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Human IL-2 Mutein with Higher Antitumor Efficacy Than Wild Type IL-2

Tania Carmenate,* Anabel Pacios,* Michel Enamorado,* Ernesto Moreno,* Karina Garcia-Martínez,* Dasha Fuente,† and Kalet León*  

IL-2 has been used for the treatment of melanoma and renal cell carcinoma, but this therapy has limited efficacy and severe toxicity. Currently, it is assumed that part of the limited efficacy is due to the IL-2–driven preferential expansion of regulatory T cells, which dampen the antitumor immunity. In this study, we characterize a human IL-2 mutant with higher antitumor efficacy and lower toxicity than wild type human IL-2 (wtIL-2). The mutant differs from wtIL-2 by four mutations at the interface with the α subunit of IL-2R. The IL-2 mutant induces in vitro proliferation of CD8+CD44hi and NK1.1 cells as efficiently as does wtIL-2, but it shows a reduced capacity to induce proliferation of CD4+Foxp3+ regulatory T cells. The IL-2 mutant shows a higher antimetastatic effect than does wtIL-2 in several transplantable tumor models: the experimental metastasis model of MB16F0 melanoma and the experimental and spontaneous metastasis models for the mouse pulmonary carcinoma 3LL-D1222. Relevantly, the IL-2 mutant also exhibits lower lung and liver toxicity than does wtIL-2 when used at high doses in mice. In silico simulations, using a calibrated mathematical model, predict that the properties of IL-2 mutein are a consequence of the reduction, of at least two orders of magnitude, in its affinity for the α subunit of IL-2R (CD25). The human IL-2 mutant described in the present work could be a good candidate for improving cancer therapy based on IL-2.

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Abbreviations used in this article: E, helper; hIL-2, human IL-2; LN, lymph node; M, memory; R, regulatory; TE, 10 mM Tris, 1 mM EDTA; Treg, regulatory T cell; wtIL-2, wild type human IL-2.

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The murine T cell line CTL.L2 was donated by Dr. A. Santos (Center of Genetic Engineering and Biotechnology, La Habana, Cuba). This line is IL-2 dependent and constitutively expresses the αβ form of IL-2R. The cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FBS, 50 IU/ml human rIL-2 (Center of Genetic Engineering and Biotechnology), 2 mM l-glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin. Murine MB16F0 melanoma cells and murine 3LL-D122 lung carcinoma cells were maintained in DMEM F12 (Life Technologies) supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin. All cells were maintained at 37°C under a humidified 5% CO2 atmosphere. Tumor cells...
were harvested using trypsin/EDTA; for in vivo experiments, cells were resuspended in PBS.

**Design and production of hIL-2 mutein**

For production of the hIL-2 mutein, the synthetic genes containing the corresponding mutations from the original hIL-2 gene, as well as the full original hIL-2 gene, were obtained from Geneart (Berlin, Germany). The genes were cloned into the commercial vector pET28a (Novagen, Darmstadt, Germany). This vector contains an N-terminal His-tag, and the genes of interest are under the control of lac operator. 

Escherichia coli cells (strain BL21(DE3); Invitrogen) were transformed with the hIL-2 mutant or wtIL-2 expression plasmids using the manufacturer’s protocol. Transformed cells were allowed to grow in 200 ml Lysogenic broth medium until the OD600nm reached 0.6; subsequently, 4 mM IPTG was added and incubation continued for 6 h, after which cells were harvested by centrifugation and stored as a pellet at −80°C.

**Purification of wtIL-2 and hIL-2 mutein from insoluble material**

The frozen pellets were suspended in 10 mM Tris, 1 mM EDTA (pH 8) (TE) and sonicated using an ultrasonic cell disrupter (IKa). In each case, the insoluble material was harvested by centrifugation (18,000 × g) and washed successively with 4 M Urea-TE and 1% Triton X-100–TE using an Ultra Turrax T8 homogenizer. For further purification, we used an HPLC system (Pharmacia, Uppsala, Sweden); the proteins were extracted with 6 M Guanidinium hydrochloride–TE at 0.1 g/ml (wet weight), and renaturation was carried out by dialysis. For further purification, proteins were applied to a reverse-phase C4 column (Vydac). In this final chromatography, the recombinant polypeptides were purified using an H2O-acetonitrile-trifluoroacetic acid system, with a linear gradient (30–85% of acetonitrile) and 0.6 ml/min flow. Finally, the proteins were dialyzed against 10 mM acetate (pH 4), filtered through 0.2 μm filters, and stored at 4°C.

