complexes from wild type IL-2 as efficiently as a mutant with disrupted IL-2Rγ affinity only. Unfortunately, our attempts to express a Q126T/V91R double mutant unexpectedly yielded a presumably misfolded protein with significantly lower IL-2Rα binding affinity.

The requirement of IL-2 signaling for many biological functions of Tregs, including activation of their suppressive functions, has been well documented in the literature.[27–30] Our antagonists inhibit IL-2 mediated proliferation in a human T cell line, and STAT5 phosphorylation in both a human T cell line and human primary Tregs. Since IL-2 does not activate the PI3K/Akt and MAPK signaling pathways in Tregs,[43] the inhibition of STAT5 phosphorylation in primary Tregs is significant, because this represents blockage of all known signaling pathways downstream of the IL-2 receptor. Therefore, the documented requirement of IL-2 signaling for Treg suppressive function, coupled with data that IL-2 signaling is blocked in Tregs by our antagonists, provide strong evidence that these antagonists are capable of inhibiting Treg suppressive function.

To measure Treg suppression *in vitro*, a coculture assay is traditionally performed, where the proliferation of effector T cells is measured in the presence of Tregs, CD3 activating antibodies and costimulation.[1] However, for testing the effects of the antagonists on Tregs, there exist many confounding and competing effects that make the results of such an assay ambiguous and not definitive of the antagonists' effects *in vivo*. First of all, the anti CD25 antibody used for Treg purification is detectably bound to the cell's surface for 48 hours or more after purification, and could inhibit binding of the antagonists to CD25 (data not shown). This was part of the reason that whole blood, instead of purified T cell subsets, was used to analyze the effects of the antagonists on gated $CD4^+Foxp3^+$ cells, without staining for CD25. Next, the usefulness of the standard *in vitro* Treg coculture suppression assay is limited, because IL-2 antagonism would also have mixed effects on activated effector T cells that are upregulated for CD25. Antagonism of wild type IL-2 may inhibit proliferation and other effector functions in activated T cells. However, there is also the possibility that the antagonists may inhibit activation induced cell death, thereby enhancing effector functions by prolonging the lifespan of the effector T cells. Given these competing effects, the results from an *in vitro* co-culture Treg suppression assay would be difficult to interpret and ambiguous at best. Furthermore, the results would likely be dependent on assay

conditions[1] such as absolute and relative Treg and effector T cell numbers used, the type and amount of costimulation, the cell isolation method, and other culture conditions; the *in vitro* assay would thus inaccurately reflect what occurs *in vivo*. Therefore, our next steps focused on testing these antagonists *in vivo*. Unfortunately, the 2-4 human IL-2 mutant does not bind with enhanced affinity to the mouse IL-2 receptor α subunit, so the antagonists developed in this study cannot be tested in a mouse model. We are currently recapitulating the approach used here for engineering human IL-2 antagonists with mouse IL-2. It will be interesting to contrast the effects of these specific IL-2 signaling antagonists to those of ablative CD25-targeted therapies such as DAB_{389}-IL-2 and anti-CD25 antibodies in an *in vivo* model system.

It will also be important to test the selectivity of the IL-2 antagonists for Tregs over activated effector T cells. The significant variability and dependence on assay conditions, as described above and previously reviewed,[1] all make an *in vitro* assay unsuitable for testing this selectivity. Nevertheless, there are several factors that would potentially favor a selective effect on Tregs *in vivo*. First, effector T cells are activated by IL-2 in an autocrine manner, whereas Tregs do not secrete IL-2 and instead require IL-2 that is secreted by neighboring effector T cells in a juxtacrine manner. Therefore, the local IL-2 concentration surrounding effector T cells would be higher than for Tregs, making it easier to antagonize IL-2 effects on Tregs. Second, activated T cells express CD25 in a transient manner, and therefore have a shorter window of time for inhibition by IL-2 antagonists than Tregs, which constitutively express CD25. Lastly, from an empirical standpoint, Tregs are selectively depleted by the DAB_{389}-IL-2 fusion protein in several studies.[19–21] Though the mechanism of this selectivity has not been elucidated, the analogy can be tentatively applied to IL-2 antagonists because both rely on the interactions between IL-2 and the high affinity trimeric IL-2 receptor.

