Interleukin 2 (IL-2) Variants Engineered for Increased IL-2 Receptor α-Subunit Affinity Exhibit Increased Potency Arising from a Cell Surface Ligand Reservoir Effect

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ABSTRACT

Proliferation of activated T cells and CD56 bright natural killer (Cytokine Growth Factor Rev 13:169–183, 1995) cells caused by interleukin-2 (IL-2) has been exploited in IL-2-based therapies for the treatment of metastatic renal cell carcinoma and melanoma (J Clin Oncol 13:688–696, 1995; J Clin Oncol 17: 2105–2116, 1999). In this study, we demonstrate the potentially improved therapeutic value of IL-2 variants engineered to gain 15- to 30-fold increased affinity for the IL-2 receptor α-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 with 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.

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 (Fyfe et al., 1995; Atkins et al., 1999). 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 min, respectively (Konrad et al., 1990). Thus, it is difficult to maintain the therapeutically effective serum concentration range (1–100 pM) over a sustained period. 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 multisubunit receptor (Nelson and Willerford, 1998). The IL-2 receptor system consists of the α (IL-2Rα), β (IL-2Rβ), and γ (IL-2Rγ) receptor subunits. IL-2Rα is not involved in intracellular signaling, whereas IL-2Rβ and IL-2Rγ are necessary and sufficient to mediate intracellular signaling. IL-2 binds with a very high affinity ($K_d = 10^{-11}$ M) to the trimeric IL-2Rαβγ complex, an intermediate affinity to IL-2Rβγ ($K_d = 10^{-9}$ M), and a low affinity to the IL-2Rα subunit ($K_d = 10^{-8}$ 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 over-expressed on the surface of activated T cells (Smith, 1989; Theze et al., 1996) and has a long half-life (~48 h) on the cell surface (Hemar and Dautry-Varsat, 1990). An IL-2 mutant

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ABBREVIATIONS: IL, interleukin; IL-2Rα, interleukin 2 receptor α-subunit; IL-2Rβ, interleukin 2 receptor β-subunit; IL-2Rγ, interleukin 2 receptor γ-subunit; NK cell, natural killer cell; PEGylation, polyethylene glycolation.
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 used previously to generate IL-2 mutants with increased affinity for IL-2Rα (Rao et al., 2003). In this article, we describe the bioassay evaluation of such mutants in a novel pulsed bioassay that more closely approximates the systemic clearance of IL-2 than conventional static bioassays do. In this assay, a T cell line shows significantly greater proliferation in response to IL-2 mutants with higher affinity for IL-2Rα than to 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). In addition, a mutant with no change in IL-2Rα affinity was implied to have higher activity in the static assay, whereas 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 administrated as an intravenous 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.

Materials and Methods

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 15- to 30-fold increased affinity for IL-2Rα compared with 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 described previously (Rao et al., 2003).

Conventional Static Bioassay. Proliferation of an IL-2-dependent cell line was used as a readout to evaluate the activity of the IL-2 mutants generated. Kit225 is a human IL-2-dependent T cell line, expressing roughly 3000 to 7000 IL-2Rαβγ and 200,000 to 300,000 IL-2Rα (Hori et al., 1987; Arima et al., 1992). A frozen stock of Kit225 cells was created using cells cultured in a humidified atmosphere with 5% CO2 at 37°C, in RPMI 1640 supplemented with 1 mM IL-2, 10% heat-inactivated fetal bovine serum, 200 mM 1-glutamine, 50 units/ml penicillin, and 50 μg/ml gentamicin. Thereafter, frozen aliquots were revived and cultured in medium containing 40 pM IL-2. Before the bioassay, Kit225 cells were cultured in medium without IL-2 for 1 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, Madison, WI). The terms used and the parameter values chosen are described in Table 1. The differential equations were solved using MATLAB.
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-2Ra 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-2Raβγ is not considered. This simplification arises primarily because there are far fewer (∼100-fold lesser) IL-2Raβγ than IL-2Rα on the T cell surface.

