Materials and Methods:

Protein production and purification

For yeast-binding studies, affinity maturation, and SPR, the ECDs of human IL-10Rα (residues 20-235) and IL-10Rβ (residues 20-220) were cloned into the pAcGP67a baculoviral vector with an N-terminal gp67 signal peptide, C-terminal 3C cleavage site followed by a biotin-acceptor peptide tag (BAP tag, GLNDIFEAQKIEW) and 6xHis tag. The baculovirus stocks were prepared by co-transfection of the BestBac^{TM} DNA (Expression Systems) and the pAcGP67a DNA into *Spodoptera frugiperda* (Sf9). Next, the viruses were used to infect the *Trichoplusia ni* (Hi5) cells. The proteins were purified from the supernatant of baculovirus infected Hi5 cells 72 hours after infection and purified with Ni-NTA resin (Qiagen) followed by size-exclusion chromatography (SEC) on a Superdex 200 column (GE). The proteins were maintained in HEPES buffered saline (HBS, 20 mM HEPES pH 7.4, 150 mM sodium chloride). IL-10Rβ ECD was site-specifically biotinylated at the C-terminal BAP tag using BirA ligase and re-purified by SEC.

For structural studies, a glyco-mutant version the IL-10Rβ ECD (N49Q, N68Q, N102Q, N161Q) (*18*) was cloned into the pAcGP67a baculoviral vector with an N-terminal gp67 signal peptide and C-terminal 6xHis tag, expressed and purified as described above. Affinity matured IL-10 (super-10, clone 5.1) and the IL-10Rα ECD were cloned into the pD649 mammalian expression vector containing an N-terminal HA signal peptide and C-terminal 6xHis-tag. DNA was transiently transfected into Expi-293F cells (Thermo) using Expifectamine transfection reagent (Thermo). 72-96 hours after transfection, cell supernatant was harvested, and proteins were purified with Ni-NTA resin (Qiagen) followed by SEC on a Superdex 200 column (GE) in HEPES buffered saline (HBS). Following SEC, the individual proteins were incubated overnight at a 1:1:1.2 molar ratio of IL-10:IL-10Rα:IL-10Rβ and re-purified by SEC on a Superdex 200 column (GE).

For signaling and functional experiments, IL-10 variants were cloned into the pD649 mammalian expression vector containing an N-terminal HA signal peptide and C-terminal 6xHis-tag. DNA was transiently transfected into Expi-293F cells (Thermo) using Expifectamine transfection reagent (Thermo). 72-96 hours after transfection, cell supernatant was harvested, and proteins were purified with Ni-NTA resin (Qiagen) followed by SEC on a Superdex 200 column (GE) in HBS.

Yeast display, library assembly, and affinity maturation

A monomeric IL-10 variant (*17*) containing a C-terminal Myc-tag was displayed on the surface of *Saccharomyces cerevisiae* strain EBY100 as a C-terminal fusion to Aga2 using the pCT302 vector. A mutant mono-IL-10 library containing seven randomized residues at the predicted IL-10Rβ contact site was generated by primer assembly PCR using degenerate codons (fig. S1D). Electroporation, rescue and expansion of the yeast library were performed as described previously (*16*).

Library selection was conducted as described previously with small modifications. Briefly, the initial selections (rounds 1-4) were conducted using magnetic-activated cell sorting (MACS). For rounds 1 and 2, 3×10^9 and 1×10^8 cells, respectively, were pre-incubated in 500 nM unlabeled IL-10Rα and selected with magnetic streptavidin microbeads (Miltenyi) that were pre-coated with 400 nM biotinylated IL-10Rβ. IL-10Rβ-binding clones were isolated using MACS LS columns (Miltenyi). For rounds 3 and 4, 1.0×10^8 yeast were pre-incubated in 500 nM IL-10Rα and stained and selected with 10 μM (round 3) or 500 nM (round 4) monomeric biotinylated IL-10Rβ, and subsequently labeled with streptavidin conjugated to Alexa Fluor 647. IL-10Rβ-binding clones were isolated using MACS LS columns (Miltenyi) in combination with Anti-Cy5/Anti-Alexa Fluor 647 MicroBeads (Miltenyi). In round 5, library selection was performed using two-color fluorescence-activated cell sorting (FACS) to normalize apparent affinity to protein expression on the cell surface. The yeast library was pre-incubated with 500 nM IL-10Rα followed by 5 nM of biotinylated IL-10Rβ. The cells were washed twice with PBE (PBS, pH 7.4 + 0.5% w/v BSA + 2 mM EDTA, pH 8.0) and co-labeled with streptavidin-Alexa Fluor 647 and anti-c-Myc-Alexa Fluor 488 (CST) for 15 min. at 4°C. Alexa Fluor 647^+ and Alexa Fluor 488^+ yeast were purified using a SH800S Cell Sorter (Sony Biotechnology). At the conclusion of the selections, post-selection library at each round was simultaneously expanded and incubated with 100 nM biotinylated IL-10Rβ for 1 hour at 4°C, washed twice with PBE, then stained with fluorescently labeled streptavidin for 10 minutes at 4°C to assess the enrichment of high-affinity clones via flow cytometry. In addition, ~4×10^6 cells from the post-round 5 library were used to extract library DNA using the Zymoprep Yeast Plasmid Miniprep II Kit (Zymo Research), according to the manufacturer's instructions. The extracted DNA was transformed into DH5α *Escherichia coli* and plated to sequence the individual clones.

To measure relative binding affinities, individual clones were displayed on the yeast surface and incubated with 500 nM IL-10Rα and increasing concentrations of biotinylated IL-10Rβ. The cells were then stained with streptavidin-Alexa Fluor 647 for 15 min. and analyzed on a CytoFLEX Flow Cytometer (Beckman Coulter).

Surface Plasmon Resonance

Dissociation constants (K_D) for IL-10Rβ-binding by affinity matured IL-10 clone 5.1 (super-10), either alone or pre-bound with soluble IL-10Rα, were determined by surface plasmon resonance (SPR) using the BIAcore T100 instrument (GE Healthcare). First, biotinylated IL-10Rβ was captured on a streptavidin-coated (SA) sensor chip (GE Healthcare) with immobilization density in the range of 100 resonance units (RU). Similarly, a control flow cell was also prepared with an off-target protein (EpoR) for reference subtraction. The binding kinetics were performed at 25°C with a flow rate of 50 µl/min. IL-10 or the IL-10–IL-10Rα complex were serially diluted in HBS buffer supplemented with 0.005% v/v P20 surfactant (GE Healthcare) and injected over the SA chip. Dissociation kinetics were monitored for 100-500 seconds, as needed, based on the ligand affinity. The affinity was determined using the BIAcore T100 evaluation software. The kinetic binding curves were generated by plotting the time-dependent response units in Prism 8 (GraphPad).

Cryo-EM specimen preparation and data collection

The hexameric IL-10–IL-10Rα–IL-10Rβ complex was concentrated to 12 mg/ml and mixed with 0.01% w/v fluorinated octyl maltoside (fOM, Anatrace, O310F) immediately prior to specimen preparation. The 0.01% w/v fOM was identified after screening a variety of detergent conditions and was found to improve particle distribution and reduce the extent of preferred particle orientation. 2.5 microliters (µl) of the sample was applied to glow-discharged 300 mesh gold grids (Quantifoil R1.2/1.3). Excess sample was blotted to a filter paper (Whatman qualitative filter paper, Grade 1) for 4 s before plunge freezing using a Leica EM GP (Leica Microsystems) at 20°C and 95% humidity. Grids were glow-discharged using an easiGlow (PELCO) at 15 mA and 0.39 mBar for 40 s.

