Exploiting a natural conformational switch to engineer an interleukin-2 ‘superkine’

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The immunostimulatory cytokine interleukin-2 (IL-2) is a growth factor for a wide range of leukocytes, including T cells and natural killer (NK) cells. Considerable effort has been invested in using IL-2 as a therapeutic agent for a variety of immune disorders ranging from AIDS to cancer. However, adverse effects have limited its use in the clinic. On activated T cells, IL-2 signals through a quaternary ‘high affinity’ receptor complex consisting of IL-2, IL-2Rα (termed CD25), IL-2Rβ and IL-2Rγ. Naive T cells express only a low density of IL-2Rβ and IL-2Rγ, and are therefore relatively insensitive to IL-2, but acquire sensitivity after CD25 expression, which captures the cytokine and presents it to IL-2Rβ and IL-2Rγ. Here, using in vitro evolution, we eliminated the functional requirement of IL-2 for CD25 expression by engineering an IL-2 ‘superkine’ (also called super-2) with increased binding affinity for IL-2Rβ. Crystal structures of the IL-2 superkine in free and receptor-bound forms showed that the evolved mutations are principally in the core of the cytokine, and molecular dynamics simulations indicated that the evolved mutations stabilized IL-2, reducing the flexibility of a helix in the IL-2Rβ binding site, into an optimized receptor-binding conformation resembling that when bound to CD25. The evolved mutations in the IL-2 superkine recapitulated the functional role of CD25 by eliciting potent phosphorylation of STAT5 and vigorous proliferation of T cells irrespective of CD25 expression. Compared to IL-2, the IL-2 superkine induced superior expansion of cytotoxic T cells, leading to improved antitumour responses in vivo, and elicited proportionally less expansion of T regulatory cells and reduced pulmonary oedema. Collectively, we show that in vitro evolution has mimicked the functional role of CD25 in enhancing IL-2 potency and regulating target cell specificity, which has implications for immunotherapy.

To engineer a CD25-independent version of IL-2, we displayed human IL-2 on the surface of yeast as a conjugate to Aga2p, and verified proper receptor-binding properties with IL-2Rβ and IL-2Rγ ectodomain tetramers that were carboxy-terminally biotinylated and coupled to phycocerythrin-conjugated streptavidin for use as a staining and sorting reagent. Yeast-displayed IL-2 bound to IL-2Rγ in the presence of IL-2Rβ, recapitulating the cooperative assembly of the heterodimeric receptor complex as seen with soluble IL-2 (Fig. 1a and Supplementary Fig. 1). We proceeded to carry out two generations of in vitro evolution (Fig. 1b and Supplementary Fig. 2). Our first generation in vitro evolution strategy was to create an error-prone PCR library of the entire IL-2 coding sequence (Supplementary Fig. 2), which resulted in selection of a predominant IL-2 variant containing an L85V mutation (Fig. 1c and Supplementary Fig. 3).

From inspection of the wild-type (WT) IL-2 structure, we were surprised to find that position 85 was not a direct IL-2Rβ contact residue, but rather resided on the internal face of the IL-2 C-helix, within the hydrophobic core of the cytokine (Fig. 2a). Thus, we surmised

![Figure 1: In vitro evolution of human IL-2 variants with high affinity for IL-2Rβ.](Image)

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that L85V may affect the structure of helix C in a way that enhances binding to IL-2Rβ. Therefore, we carried out a second generation selection where we made a biased library that contained F/I/I/V at amino acids L80, L85, I86, I89, I92 and V93, which are contained within the hydrophobic core and linker region on helix C (Fig. 1b, c). To rapidly select the most active variants, we used the yeast-displayed cytokines themselves to stimulate STAT5 phosphorylation in the cytokine/IL-2Rβ receptor heterodimeric architecture and mode of cytokine/IL-2Rβ contact in the D10 ternary complex were essentially identical to the previously reported IL-2 quaternary assembly (root mean squared deviation (r.m.s.d.) = 0.43 Å) (Supplementary Fig. 7b).

