Supporting Information

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SI Discussion

The high affinity of H11 for CTLA-4 also enables imaging of the target protein in animals cotreated with full-sized antibody at therapeutic doses, a critical feature for imaging of patients who receive immunotherapy with antibodies that recognize the imaging target.

The distribution of CTLA-4 in naïve and tumor-bearing mice may give insights into why CTLA-4 antibodies selectively target the tumor microenvironment in mice. We and others have found that anti–CTLA-4 antibody treatment leads to a relative reduction in Tregs in the tumor, but not in tumor-draining lymph nodes or the spleen, despite expression of CTLA-4 at these sites (23–25). CTLA-4 predominantly resides within endocytic vesicles before T cell activation, shuttling between these vesicles and the cell surface. CTLA-4 is surface-exposed in substantial quantities only in properly antigen-triggered cells (14). For this reason, staining of CTLA-4 on cells in vitro requires cell permeabilization or prolonged incubations with live cells at physiologic temperatures. In endocytic compartments, CTLA-4 should be inaccessible to antibodies and VHH H11. We therefore do not expect to record an immuno-PET signal for the immune organs, despite the known presence of CTLA-4. This is in contrast to PD-L1, for which a weak PET signal is apparent in the spleen of naïve mice, in which PD-L1 expression is largely confined to a small number of myeloid dendritic cells. In tumor-bearing mice, we do observe an intratumoral CTLA-4 signal. This tissue must therefore contain a larger fraction of cells that express CTLA-4 on their surface, presumably because of ongoing antigen exposure. Translation of this imaging technique to the clinical setting may help identify patients whose tumors or tissues contain surface-exposed CTLA-4. This, in turn, might improve prediction of therapeutic efficacy or treatment-related immune toxicities.

We attribute the superiority of H11–IgG2a compared with the monoclonal antibody 9H10 to two factors. Because H11–IgG2a lacks a light chain as well as the heavy-chain C_H1 domain, the construct is approximately half the size of a conventional antibody, which should improve tissue penetration. Although this is not likely a major factor in the highly vascularized B16 model, the ability to penetrate tissue may be more important in fibrotic, denser tumors such as tumors of the pancreas. H11 also blocks CTLA-4 interactions with B7-1 at much lower concentrations than does the 9H10 antibody, thereby providing more potent CTLA-4 blockade. This higher affinity likely accounts for its enhanced activity in vivo, although we cannot exclude an effect of altered FcγR affinity as a result of differences in Fc sequence or glycosylation patterns, as 9H10 is a Syrian Hamster IgG whereas we used native murine IgG2a. We do not observe further relative reductions in Tregs or changes in peripheral Tregs when comparing 9H10 and H11–IgG2a. Instead, we observed a relative expansion of effector CD4 T cells (43). H11–IgG2a may therefore act not only by blockade of CTLA-4 to expand CD4 effector cells, but also facilitates Treg depletion through engagement of the FcγR (43). Even though CTLA-4–directed therapies can benefit from improved receptor blockade, this effect is far outweighed by the requirement for the inclusion of an Fc domain. Modifications of the Fc domain may be the most effective way to improve the efficacy of CTLA-4–directed antibodies. Indeed, low-affinity H11–IgG2a demonstrated substantially higher efficacy than the single-domain antibody with WT sequence, underscoring the central importance of this Fc-mediated effect.

SI Methods

Animal Care. Animals were housed at the Whitehead Institute for Biomedical Research or DFCI and were maintained according to protocols approved by the MIT Committee on Animal Care or the DFCI Institutional Animal Care and Use Committee, respectively. C57BL/6 WT mice were purchased from Jackson Labs. Access to CTLA-4 conditional-KO mice was made possible by Arlene Sharpe (Harvard Medical School, Boston, MA). A male alpaca (*Vicugna pacos*) was purchased locally, maintained in pasture, and immunized following a protocol authorized by the Tufts University Cummings Veterinary School Institutional Animal Care and Use Committee.

VHH Library Generation. A male alpaca (*V. pacos*) was immunized with a mixture of recombinant mouse and human proteins, including mouse CTLA-4. A primary immunization was followed by four boosts, spaced 2 wk apart, each containing 200 μg of mCTLA-4 mixed 1:1 in alum (Thermo Scientific). Following immunization, total RNA was isolated from ∼10^6 fresh peripheral blood lymphocytes by using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's instructions. First-strand cDNA synthesis was performed by using SuperScript III reverse transcriptase (Life Technologies) and a combination of oligo dT, random hexamer, or Ig-specific primers AlCH2 and AlCH2.2 as previously described (45). PCR amplification of VHH sequences and phage library generation followed a previously published procedure. Following transformation into TGI cells (Agilent), the total number of independent clones was estimated to be 3.2×10^6. Ninety-six clones were selected at random and sequenced to assess library diversity. The resulting phagemid libraries were stored at −80°C.

Selection of VHHs by Phage Display. Two hundred microliters of the immunized VHH phage library was inoculated in 100 mL super optimal broth (SOC) with 50 μg/mL ampicillin. The culture was grown to midlog phase and infected with 100 μL 10^{14} pfu/mL VCSM13 helper phage. The culture was then incubated for 2 h at 37 °C and the cells were harvested by centrifugation and resuspended in 100 mL 2YT, 0.1% glucose, 50 μg/mL kanamycin, and 50 μg/mL ampicillin. Cultures were incubated overnight at 30 °C and then centrifuged for 20 min at 7,700 × *g* followed by phage precipitation from the resulting supernatant with 20% PEG-6000/500 mM NaCl at 4 °C and resuspension in PBS solution.