Cryo-EM data were collected at the CryoEM facility at the HHMI Janelia Research Campus. Images were collected on a Titan Krios operated at 300 kV equipped with a Gatan

imaging filter and K3 camera in correlated double sampling super-resolution mode at a nominal magnification of 81,000X, corresponding to a physical pixel size of 1.078 Å. Each movie was recorded for a total of 6.325 s with 0.1265-s exposure per frame at an exposure rate of 9.2 electrons/pixel/second (7.9 e/Å^2/s) at the specimens, that yielded an electron exposure of 7.5~ e/pixel/s (6.5~ e/Å^2/s) at the camera. The dataset was collected using SerialEM (*22*) with defocus range set between −0.8 and −2.0 μm and beam-image shift to collect nine movies from nine holes per stage shift and focusing.

Cryo-EM Data processing and 3D reconstruction

A total of 9413 raw image stacks were subjected to motion correction with binning (2 x) to a pixel size of 1.078 Å and dose weighting with default parameters using MotionCor2 (*23*). Dose-weighted micrographs were imported into the cryoSPARC software package (*24*) for all subsequent processing steps. Reference-free particle picking on a small subset of data was used to generate 2D classes for reference-based picking. A total of 6,701,298 "particles" were picked initially, on which extensive 2D classification was performed. During the 2D classification process, as well as our previous screening attempts, it became apparent that the molecules showed moderate preferred orientation. To mitigate artifacts in the final reconstruction, we selectively discarded lower quality 2D classes of the same views. After ab initio reconstruction and heterogeneous refinement for 3D classification, the final reconstruction contained 86,725 particles. While we attempted applying C2 symmetry in the 3D refinement step, these resulted in poor reconstructions. Finally, it became apparent that there was a symmetry break in the molecule due to difference in occupancy of the IL-10Rβ, as well as significant flexibility in the center of the molecule. We then used non-uniform refinement in combination with local refinement with a "fulcrum" (i.e. a pivot point between two flexibly connected sub-volumes, used by CryoSPARC to re-calculate the local angular search grid for improved accuracy) on the center of the complex, which yielded a 3.49-Å resolution reconstruction determined by gold-standard Fourier shell correlation using the 0.143 criterion. Anisotropy of the reconstruction was assessed using the 3DFSC program (*25*) with default settings to generate directional FSC curves.

Model building and refinement

Models derived from crystal structures of IL-10 (PDB ID: 1Y6K), IL-10Rα (PDB ID: 1Y6K), and IL-10Rβ (PDB ID: 3LQM) were docked into the EM map using UCSF Chimera X (*26*). Due to significant differences in map quality between the two IL-10–IL-10Rα–IL-10Rβ subcomplexes, iterative rounds of manual rebuilding were carried out in COOT (*27*) for one ternary subcomplex, followed by iterative rounds of real space refinement using PHENIX with secondary structure restraints and Ramachandran restraints (*28, 29*) first for the trimeric subcomplex complex alone, followed by refinement of the full hexameric complex with the addition of NCS restraints, with a resolution of 3.9 Å used in refinement and resolution factor of 0.25. Due to the poorly resolved density for much of the second trimeric complex, parts of chains E, F, and D were truncated to poly-Ala. Model statistics for both the trimeric and hexameric models were generated using the comprehensive validation (cryo-EM) module in PHENIX (*28, 29*) and are listed in Table S1. All structure figures were made using ChimeraX (*26*).

Cell Culture

THP-1 (ATCC TIB-202), YT-1 (RRID: CVCL_EJ05), Jurkat (ATCC TIB-152), and Daudi (ATCC CCL-213) cells were all grown in RPMI 1640 Medium (Gibco) supplemented with 10% v/v fetal bovine serum (FBS), penicillin–streptomycin, and 2 mM GlutaMAX™ (Gibco) and maintained at 37°C with 5% CO_2. HEK-293T cells (ATCC CRL-3216) were grown in DMEM (Dulbecco's Modified Eagle Medium, Gibco) supplemented with 10% v/v FBS, penicillin–streptomycin, and 2 mM GlutaMAX™ (Gibco) and maintained at 37°C with 5% CO_2. Expi293F cells were grown in serum free Expi293 expression media (Thermo) and maintained at 37°C with 5% CO_2. MC/9 cells (ATCC CRL-8306) were grown in DMEM (Gibco) supplemented with 10% v/v FBS, penicillin–streptomycin, and 2 mM GlutaMAX™ (Gibco) and 10% v/v Rat T-stim (BD) and maintained at 37°C with 5% CO_2

Signaling assays in human cell lines

For phospho-flow cytometry experiments, THP-1, YT-1, Jurkat, and Daudi cells were plated in 96-well plates and stimulated with WT or mutant IL-10 for 20 min. at 37°C, followed by fixation with paraformaldehyde (Electron Microscopy Sciences) for 10 minutes at room temperature. The cells were permeabilized for intracellular staining by treatment with ice-cold

methanol (Fisher) for 30 min. at –20°C. The cells were then incubated with Alexa Fluor 647 conjugated Anti-Stat3 (pY705) antibody (1:50, BD, clone 4/P-STAT3) for 1 hour at room temperature in autoMACS buffer (Miltenyi). The background fluorescence of the unstimulated samples was subtracted from all samples. Data was acquired using CytoFlex flow cytometer instrument (Beckman Coulter). The (mean fluorescence intensity) MFI values were background subtracted and normalized to the maximal WT IL-10 value (maximal WT signal set to 100%) within each experiment and plotted in Prism 8 (GraphPad). The dose-response curves were generated using the "sigmoidal dose-response" analysis.

Receptor surface expression was analyzed by resuspending live cells in autoMACS buffer and incubating with Human TruStain FcX (Biolegend) followed by anti-human IL-10Rα Alexa Fluor 647 (1:20, BD, clone 3F9) and anti-human IL-10Rβ Alexa Fluor 488 (1:20, R&D, clone #90220) antibodies for 30 min. at 4°C. Fluorescent intensities were measured by flow cytometry on an Accuri C6 cytometer (BD), and data were analyzed using FlowJo software (BD).

For signaling assays in HEK-293T cells, cells 0.7×10^6 cells were plated in six-well culture dishes coated with fibronectin (Millipore). Twenty-four hours later, cells were transfected using FuGene 6 (Promega) with pLV-EF1a-IRES-Puro vector containing HA-tagged IL-10Rβ, WT or E141K mutant along with pD649 vector containing Myc-tagged IFN-λR1 or IL-10Rα. Twenty-four hours after transfection, cells were treated with IFN-λ and/or IL-10 (WT or mutant) for 20 minutes at 37°C. Cells were then rinsed once with ice-cold PBS and immediately lysed with Triton lysis buffer (1% v/v Triton, 20 mM HEPES pH 7.4, 150 mM NaCl, one tablet of PhosSTOP phosphatase inhibitor cocktail (Roche), and one tablet of EDTA-free protease inhibitor (Roche) (per 10 ml buffer). The cell lysates were cleared by centrifugation at 15,000 *g* at 4°C for 10 min. Cell lysates were denatured by the addition of SDS sample buffer and boiling for 5 min., resolved by SDS-PAGE, and analyzed by immunoblotting.

For immunoblot-based signaling in THP-1 cells, 5×10^6 cells were seeded at 2×10^6 cells/ml in six-well culture dishes and treated with 10 nM IL-10 (WT or mutant) for 20 minutes at 37°C. Cells were then rinsed one time with ice-cold PBS and immediately lysed and analyzed as described above.

