An engineered lipocalin specific for CTLA-4 reveals a combining site with structural and conformational features similar to antibodies

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Biomolecular reagents that enable the specific molecular recognition of proteins play a crucial role in basic research as well as medicine. Up to now, antibodies (immunoglobulins) have been widely used for this purpose. Their predominant feature is the vast repertoire of antigen-binding sites that arise from a set of 6 hypervariable loops. However, antibodies suffer from practical disadvantages because of their complicated architecture, large size, and multiple functions. The lipocalins, on the other hand, have evolved as a protein family that primarily serves for the binding of small molecules. Here, we show that an engineered lipocalin, derived from human Lcn2, can specifically bind the T cell coreceptor CTLA-4 as a prescribed protein target with subnanomolar affinity. Crystallographic analysis reveals that its reshaped cup-like binding site, which is formed by 4 variable loops, provides perfect structural complementarity with this “antigen.” Furthermore, comparison with the crystal structure of the uncomplexed engineered lipocalin indicates a pronounced induced-fit mechanism, a phenomenon so far considered typical for antibodies. By recognizing the same epitope on CTLA-4 that interacts with the counterreceptors B7.1/B7.2 on antigen-presenting cells the engineered Lcn2 exhibits strong, cross-species antagonistic activity, as evidenced by biological effects comparable with a CTLA-4-specific antibody. With its proven stimulatory activity on T cells in vivo, the CTLA-4-blocking lipocalin offers potential for immunotherapy of cancer and infectious disease. Beyond that, lipocalins with engineered antigen-binding sites, so-called Anticalins, provide a class of small (~180 residues), structurally simple, and robust binding proteins with applications in the life sciences in general.

Whereas members of the Ig superfamily, either as soluble antibodies or as membrane receptors, are primarily responsible for the molecular recognition of protein antigens in higher organisms, the lipocalins comprise a class of proteins that exclusively bind small molecules (1). Lipocalins are found in most phyla of life, from bacteria to man, and generally serve for the transport, storage, or sequestration of endogenous or exogenous compounds of low molecular weight. They are secretory molecules—usually compact monomers with 150 to 180 residues—and in vertebrates, they mostly occur in the body fluids, including blood and secretions.

The lipocalins share a common architecture with a strongly conserved 8-stranded antiparallel β-barrel and an α-helix attached to its side (2). At one end, the β-barrel is closed by short loops and densely packed side chains, forming a hydrophobic core. At the other end, 4 loops connect the β-strands in a pairwise fashion and shape the entrance to the ligand pocket. Among the natural lipocalins, these loops are remarkably variable in terms of length, conformation, and amino acid sequence, which reflects the variety of observed binding specificities for vitamins, hormones, and secondary metabolites or even catalytic functions in some cases.

In the human body, ~10 different lipocalins are abundant (3), including the neutrophil elastase-associated lipocalin (NGAL or Lcn2, Lipocalin 2) (4). Lcn2 plays a role in innate immunity by scavenging iron-charged microbial siderophores, in particular FeIII-enterobactin, and thus inhibiting bacterial infection (5). Whereas Lcn2 binds its ligand with high affinity [Kp = 0.4 nM (4)] and specificity, other lipocalins are more promiscuous and exhibit moderate Kp values in the low-micromolar range. A striking example is α1-acid glycoprotein (AGP) (6), which can bind hundreds of different low-molecular-weight compounds, including many pharmacologically active substances, and thus influences their plasma circulation and distribution. However, lipocalins lack mechanisms of somatic variation such as those known for Igs (7). Hence, we set out to investigate whether the binding site of a lipocalin can be reshaped by in vitro mutagenesis and selection to specifically recognize a prescribed protein “antigen” with biomedical relevance.

The cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, CD152) is a critical T cell regulatory molecule (8, 9) that has attracted attention as a target for immunotherapy of cancer (10, 11) as well as HIV (12) and other infectious diseases (13, 14). A few days after antigen-dependent stimulation, CTLA-4 becomes up-regulated and effectively competes with binding of the costimulatory molecule CD28 to CD80 (B7.1) and CD86 (B7.2) on antigen-presenting cells (APCs), thereby attenuating the T cell. Blockade of the physiological interaction between CTLA-4 and these counterreceptors abolishes its negative immunoregulatory function. Accordingly, application of antibodies that bind to human CTLA-4 and prevent its interaction with B7.1 and B7.2 appears effective in cancer treatment (9), especially in combination with tumor vaccination (15). Because an Fc effector region is most likely not required for the functional blockade, engineered lipocalins with antagonistic activity could provide alternative therapeutic agents.


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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 3BX7 and 3BX8).

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Results

We have constructed a mutant genetic library of Lcn2 by randomizing 20-aa positions in the structurally variable loop region (Fig. 1). This lipocalin library with \( \approx 2 \times 10^{10} \) different combinatorial variants [see supporting information (SI) Text] was subjected to several cycles of bacterial phage display selection (16) against human CTLA-4-Fc, a soluble recombinant fusion protein of the extracellular domain of this receptor. Enriched clones were individually expressed in the periplasm of E. coli LE392 as a recombinant (see SI Text).