One hundred micrograms of recombinant mouse CTLA-4-Fc was biotinylated by coupling Chromalink NHS-biotin reagent (Solulink) to primary amines for 90 min in 100 mM phosphate buffer, pH 7.4, 150 mM NaCl. The reaction was then run through an Amicon 10-kDa MWCO concentrator (EMD Millipore) to remove remaining NHS-biotin. Incorporation of biotin was monitored spectrophotometrically following the manufacturer's guidelines. MyOne Streptavidin-T1 Dynabeads (100 μL; Life Technologies) were blocked in PBS solution plus 2% (wt/vol) mouse serum in a microfuge tube for 2 h at 37 °C. Following blocking, 20 μg biotinylated CTLA-4-Fc in mouse serum was added to the beads and incubated for 30 min at room temperature with agitation. The beads were then washed 3× in PBS solution, and 200 μL of 10^{14} pfu/mL M13 phage displaying the VHH library was added in PBS solution/mouse serum for 1 h at room temperature. The beads were then washed 15× with PBS solution, 0.1% Tween-20. Phage was eluted by the addition of *E. coli* ER2738 (NEB) for 15 min at 37 °C, followed by elution with

200 mM glycine, pH 2.2, for 10 min at room temperature. The glycine elution was neutralized and pooled with the *E. coli* ER2738 culture, plated onto 2YT agar plates supplemented with 2% glucose, 5 μg/mL tetracycline, and 10 μg/mL ampicillin, and grown overnight at 37 °C. A second round of panning was performed with the following modifications: 2 μg of biotinylated CTLA-4-Fc was used as bait and incubated with 2 μL 10^{14} pfu/mL M13 phage displaying the first-round VHH library for 15 min at 37 °C, followed by extended washes in PBS solution with 0.1% Tween-20.

Following two rounds of phage panning, 96 colonies were isolated in 96-well round-bottom plates and grown to midlog phase at 37 °C in 200 μL 2YT, 10 μg/mL ampicillin, and 5 μg/mL tetracycline, induced with 3 mM IPTG, and grown overnight at 30 °C. Plates were centrifuged at 12,000 × *g* for 10 min, and 100 μL of supernatant was mixed with an equal volume of PBS solution, 5% (wt/vol) nonfat dry milk. This mixture was added to an ELISA plate coated with 1 μg/mL CTLA-4-Fc. Following four washes in PBS solution, 1% Tween-20, anti-Etag antibody (Bethyl) was added at 1:10,000 dilution in PBS solution, 5% (wt/vol) nonfat dry milk for 1 h at room temperature. The plate was developed with fast kinetic TMB (Sigma) and quenched with 1 M HCl. Absorbance at 450 nm was determined in a plate reader (Spectramax; Molecular Devices).

Cloning and Expression of H11. H11 coding sequence was subcloned into the *E. coli* periplasmic expression vector pHEN6 by using the PCR primers 189 (5′-tactcgcggcccagccGGCCCAACCGGCCATGGC-3′) and 190 (5′-agtcctcctgaggagacggtgaccGAGACGGTGACCTGGGTCCCC-3′) to allow for Gibson cloning and the inclusion of a C-terminal sortase motif and 6x His tag. WK6 *E. coli* containing the plasmid was grown to midlog phase at 37 °C in Terrific Broth plus ampicillin and induced with 1 mM IPTG overnight at 30 °C. Cells were harvested by centrifugation at 5,000 × *g* for 15 min at 4 °C and then resuspended in 25 mL 1× TES buffer (200 mM Tris, pH 8, 0.65 mM EDTA, 0.5 M sucrose) per liter culture and incubated for 1 h at 4 °C with agitation. Resuspended cells were then submitted to osmotic shock by 1:4 dilution in 0.25× TES buffer and incubation overnight at 4 °C. The periplasmic fraction was isolated by centrifugation at 9,715 × *g* for 30 min at 4 °C and then loaded onto Ni-NTA (Qiagen) in 50 mM Tris, pH 8, 150 mM NaCl, and 10 mM imidazole. Protein was eluted in 50 mM Tris, pH 8, 150 mM NaCl, 500 mM imidazole, and 10% glycerol and then loaded onto a Superdex 75 10/300 column in 50 mM Tris, pH 8, 150 mM NaCl, 10% glycerol. Recombinant VHH purity was assessed by SDS/PAGE, and peak fractions were recovered and concentrated with an Amicon 10,000-kDa MWCO filtration unit (Millipore) and stored at −80 °C.

VHH-Fc Production. H11–IgG2a, GGG–mIgG2a, and mH11–IgG2a were each cloned into the mammalian expression vector pVRC and transiently transfected by using polyethylenimine into Expi293F cells cultured in ExpiFreeStyle media (ThermoFisher). Secreted protein was harvested 6 d after transfection by centrifugation at 8,000 × *g* for 20 min at 4 °C, followed by HisTrap HP (GE Healthcare) and size-exclusion chromatography on a Superdex 200 16/600 column (GE Healthcare).

Protein Ligation and Purification of H11–GGG–IgG2a. To generate H11–GGG–IgG2a, 200 μM bacterially expressed H11–LPETGG–6xHis was incubated with 400 μM GGG-IgG2a and 5 μM pentamutant (5M) sortase overnight at 4 °C in 100 mM Tris, pH 8, 150 mM NaCl, 10 mM $CaCl_2$. The reaction was stopped with the addition of 100 μM EDTA, and the resulting product was isolated by size-exclusion chromatography on a Superdex 200 16/600 column (GE Healthcare).