Previously, we found that the C-helix of IL-2 seems to undergo subtle repositioning upon binding to IL-2Rγ1 (Fig. 2c and Supplementary Fig. 8a). Inspection of three wild-type unliganded IL-2 structures revealed conformational variability in the C-helix position, consistent with higher crystallographic B-factors in this helix relative to the rest of the molecule (Supplementary Fig. 8b). We compared the structure of our D10 IL-2 superkine to that of an unliganded structure of IL-2, and IL-2 in the receptor complexes. We found that the C-helix in D10 was more similar to that seen in the two receptor-bound conformations of IL-2 than the free forms, having undergone a relatively small shift towards the helical core as a consequence of the stabilizing mutations (Fig. 2c).

We used molecular dynamics simulations of IL-2 and D10 to further interrogate the mechanism responsible for higher binding affinity to IL-2Rβ by the IL-2 superkine (Fig. 2d, e). We constructed an atomically detailed Markov state model (MSM) to probe the relative conformational flexibility of IL-2 versus D10 directly. Analysis of the MSM clearly demonstrated that D10 was more stable than IL-2, and that IL-2 visited nearly twice as many clusters as D10. For example, the most populated state of D10 had an equilibrium probability of approximately 0.20, compared to approximately 0.05 for IL-2, demonstrating that the equilibrium population of D10 was far more localized than IL-2. Helix B, the B-C loop and helix C appeared rigidified in D10 compared to IL-2 as evidenced by reduced r.m.s.d. from the starting conformations (Fig. 2d and Supplementary Movies 1, 2). F92 seemed to act as a molecular wedge between helix C and helix A, stabilizing the more C-terminal end of the helix (Fig. 2a). We also simulated both D10 and IL-2 starting in a receptor-bound-like structure and monitored the divergence in r.m.s.d. of the B-C loop and helix C from the actual receptor-bound structure. IL-2 (Fig. 2e, left, and Supplementary Fig. 8c) quickly ‘wandered’ from the receptor conformation and experienced drastic fluctuations compared to D10 (Fig. 2e, right, Supplementary Fig. 8c and Supplementary Movies 1 and 2). Based on these observations, we propose a mechanism whereby the reduced flexibility of helix C in the IL-2 superkine, as a result of its improved core packing with helix B, results in a superior receptor-binding poise that increases its affinity for IL-2Rβ, and consequently mimics a functional role of CD25. The structural and molecular dynamics results indicate that the evolved mutations in the IL-2 superkine cause a conformational stabilization of the cytokine that reduces the energetic penalties for binding to IL-2Rβ.
We asked if the IL-2 superkines demonstrated signalling potencies on cells in accordance with their IL-2Rβ-binding affinities, and whether their activities depended on cell surface expression of CD25. We determined the dose–response relationships of wild-type IL-2 versus the IL-2 superkines 6-6, D10 and H9 on both CD25+ and CD25− human YT-1 NK cells by assaying STAT5 phosphorylation with flow cytometry (Fig. 3a–d and Supplementary Fig. 9). On CD25− YT-1 cells, the half-maximum effective concentration (EC50) of H9 and D10 were decreased over tenfold (EC50 = 2.5 and 1.8 ng ml−1, respectively) compared to IL-2 (EC50 = 39 ng ml−1), with the 6-6 mutein yielding an EC50 intermediate between IL-2 and H9/D10 (EC50 = 15 ng ml−1), consistent with the improved affinity of the IL-2 superkines for IL-2Rβ (Fig. 3a). On CD25+ YT-1 cells, the EC50 of IL-2 decreased over 50-fold relative to CD25− YT-1 cells, from 39 to 0.66 ng ml−1 (Fig. 3b). In contrast, the EC50 of H9 and D10 improved only modestly in the presence of CD25 (EC50 of 0.47 and 0.52 compared to 2.5 and 1.8 ng ml−1, respectively) (Fig. 3b).