The endocytic sink caused by the IL-2-IL-2Raβγ complexes under conditions of maximal endocytic degradation was calculated and found to be negligible at times less than 400 min. The details of this calculation are as follows. All IL-2Rβ and IL-2γ subunits are assumed to be associated with the IL-2Rα subunit. All IL-2Raβγ trimers are assumed to be associated with IL-2. Maximal endocytic degradation of IL-2-IL-2Raβγ complexes will occur under these conditions. At steady state, the endocytic rate should equal the rate of synthesis of IL-2Raβγ.

The rate of synthesis of IL-2Raβγ can be estimated as \( V_R + k_{syn} C \) (Fallon and Lauffenburger, 2000), where \( V_R \) is the constitutive rate of IL-2Raβγ synthesis and is 11/min, \( k_{syn} \) is the induced rate of IL-2Raβγ synthesis and is 0.0011/min, and \( C \) is the total number of IL-2-IL-2Raβγ complexes. A value of 3000 is used as the maximum estimate of IL-2-IL-2Raβγ complexes.

The maximal endocytic degradation rate is thus estimated as 14/min/cell. Considering 10^8 cells/l, this translates to a decrease of 2 pM/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 caused by endocytic degradation is negligible relative to systemic clearance of IL-2. In addition, 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 h (480 min).

### Results

**A Pulse Assay Approximates Renal Clearance.** We earlier reported the generation of mutants with increased affinity for IL-2Ra (Rao et al., 2003). Mutants M6 and M1 exhibit increased affinity for IL-2Ra, whereas mutant C1 has wild-type IL-2Ra affinity. In static bioassays, in the 0.5-to-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 because the total number of IL-2Ra 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 1A shows the viable cell density in response to varying concentrations of wild-type IL-2 or the IL-2 mutants, assayed at 60 h 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-2Ra, whereas 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-2Ra.

When administered as an intravenous bolus, IL-2 is rapidly cleared from the body, with half-lives of 12.9 and 85 min (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 min, then washed and resuspended in IL-2-free media, and the viable cell density was measured as a function of time. When exposed to a 1 nM concentration of cytokine for 30 min in the pulse assay, M6 and M1 showed significantly improved activity compared with wild-type IL-2. C1 and wild-type IL-2 showed similar activity. This is shown in Fig. 1B.

We assayed the effect of varying the concentration of cytokine in the pulse assay on the viable cell density. Figure 1C is a snapshot of viable cell density as a function of pulse concentration, at 60 h after the cytokine pulse, and Fig. 2 shows the effect on 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. In addition, the level of proliferation obtained using less than a 100 pM pulse of mutants with higher affinity for IL-2Ra (M6 and M1) cannot be achieved by using any concentration of C125S or C1. It is interesting to note

<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
<th>Value</th>
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<tr>
<td>[IL-2]</td>
<td>Serum concentration of IL-2 (molar units)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[IL-2 · α]</td>
<td>IL-2 in complex with IL-2Ra (molar units)</td>
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<tr>
<td>[IL-2Ra]</td>
<td>Total IL-2Ra on T cell surface</td>
<td>100,000/cell</td>
<td></td>
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<tr>
<td>t</td>
<td>Time (min)</td>
<td></td>
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</tr>
<tr>
<td>A</td>
<td>Magnitude of fast component of double exponential clearance</td>
<td>0.866</td>
<td>Konrad et al. (1990)</td>
</tr>
<tr>
<td>k_1</td>
<td>Rate constant for fast component of double exponential clearance</td>
<td>0.0537 min⁻¹</td>
<td>Konrad et al. (1990)</td>
</tr>
<tr>
<td>B</td>
<td>Magnitude of slow component of double exponential clearance</td>
<td>0.134</td>
<td>Konrad et al. (1990)</td>
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<tr>
<td>k_2</td>
<td>Rate constant for slow component of double exponential clearance</td>
<td>0.00815 min⁻¹</td>
<td>Konrad et al. (1990)</td>
</tr>
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<td>Association rate constant for IL-2-IL-2Ra interaction (wild-type)</td>
<td>6 × 10⁸ M⁻¹ min⁻¹</td>
<td>Liparoto et al. (2002)</td>
</tr>
<tr>
<td>k_{off}</td>
<td>Dissociation rate constant for IL-2-IL-2Ra interaction (wild-type)</td>
<td>18 min⁻¹</td>
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<td>k_{ec}</td>
<td>Rate constant for endocytosis and degradation of IL-2-IL-2Ra complex</td>
<td>0.00024 min⁻¹</td>
<td>Heman and Dautry-Varsat (1990)</td>
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<td>n</td>
<td>Total number of activated T cells in circulation</td>
<td>10^8 1⁻¹</td>
<td>Hodge et al. (2000); Storek et al. (2000)</td>
</tr>
<tr>
<td>N_{av}</td>
<td>Avogadro number</td>
<td>6.023 × 10²₃</td>
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that C1 and M6 show similar activity in a static assay (Fig. 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. On the other hand, M1 does not show significantly improved activity in the static assay, but has an activity similar to that of 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.