LPS Removal. All therapeutic agents were depleted of LPS (<2 IU/mg) or purchased LPS-free from the manufacturer. To remove LPS, VHHs were immobilized on HisTrap HP 1 mL columns (GE Healthcare) in PBS solution, washed with 40 column volumes of PBS solution + 0.1% Triton X-114, and eluted in 2.5 column volumes of endotoxin-free PBS solution (Teknova) with 500 mM imidazole. Imidazole was removed by PD10 column (GE Healthcare). LPS content was tested by using the LAL Chromogenic Endotoxin Quantitation Kit (Pierce) according to the manufacturer's instructions.

C-Terminal Labeling of VHHs with Biotin or Alexa647. A heptamutant variant of *Staphylococcus aureus* Sortase A was used to label H11, H11Fc, or control by incubating 30 μM of purified VHH protein with 5 μM 7M SrtA and 100 μM GGGK-Biotin or GGGK-Alexa 647 nucleophiles in 50 mM Tris, pH 8, 150 mM NaCl for 2 h at room temperature. Unreacted VHH and 7M SrtA were removed by adsorption onto Ni-NTA agarose beads (Qiagen). After removal of the unreacted VHH and 7M SrtA by Ni-NTA agarose beads, the unbound fraction was concentrated and separated from excess nucleophile (biotin or alexa647) with an Amicon 3,000-kDa MWCO filtration unit (Millipore), and stored at −80°C.

Immunoblots. Fifty nanograms per lane of mPD-L1 or mCTLA-4 were diluted in 1× SDS/PAGE loading buffer and run on SDS/PAGE and transferred to PVDF. The membrane was blocked overnight at 4 °C in PBS solution, 5% (wt/vol) milk. VHH H11 was diluted to 1 μg/mL in blocking solution and incubated for 1 h at room temperature. Anti–llama-IgG-HRP antibody (Bethyl) was used as a secondary reagent at a 1:25,000 dilution in blocking solution. Blots were developed with Western Lightning Plus-ECL (Perkin-Elmer) and exposure to autoradiography film (Labscientific). Blots were stripped and reprobed with anti–5xHis-HRP antibody (Qiagen) at 1:10,000 dilution in blocking solution as a loading control and imaged as described earlier.

ELISAs. High-binding microtiter plates (Corning) were coated with recombinant Fc fusion proteins (produced by S.C.A.) overnight at 25 ng/mL in carbonate buffer. Biotinylated VHHs or antibodies (BD Biosciences) were incubated on the coated plates in 10% inactivated FCS in PBS solution for 1 h in the presence or absence of unlabeled VHH or antibody across a range of concentrations, washed in 0.5% Tween in PBS solution, and then developed by using streptavidin-HRP (BD Biosciences) and TMB. For plate-bound ligand-binding assays, B7-1 Fc-fusion (R&D Systems) was incubated with plate-bound ligand for 1 h in the presence of VHHs or antibodies. Plates were then washed, and binding was determined by using biotinylated polyclonal antibody against B7-1 (1:1,000 dilution; R&D Systems), followed by incubation with streptavidin-HRP and then development with TMB.

Flow Cytometry. To prepare tissues for flow cytometry, tumor samples were digested by using a tumor dissociation kit (MACS) for 45 min at 37 °C. Digested tumors, spleens, and lymph nodes were mechanically disrupted into single-cell suspensions through a 40-μm cell strainer. When indicated, RBCs were lysed in ammonium-chloride-potassium lysis buffer. Cells were washed, pelleted, and resuspended in PBS solution. Cells were stained in 100 μL PBS solution with various staining mix for 20 min unless indicated otherwise. Cells were washed, pelleted, and fixed in 1% formalin. All antibodies were purchased from BD Pharmingen or eBiosciences as indicated. Data were measured on a FACS LSR or FORTESSA or a Sony SP6800 device and analyzed by using FlowJo software. Flow cytometry antibodies used in this study were purchased from BD Pharmingen (αCD4 [RM4-5], αCD8 [53-6.7], CD3 [145-2C11], CD19 [1D3]) or BioLegend (CD45 [30-F11], CD8a [53-6.7], CD4 [RM4-5], TCRβ [H57-597], CD25 [PC61], PD-1 [29F.1A12], FoxP3 [MF-14]).

Imaging. The ^{18}F radiolabeling was performed following an established procedure (9). To label purified VHH with tetrazine, reactions were performed in 50 mM Tris·HCl, pH 7.5, supplemented with 10 mM $CaCl_2$, 150 mM NaCl, 750 μM triglycine-containing probe, 100–200 μM VHH, and 5 μM sortase. After incubation at 4 °C with agitation for 1 h, reaction products were analyzed by liquid chromatography/MS, with yields generally >90%. The product was purified from excess of the tryglycine substrate by size-exclusion chromatography in PBS solution by using a PD-10 column. Ni-NTA beads were added to the collected fractions with agitation for 5 min at 25 °C, followed by centrifugation to remove sortase and any remaining unreacted His-tagged substrate. VHH-Tz (40 μL, 150 μM), PBS solution (300 μL), and ^{18}F-TCO (transcyclooctene) in DMSO [4.0 mCi (148.0 MBq), 100 μL] were mixed in a microfuge tube. The tube was sealed and shaken at room temperature for 20 min. The mixture was analyzed by radio-TLC (thin layer chromatography) [instant TLC, 100% MeCN, Rf (retardation frequency) ^{18}F-TCO = 0.9, Rf ^{18}F-VHH = 0.0], showing 90% conversion to ^{18}F-VHH. The reaction mixture was loaded onto a PD-10 size-exclusion cartridge (GE Healthcare) and eluted with PBS solution. Imaging was performed on a Siemens Inveon PET/CT imager. All PET/CT imaging procedures were approved by the Massachusetts General Hospital subcommittee on research animal care.