We sought to further probe the CD25-independence of the IL-2 superkines by taking advantage of a previously characterized mutation in IL-2, Phe42 to Ala (F42A), which showed reduced binding to CD25 by approximately 220-fold for H9 (KD 6.6 nM versus 1.4 μM) and approximately 120-fold for IL-2 (KD 6.6 nM versus 0.8 μM) (Supplementary Fig. 10). The F42A mutation is an alternative diagnostic probe of the relative CD25 dependence of IL-2 and the IL-2 superkine. The F42A mutation right-shifted the dose–response curve of wild-type IL-2 on CD25− cells by about 1 log, but had no effect on CD25+ cells (Fig. 3c). In contrast, H9 was far less sensitive to the F42A mutation, with the dose–response curves of H9 versus H9 F42A being very similar on both CD25− and CD25+ cells (Fig. 3d).

We assessed the activity of several IL-2 superkines on T cells that were either deficient in, or expressed CD25. For the former experiment, CD4+ T cells were isolated from CD25-knockout mice, followed by stimulation by either wild-type IL-2 or six IL-2 superkines and assaying for STAT5 phosphorylation at a range of cytokine concentrations (Fig. 3e and Supplementary Fig. 11). Increased proliferation effects on naive human T cells correlated with increased affinity for IL-2Rβ and STAT5 phosphorylation shown earlier, as the rank order of potency was D10 = H9 > 6-6 > wild-type IL-2 (see Supplementary Fig. 12 for the complete titration).

We next tested the IL-2 variants for their ability to induce STAT5 phosphorylation on experienced human CD4+ T cells (Supplementary Fig. 13), which highly express the trimeric IL-2Rβγc complex. Human CD4+ T cells were activated in vitro by T cell receptor (TCR) stimulation and rested to generate ‘experienced’ human CD4+ CD25+ T cells. As for the CD25− YT-1 cells, we observed a much smaller difference between IL-2 and the IL-2 superkines.

We assessed the potency of the IL-2 superkine H9 on expansion of CD25low versus CD25high T cells, in comparison to wild-type IL-2 and IL-2–anti-IL-2 monoclonal antibody (mAb) complexes, which have been shown to exert reduced pulmonary oedema yet very potent antitumour responses in vivo.14–16. On antigen-experienced (memory-phenotype, MP) CD8+ T cells, expressing only low levels of CD25 but high levels of IL-2Rβγc, H9 induced more than three times the rate of proliferation and expansion as wild-type IL-2 (Fig. 4a and Supplementary Fig. 14a). However, on CD4+ CD25high T regulatory (Treg) cells, we found that the CD25–competent wild-type IL-2 and H9 achieved comparable maximal expansion, demonstrating again that expression of CD25 mitigates the difference between the IL-2 superkine and wild-type IL-2 (Fig. 4a and Supplementary Fig. 14b). Thus, the H9 has the desired property that it shows enhanced stimulation towards CD8+ T cells, but not towards Treg cells, compared to wild-type IL-2.

As previously reported, administration of high-dose wild-type IL-2 for 5 days induced substantial pulmonary oedema, which is known to be CD25–dependent15 (Fig. 4b). Although significantly more stimulatory for cytotoxic CD8+ T cells (Fig. 4a), the H9 IL-2 superkine caused substantially less pulmonary oedema (Fig. 4b).

Given the more favourable properties of H9 in comparison to IL-2, we assessed its ability to stimulate effector functions of cytotoxic T cells in four different tumour models in vivo, where high-dose IL-2 administration has been previously shown to result in tumour regression.15,17

To this end, C57BL/6 mice were injected subcutaneously with B16F10 melanoma cells, followed by administration of either high-dose IL-2, IL-2–anti-IL-2 mAb complexes, or the H9 IL-2 superkine, once tumour nodules became visible and palpable. PBS-treated control mice rapidly developed large subcutaneous tumours reaching a volume of about 1,500 mm3 on day 18 (Fig. 4c). As previously shown, high-dose IL-2 treatment was able to delay tumour growth by as much as 39% on
IL-2 can be exploited for therapy. The IL-2 superkine robustly activates B16F10 cells administered intravenously (Fig. 4f and Supplementary Fig. 14). Shown is mean tumour volume in mm$^3$ (± s.e.m.) versus time upon tumour inoculation. Error bars represent s.e.m., $P$ values refer to comparisons of wild type with the other treatment modalities. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