**Abs and flow cytometry**

For flow cytometry, cell suspensions from spleen and lymph nodes (LNs) were prepared according to standard protocols. All fluorochrome-conjugated mAbs were purchased from eBioscience, unless otherwise stated; FITC-conjugated anti-CD3 (145-2C11), PECy5.5-conjugated anti-CD4 (L3T4), PE-conjugated anti-NK1.1 (PK136), PE-conjugated anti-CD8 (eBio H35-17.2), PECy5.5-conjugated anti-B220(RA3-6B2), PE-conjugated anti-Foxp3 (NRRF-30), PE-conjugated anti-CD25 (3C7), and PE-conjugated anti-His-tag were from R&D Systems. Intracellular Foxp3 staining sets were purchased from eBioscience. Samples were measured using a FACScan (Becton Dickinson) flow cytometer and analyzed using FlowJo software (TreeStar). Anti-CD3 (YTS169) and anti-NK1.1 (PK136), both produced at the Center of Molecular Immunology, were mAbs used for in vivo Ab-depletion experiments.

**Mice**

Seven- to eight-week-old female C57BL/6 and BALB/c mice were obtained from the National Center for Laboratory Animal Breeding (Havana, Cuba). Food and water were provided ad libitum. The experiments were performed according to guidelines of the International Laboratory Animals Resources using standardized procedures in the Center of Molecular Immunology. All animal studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee.

**Cell suspension preparation**

CD8+ T cells and CD4+FOXP3+ Tregs were purified from inguinal and mesenteric LN cell suspensions using CD8+ T Cell and CD4+CD25+ Regulatory T Cell Isolation kits (Miltenyi Biotec), following the manufacturer’s recommendation. In both cases, purity was tested by flow cytometry. In the case of NK cell expansion from spleen cells, spleens from two mice were collected, disaggregated, and RBC lysed.

**Proliferation assays**

CTLL2 cells were harvested by centrifugation, washed two times, and incubated in RPMI 1640 without IL-2 for 5 h prior to the proliferation assay. A total of 10^7 cells/well was incubated with serial dilutions of either wtIL-2 or hIL-2 mutein in RPMI 1640 and allowed to grow for 48 h. After that, 20 μl alamarBlue dye (Invitrogen) was added per well, and plates were incubated for 12 h. Finally, plates were read at 540 and 630 nm, and the percentage of reduction of alamarBlue was calculated following the manufacturer’s recommendation.

For lymphocyte populations, 10^5 cells/well purified CD8+ cells or CD4+ CD25+ Tregs were cultivated on 96-well plates. In the case of CD4+CD25+ Tregs, 96-well plates were precoated with 5 μg/ml anti CD3 mAb 2C11, and cells were cultivated in the presence of 100 μg/ml anti mAb 2C11 S45B6. For the NK1.1+ cell expansion assay, 2×10^3 spleen cells/well were cultivated on 24-well plates. In all cases, cells were incubated with different concentrations of wtIL-2 or hIL-2 mutein; cells were harvested after 5 d and counted by flow cytometry. For determination of the number of cells reference fluorospheres Flow-Check (Beckman Coulter) were used.

**Lung metastasis models**

For experimental metastasis models, C57BL/6 mice were inoculated with 1×10^6 B16F0 or 3LL-D122 cells via the tail vein on day 0. Treatments were given on days 1–4 by i.p. injections of saline solution, 20 μg wtIL-2, or 20 μg IL-2 variant, twice a day. When necessary, 1 mg depleting mAbs specific for CD8 or NK1.1 molecules was injected i.p. into mice) on days 1, 4, and 7. For the spontaneous metastasis model, C57BL/6 mice were inoculated with 2×10^5 3LL-D122 cells in the footpad; when primary tumors reached 0.9 mm in diameter, they were removed surgically. Treatments were given on days 1–4 after challenge and after surgery by i.p. injection of saline solution, 20 μg wtIL-2, or 20 μg hIL-2 variant. Mice were sacrificed on day 21, lungs were removed and embedded in Bouin’s solution, and metastatic nodules were counted under a binocular microscope.

**Toxicity**

For toxicity assessment, five BALB/c mice/group were inoculated with 80 μg wtIL-2 or hIL-2 mutein, whereas the control group received PBS. The treatments were administered twice a day for 5 d. Mice were sacrificed, and lungs and livers were weighed. For histologic study, organs were fixed in 10% formalin solution, and paraffin-embedded sections were stained with H&E.

**Statistical analysis**

We used Graph Pad Prism 4.0 software for statistical analysis. In vitro proliferation curves were adjusted to sigmoid dose-response curves. In the case of antitumor assays, a parametric ANOVA, followed by a Bonferroni multiple-comparison test, was applied. For toxicity assessment, organ weights were compared using the nonparametric Kruskal–Wallis test, followed by the Dunn multiple-comparison test.