While these antagonists were originally designed as a novel class of Treg inhibitors, their use is not necessarily limited to biological inhibitors of regulatory T cell function. For example, these antagonists may be of use for inhibiting the effects of soluble CD25 in the body. Another potential use may be for targeting CD25 expressing cells for the delivery of drug payloads or for gene delivery, where IL-2 receptor activation, antibody dependent cell cytotoxicity or complement-mediated elimination may be undesirable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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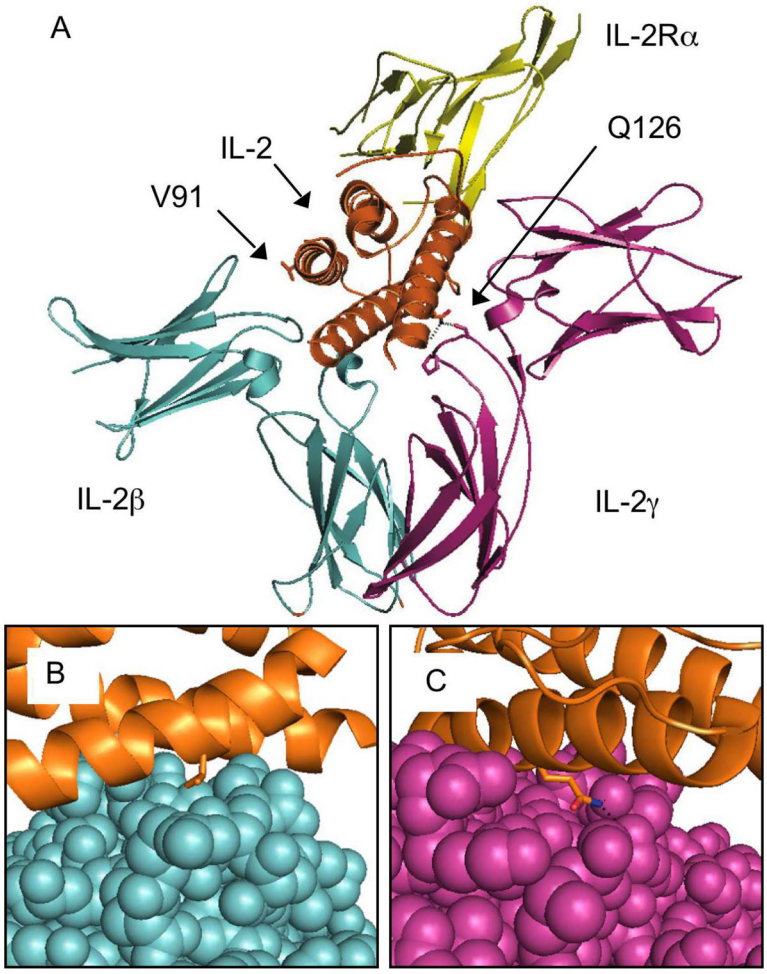

Figure 1.

Interleukin-2 Antagonist Design

The crystal structure of IL-2 (orange) complexed with the full IL-2 receptor complex, IL-2Rα (yellow), IL-2Rβ (blue), and IL-2Rγ (magenta), is shown with the valine 91 and glutamine 126 residues highlighted (A). Close-ups are shown of the IL-2 / IL-2Rβ interface with V91 (B), and the IL-2 / IL-2Rγ interface with Q126 (C). The crystal structure was calculated by Wang et al.[32]

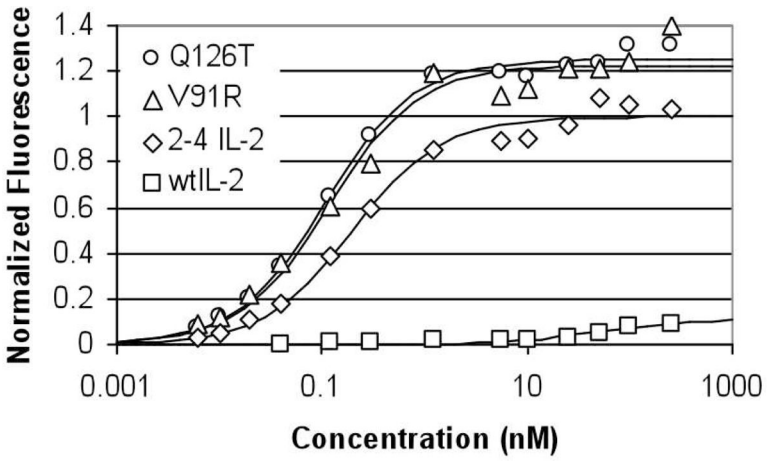

Figure 2.

Kit225 cell surface titrations to measure IL-2Rα binding affinity. The binding isotherms are shown for: Q126T (K_D = 109±19 pM), V91R (K_D = 119±45 pM), 2-4 IL-2 (K_D = 199±56 pM), and wild type IL-2 (K_D = 46±36 nM). Fluorescence is normalized to the maximum fluorescence of 2-4 IL-2 as determined by least squares regression, and K_D values are reported with 95% confidence intervals.