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day 18 ($P<0.05$), whereas IL-2–anti-IL-2 mAb complexes exerted very effective tumour control, reducing tumour growth by more than 80% on day 18 ($P<0.005$) (Fig. 4c). Significantly, similar to IL-2–anti-IL-2 mAb complexes, mice receiving high-dose H9 showed a dramatic decrease of tumour load on day 18, which was reduced by more than 80% compared to PBS ($P<0.005$) and by more than 70% compared to wild-type IL-2 ($P<0.001$) (Fig. 4c). Similar results were obtained using three other tumour models, including murine colon carcinoma and Lewis lung carcinoma injected subcutaneously (Fig. 4d, e) and B16F10 cells administered intravenously (Fig. 4f and Supplementary Fig. 15). Collectively, these data show that the H9 IL-2 superkine is very effective against different tumours, albeit inducing reduced pulmonary oedema.

The practical implications are that this conformational nuance in IL-2 can be exploited for therapy. The IL-2 superkine robustly activates cytotoxic CD8$^+$ T cells and NK cells for potent antitumour immune responses, yet it elicits minimal toxicity, suggesting that the IL-2 superkine could warrant reconsideration for clinical applications of IL-2.

**METHODS SUMMARY**

**Yeast display and selection of IL-2.** Error-prone and site-directed libraries of IL-2 were displayed on yeast as previously described$^{11}$ and stained with biotinylated IL-2R at successively decreasing concentrations. Staining was detected with streptavidin–phycoerythrin and yeast were separated using paramagnetic anti-phycoerythrin microbeads (Miltenyi; MACS). Enrichment of positively-staining yeast was monitored by flow-cytometry.

**Protein expression, purification and structural determination.** Human IL-2 variants and the ectodomains of IL-2R$\alpha$, IL-2R$\gamma$ and CD25 were expressed in H15 cells and purified as previously described$^{11}$. Proteins were concentrated to 8–20 mg ml$^{-1}$ and crystallized by vapour diffusion in sitting drops. Diffraction studies were performed at the Stanford Synchrotron Radiation Laboratory and the Advanced Light Source. Crystal structures were solved by molecular replacement with Phaser$^{19}$ and refined using PHENIX$^{20}$ and COOT$^{21}$.

**Mice.** C57BL/6 and Thy1.1-congenic mice on a C57BL/6 background were maintained under specific pathogen-free conditions and used at 3–6 months of age. Experiments were performed in accordance with the Swiss Federal Veterinary Office guidelines and approved by the Cantonal Veterinary Office.

**In vivo T-cell proliferation.** Carboxyfluorescein succinimidyl ester (CFSE)-labelled CD44$^{hi}$CD8$^+$ T cells (2 × 10$^5$ to 3 × 10$^6$) from Thy1.1-congenic mice were injected intravenously to Thy1.2-congenic animals. Mice received daily intraperitoneal (i.p.) injections of PBS, 20 µg IL-2, 1.5 µg IL-2–anti-IL-2 mAb complexes, or 20 µg H9 for 5 days. On the sixth day, spleens were removed and analysed by flow cytometry.

**Toxicity.** Pulmonary oedema was determined by measurement of pulmonary wet weight on the sixth day after five daily i.p. injections of PBS, 20 µg IL-2, 1.5 µg IL-2–anti-IL-2 mAb complexes, or 20 µg H9 as previously described$^{11}$. Treatment consisted of five daily i.p. injections of PBS, 20 µg IL-2, 1.5 µg IL-2–anti-IL-2 mAb complexes, or 20 µg H9.

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Author Contributions A.M.L. performed in vitro evolution and contributed to preparation of the manuscript. D.L.B. produced recombinant proteins, determined crystal structures, and carried out surface plasmon resonance analysis. A.M.R. carried out cellular and signalling assays, biophysical measurements and contributed to preparation of the manuscript. C.K. carried out in vivo experiments, analysed data and contributed to preparation of the manuscript; M.E.R. carried out in vivo experiments in mice. I.M. analysed cell-signalling data. G.R.B., P.N. and V.S.P. carried out and analysed molecular dynamics simulations. J.T.L., L.S. and C.G.F. performed and analysed T-cell signalling experiments. O.B. designed and supervised in vivo experiments, analysed data and contributed to preparation of the manuscript. K.C.G. conceived of the project, analysed data, supervised execution of the project, and prepared the manuscript.