**Mathematical model of the interplay between IL-2 and IL-2 muteins with T lymphocytes**

The mathematical model used in this study was developed and calibrated to describe the interaction between IL-2 and Th cells (E), regulatory (R) CD4+ T cells, and memory CD8+ T cells (17). The model includes several compartments, which represent different LNs, in which T cells are confined interacting with each other, with the APCs and available soluble molecules. It also includes a compartment representing the blood (i.e., the circulatory system), which contains only soluble molecules, IL-2- or IL-2 mutants. LNs are connected to the blood compartment, allowing the free exchange of soluble molecules. The concentration of IL-2- and muteins in the blood is assumed to decay with a constant rate, which represents renal elimination. An external source term for these molecules is added in this compartment to simulate particular treatment applications. The model includes the dynamics of helper (E), regulatory (R), and memory (M) T cells on the different functional states of their life cycle: resting, activated, and cycling cells.

No α IL-2 mutein is modeled as a soluble molecule that bears all of the properties of IL-2 in the model but whose conjugation affinity for the α-chain of IL-2R is reduced by the factor f (parameter f = (0 to 1)). Treatments are simulated to represent a continuous infusion of this molecule in the blood for a defined period of time. This is implemented by setting on, transiently, the external source term in the blood compartment of this molecule. Two parameters always control treatment application: the “dose,” which set up the total amount per day of IL-2 mutein infused in the blood, and the “treatment duration,” which set the length of time for which continuous infusion is maintained. We explore how the dose and treatment duration determine the outcome of the system simulation, as well as whether this treatment can condition a significant preferential expansion (dominance) of Th cells, Tregs, or M cells in the LN compartment.

For calibration of the mathematical model, the majority of the parameters were fixed to values taken directly or derived from available independent experimental data; just a few parameters remain unknown, and their influence on the result was explored within a range of biologically reasonable values.
Results

Mutein production

An hIL-2 mutein was designed that differs from wtIL-2 only at positions R38, F42, Y45, and E62; all of these residues were substituted by alanine. For production convenience, Cys325 was substituted by Ser, because this mutation was shown not to change the biological activity of hIL-2 (18). The synthetic genes encoding either the mutein or wtIL-2 were cloned into the pET28a vector and expressed in the BL21 (DE3) E. coli strain. Proteins were expressed as inclusion bodies and were purified to homogeneity following the purification protocol described by Moya et al. (19) for human rIL-2 purification.

IL-2 mutant behaves as an IL-2 agonist in vitro

Fig. 1 shows the molecular structure of hIL-2 mutein with the positions of mutated residues. Point mutations on the mutant are concentrated in the region in contact with the α subunit of hIL-2R. We used flow cytometry to assess the capacity of the new molecule to bind to the CTLL-2 cells. This cell line expresses the high-affinity αβγ IL-2R just as the activated T cells do, with 4.0–10-fold excess of the free α chain (20). For detection of His-tagged recombinant proteins bound to the cell surface, we used a PE-conjugated anti–His-tag mAb. At the high concentrations used, wtIL-2 and hIL-2 mutein bind in the same way to the cell surface. Furthermore, a competition assay was performed to establish whether the hIL-2 variant is able to inhibit the binding of the anti–CD25 mAb, clone 3C7, which competes with wtIL-2 (Fig. 2A). As a result, when cells were preincubated with hIL-2 mutein, binding of anti-CD25 mAb was not inhibited, showing the same mean fluorescence intensity 275 ± 5.74 for cells incubated with anti-CD25 mAb alone versus 270 ± 35.7 for cells preincubated with the hIL-2 mutant. As expected, the preincubation of cells with wtIL-2 decreased the mean fluorescence intensity of CD25 labeling to 108 ± 9.71. The anti-CD25 mAb is able to bind to CTLL-2 cells even after wtIL-2 preincubation; the excess of α subunit not assembled with the rest of receptor chains must explain this residual binding. This result indicates that the hIL-2 mutant has, at least, less capacity than wtIL-2 for binding the α subunit receptor, and it was termed no-α mutein.

We used the CTLL-2 cell line–proliferation assay to determine the signaling capacity of no-α mutein in vitro. This cell line is highly sensitive to IL-2 and is commonly used for IL-2 activity determination (21). Cells were allowed to grow in the presence of either wtIL-2 or no-α mutant. Fig. 2B shows the proliferation curves, after 48 h of incubation; the no-α mutant was able to induce cell proliferation by behaving as an IL-2 agonist. The two proteins induced the same maximal level of proliferation; however, a 38-fold reduction in no-α mutant activity was observed when comparing EC50. In contrast, the mutations do not seem to influence the global conformation of the molecule, because no-α mutant is able to bind to the cell and induce proliferation.