Phospho-flow signaling assays in human PBMC

LRS chambers (leukoreduction chambers) of healthy donors recovered after plateletpheresis were obtained from the Stanford Blood Bank. Healthy donor derived human Peripheral blood mononuclear cells (PBMC) were isolated from the LRS chambers and stored as frozen aliquots. PBMCs were thawed in warm media, washed twice and resuspended at 5×10^6 viable cells/mL. Two hundred microliters of cells were plated per well in 96-well plates. After resting for 2 hours at 37°C, cells were stimulated with varying concentrations of WT or mutant IL-10 and incubated at 37°C for 20 min. The PBMCs were then fixed with paraformaldehyde (Electron Microscopy Sciences) for 10 min. at room temperature, and permeabilized for intracellular staining by treatment with ice cold methanol (Fisher) for at least 30 min. at –20°C. The cells were washed with autoMACS buffer (Miltenyi), incubated with human TruStain FcX (Biolegend), and then stained with the following antibodies: CD3 Pacific Blue (1:50, BD, clone UCHT1), CD4 PerCP-Cy5.5 (1:20, BD, clone SK3), CD20 PerCp-Cy5.5 (1:20, BD, clone H1), CD33 PE-Cy7 (1:50, BD, clone P67.6), and pSTAT-3 AlexaFluor 488 (1:50, BD, clone 4/P-STAT3) or pSTAT-1 AlexaFluor 488 (1:50, BD, clone 4a). The samples were then washed and resuspended in autoMACS buffer. Data was acquired using CytoFlex flow cytometer instrument (Beckman Coulter) and analyzed using FlowJo software (BD). The MFI values were background subtracted and normalized to the maximal WT IL-10 value within each cell type and plotted in Prism 8 (GraphPad). The dose-response curves were generated using the "sigmoidal dose-response" analysis. Cells were gated on live cells based on forward versus side scatter profiles, followed by cell subset-specific gating as described previously (*30*).

For signaling in primary human Tregs, $CD4^+$ T cells were isolated from human PBMCs using MACS LS columns (Miltenyi) in combination with human $CD4^+$ isolation kit (Miltenyi) per the manufacturer's instructions. Cells were washed and resuspended in autoMACS buffer, incubated with CD127 FITC (1:20, BD, clone HIL-7R-M21) and CD25 BV605 (1:20, clone BC96) antibodies for 20 min. at 4°C and washed once in autoMACS buffer before being resuspended in RPMI and seeded in 96-well tissue culture plates and stimulated with 10 nM IL-10 (WT or mutant) at 37°C for 20 minutes. Cells were then fixed and permeabilized. pSTAT3 signaling was then analyzed as described above.

Stimulation of human PBMCs, monocytes, and macrophages

PBMCs were thawed in warm media, washed twice and resuspended in complete RPMI at 1×10^7 cells/ml. For stimulation with lipopolysaccharides (LPS), cells were plated at 500 µl/well in a 24-well culture dish and rested for 2 hours at 37°C. Cells were then incubated with 1 ng/ml of LPS (Sigma) alone or with 10 nM IL-10 (WT or mutant) for 24 hours. For T cell activation in the context of bulk PBMCs, cells were plated at 500 µl/well in a 24-well tissue culture plate pre-coated with anti-CD3 antibody (clone OKT1) for 72 hours. For cytokine measurements, cell supernatants were isolated and levels of TNF-α (Biolegend), IL-6 (R&D), IL-8 (R&D), IL-1β (Biolegend) and IFN-γ (Biolegend) were measured by ELISA as per the manufacturer's instructions.

For monocyte HLA-DR and CD86 surface expression analysis, PBMCs were washed and resuspended in autoMACS buffer, incubated with human TruStain FcX (Biolegend) and stained with CD14 PE-Cy7 (1:50, BD, clone M5E2), HLA-DR BV605 (1:50, Biolegend, clone L243), and CD86 APC (1:50, clone GL-1) antibodies for 30 minutes at 4°C. Fluorescent intensities were measured using CytoFlex flow cytometer instrument (Beckman Coulter) and analyzed using FlowJo software (BD). Cells were gated on live cells based on forward versus side scatter profiles, followed by monocyte specific gating ($FSC^{hi}CD14^+$).

For analysis of primary human macrophages, PBMCs were thawed in warm media, washed twice, and resuspended in complete RPMI. Monocytes were isolated using MACS LS columns (Miltenyi) in combination with human monocyte ($CD14^+$) isolation kit (Miltenyi). Isolated monocytes were plated in a 12-well tissue culture dish and incubated in human M-CSF at 20 ng/ml. Cells were incubated for 24 hours at 37°C to allow monocytes to adhere to the plate. Media was aspirated to remove non-adherent cells, and cells were incubated in fresh media containing 20 ng/ml M-CSF at 37°C for an additional 5 days, when the vast majority of cells adopted a distinct macrophage morphology by visual inspection. Cells were then stimulated with 1 ng/ml of LPS alone or with 10 nM IL-10 (WT or mutant) for 24 hours. Cell supernatants were isolated and levels of TNF-α (Biolegend), IL-6 (R&D), and IL-8 (R&D) were measured by ELISA as per the manufacturer's instructions.

Stimulation of human $CD4^+$ and $CD8^+$ T cells

$CD8^+$ T cells were isolated from human PBMCs using MACS LS columns (Miltenyi) in combination with human $CD8^+$ isolation kit (Miltenyi) per the manufacturer's instructions. The potentiation of IFN-γ and granzyme B production by IL-10 was performed as described previously

(*13*). Briefly, isolated $CD8^+$ T cells were seeded at 2×10^6 cells/ml in a 12-well tissue culture dish pre-coated with anti-CD3 antibody (clone OKT1) and incubated with soluble anti-CD28 antibody (5 μg/ml, Biolegend, clone CD28.2) for 3 days. Cells were then collected and re-seeded in fresh media at 1×10^6 cells/ml in a 24 well tissue culture dish with or without 10 nM IL-10 (WT or mutant) and incubated for an additional 3 days. Cells were then restimulated with soluble anti-CD3 (clone OKT1, 2 μg/ml) for 4 hours. Supernatant was then isolated and levels of IFN-γ (Biolegend), IL-9 (Biolegend) and Granzyme B (R&D) were measured by ELISA as per the manufacturer's instructions.

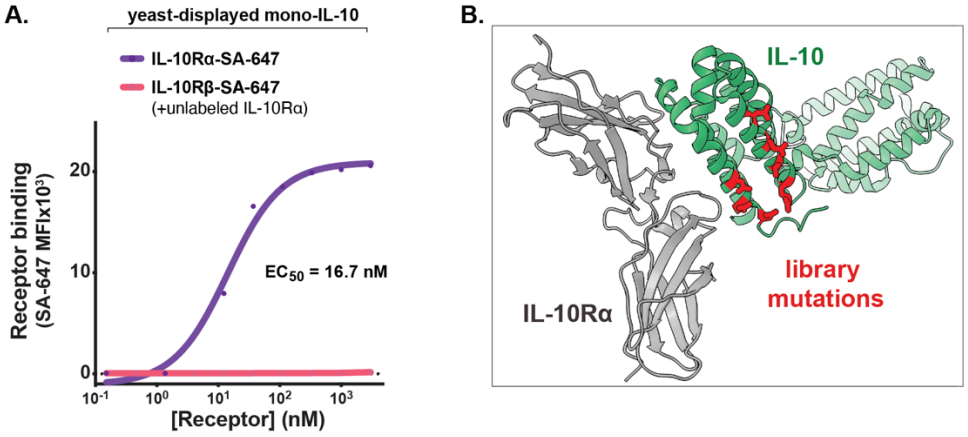
For transcriptomic analysis, $CD8^+$ T cells were isolated and activated as described above, then treated with 10 nM IL-10 (WT or mutant) for 24 hours. Cells were then collected and washed in PBS. RNA was then isolated using the RNAeasy mini kit (Qiagen). RNA-quality control was performed using Qubit and Bioanalyzer 2100 (Agilent). cDNA libraries were constructed using NEBnext® Ultra™ II RNA Library Prep Kit for Illumina®, using 0.2 μg of RNA input, and the mRNA enrichment method. Library QC was performed using Qubit, LabChip, and qPCR (KPA library quantification kit). cDNA libraries were loaded onto an Illumina NovaSeq 6000 sequencer, PE150 platform. RNA samples from two biological replicates were used for sequencing. Reference genome and gene model annotation files were downloaded from genome website browser (NCBI/UCSC/Ensembl) directly. Indexes of the reference genome was built using STAR and paired-end clean reads were aligned to the reference genome using STAR (v2.5) (*31*). The FPKM of each gene was calculated based on the length of the gene and reads count mapped to the gene and used to quantify relative gene expression levels (*32*).