The ^{89}Zr radiolabeling was performed following an established procedure (11). In a typical reaction, a solution of 25–50 μg of PEGylated-VHH-H11-DFO in 200 μL of 0.5 M Hepes buffer, pH 7.5, was prepared. Then, a volume of the 89Zr4+ stock solution (typically supplied in 1.0 M oxalic acid) corresponding to 1.0–1.5 mCi was added to a 2-mL plastic screw-cap microcentrifuge tube. The volume of this solution was adjusted to a total of 200 μL by using 1.0 M oxalic acid. The pH of the 89Zr4+ solution was adjusted to 6.8–7.5 by using 2.0 M Na_2CO_3. This solution was added to PEG-VHH-DFO. The reaction mixture was incubated for 60 min at room temperature on an agitating block, loaded onto a PD-10 size-exclusion cartridge (GE Healthcare), and eluted with 1×PBS solution, yielding >80% (~0.8–1.3 mCi) of ^{89}Zr-PEG-VHH (decay-corrected radiochemical yield). Imaging was performed on a PerkinElmer G8 PET/CT imager. All PET/CT imaging procedures were approved by the Massachusetts Institute of Technology Subcommittee on Research Animal Care. Mice were imaged 90 min after injection with ^{18}F-labeled VHHs and 24 h after injection of ^{89}Zr-PEG–labeled VHHs.

Murine CTLA-4 Antigen Production in HEK293 Cells. Coding sequences were cloned into a modified version of the Daedalus lentiviral transfer vector, which supported ligation-independent cloning and expressed targets as a fusion with a variant of mIgG2a. Transduction of HEK-293F cells (Gibco) was carried out at a scale of 30 mL in Freestyle 293 Expression media (Gibco), and cultures were expanded to a 3L production scale. Supernatant from 3L culture was harvested by centrifugation (2,000 × *g*, 10 min), 15 mL 1 M MES, pH 6.5, and AEBSF (final concentration 20 μM) was added, and the supernatant swirled at 4 °C for 1 h with a 30-mL bed volume (bv) of His60 Ni-IDA resin (Clontech). Resin was poured into a gravity column, washed with 10 bv wash buffer (25 mM MES, pH 6.5, 150 mM NaCl, 10% glycerol, 50 mM arginine-Cl and 5 mM imidazole), and eluted with 5 bv elution buffer (wash buffer with 100 mM arginine-Cl and 0.5 M imidazole). Protein was concentrated by using centrifugal units (Amicon Ultracel-10; Millipore) and applied to a gel-filtration column (Sephacryl S200 26/60; GE Lifesciences) equilibrated in 25 mM MES, pH 6.5, 150 mM NaCl, 10% glycerol, 100 mM Arg-Cl. Peak fractions were collected and concentrated as described earlier.

Murine CTLA-4 Production for Crystallography. The murine CTLA-4 ectodomain was prepared as previously described (46). In brief, the ECD of murine CTLA-4, encompassing residues 38–154, was expressed in BL21(DE3), refolded from inclusion bodies, and purified by anion exchange and size-exclusion chromatography. Purity was assessed by SDS/PAGE, and peak fractions were concentrated with an Amicon 10,000-kDa MWCO filtration unit (Millipore).

Crystallization and Data Collection. Crystals of murine CTLA-4, the anti–CTLA-4 VHH, and the CTLA-4:VHH complex were grown by sitting drop vapor diffusion at room temperature. Crystals of murine CTLA-4 were produced by equilibrating protein (29 mg/mL) in 20 mM Hepes, pH 7.0, 150 mM NaCl, and 1.0 mM EDTA, pH 8.0, and 5% glycerol with a precipitant solution containing 1.4 M sodium/potassium phosphate, pH 5.6. Crystals appeared in 6–7 d and exhibited diffraction consistent with the space group C2, with one molecule of the protein per asymmetric unit (Table S1). VHH crystals were grown by equilibrating protein (12 mg/mL) in 20 mM Hepes, pH 7.0, 150 mM NaCl, 1.0 mM EDTA, pH 8.0, and 5% glycerol with a precipitant solution composed of 1.5 M lithium sulfate and 0.1 M Hepes, pH 7.5. Crystals appeared in 1 mo and exhibited diffraction consistent with space group $P6_122$, with one protein molecule per asymmetric unit. Crystallization of the CTLA-4:VHH complex was performed by equilibration of protein solutions containing a 1:1 stoichiometry of CTLA-4 and H11 against 0.8 M sodium/potassium phosphate, pH 8.2. Crystals appeared in 1 wk and exhibited diffraction consistent with space group $P2_12_12_1$, with eight protein molecules (four copies of the complex) per asymmetric unit (Table S1). For consistency with the conventions used in earlier structures, the first residue in CTLA-4 is referenced as residue 1.

Before data collection, crystals were transferred to cryoprotectant solutions composed of their mother liquids supplemented with 20% glycerol. After incubation for ~10 s, the crystals were flash-cooled in a nitrogen stream. Diffraction data were collected on a Rayonix 225-HE detector (Rayonix) at APS beamline 31-ID (Advanced Photon Source, Argonne National Laboratory). Diffraction intensities were integrated and scaled with programs DENZO and SCALEPACK; data collection statistics are given in Table S1.