No-α mutein conserves the capacity to direct stimulate effector cells, but not Tregs, in vitro

We compared no-α mutein and wtIL-2 with regard to their ability to stimulate effector cells or CD4+Foxp3+ Tregs. As effector cells we chose CD8+ T cells and NK cells, which mainly express the βγ intermediate-affinity form of IL-2R. First, CD8 cells purified from mesenteric LN s of C57/BL6 mice were cultured with different concentrations of wtIL-2 or mutated hIL-2. After 5 d, a considerable increase in the percentage of CD8+CD44hi cells was observed, reaching 62.13% for wtIL-2 and 64.15% for no-α mutein. The mutant and wtIL-2 were able to stimulate the proliferation of CD8+ T cells; the total numbers of CD8+CD44hi cells were determined, and the proliferation curves obtained were similar for the two molecules (Fig. 3A).

The capacity to induce the differentiation of NK cells from mouse splenocytes was also tested (Fig. 3B). At the highest concentration, the no-α mutein was as effective as wtIL-2, inducing the same percentages of NK1.1+ B220+ cells. Nevertheless, this effect decreased faster for no-α mutant, with an EC50 of 5.96 μg/ml for the mutant and 0.48 μg/ml for wtIL-2. The differences observed might be due to the 10% of NK cells that express the high-affinity form of IL-2R, because they respond better to wtIL-2 than to the no-α mutant.

We also evaluated the in vitro expansion of Tregs driven by either wtIL-2 or no-α mutein (Fig. 3C). After Treg enrichment, the sample was analyzed by flow cytometry; CD4+Foxp3+ Tregs accounted for 80% of the sample, and the most important contaminant was CD4+Foxp3− T cells (up to 16%). The cells were activated with plate-bound anti–CD3 mAb, and we used 100 μg/ml of anti-murine IL-2 mAb S4B6 to inhibit the IL-2 that effector T cells produce after activation. After culture, the total number of Tregs was determined, and proliferation curves were obtained (Fig. 3C, right panel). As expected, the activity of the mutein over Tregs was significantly lower than was wtIL-2 activity, with an EC50 of 1.58 ± 0.9 μg/ml for the mutant and 1.8 ± 1.2 ng/ml for wtIL-2; almost 1000-fold more no-α mutant was required to achieve the same expansion level. Also, when the expanded population was assayed by flow cytometry, expansion of a population of CD4+Foxp3− T cells was seen in the samples treated with different concentrations of the no-α mutein. This population, which accounted for <2% in the initially purified lymphocytes, accounted for >30% at the end of culture. This in vitro–expanded population was later identified as CD8+CD44hi T cells. This result indicates that, in the presence of different lymphocyte populations, the mutant is available for either αβ γ IL-2R− or for βγ IL-2R-expressing cells. In conclusion, the hIL-2 mutein nearly completely conserves the capacity of wtIL-2 to induce proliferation of βγ IL-2R-bearing cells, whereas its capacity to induce the in vitro proliferation of αβ γ IL-2R–expressing cells is severely affected. Particularly, the decrease in signaling capacity through the high-affinity IL-2R was notable for Tregs. We do not expect that the same behavior takes place in vivo, where different T cell subsets are able to produce IL-2, and, in turn, the cytokine may stimulate Tregs. For this reason, we performed the following in vivo experiments to study the antitumor effect of the mutein in comparison with wtIL-2.

No-α mutein shows greater antitumor effect than wtIL-2

The antitumor effect induced by no-α mutant was compared with that induced by wtIL-2 in the experimental metastases model of the mouse melanoma line MB16F0 (Fig. 4). This is a very significant experimental model because human melanoma is one of
the diseases treated with wtIL-2. Previously, it was demonstrated that MB16F0 cells do not express IL-2R and do not respond to wtIL-2 exposure in vitro. C57/BL6 mice were inoculated i.v. on day 0 with \(2 \times 10^5\) tumor cells and were treated on days 1–4 with saline as control, wtIL-2, or no-α mutein. Considering that anti-tumor effector cells, mainly CD8 and NK cells, express IL-2Rβγ, and that the no-α mutein fully signals through this receptor, a 20-μg dose was used in both cases. Mice were sacrificed on day 21, lungs were removed, and metastases were counted under a binocular stereoscope. Native hIL-2–treated and no-α mutant–treated mice exhibited a reduction in the number of lung metastases; however, the antitumorous effect of no-α mutein was superior, being different from the control group (\(p < 0.01\)), as well as the group treated with wtIL-2 (\(p < 0.05\)).