Author Information Atomic coordinates and structure factors for the reported crystal structures have been deposited with the Protein Data Bank under accession codes 3QAZ and 3QB1. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to K.C.G. (kcgarcia@stanford.edu) or O.B. (onur.boyman@uzh.ch).
METHODS

Yeast display of wild-type IL-2. Human IL-2 DNA was cloned into the vector pCT302 and displayed on the Saccharomyces cerevisiae strain EB100 as previously described46. Individual colonies of IL-2 yeast were grown overnight at 30 °C in SDCAA liquid media and induced in SGCAA liquid media for 2 days at 20 °C. The yeast were stained with tetrazolium biotinylated IL-2Rβ (b-II-2Rβ), biotinylated IL-2Rγ (b-II-2Rγ), or b-II-2Rβ in the presence of b-II-2Rγ. IL-2Rβ tetramers were formed by incubating 2 μM b-II-2Rγ with 470 mM SA-PE (streptavidin–phycocyrin conjugate, Invitrogen) in phosphate buffered saline supplemented with 0.5% BSA and 2 mM EDTA (PBE) for 15 min on ice. Analysis was performed on an Accuri C6 flow cytometer.

Error-prone PCR IL-2 library construction and selection. Human IL-2 DNA was subjected to error-prone PCR using the GeneMorph kit (Stratagene). The two user-determined variables in the kit were the starting concentration of DNA template and the number of cycles. We used 100 ng template and 30 cycles in an effort to maximize the number of errors. The primers used for error-prone PCR were 5′-GCACTACTTACAGTTCTAC-3′ for the forward primer and 5′-GC CACAGAGGATC-3′ for the reverse primer. The PCR product was further amplified using primers containing sequence homology to pCT302 for recombinant IL-2 cDNA. The PCR products were ligated into yeast and amplified using primers containing sequence homology to pCT302 for recombinant IL-2 cDNA. The PCR products were ligated into yeast and electroporated of insert DNA and linearized vector into EBY-100 yeast. CD25 (amino acids 1–217) were expressed and purified from Hi5 cells as previously described.

Protein expression and purification. Concentrations of monomeric IL-2Rβ were determined by molecular replacement with the program PHASER47 using the coordinates of IL-2 (PDB ID 1M47) and the quaternary complex (PDB ID 2B5I), respectively, and refined with PHENIX48 and COOT21 (Supplementary Table 1). Bulk solvent flattening was used for solvent correction in both structures. For the IL-2 D10 free structure and the ternary complex non-crystallographic symmetry (NCS) restraints (not constraints) were used for initial stages of the refinement. Coordinate refinement strategies included rigid body, restrained individual, group atomic displacement parameters (ADP) and torsion-simulated annealing. The final rounds of refinement removed all NCS restraints for minimization and a round of individual ADP refinement.

Cryostalisation and data collection. IL-2 D10 crystals were grown in sitting drops at 22 °C from 50 mM HEPES (pH 7.2) and 30% PEG-400. A 3.1 Å data set was collected under cryo-cooled conditions (20% glycerol) at beamline 11-1 at the Stanford Synchrotron Radiation Laboratory. IL-2 D10 ternary crystals were grown from 100 mM Bis-Tris (pH 5.5), 200 mM NH4SO4 and 25% PEG-3350. A cryo-cooled 3.8 Å data set was collected at beamline 8-2 at the Advanced Light Source. Diffraction data were processed using HKL2000. Data processing statistics can be found in Supplementary Table 1.