Structure Determination and Model Refinement. The structure of murine CTLA-4 was determined by molecular replacement with PHENIX, with the previously reported murine CTLA-4 structure (PDB ID code 1DQT) used as the search model. Iterative cycles of refinement were performed, including model rebuilding with COOT, refinement with PHENIX, and automated model rebuilding with ARP/wARP. The final model refined with an R_{cryst} of 0.180 and an R_{free} of 0.203 at 1.5-Å resolution. The structure of anti–CTLA-4 VHH was determined by molecular replacement with the BALBES pipeline using PDB ID code 1OHQ as the search model. Following a similar strategy to that described earlier, this structure was refined to an R_{cryst} of 0.171 and an R_{free} of 0.189 at 1.68 Å. The structure of the murine CTLA-4:VHH complex was determined by molecular replacement using PHENIX and the two structures described earlier as search models. This structure contains eight protein molecules, which form four independent copies of the CTLA-4:VHH complex in the asymmetric unit. The structure refined with an R_{cryst} of 0.193 and an R_{free} of 0.247 at 2.2 Å. Final crystallographic refinement statistics for all three structures are provided in Table S1.

In Vitro Killing Assays. Spleen cells were isolated from OT-I RAG KO mice and were stimulated with αCD3/CD28 beads (Dynabeads) for 48 h at 2×10^6 cells per milliliter in RPMI medium per manufacturer instructions. Beads were then removed by magnetic separation, and cells were incubated for an additional 24 h in the culture supernatant. In parallel, 2.5×10^3 B16-ova

cells were incubated for 18 h in 250 μL RPMI containing 20 ng/mL IFN-γ. IFN-γ–containing media was exchanged for fresh media, and the T cells were cocultured with the B16 cells for 48 h at a ratio of 2:1. VHHs or antibodies were added at the start of the culture. After 48 h, T cells were removed from the cultures by washing, and B16 viability was assessed by using the CellTiterGlo bioluminescence assay according to the manufacturer's instructions. Percent survival was calculated by using untreated B16 cells cultured without T cells for normalization.

Melanoma Models. B16 cells were purchased from American Type Culture Collection. B16 GM-CSF and B16-ova cells were a gift from Glenn Dranoff (Novartis Institute for Biomedical Research, Cambridge, MA). For in vivo challenge experiments, 5×10^5 B16 cells were inoculated by s.c. injection in 500 μL of HBSS. For vaccinations, 5×10^5 irradiated (3,500 rads) GM-CSF–secreting B16 cells (GVAX) were administered as an s.c. injection in 250 μL HBSS. VHHs (200 μg per dose), anti–CTLA-4 (200 μg/dose, 9H10; BioXCell), and H11Fc (100 μg per dose), were administered in 200 μL LPS Free PBS solution (TekNova) by i.p. injection. Tumor size was measured in two dimensions by using precision calipers. Mice were euthanized when the total tumor volume exceeded 125 mm^2.

Statistics. Two-sample comparisons used the *t* test with pooled variance if there was no evidence of inhomogeneity of variances between groups. If the variances were unequal, the exact Wilcoxon rank-sum test, a nonparametric alternative to the *t* test, was used. Every effort was made to keep testing consistent across related experiments. For comparisons of more than two groups, ANOVA was used if there was no evidence of inhomogeneity of variance; the Kruskal–Wallis test was the nonparametric alternative. Tumor growth studies were analyzed by using mixed-model ANOVA.