It is known that effector lymphocytes, CD8+ T cells and particularly NK cells, are relevant effectors for the antitumorous response. Therefore, we tested whether depletion of such populations abrogates the antitumor effect observed in vivo after no-α mutant treatment. Depletion capacities for all Abs were assessed by flow cytometry analysis of mouse spleen cell suspensions. Depletion with both mAbs, anti CD8 and anti-NK1.1, abrogated the antitumorous effect induced by treatment with no-α mutein (Fig. 4D); consequently, this population seems to be the major effector cells mediating the delayed growth of lung metastases induced by mutein treatment.

The in vivo antitumor effect of no-α mutein treatment was also assayed in the experimental metastases model of the 3LL-D122 cell line (Fig. 5). We verified that 3LL-D122 cells do not express IL-2R and do not respond to wtIL-2 exposure in vitro, just like MB16F0 cells. As described above, C57BL6 mice were inoculated i.v. with \(2 \times 10^5\) tumor cells and treated on days 1–4 with saline as control, wtIL-2, or no-α mutein. Mice treated with no-α mutein showed a marked reduction in nodule numbers, being statistically different from the control group, whereas mice treated with wtIL-2 did not show any reduction compared with the control group. Furthermore, using the same tumor cell line, we compared the effect of no-α mutein with that of wtIL-2 in the model of spontaneous metastases, which better resembles the clinical setting for human disease. As described in the previous section, mice were inoculated in the footpad with \(2 \times 10^3\) 3LL-D122 cells and treated for 4 d after challenge. Tumor growth was monitored; when tumors reached 0.8–0.9 mm in the control group, they were surgically removed, and mice were treated for 4 d with the same treatments. The no-α mutein also showed a clear antitumorous effect in this therapeutic setting, being better than wtIL-2 and statistically different from the control group treated with PBS (\(p < 0.05\)).

**No-α mutein induces less toxicity than wtIL-2**

The toxicity induced by the high doses of IL-2 used is one of the limitations of IL-2–based therapy. The most frequent complication induced by IL-2 therapy is vascular leak syndrome, resulting in edema and lymphocyte infiltration in several organs. We decided to compare the toxicity induced by wtIL-2 and the no-α mutein. BALB/c mice were injected i.p. with 80 μg of each protein, twice a day, for 5 d; this dose is four times higher than that used to achieve antitumor effect. After treatment, a significant increment in the weights of lungs and livers was observed in the group treated with wtIL-2 but not in the groups treated with no-α mutein or PBS. Moreover, histopathological studies of the lungs and livers of IL-2–treated mice revealed perivascular lymphocytic infiltrations. Such infiltrations were not observed in organs from no-α mutein–treated mice, which were similar to organs from the control group (Fig. 6). These results suggest that, because the no-α mutein characterized in this work induces less toxicity than does wtIL-2, it could be used at higher doses than wtIL-2 to obtain better efficacy.

**Modeling the therapeutic impact of IL-2 muteins with a reduced capacity to bind to CD25**

We resorted to mathematical modeling to theoretically address the therapeutic impact of eliminating or severely reducing IL-2’s capacity to interact (bind) with the α-chain of its receptor (CD25). We used a mathematical model that was developed and calibrated by our group (17). This model focuses on the complex interplay of IL-2 with the dynamics of regulatory CD4+CD25+FoxP3+ T cells (R), CD4+ Th cells (E), memory CD8+CD44+ T cells (M), and NK cells (M). In particular, it takes into account our current knowledge of the differential and dynamical expression of the α-, β-, and γ-chains of IL-2R. To address the immune-stimulating po-
potential of different therapies, the model is set to a steady-state equilibrium, where Tregs (R) effectively (dominate) regulate the expansion of autoreactive effector T lymphocytes (E cells) and memory cells (M cells). Such a steady state is interpreted in the model as natural tolerance. Then the system dynamics is perturbed by simulating the injection of a desired agent, further evaluating whether it is driven away from the natural-tolerance steady state.