**IL-2Rα Acts as a Ligand-Reservoir in a Pulse Assay.**

We hypothesized that M6 and M1 exhibit increased activity in a pulse bioassay because of 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 Fig. 3A, surface-associated wild-type IL-2 is negligible after the wash steps in the pulse bioassay. However, even after 6 h, mutant M6 persists on the cell surface. The closed symbols in Fig. 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 rebind subsequently. We used soluble IL-2Rα as a reagent to capture any dissociated IL-2, to detect any rebinding occurring in the pulse assay. The cell surface-associated M6 was probed using flow cytometry. As shown by the open symbols in Fig. 2B, there is greater decrease in cell surface-bound IL-2 in the presence of the soluble IL-2Rα, indicating a certain amount of rebinding 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-2Rβ species on the cell surface. This aspect of the mechanism is currently under investigation. It should be pointed out that rebinding and equilibration could also occur in the physiological context of systemic clearance, as is evident from the mathematical model described below.

**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). It is conceivable that 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 (Fig. 4). 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 10-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 with conjugation of polyethylene glycolation (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, in-

![Fig. 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 are representative of three separate experiments.](image-url)

A, viable cell density as a function of IL-2 concentration, in a steady-state assay, after 60 h. 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 h.
asmuch as these are governed by the affinity of the cytokine-receptor binding interaction. Furthermore, PEGylated IL-2 remains available for interaction with NK cells in the circulation, with attendant side effects. By contrast, mutant IL-2s such as M1 or M6 are sequestered to the surfaces of those cells specifically targeted for stimulation.

**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 used 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. In addition, the lowered dosage concentration would help in 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 approximately 48 h (Hemar and Dautry-Varsat, 1990). In the pulse assay, the half-life of M6, an IL-2 mutant with 15- to 30-fold enhanced IL-2Rα affinity, on the surface of Kit225 cells is on the order of 4 h. 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.

![Fig. 2](image1.png)

**Fig. 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.

![Fig. 3](image2.png)

**Fig. 3.** IL-2Rα acts as a ligand-reservoir and mediates prolonged persistence of M6 on the cell surface of Kit225 cells relative to wild-type IL-2 (C125S). A, phycoerythrin (PE) fluorescence is a measure of cell surface-bound IL-2. Data shown are a representative set of histograms for three different experiments. B, different colored closed symbols denote data from different experiments in which no capture reagent is present. Colored open symbols denote data from different experiments in which soluble IL-2Rα was used as a capture reagent for dissociated M6.
Increased Activity of Enhanced IL-2Ra Affinity IL-2 Variants

Fig. 4. Model for persistence of IL-2 mutants with decreased off-rate for IL-2Ra on activated T cells, in blood circulation. Decreased off-rate of IL-2 mutants for IL-2Ra results in greater levels and longer persistence of IL-2 on activated T cells in blood circulation.

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-2Rα subunit, whereas each has its own private α-receptor subunit (Fehniger et al., 2002). IL-15 has a high affinity for its private α-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-2Ra 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 agent 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 multisubunit receptors, to generate superagonists or superantagonists. Examples include interleukin-3 (IL-3), interleukin-5 (IL-5), and granulocyte macrophage colony-stimulating factor. IL-3, IL-5, and granulocyte macrophage colony-stimulating factor use private α-receptor subunits and a common β-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 α-subunits would conceivably increase persistence of these cytokines in circulation and hence lead to improved cytokine superagonists (through persistent signaling) or superantagonists (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.

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References


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