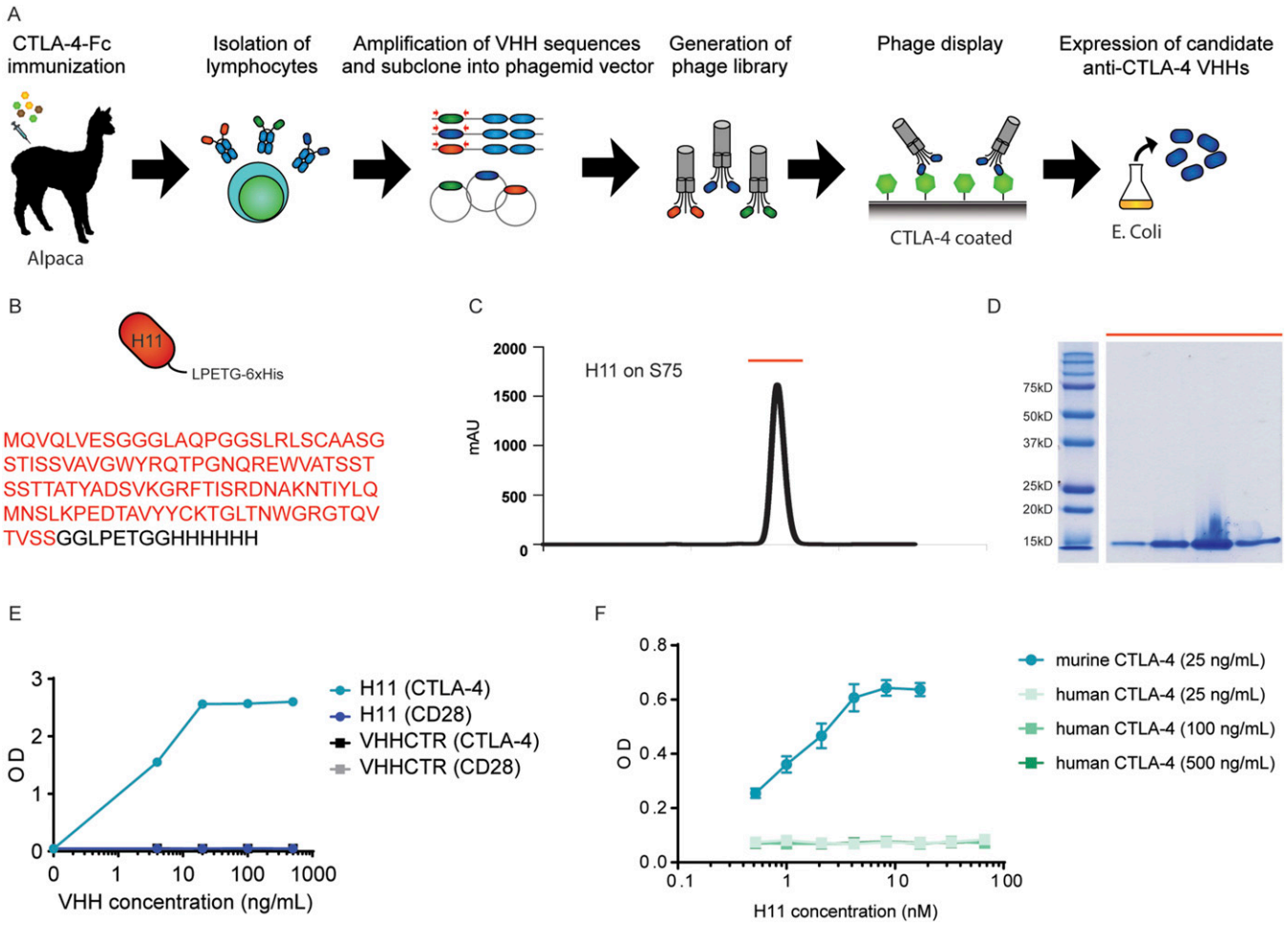

Fig. S1. Isolation of a murine CTLA-4–specific VHH. (*A*) Schematic diagram of the isolation of candidate VHHs specific for murine CTLA-4. (*B*) Schematic and amino acid sequence of the H11 construct. (*C*) Size-exclusion chromatography profile of eluted H11. (*D*) SDS/PAGE of peak fractions of H11 from size-exclusion chromatography. In *C* and *D*, the red bar indicates the collected fractions. (*E*) Biotinylated VHH clone H11 was incubated with plate-bound murine CTLA-4-Fc or CD28-Fc at the indicated concentrations. (*F*) Biotinylated VHH clone H11 was incubated with plate-bound murine CTLA-4 or human CTLA-4 at the indicated concentrations. (*E* and *F*) Binding was detected by using streptavidin/HRP developed with TMB. *y* axis shows OD at 450 nm.