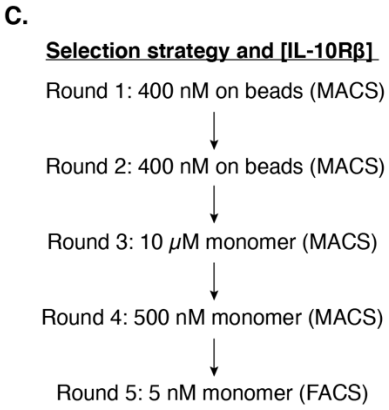
For $CD4^+$ Th1 differentiation assays, naïve $CD4^+$ T cells were isolated using MACS LS columns (Miltenyi) in combination with human naïve $CD4^+$ T cell isolation kit (Stem Cell Technologies). Isolated naïve $CD4^+$ T cells were seeded at 1×10^6 cells/ml in a 24-well tissue culture dish pre-coated with anti-CD3 antibody (clone OKT1) and incubated with Th1 differentiation media containing soluble anti-CD28 antibody (2 μg/ml, Biolegend, clone CD28.2), anti-IL-4 antibody (1 μg/ml, Biolegend, clone MP4-25D2), IL-2 (5 ng/ml), and IL-12 (10 ng/ml) in RPMI1640 supplemented with 10% v/v FBS, NEAA, antibiotics, and 55 μM β-mercaptoethanol), with or without IL-10 (10 nM, WT or mutant). After 5 days, cell supernatant was harvested and levels of IFN-γ (Biolegend) were measured by ELISA per the manufacturer's instructions.

LPS Sepsis

Endotoxemia was induced in 4-6 week old female C57BL/6 mice by intraperitoneal injection of 200 µl of LPS derived from *E. coli* O111:B4 diluted in PBS at either a lethal (15 mg/kg) or sub-lethal (4 mg/kg) dose, in combination with a single injection of 200 µl of PBS or IL-10 (WT or 10-DE, 10 µg/mouse). Survival was monitored for 120 hours. Kaplan–Meier survival curves were compared using the log-rank Mantel–Cox test. For serum cytokine measurements, blood was drawn by submandibular puncture from mice treated with a sub-lethal LPS dose at 4 hours and 24 hours post LPS injection. Serum was isolated by centrifugation at 2,000 *g* for 10 min. at 4°C and TNF-α (Biolegend), IL-6 (Biolegend), and haptoglobin (R&D) were measured by ELISA per the manufacturer's instructions.

C57BL/6J mice were purchased from Jackson Labs (Cat 000664). All animals were housed at Stanford University according to protocol and guidelines approved by the Administrative Panel on Lab Animal Care (APLAC).





D. **IL-10 yeast-displayed library**

Theoretical diversity = 5.66x10⁷

IL-10	library codon	clone 5.1	clone 5.2	clone 5.3
N18	random (NNK)	Tyr	Tyr	Tyr
N21	(MRK) N,R,Q,H,K,S	Asn	His	His
D25	(VWT) N,D,H,I,L,V	Asp	Asp	Asp
N92	(VAW) N,D,Q,E,H,K	Gln	Gln	Asn
E96	(VAW) N,D,Q,E,H,K	Glu	Asp	His
T100	random (NNK)	Asp	Val	Val
R104	random (NNK)	Trp	Trp	Trp

Fig. S1: Affinity maturing the IL-10–IL-10Rβ interaction via directed evolution. (**A**) Binding titration of SA-647-IL-10Rα (purple) SA-647-IL-10Rβ (pink) on yeast displaying WT monomeric IL-10. For IL-10Rβ titration, yeast were pre-bound with 500 nM unlabeled IL-10Rα. (**B**) Structure of the IL-10–IL-10Rα partial complex (PDB ID:1J7V), with corresponding residues selected for randomization in monomeric-IL-10 yeast-display library shown in red. (**C**) Selection strategy for each round of IL-10 selections, indicating concentration of IL-10Rβ ECD used and sorting method (MACS, magnetic-activated cell sorting; FACS, fluorescence-activated cell sorting). (**D**) Table showing the seven residues targeted for randomization, the codons used for each site in library generation, and the amino acids present at each location in clones 5.1 (super-10), 5.2, and 5.3.

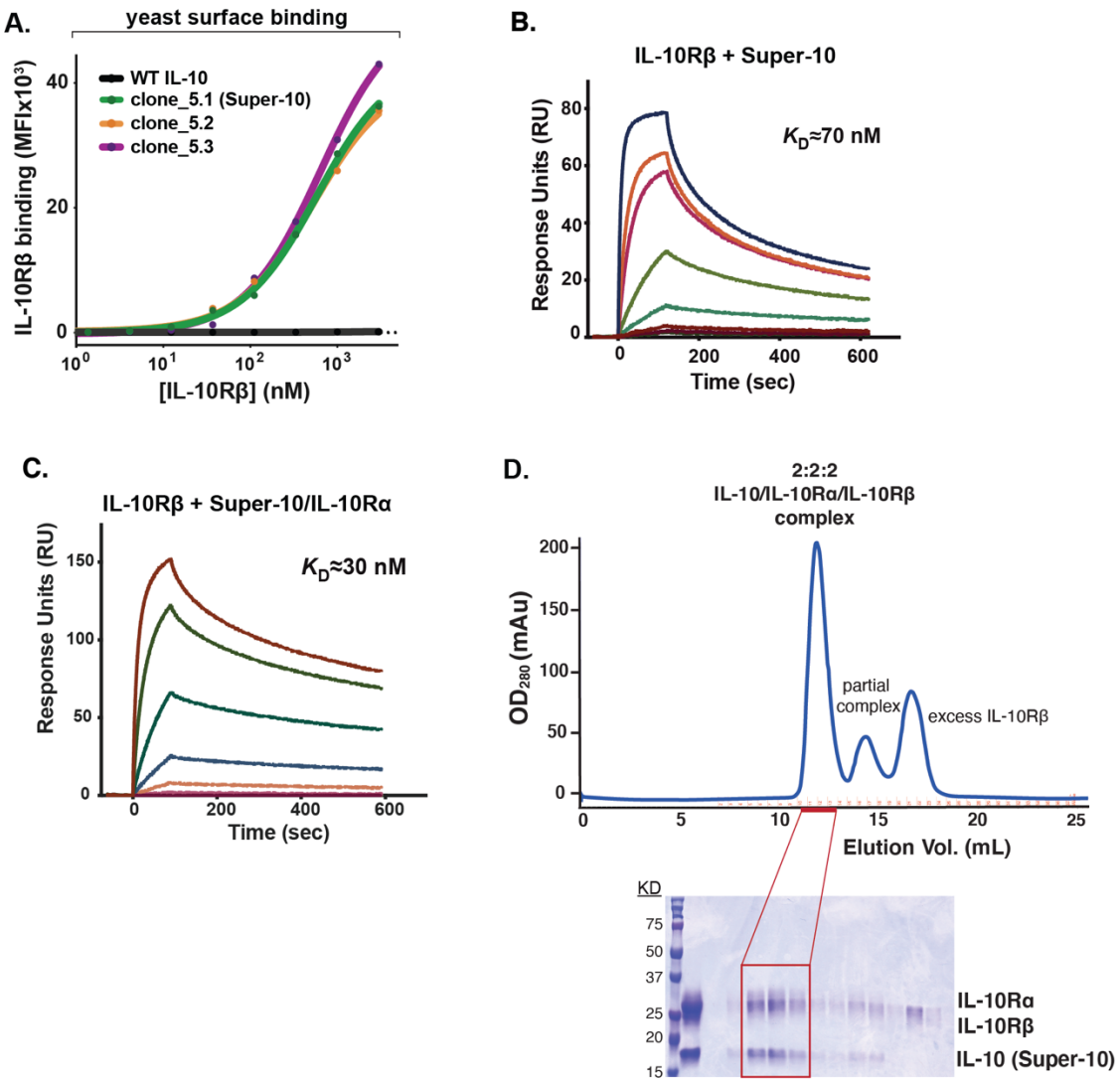


Fig. S2. Biophysical characterization of affinity-matured IL-10 variants. (**A**) Binding titration of SA-647-IL-10Rβ on yeast displaying WT monomeric IL-10 or affinity matured clones. Yeast were pre-bound with 500 nM unlabeled IL-10Rα. (**B, C**) Surface plasmon resonance sensograms of soluble IL-10Rβ binding to immobilized affinity matured IL-10 clone 5.1 either alone (**B**) or pre-bound with saturating soluble IL-10Rα (**C**). K_D, dissociation constant. (**D**) Size-exclusion chromatography profile and corresponding SDS-PAGE gel of the assembled hexameric IL-10–IL-10Rα–IL10Rβ complex.