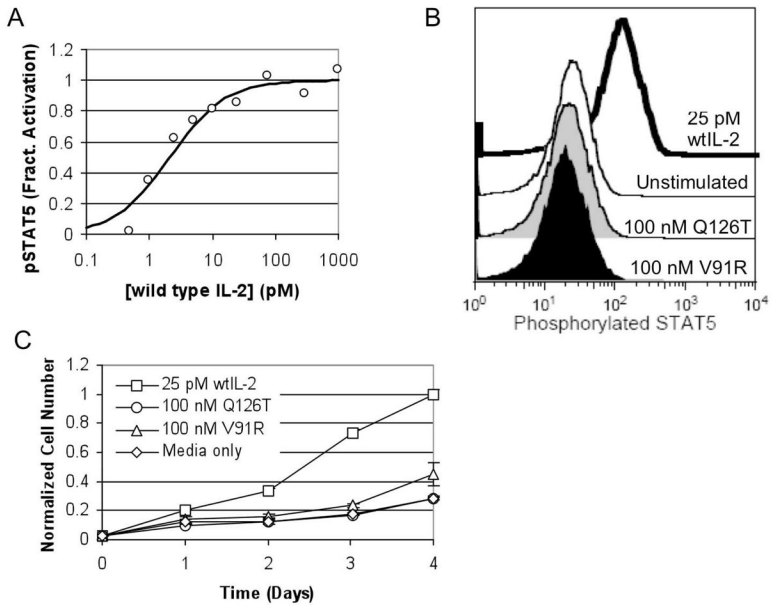

Figure 3.

Lack of Agonism by Q126T and V91R. STAT5 phosphorylation in Kit225 cells is highly sensitive to IL-2. In an IL-2 dose response curve (A) in the absence of antagonist, the measured EC_{50} is 2.1±1.2pM, and these data are representative of two independently repeated experiments. In both a STAT5 phosphorylation assay (B) and a cell proliferation assay (C) in Kit225, Q126T and V91R were significantly inhibited in their ability to activate the IL-2 receptor. Error bars represent the standard deviation of the live cell number at each data point measured in triplicate. Cell number is normalized to the mean cell number of the 25 pM wild type IL-2 group on day 4. These data are representative of three independently repeated experiments.

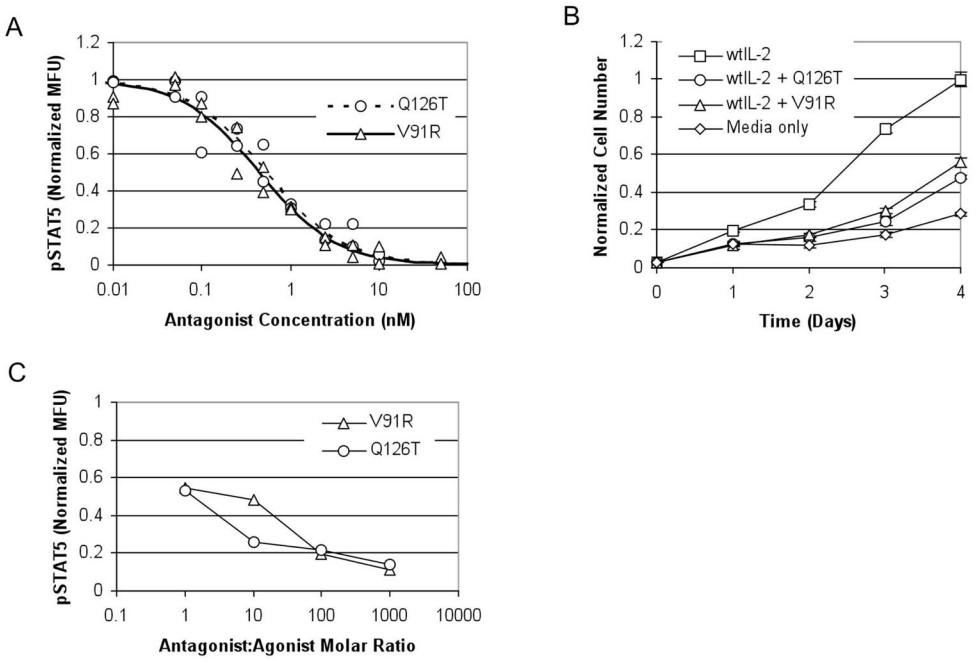

Figure 4.

Antagonism by Q126T and V91R. The two mutants, Q126T and V91R, were assayed for antagonism in the presence of 3 pM wild type IL-2 in a phosphorylated STAT5 assay (A), where the IC_{50} values and 95% confidence intervals were determined to be 525±252 pM Q126T, and 445±90 pM V91R. Data for each antagonist are combined from two independent experiments. Fluorescence is normalized to the maximum fluorescence of each antagonist as determined by least squares regression. In the Kit225 cell proliferation assay (B), 100 nM of Q126T or V91R was able to antagonize 25pM wild type IL-2. These data are representative of three independently repeated experiments. Cell number is normalized to the mean cell number of the 25 pM wild type IL-2 group on day 4. Error bars represent the standard deviation of the cell number at each data point measured in triplicate. Antagonism of STAT5 phosphorylation in primary human Treg cells *ex vivo* in the presence of 40 pM wild type IL-2 was also measured (C). Fluorescence was normalized to a value of 1.0 for 40 pM IL-2 in the absence of antagonist, and 0.0 in the absence of either antagonist or agonist.

Table I

Mutations for Disrupting IL-2 Receptor Subunit Binding

Subunit Binding Disrupted	Mutation
IL-2Rβ	D88R*, V91R
IL-2Rγ	Q126T, Q126I
IL-2Rβ and IL-2Rγ	E15W

*Wild type IL-2 has an asparagine at position 88, but 2-4 IL-2 has an asparagine to an aspartic acid substitution