We explored, through mathematical modeling, the effect of injections with no-α mutants on T lymphocytes. The no-α mutants are simulated as molecules that share all of the quantitative properties of wtIL-2 in the model, but their affinity for binding CD25 is reduced by the factor $f$ (parameter $f = \{0 \text{ to } 1\}$). Fig. 7A shows the effects of a dose of $40 \mu g$ of wtIL-2 (thin lines) or no-α IL-2 ($f = 0$) for 5 d on different lymphocyte populations. Note how, in these simulations, the system dynamics starts in a steady state, with a significantly high number of Tregs (R) and a low number of autoreactive effector T cells (E) and memory cells (M). This is the natural-tolerance steady state. The system is perturbed for 5 d; afterward, the dynamic evolution differs for treatment with IL-2 mutant or wtIL-2. The injection of wtIL-2 seems to preferentially expand the Treg population, whereas injection of IL-2 mutein barely expands the Tregs, favoring instead a significant expansion of the NK and memory CD8+CD44+ T cells (M cells in the model). In both cases, the effect of the treatment is transient, and the system returns to the natural-tolerance steady state. To quantify this transient differential effect on lymphocytes, we calculated, at day 6 (just after the treatment is finished), the ratio of the increase in effector cells (E+M cells in the model) to the increase in Tregs, as a function of the injected dose for IL-2 muteins, with different values of $f$ (Fig. 7B). It seems that IL-2 muteins with a more severely reduced capacity to bind CD25 ($f$, 0.01) have a wider range of low-intermediate treatment doses with which they exhibit a higher capacity than wtIL-2 to preferentially expand the effector lymphocytes. Moreover, treatment with higher and sustained doses of IL-2 or muteins could induce a permanent change in the system dynamics, leading to a full breakdown of the natural-tolerance steady state. Fig. 7C illustrates this result for treatment with no-α mutein ($f = 0$) at a dose of 0.5 mg/d for 15 d. Note how the system evolves there into a new steady state, which is characterized by a significant expansion of the autoreactive effector T cells (M and E cells) and a net reduction in the number of Tregs (R). Such a steady state was interpreted in the work of García-Martínez et al. (17) as a steady state of autoimmunity. In Fig. 7D, the minimal dose of no-α mutant required in the model simulation to achieve the latter effect is quantified. This is shown as a function of the mutant's
capacity to bind to CD25 ($f$) for two mutant decay rates in the blood. The solid line represents the normal IL-2 decay rate (half-life of 9 min) (22), and the dashed line represents the decay rate reported for an IL-2 molecule fused to Fc (half-life of 7 h) (23). As can be seen in Fig. 7, wtIL-2 ($f = 1$) requires higher doses than does mutant IL-2 to effectively destroy the natural-tolerance steady state. The minimal dose required for the mutant IL-2 is reduced significantly by increasing its half-life in blood. Interestingly, the model predicts that a significant reduction, of at least two orders of magnitude ($f, 0.01$), in the capacity of IL-2 to bind CD25 is required to see a clear differentiation of mutein properties with respect to wtIL-2.

Discussion
In the 1990s, IL-2 was approved by the U.S. Food and Drug Administration for the treatment of melanoma and renal cell carcinoma. The clinical efficacy achieved with IL-2 therapy has not met expectations, because only 15–20% of patients respond to the therapy. But it is relevant that some of those patients experience total or long-lasting responses (5). The IL-2–driven expansion of Tregs in vivo has been related to this poor efficacy. In addition, high-dose treatment with IL-2 induces severe toxicity, with vascular leak syndrome being the major symptom. Therefore, the search for improvements in IL-2 as a therapeutic agent remains a very attractive topic.

FIGURE 4. Antitumor effect of IL-2 and IL-2 mutein in the MB16F0 experimental metastases model. (A) Schedule of treatment for mice bearing MB16F0 cells. (B) Photographs of representative lungs of each group. (C) MB16F0 nodule counts. Cumulative data from three different experiments ($n = 8$ mice/group). Bars represent means; error bars represent SEM. IL-2 mutein shows greater ant metastatic effect than does native IL-2. **$p < 0.001$, *$p < 0.05$, Bonferroni multiple-comparison test. (D) In vivo CD8+ cell depletion abrogates the ant metastatic effect on IL-2 mutein. (E) In vivo NK1.1+ cell depletion abrogates the ant metastatic effect on IL-2 mutein. *$p < 0.01$, Bonferroni multiple-comparison test.

FIGURE 5. Antitumor effect of IL-2 and IL-2 agonist in the 3LL-D122 mouse lung carcinoma model. (A) Treatment schedule for mice bearing 3LL-D122 cells: experimental metastases model (upper panel) and spontaneous metastases model (lower panel). (B) Photographs of representative lungs for each group in the experimental metastases model. (C) 3LL-D122 nodule counts from the experimental metastases model. (D) 3LL-D122 nodule counts from the spontaneous metastases model. Figures show cumulative data from three different experiments. Error bars represent SEM. *$p < 0.05$, versus control, Bonferroni test.
In this study, a new IL-2 mutant, termed no-α, was designed for its noticeably reduced capacity to stimulate Tregs and its capacity to expand CD8+ cells and NK cells as well as does wtIL-2. In contrast, the hIL-2 mutein shows an ∼40-fold reduced capacity to stimulate the proliferation of the CTLL2 cell line, an αβγ IL-2R-expressing cell line, and an even greater reduced capacity (1000-fold) to stimulate CD4+CD25+Foxp3+ cells in vitro. The no-α mutant was also assayed in two tumor models in mice. After treatment, the number of lung nodules was reduced in the mice depleted. In addition, in preliminary studies, the no-α mutein seems to induce less toxicity in mice than does wtIL-2.