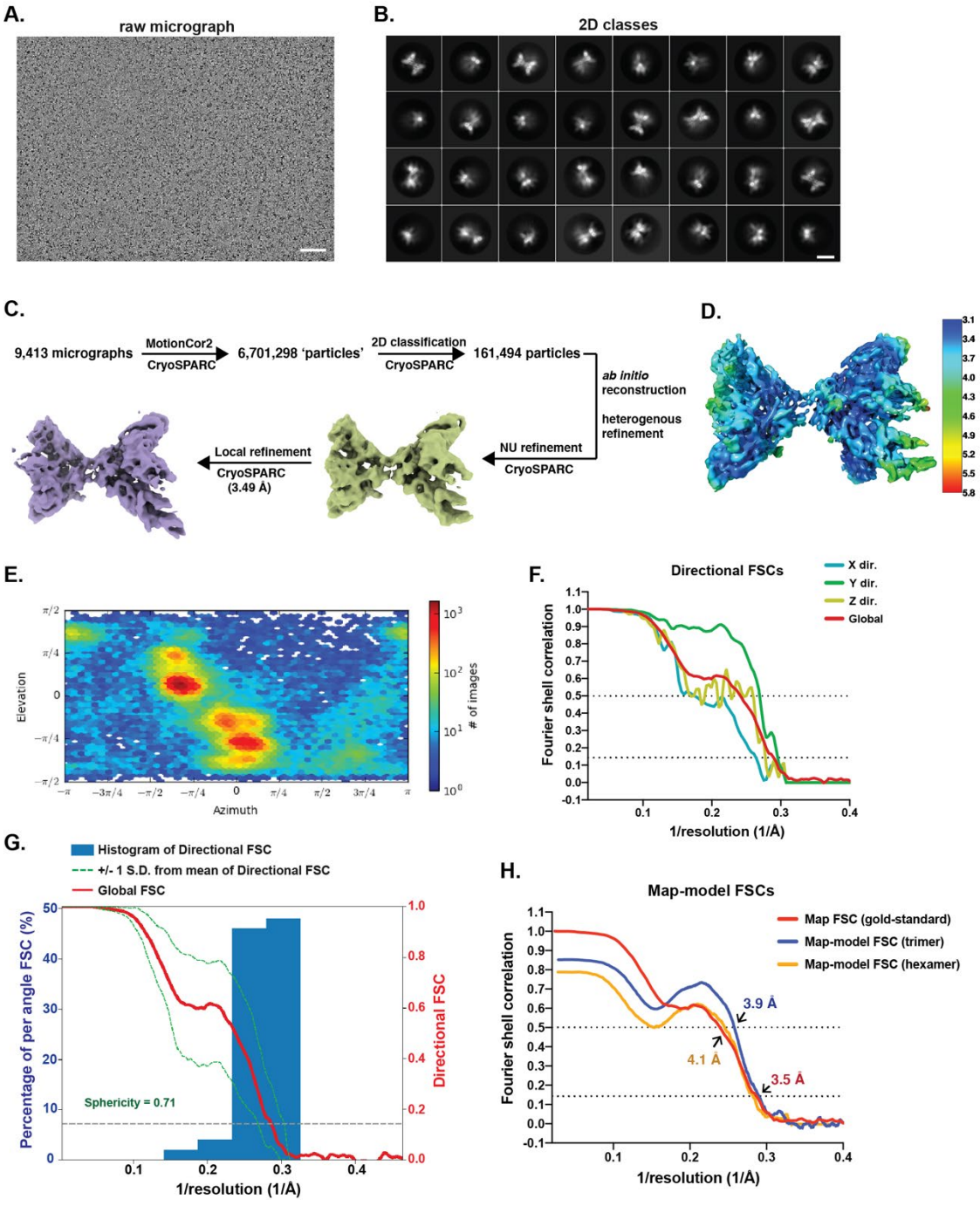


Fig. S3. Single-particle cryo-EM analysis of the IL-10 receptor complex. (A) Cryo-EM micrograph (scale bar = 50 nm). (**B**) 2D class averages for the IL-10 receptor complex (scale bar = 10 nm). (**C**) Workflow for processing IL-10 receptor complex cryo-EM dataset. (**D**) Local resolution of the IL-10 receptor complex cryo-EM density map, ranging from 3.1 Å to 5.8 Å. (**E**) Euler angle orientation distribution for IL-10 receptor complex cryo-EM dataset. **(F)** Directional and global half-set Fourier shell correlation (FSC) curves, reflecting anisotropy due to preferred particle orientation. **(G)** Global FSC curve (red) and histogram of directional resolutions sampled over the 3DFSC (blue). **(H)** Half-set gold-standard

FSC curve (red) and map-model FSC curves for trimeric IL-10 receptor subcomplex (blue) and hexameric complex (yellow).