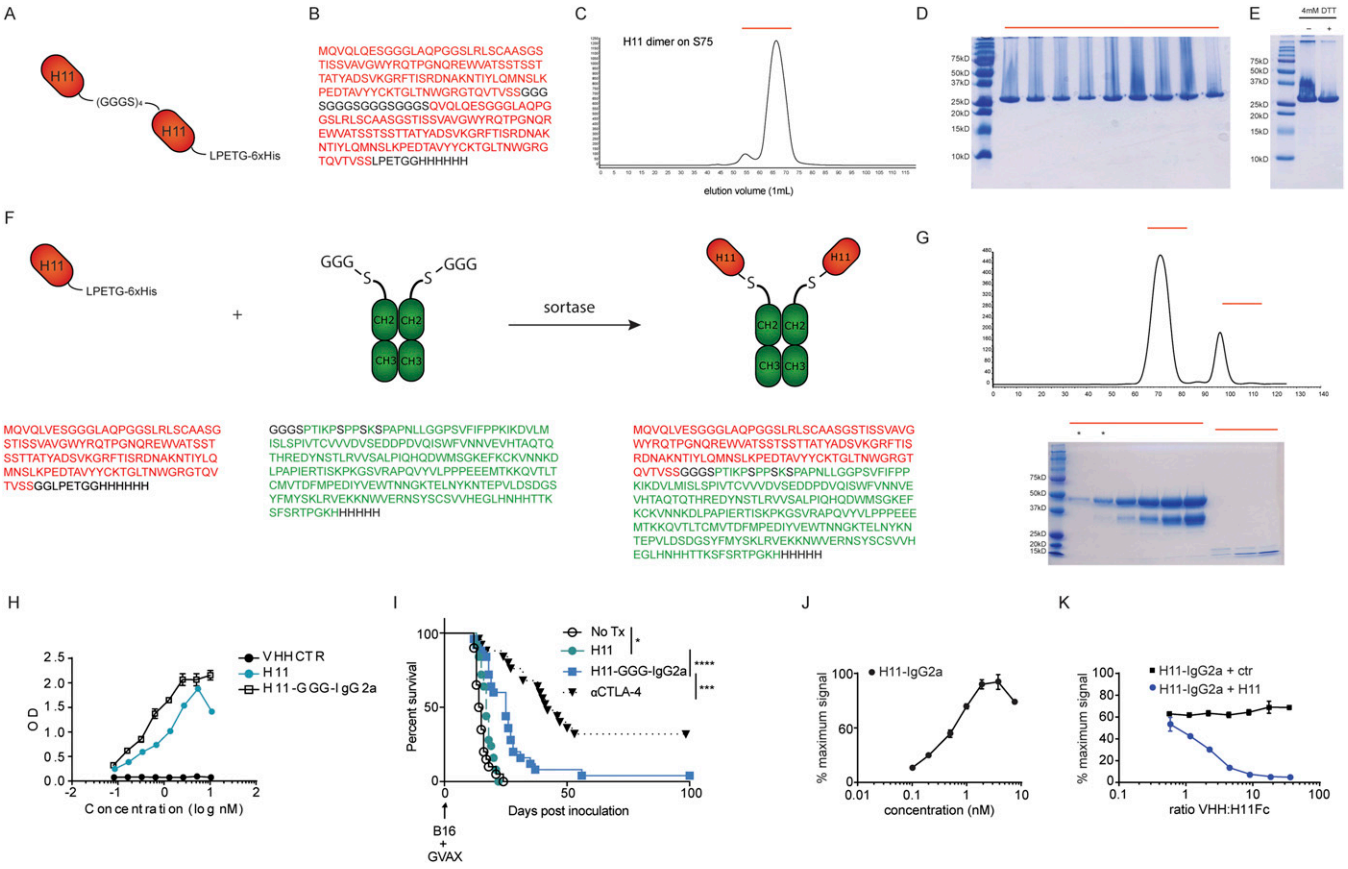

Fig. S2. Characterization of H11 fusion constructs. Schematic depiction (*A*) and amino acid sequence (*B*) of H11 dimer ($H11_2$). (*C*) Size-exclusion chromatography profile of eluted H11 dimer. (*D*) SDS/PAGE of peak fractions of H11 dimer from size-exclusion chromatography. (*E*) Aggregation of H11 dimer at high concentrations was resolved with increased concentration of reducing agent. (*F*) Schematic diagram of the generation of H11–GGG–mIgG2a by using sortase-mediated protein ligation, and amino acid sequences of the substrates and final product. (*G*) Size-exclusion chromatography profile of eluted H11–GGG–mIgG2a. (*F*) SDS/PAGE of peak fractions of H11–GGG–mIgG2a from size-exclusion chromatography. Asterisks indicate fractions used for subsequent studies. (*H*) Biotinylated VHHCTR, H11, or H11–IgG2a was incubated with plate-bound murine CTLA-4 at the indicated concentrations. Binding was detected and analyzed as in Fig. 1*E*. (*I*) Mice were inoculated with 5×10^6 B16 cells s.c. and vaccinated with GVAX on day 0. Mice were treated daily with 100 μg H11, twice weekly with H11–GGG–IgG2a (100 μg) or anti–CTLA-4 (100 μg) clone 9H10, or left untreated. Graph shows a survival curve for mice euthanized when their tumors reached 125 mm^2. (*J* and *K*) Biotinylated H11–IgG2a was incubated with plate-bound CTLA-4 at the indicated concentrations (*H*) or 1 nM H11–IgG2a was incubated with plate-bound CTLA-4 in the presence of increasing concentrations of unlabeled control VHH (ctr) or H11 (*I*). Binding was detected as in Fig. 1*E*; *y* axis depicts the OD at 450 nm normalized to the maximum value in the assay (% maximal binding). $*P < 0.05$, $***P < 0.001$, $****P < 0.0001$.

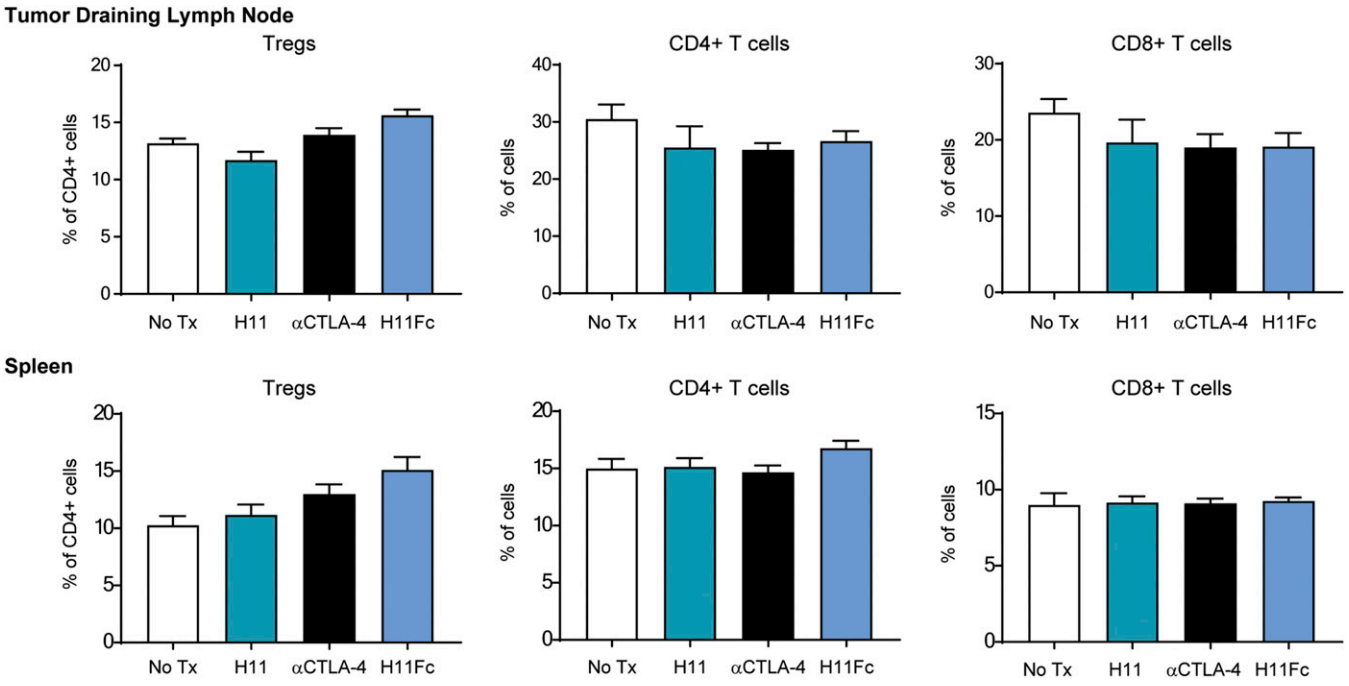

Fig. S3. Treg populations do not change in the spleen or draining lymph nodes of H11-IgG2a–treated mice. Mice were treated and analyzed as in Fig. 4 *D*–*G*. (*Top*) Treg, $CD4^+$, and $CD8^+$ cell percentages from the tumor-draining lymph node harvested from mice treated as indicated. (*Bottom*) Treg, $CD4^+$, and $CD8^+$ cell percentages from the spleens harvested from mice treated as indicated. Tx, treatment.