From the three-dimensional structure of the quaternary IL-2/IL-2Rαβγ complex solved by Wang et al. (24), and using PyMol software for structure visualization and calculation, we located the mutated residues on the hIL-2 molecule surface. All mutated residues are separated by ≤ 5 Å from the α receptor subunit. When a computational alanine scanning of the IL-2/IL-2Rα interface was performed, we found that all of the mutated residues make significant contributions to the IL-2/IL-2Rα interaction, suggesting that the hIL-2 mutein does not bind to, or has a reduced capacity to bind to, the α receptor subunit. This suggestion was verified, in part, by the fact that IL-2 mutein is not able to displace the binding of anti-CD25 mAb (3C7) to the CTLL2 surface, whereas wtIL-2 competes with 3C7 mAbs and induces a marked reduction in labeling intensity. Individual mutations in the residues R38 and F42 were reported by Heaton et al. (25) as being important for disrupting IL-2 interactions with the α-chain. In this case, we included mutations in these positions and at Y45 and E62 at the same time: consequently, we expected a dramatic decrease in IL-2 affinity by the α-chain. In the study by Heaton et al. (26), R38A and F42K variants showed only a 50% reduction in sp. act.; in our work, the introduction of four mutations in the same molecule induced a stronger (40-fold) decrease in sp. act. over the αβγ IL-2R–bearing CTLL 2 cell line.

Several hIL-2 agonists, with mutations on different regions of the molecule surface, have been described; most of them were designed and tested in vitro for structural characterization of the IL-2 molecule. Nevertheless, some previous studies with IL-2 muteins aimed to obtain variants with a greater therapeutic index than hIL-2. The work of Shanafelt et al. (15) is one of the most significant; a mutant, named BAY 50-4798, exhibited antitumor activity and was better tolerated than hIL-2. Different from the mutein described in the present study, BAY 50-4798 was mutated.
at the interface with either IL-2Rβ or IL-2Rγ, but the residues implicated in the interaction with IL-2Rα remained untouched.

Other investigators also mutated the residues implicated in the interaction with IL-2Rα. In 2003, Rao et al. (16) tested the hypothesis that a mutein with increased affinity for IL-2Rα must have greater biological potency; nevertheless, these mutants did not exhibit increased activity in vitro. In contrast, Heaton et al. (25) tested whether R38 or F42 variants, which must have affected the interaction with IL-2Rα, were able to reduce the secretion of IL-1β, TNF-α, TNF-β, and IFN-γ. PBMCs stimulated with both mutants induce the same lytic activity as does wtIL-2, but the concentration of inflammatory cytokines tested in the supernatant of such culture decreased compared with that produced by wtIL-2 stimulation. The investigators postulated that such single-point mutated variants could be used for immunotherapeutic purposes, and they must evoke less systemic toxicity, based on the idea that the major side effects of IL-2 treatment are caused primarily by the secretion of IL-1β and TNF-α. More recently, Krieg et al. (27) postulated that IL-2−mediated pulmonary edema depends on the direct effect on lung endothelial cells that express IL-2Rαβγ in a functional form and that blocking the α-chain is enough to reduce the pulmonary toxicity induced by IL-2. This idea also explains a possible low toxicity induced by mutants described by Heaton and even more for the mutein described in the present work, in which the interaction with the α subunit seems to be totally disrupted. Indeed, in this study, we obtained primary in vivo evidence showing lower toxicity induced in mice by the no-α mutein. This latter property is important because the high toxicity associated with IL-2−based therapy is one of its major drawbacks.

Furthermore, we used a previously calibrated mathematical model to assess the impact of treatment with a mutein of IL-2 with a reduced capacity to bind to CD25 on lymphocyte dynamics. The results of our simulations suggest that an IL-2 mutein with severely reduced or null capacity to bind to α-chain of IL-2R (CD25) is a more potent inducer of immunotaxis than is wtIL-2. Such increased capacity relies on the quantitative nature of the relationship between IL-2 and lymphocyte dynamics, particularly on the regulated differential expression of the α- and β-chains of IL-2Rs among lymphocytes. A significant reduction in the interaction of IL-2 with CD25 (of at least two orders of magnitude, \( f < 0.01 \)) reduces its preferential use by Tregs (a fact characteristic of wtIL-2 in vivo dynamics), making the IL-2 mutant equally accessible to Tregs and Th cells and even more preferentially used by NK and memory CD8 T cells. Such an imbalance is sufficient to drastically alter the overall T cell dynamic response. Furthermore, our simulations predict that, in the no-α muteins, the lower the capacity to bind to CD25 and the smaller their decay rate in vivo, the greater their immune-stimulatory potential.