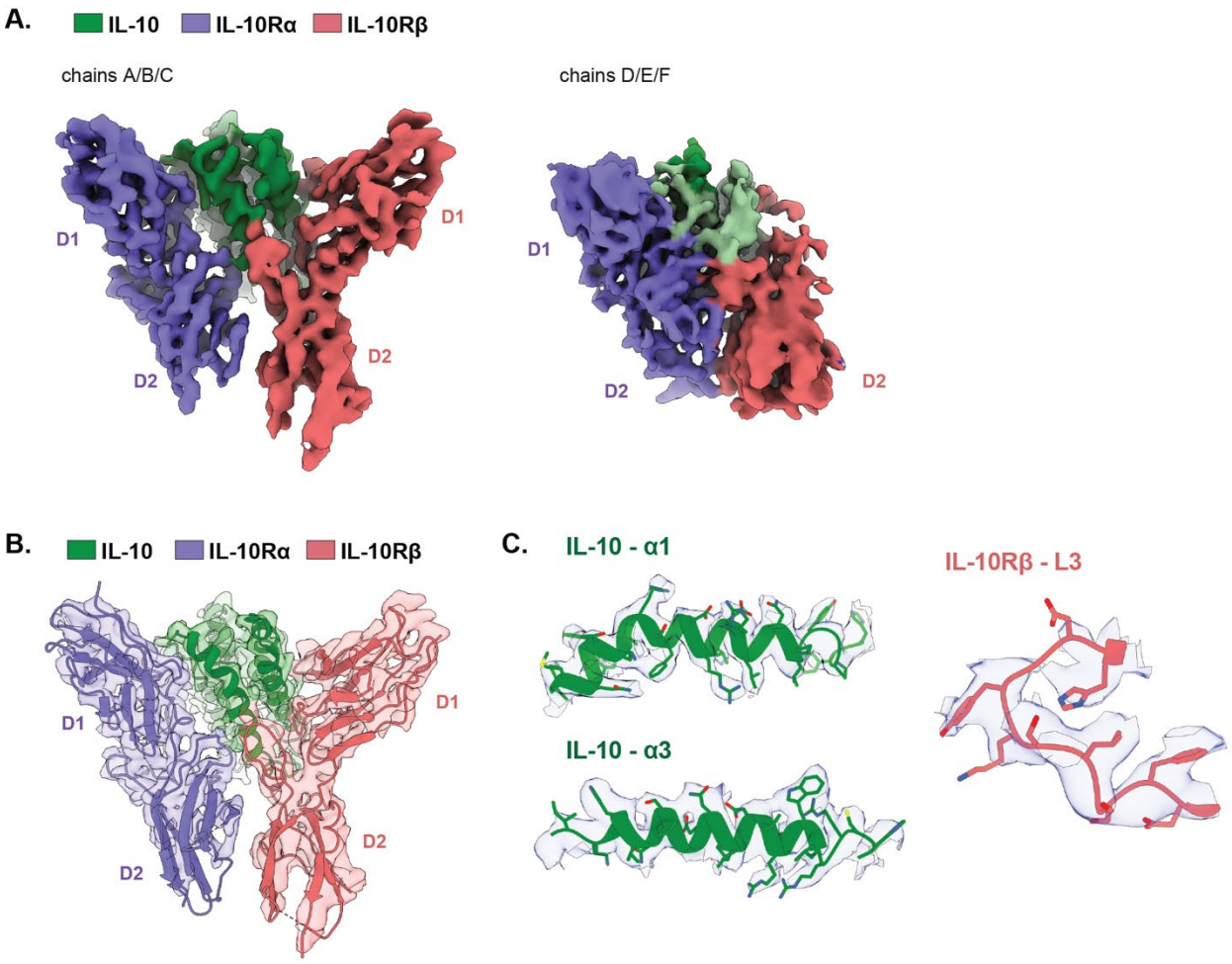


Fig. S4. Fitting of the IL-10 receptor complex structure into the cryo-EM density map. (**A**) Segmented cryo-EM density maps of each IL-10–IL-10Rα–IL-10Rβ subcomplex within the hexameric IL-10 receptor complex. One subcomplex is well-resolved while the other is poorly resolved, possibly due to lower IL-10Rβ occupancy and flexibility between IL-10 subunits. Map threshold used in ChimeraX set to 0.4 (**B**) Molecular model showing the front view of the IL-10–IL-10Rα–IL-10Rβ ternary sub-complex within its segmented cryo-EM density map. Map threshold used in ChimeraX set to 0.4. IL-10 is shown in green, IL-10Rα in purple, and IL-10Rβ in salmon. (**C**) Sample cryo-EM density map and fitted molecular model for key regions at the IL-10–IL-10Rβ interface, showing that secondary structure and bulky side chains can be resolved at the current resolution.

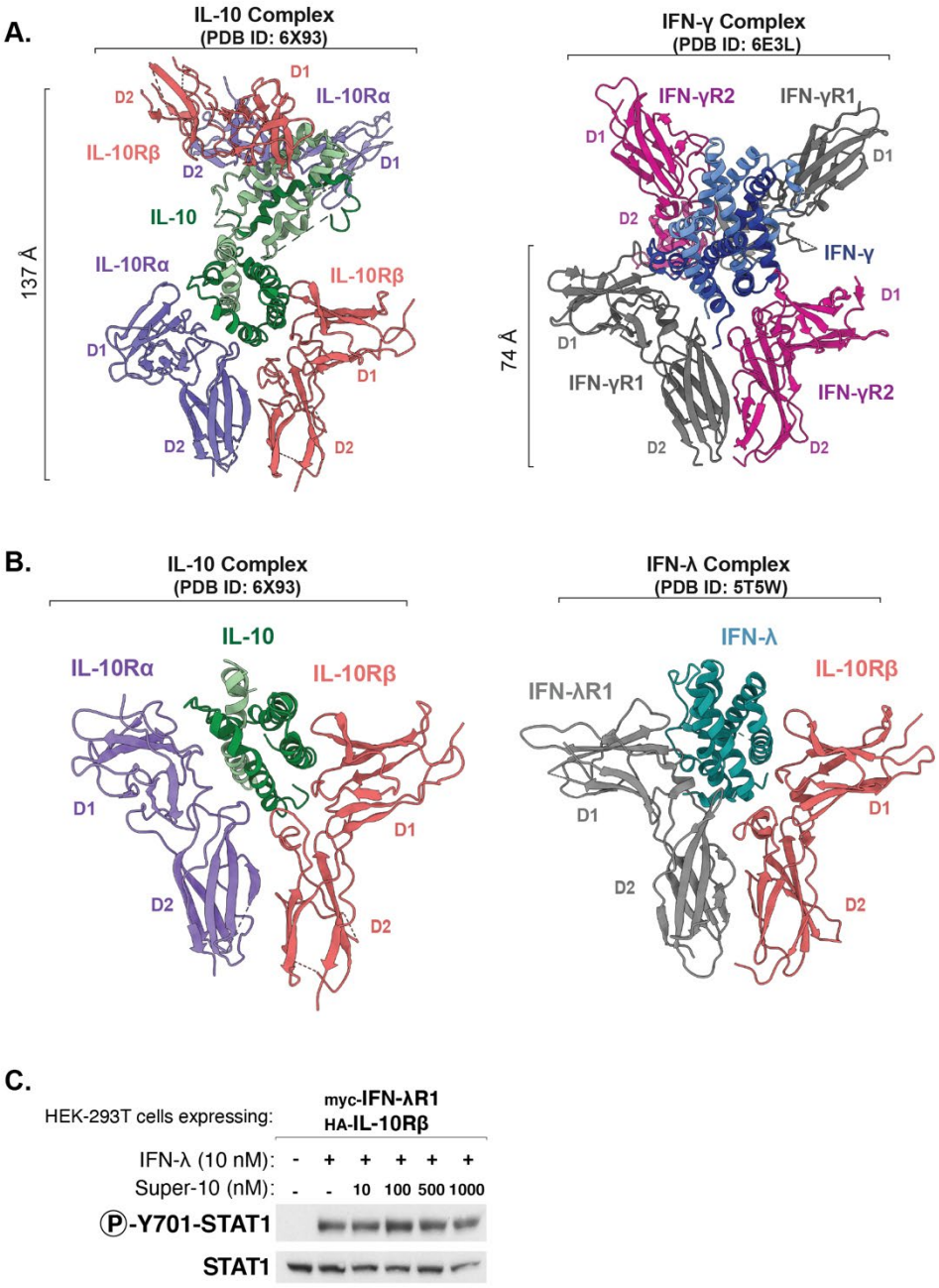


Fig. S5. Comparison of the IL-10 complex structure with type II and type III interferon complexes. (**A**) Side-by-side views of the hexameric IL-10 receptor complex structure (left) and the hexameric IFN-γ complex structure (right, PDB ID: 6E3L). (**B**) Side-by-side views of half of the IL-10-receptor complex (left) and IFN-λ receptor ternary complex (right, PDB ID: 5T5W). (**C**) Immunoblot of cell lysates prepared from HEK-293T cells transiently expressing IFN-λR1 and IL-10Rβ and treated with the indicated cytokines for 20 min.