Structure determination and refinement. The IL-2 D10 and IL-2 D10 ternary crystal structures were solved by molecular replacement with the program PHASER47 using the coordinates of IL-2 (PDB ID 1M47) and the quaternary complex (PDB ID 2B5I), respectively, and refined with PHENIX48 and COOT21 (Supplementary Table 1). Bulk solvent flattening was used for solvent correction in both structures. For the IL-2 D10 free structure and the ternary complex non-crystallographic symmetry (NCS) restraints (not constraints) were used for initial stages of the refinement. Coordinate refinement strategies included rigid body, restrained individual, group atomic displacement parameters (ADP) and torsion-simulated annealing. The final rounds of refinement removed all NCS restraints for minimization and a round of individual ADP refinement.

Ramachandran analysis was performed with MolProbity49. Buried surface area values were calculated using the Protein Interfaces, Surfaces, and Assemblies (PISA) software50. IL-2 D10 consisted of eight chains with chain A displayed in the paper. The IL-2 D10 ternary complex contained 36 chains comprising 12 ternary complexes. The paper figures are from chains A, B and C. All structural figures and overlays were prepared using PyMOL51.

Tissue culture and magnetic purification of CD25+ YT-1 cells. YT-1 and CD25+ YT-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, minimum non-essential amino acids, sodium pyruvate, 25 mM HEPES, and penicillin-streptomycin (Gibco). CD25+ YT-1 cells were purified as follows: 1×10^7 cells were washed with FACS buffer (phosphate buffered saline + 2% bovine serum albumin) and stained with PE-conjugated anti-human CD25 (1:20; Biologend) in 1 ml FACS buffer for 20 min at 4 °C. The stained cells were collected with 200 μl paramagnetic microbeads coupled to anti-PE IgG and separated with an LS MAGS separation column according to the manufacturer’s instructions (Miltenyi Biotech). Eluted cells were re-suspended in complete RPMI medium at a concentration of 1×10^6 cells per ml and expanded for subsequent experiments. Enrichment of cells was monitored by flow cytometry with the FL-2 channel using an Accuri C6 flow cytometer.

YT-1 dose–response experiments and phospho-flow cytometric analysis. 2×10^5 CD25+ or CD25− YT-1 cells were washed with FACS buffer and suspended in 0.2 μM FACS buffer with the indicated concentration of IL-2 variant per well in a 96-well plate. Cells were stimulated for 20 min at room temperature and then fixed by addition of formaldehyde to 1.5% and incubated for 10 min. Cells were permeabilized with 100% ice-cold methanol for 20 min on ice, followed by incubation at −80 °C overnight. Fixed, permeabilized cells were washed with excess FACS buffer and incubated with 50 μl Alexa647-conjugated anti-STAT5 pY199 (BD Biosciences) diluted 1:20 in FACS buffer for 20 min. Cells were washed twice in FACS buffer and mean cell fluorescence determined using the FL-4 channel on an Accuri C6 flow cytometer. Dose–response curves and EC50 values were calculated in GraphPad Prism after subtracting the mean cell fluorescence of unstimulated cells.

For ‘on-yeast’ stimulation experiments, the same protocol was used with the following modifications. Induced yeast were washed twice in FACS buffer and...
mixed with the 2 × 10^5 YT cells at the indicated ratios for 20 min in FACS buffer at room temperature. Cells were then fixed, permeabilized and stained as described above.

**T cell isolation and proliferation for phospho-flow cytometric analysis.** Human and mouse CD4 T cells were prepared from peripheral blood mononuclear cells (PBMC, Stanford Blood Bank) and spleens and lymph nodes of BALB/c mice, respectively, using antibody-coated CD4 T-cell isolation magnetic beads ( Stem Cell Technologies and Miltenyi Biotec). For naive cell stimulation assays, cells were used immediately. For generation of in vitro 'experience' T cells, wells were pre-coated with secondary antibody (Vector Labs) in bicarbonate buffer, pH 9.6 before coating with anti-CD3 (OKT3 for human, 2C11 for mouse, eBioscience) at 100 ng ml⁻¹. T cells were seeded at 0.1 × 10⁶ cells per well with soluble anti-CD28 (CD28.2 for human, 37.51 for mouse, eBioscience). Cells were cultured for 3 days with full T-cell receptor stimulation, followed by 2 days rest in conditioned media and 2 days rest in fresh culture media. Prior to use, live cells were collected by Lympholyte-M (Cederlane) centrifugation and counted.