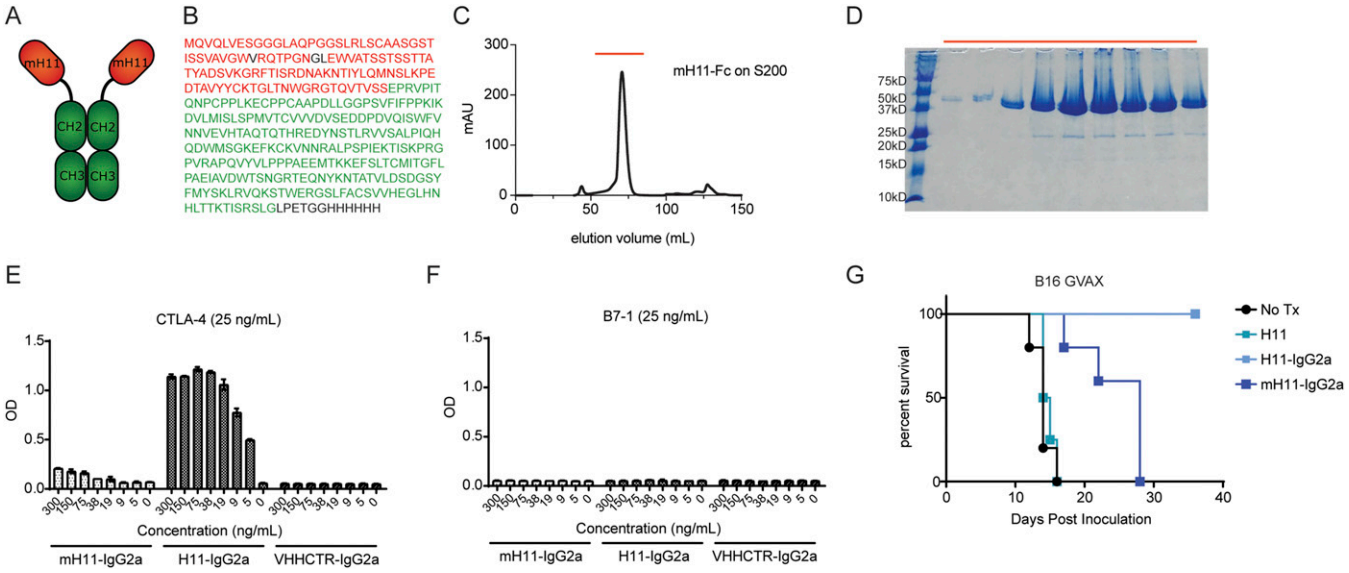

Fig. S4. H11–IgG2a CTLA-4 binding mutant has reduced therapeutic efficacy. Schematic depiction (*A*) and amino acid sequence (*B*) of mH11–IgG2a. (*C*) Size-exclusion chromatography elution profile of mH11–IgG2a. (*D*) SDS/PAGE of peak fractions of mH11–IgG2a from size-exclusion chromatography. (*E*) Biotinylated control VHH Fc fusion (VHHCTR–IgG2a), H11–IgG2a, or CTLA-4–binding mutant mH11–IgG2a were incubated with plate-bound CTLA-4 (*E*) or B7-1 (*F*) at the indicated concentrations. Binding was detected and analyzed as in Fig. 1*E*. (*G*) Mice were inoculated with 5×10^6 B16 cells s.c. and vaccinated with GVAX on day 0. Mice were treated daily with 100 μg H11, twice weekly with H11–IgG2a (100 μg) or mH11–IgG2a (100 μg), or left untreated. Graph shows a survival curve for mice euthanized when their tumors reached 125 mm^2. Tx, treatment.

Table S1. Data collection and refinement statistics of mCTLA:H11 structure

	Murine CTLA-4 native	Murine α-CTLA-4 nanobody	Complex CTLA-4:nanobody
Data collection			
Space group	C2	$P6_122$	$P2_12_12_1$
No. of molecules in asymmetric unit	1	1	8 (4 independent complexes)
Cell dimensions			
a, *b*, *c*, Å	47.54, 36.60, 65.15	42.15, 208.52	78.22, 111.57, 112.88
β,°	105.13		
Resolution, Å	1.50	1.68	2.18
No. of unique reflections	17,192	23,330	98,782
R_{merge}	0.059	0.056	0.089
$I/\sigma I$	12.5	11.6	9.2
Completeness, %	99.1	99.8	99.7
Refinement			
Resolution, Å	25.0–1.50	25.0–1.68	25.0–2.18
R_{cryst}	0.180	0.171	0.193
R_{free}	0.203	0.189	0.247
rmsds			
Bond lengths, Å	0.006	0.007	0.008
Bond angles, °	1.01	1.07	1.09
No. of atoms			
Protein	946	835	6,883
Waters	72	123	102
Ligands	$1Na^{1+}$	$1SO_4^{2-}$	1GOL
PDB entry	5E56	5E03	5E5M