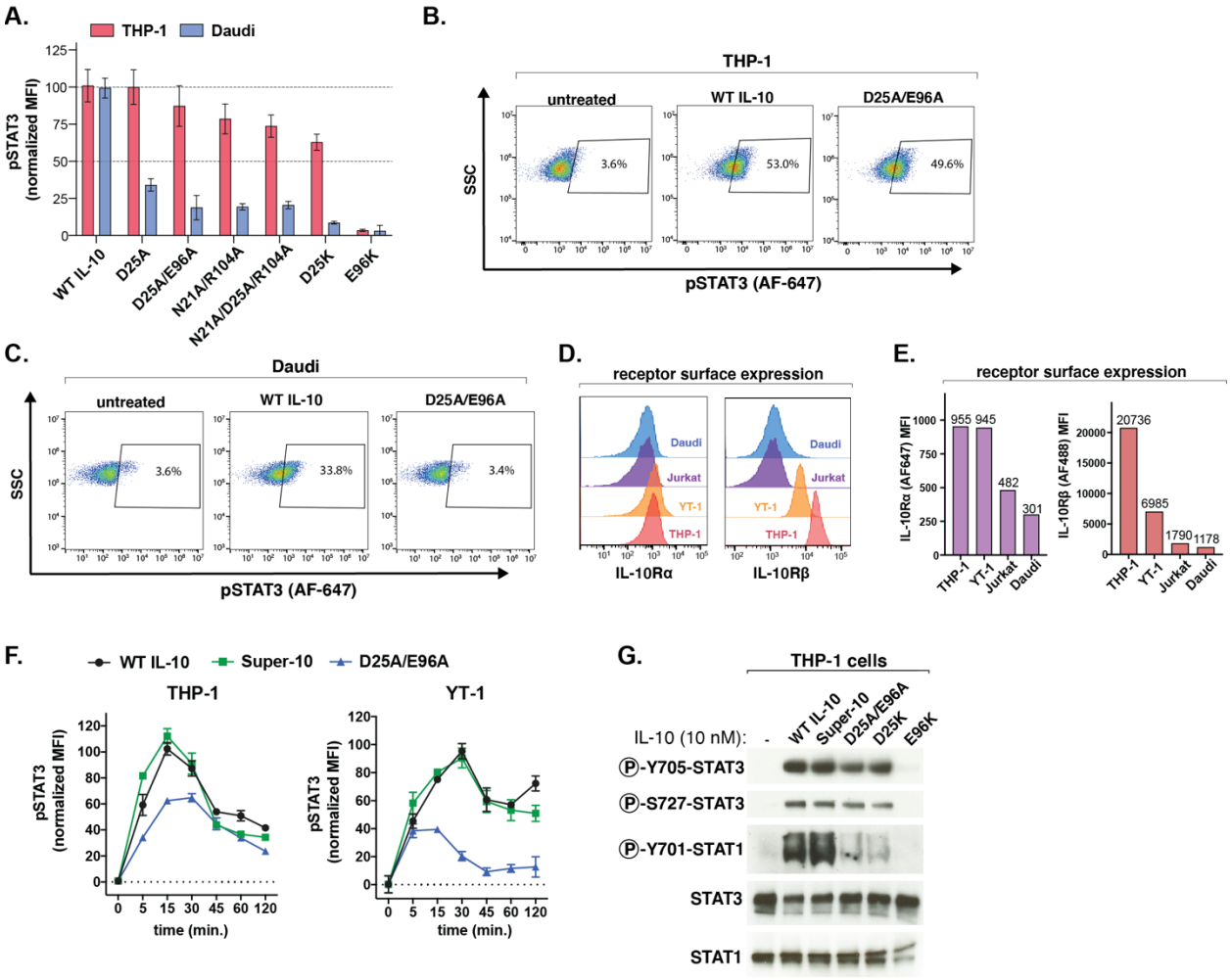


Fig. S6. IL-10 partial agonists elicit differential STAT3 signaling across cell lines. (**A**) Normalized E_{max} values for phospho-STAT3 calculated from sigmoidal dose-response curves in the indicated cell lines analyzed by flow cytometry. (**B, C**) Representative flow cytometry plots gated on live cells for THP-1 or Daudi cells treated with the indicated IL-10 variant for 20 min. (**D**) Histograms and (**E**) bar graphs showing IL-10Rα and IL-10Rβ surface expression on the indicated cell lines analyzed by flow cytometry. (**F**) Time-course of phospho-STAT3 signaling in THP-1 or Daudi cells treated with 10 nM of the indicated IL-10 variant. Data are mean ± SD for two replicates, shown as a percent of maximal WT IL-10 MFI. (**G**) Immunoblot of cell lysates prepared from THP-1 cells treated with 10 nM of the indicated IL-10 variants for 20 min.

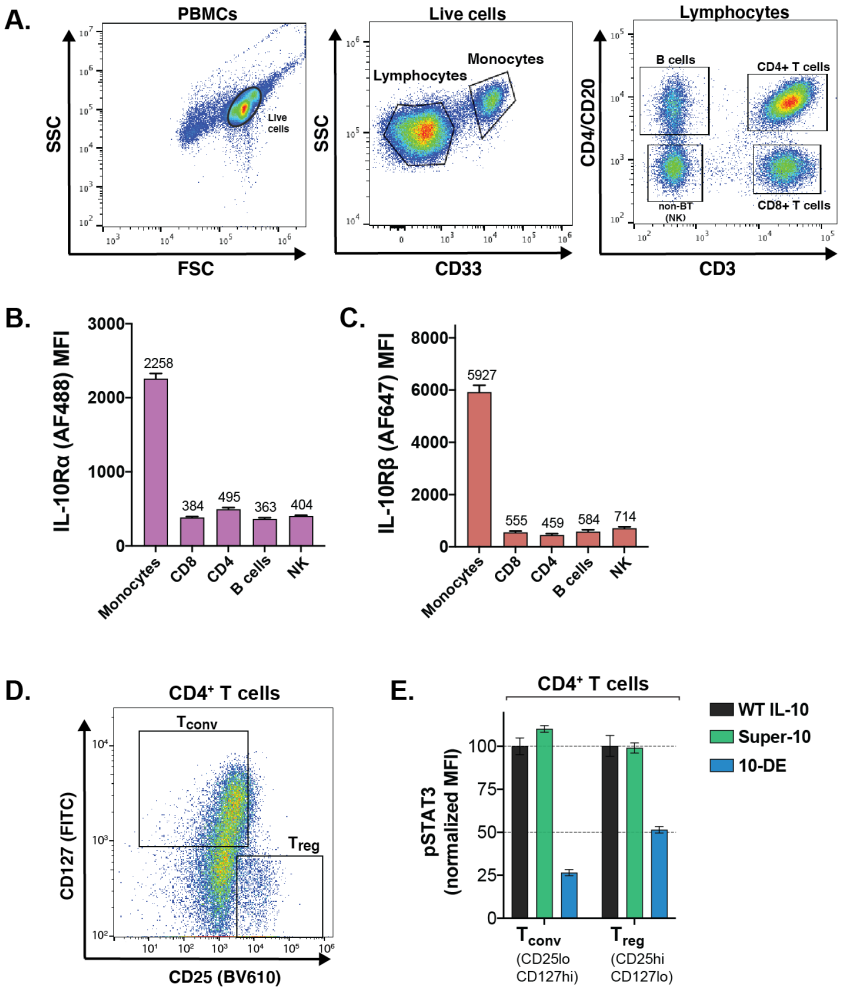


Fig. S7. IL-10 partial agonists elicit differential STAT3 signaling activity across primary human immune cell populations. (**A**) Gating strategy for PBMC phospho-flow cytometry signaling experiments. (**B, C**) Bar graphs showing IL-10Rα and IL-10Rβ surface expression on the indicated cell types from human PBMCs analyzed by flow cytometry. (**D**) Gating strategy for analysis of conventional $CD4^+$ T cells (T_{conv}) and regulatory T cells (T_{reg}) using isolated human $CD4^+$ T cells. (**F**) Normalized MFI values for phospho-STAT3 from $CD4^+$ T cells treated with 10 nM WT IL-10, Super-10, or 10-DE and analyzed by flow cytometry (mean ± SEM, *n*=4, *N*=2*)*.