The experimental evidence reported in this article shows that an IL-2 mutein, with a severely reduced binding capacity to the IL-2Rα subunit, has a higher therapeutic efficacy than does wtIL-2 in two experimental metastasis models in mice. Such increased therapeutic efficacy might be explained, following our in vitro experiments and the in silico simulations, by the preferential use of the mutein by NK cells and CD8 memory T cells and not by the CD4+CD25+ Tregs. The dynamic effects predicted in silico for this type of IL-2 mutein seem to be qualitatively similar to those predicted in the same calibrated mathematical model for treatments with immune complexes of IL-2 and anti–IL-2 mAbs, as far as the mAb block the binding of IL-2 to CD25 (17). From a more quantitative point of view, the predicted effects are clearly stronger for mutants with a greater reduced capacity to bind CD25 and longer expected life spans in vivo.

Consistent with the latter ideas, Boyman et al. (28) reported a substantially higher immune-stimulatory effect in vivo for some IL-2/IL-2 mAbs complexes compared with wtIL2. They described the unexpected property of the so-called “stimulator IL-2 mAbs” to dramatically expand, in vitro and in vivo, the memory CD8+CD44hi T cells, which express IL-2Rβγ in higher amounts than do CD4+ T cells. Subsequently, Krieg et al. (27) demonstrated that treatment with such immune complexes, which they called “IL-2/mAbCD122,” reduced the number of lung nodules in the MB16F0 experimental metastasis model. Furthermore, it was demonstrated that the increase in life span of IL-2 in vivo, which the mAbs confer, is not sufficient to achieve the superagonist effect of stimulator immune complexes. In contrast, treatment of mice with IL-2–IgG fusion protein and concomitant blocking of the CD25 molecule with the mAb PC-61 was the only way to reproduce the extensive proliferation of CD8+CD44hi T cells induced by the IL-2/S4B6 mAb immune complex (29). Although the direct binding of stimulator anti–IL-2 mAbs, S4B6 and 5H4, was not demonstrated in the original work, Boyman et al. (28) postulated that those mAbs bind to the IL-2 molecule at the IL-2Rα interface. However, the hypothesis was recently demonstrated by Rojas et al. (30) via fine epitope mapping of the anti–mIL-2 mAbs using phage display technology. The above-mentioned mAbs indeed bind to the IL-2 molecule by the same region of interaction with α receptor chain. The IL-2 mutein described in the present study is a direct demonstration that blocking the interaction of IL-2 with the CD25 chain is sufficient to enhance IL-2 action over CD8+CD44hi cells, as well as other IL-2Rβγ−expressing cells, such as NK cells. In the case of no-α mutein, a negligible increment in IL-2 life span is expected, because the mutein has the same molecular weight as does wtIL-2. However, their fusion to Fc molecules or other carrier proteins might substantially increase their life span, as well as their in vivo activity.

To our knowledge, this is the first time that an IL-2 mutein, which binds only IL-2Rβγ, has been tested in vivo for antitumor activity, showing a stronger effect than wtIL-2. Different from other studies, we mutated four residues on the same molecule; nevertheless, the purified mutant was stable and conserved the capacity to bind to the cell surface. Multiple mutations of relevant residues at the interface with the α subunit seem to produce a drastic disruption in the interaction with this IL-2R subunit and seem to be related to better antitumor activity. Based on our in vitro and in silico results, we postulate that, in vivo, the no-α mutein must behave in a similar way to the IL-2/S4B6 immune complex, preferentially stimulating the proliferation of memory phenotype CD8+ cells and NK cells that express IL-2Rβγ in large amounts. This direct effect of no-α mutein on lymphocyte populations remains to be tested. The dual role of IL-2—promoting the expansion of effector cells while maintaining the generation and homeostatic proliferation of Tregs—is a limitation for its use in cancer and HIV therapy. The realization that it is possible to separate the two functions, by selectively disrupting the interaction of IL-2 with CD25, is a new concept that holds promise for novel therapeutic developments. The basic idea of segregating different functions of a cytokine by selectively disrupting its interaction with the different subunits of its receptor is quite appealing. It might be relevant for future drug designs using IL-2, as well as other cytokines with multimeric receptors.

Disclosures
The authors have no financial conflicts of interest.

References
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