**In vivo studies.** C57BL/6 and Thy1.1-congenic mice on a C57BL/6 background (both from Charles River) were maintained under specific pathogen-free conditions and used at 3–6 months of age. Experiments were performed in accordance with the Swiss Federal Veterinary Office guidelines and approved by the Cantonal Veterinary Office.

Cell suspensions of spleen were prepared according to standard protocols and stained for analysis by flow cytometry using phosphate-buffered saline (PBS) containing 4% fetal calf serum and 2.5 mM EDTA. Fluorochrome-conjugated monoclonal antibodies (mAbs) (from BD Biosciences unless otherwise stated) were used against: CD3 (145-2C11, eBioscience), CD4 (RM4-5, Caltag Laboratories), CD8α (53-6.7, BD Pharmingen), CD25 (PC61, eBioscience), CD44 (IM7, eBioscience), NK1.1 (PK136), and Thy1.1 (HS51, eBioscience). At least 100,000 visible cells were acquired on a BD FACS Canto II flow cytometer and analysed using FlowJo software (TriStar Inc.).

To prepare IL-2–anti-IL-2 mAb complexes, recombinant human IL-2 (rhIL-2) and anti-human IL-2 mAb were premixed at a 2:1 molar ratio using 15,000 international units of recombinant human IL-2, as previously described. Recombinant human IL-2 and anti-human IL-2 mAb clone 5355 (MAB602) were obtained from R&D Systems.

T-cell subsets were obtained by negative T-cell enrichment (StemCell Technologies). Where indicated, purified cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes), as previously published. Recombinant human IL-2 and anti-human IL-2 mAb clone 5355 (MAB602) were obtained from R&D Systems.

**In vivo tumor studies.** To generate subcutaneous tumours, as indicated either 10^6 B16F10 melanoma (provided by R. Schwendener), or 1.0 × 10^5 Lewis lung carcinoma (provided by R. Schwendener) cells were injected into the upper dermis of the back of mice (3–4 mice per group), as previously established. Treatment consisted of five daily injections of PBS, 20 μg IL-2, 1.5 μg IL-2–anti-IL-2 mAb complexes, or 20 μg H9, and 1.5 μg IL-2–anti-IL-2 mAb complexes for 5 consecutive days as described above. On day 6, lungs were removed and weighed before and after drying overnight at 58°C. Pulmonary wet weight was calculated by subtracting initial pulmonary weight from lung weight after dehydration.

To generate subcutaneous tumours, as indicated either 10^6 B16F10 melanoma (from ATCC), 10^6 Lewis lung carcinoma (provided by R. Schwendener), or 2.5 × 10^5 murine colon carcinoma 38 (provided by R. Schwendener) cells were injected into 100 μl DMEM into the upper dermis of the back of mice (3–4 mice per group), as previously established. Treatment consisted of five daily injections of either PBS, 20 μg IL-2, 1.5 μg IL-2–anti-IL-2 mAb complexes, or 20 μg H9, and was started 1 day after tumour nodules were clearly visible and palpable at a volume of approximately 50–55 mm³. For the generation of lung metastases, 3 × 10^5 B16F10 cells in 300 μl DMEM were injected into the tail vein, as previously shown. Treatment was as above and was started on day 3 after tumour inoculation. On day 16 after injection, lungs were perfused, harvested and fixed in Fekete’s solution (70% ethanol, 3.7% paraformaldehyde, 0.75 M glacial acetic acid), followed by dissection of lungs and counting of pulmonary micrometastases. Differences between groups were examined for statistical significance by using a one-way analysis of variance (ANOVA) with Bonferroni post-test correction.

**Molecular dynamics simulations and MSM.** We used MODELLER with the default settings to create five starting conformations for simulations from each of three IL-2 structures (PDB ID 3INK, 1M47 and 1Z92; refs 27–29), and D10 with mutated. Noe, F. & Fischer, S. Transition networks for modeling the kinetics of biological processes. *J. Chem. Phys.* 124, 197101 (2006).

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