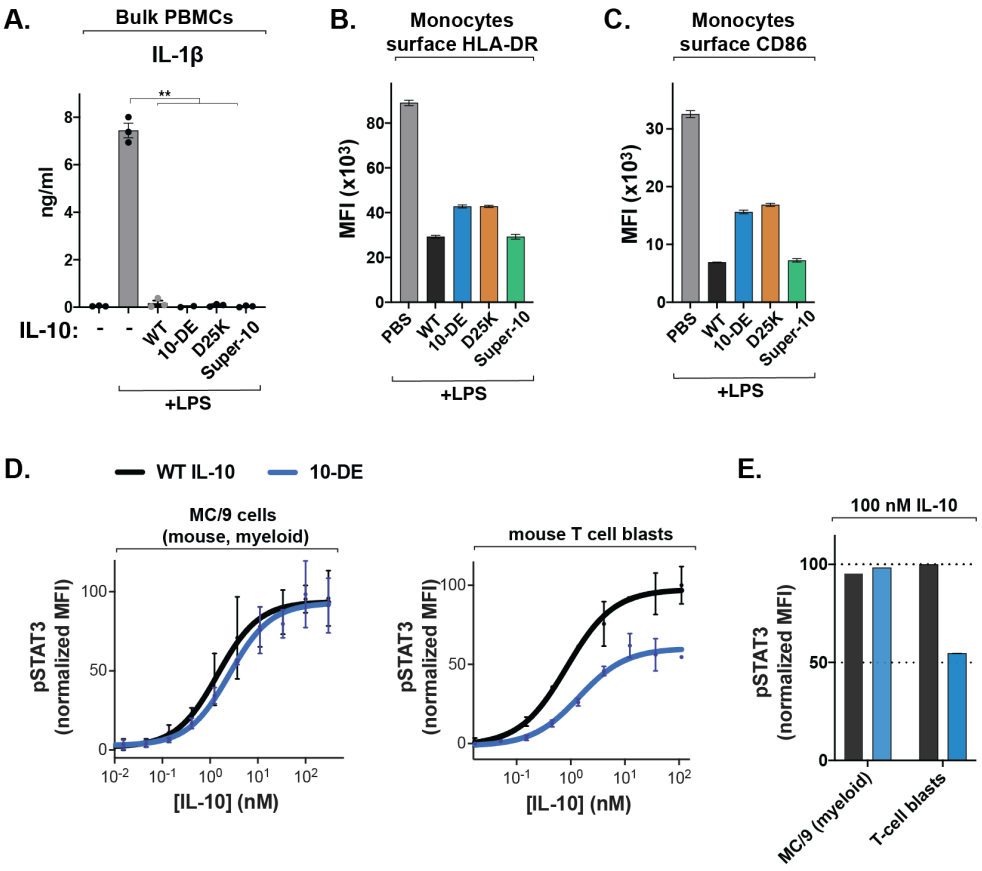


Fig. S8. IL-10 partial agonists retain anti-inflammatory functions of IL-10. (**A**) Levels of IL-1β from bulk human PBMCs treated with LPS and the indicated IL-10 variant, measured by ELISA (mean ± SEM, n=3, N=2, $^{**}P$<0.01, two-sided Student's t test). (**B**) Surface HLA-DR and (**C**) CD86 surface expression on monocytes activated with LPS alone or in combination with 10 nM of the indicated IL-10 variant, analyzed by flow cytometry (mean ± SD, n=4, N=2). (**D**) Dose response curves for phospho-STAT3 in the MC/9 mouse myeloid cell line or primary mouse T cell blasts (right) stimulated with WT IL-10 or 10-DE for 20 minutes and analyzed by flow cytometry shown as a percent of maximal WT IL-10 signal (mean ± SD, n≥2, N=2). (**E**) Normalized MFI values for phospho-STAT3 from the indicated cell types treated with 100 nM WT IL-10 or 10-DE and analyzed by flow cytometry (n=2, N=2).

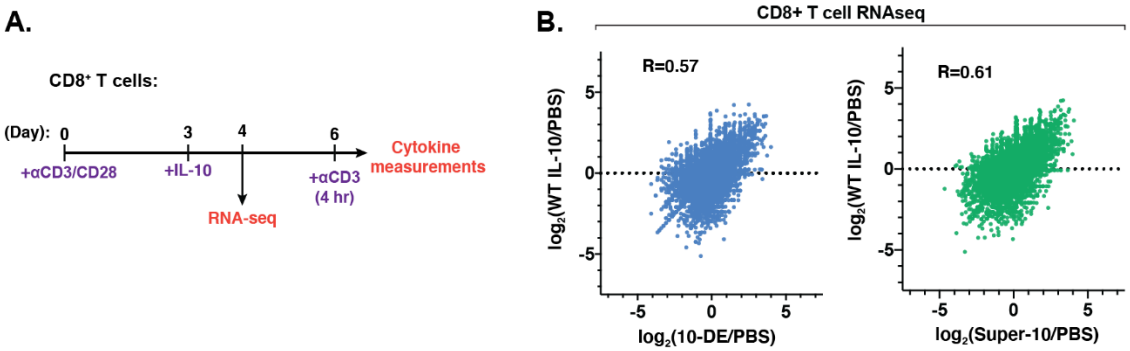


Fig. S9. Gene expression profiling of CD8+ T cells treated with WT IL-10 and 10-DE. (**A**) Schematic of experimental strategy for analyzing IL-10 effects on primary human $CD8^+$ T cells. (**B**) Correlation analysis of gene expression changes induced by 10-DE (left) and Super-10 (right) compared to WT IL-10, analyzed by RNA-seq (n=2 biological replicates).

Table S1. Cryo-EM data collection, refinement, and validation statistics

Data collection and processing	**IL-10 receptor complex**
EMDB	EMD-22098
Camera	Gatan K3 with GIF BioQuantum
Nominal magnification	81,000X
Calibrated magnification	46,382X
Voltage (kV)	300
Defocus range (μm)	–0.8 to –2.0
Total electron exposure (e/Å^2)	50
Exposure rate (e/Å^2/s)	1
Number of frames (frames/movie)	50
Pixel size (Å)	1.078
Energy filter slit width (eV)	20
Automation software	SerialEM
Symmetry imposed	C1
Initial particle images	6,701,298
Final particle images	86,725
Map resolution, masked (Å) (FSC threshold)	3.5 (0.143), 4.2 (0.5)
Map resolution, unmasked (Å) (FSC threshold)	4.6 (0.143), 9.1 (0.5)
Map resolution range (Å)	3.1–5.8
Resolution range due to anisotropy (Å)	3.2–6.1

Refinement	Trimeric complex	Hexameric complex
PDB ID		6X93
Initial models used (PDB ID)	1Y6K, 3LQM	Trimeric complex
Map sharpening B factor (Å^2)	–79	–79
Model composition		
Non-hydrogen atoms	4,301	6,846
Protein residues	529	1,042
B factors (Å^2)		
Protein	69	76
R.m.s. deviations		
Bond lengths (Å^2)	0.008	0.007
Bond angles (°)	1.05	1.13
Validation		
MolProbity score	2.14	2.07
Clashscore	20.2	16.7
Rotamer outliers (%)	0.4	0.4
C-beta outliers (%)	0.0	0.0
CaBLAM outliers (%)	3.0	3.1
CC_{mask}	0.73	0.62
CC_{volume}	0.73	0.65
EM Ringer score	1.9	1.9
Ramachandran plot		
Favored (%)	95.2	95.0
Allowed (%)	4.8	5.0
Outliers (%)	0.0	0.0

Table S2. Signaling properties of IL-10 variants in human cell lines

	WT IL-10		Super-10		D25A/E96A		D25K		E96K	
	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}
THP-1	1.61	102.7%	0.38	93.46%	2.86	87.3%	3.05	62.9%	-	3.5%
YT-1	1.81	98.6%	1.27	101.5%	1.02	53.3%	1.40	38.61%	-	5.2%
Jurkat	1.14	100.6%	1.07	117.7%	1.93	22.5%	0.41	13.9%	-	3.9%
Daudi	0.55	97.8%	0.24	199.3%	11.09	18.8%	2.24	8.7%	-	7.6%

Table S3. Signaling properties of IL-10 variants in primary immune cells

	WT IL-10		Super-10		D25A/E96A		D25K		E96K	
	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}
Mono	0.41	100.7%	0.32	95.2%	0.75	94.4%	1.38	71.7%	1.733	15.5%
$CD8^+$	0.40	100.1%	0.37	98.6%	1.52	55.2%	1.40	40.3%	-	4.4%
$CD4^+$	0.42	100.3%	0.42	97.8%	1.05	31.1%	1.40	41.1%	-	2.3%
B cells	0.38	99.7%	0.29	99.4%	1.05	52.8%	1.03	31.3%	-	7.6%
NK cells	0.58	101.7%	0.47	117.3%	1.51	37.8%	0.91	21.0%	-	8.2%

Movie S1. Cryo-EM structure of the IL-10 receptor complex
Model of the hexameric IL-10 receptor complex inside its 3.5-Å cryo-EM density map. IL-10 is shown in green, IL-10Rα in purple, and IL-10Rβ in salmon. Map threshold used in ChimeraX set to 0.4