One lipocalin candidate (PRS-010#001) was found to competitively inhibit the interaction between CTLA-4 and its recombinant ligands B7.1 and B7.2 in ELISA as well as Biacore petitively inhibit the interaction between CTLA-4 and its re-

Fig. 1. Amino acid sequences of CTLA-4-specific engineered lipocalins in comparison with the human wild-type Lcn2 scaffold. Randomized positions are indicated by x, whereas 3 fixed conservative amino acid exchanges (Q28H, L137I, T145A) allowing the introduction of unique cloning sites for the central gene cassette are indicated as lowercase letters. Additional side chains were removed to expose free Cys residue of Lcn2 (C87S) (3) as well as the 3 positively charged side chains that play a role for recognition of the natural ligand Fe\(^{3+}\)-enterobactin (R81A, K125S, K134A). Dots represent amino acids identical with the wild-type Lcn2 sequence (SWISS-PROT entry P800188). The 8 structurally conserved strands A–H of the \( \beta \)-barrel (2) and the 4 loops #1–4 that form the binding site are labeled. C76 and C175 are connected by a disulfide bond. Affinity values (K\(_D\)) measured via kinetic binding analysis of the soluble lipocalin variants by using human CTLA-4-Fc immobilized on a surface plasmon resonance chip are given at the end of each amino acid sequence.

The latter engineered lipocalin has an affinity for the protein sequence of 32 nonmalignant human tissues (according to FDA recommendations for testing of monoclonal antibody products for human use). Notably, the engineered lipocalin shows no cross-reactivity with the structurally related T cell coreceptor CD28 (18) because no binding activity was detectable in corresponding Biacore measurements (Fig. S1) for the entire series of variants obtained in the course of the affinity maturation.

Finally, a PEGylated Lcn2 variant (PRS-010#004a) was demonstrated to have similar in vivo efficacy as a CTLA-4-specific monoclonal antibody in promoting resistance to the intracellular pathogen Leishmania donovani (Fig. 2C), an infection previously shown to be under negative regulatory control by CTLA-4 (19).

The Lcn2 variant PRS-010#003 was successfully crystallized, and its X-ray structure was elucidated both as apo-protein and as complex with the bacterially produced extracellular domain (residues 3–126) of human CTLA-4 (Table S2). In the crystal structure of the complex, 1 molecule of the engineered lipocalin is bound to 1 subunit of the CTLA-4 target. The extracellular receptor domain shows the known Ig V-type fold, whereby its C terminus is connected—via the native disulfide bridge arising from Cys-122—to a symmetry-related subunit (Fig. 3 and Fig. S3). The interface between the 2 CTLA-4 monomers exhibits essentially the same mainly hydrophobic contact area as in previous (lower-resolution) crystallographic analyses of this protein (20, 21).

In the complex, each CTLA-4 monomer rests with its wedge-like tip—formed at one end of the \( \beta \)-sandwich—in the binding site of the engineered lipocalin. The arrangement resembles that of an egg in a cup (Fig. 4), whereby contacts with all 4 reshaped loops at the open end of the \( \beta \)-barrel are involved. Because the crystallized CTLA-4 dimer represents a similar quaternary structure as it is anticipated for the native receptor on the T cell surface (20, 21), this mode of interaction should allow the simultaneous attachment of 2 copies of the engineered lipocalin without steric hindrance.

Similarly to the complex, the apo-structure of PRS-010#003 reveals the characteristic lipocalin fold and is thus closely related to the crystal structure of the parental human Lcn2 (4). Superposition of 58 conserved C\(_\alpha\) positions in the \( \beta \)-barrel (2) with the wild-type lipocalin results in a remarkably small root mean-square deviation of 0.34 Å. In contrast, all 4 loops at the open
end of the β-barrel, which carry most of the substituted side chains in the engineered protein, show significant conformational deviations from Lcn2 (Fig. 4).

In the complex of PRS-010#003 with the extracellular domain of CTLA-4, the 4 loops provide an almost perfectly complementary shape for the target protein, covering its twisted β-sandwich from one side. The “epitope,” comprising the FG loop (also known as CDR3-like segment) with its center around positions 100–104 as well as the BC loop and strand C’ of CTLA-4, points directly into the binding site of the engineered lipocalin (Fig. S4). Remarkably, this epitope coincides with the surface region that is involved in the physiological interaction between CTLA-4 and the V-type domains of B7.1 and B7.2 (20, 21) (Fig. 3 A and B), hence explaining the experimentally observed antagonistic activity of the engineered lipocalin.

Overall, a protein surface of 2,378 Å² becomes buried upon complex formation between PRS-010#003 (1,155 Å²) and CTLA-4 (1,225 Å²). The resulting interface has more than double the size described for the CTLA-4/B7.1 complex (1,255 Å²) (21), which reflects the up to 1,000-fold stronger affinity of the lipocalin variants for CTLA-4 compared with the B7.1 receptor (K_D = 0.4 µM) (22). A large number of protein–protein contacts via hydrogen bonds and hydrophobic as well as aromatic interactions are involved, whereby 10 of the 20 exchanged residues participate in specific contacts with the CTLA-4 target (Fig. 3B and Fig. S5).

The bound FG loop of CTLA-4 contains the 99MYPPPYY105 motif, whose 3 consecutive Pro residues adopt a cis-trans-cis configuration with important implications for B7.1 binding (21). The PPP segment is directly centered at the lipocalin cavity (Fig. S4; for a detailed description, see SI Text), leading to contacts with several core residues in the β-barrel (Fig. 4). Notably, in light of the proven antagonistic activity of the engineered lipocalin, the observed structural mode of interaction with CTLA-4, which involves similar interfaces with the ectodomains of B7.1 and B7.2 as described in previous structural studies (20, 21), lends further support to the assumed signaling mechanism between APCs and T cells.

The extracellular domain of CTLA-4 shows close structural relationship to CD28, the stimulatory coreceptor on T cells (18). In particular, the FG loop with the central sequence motif MYPPPYY is fully conserved. In addition, CD28 and CTLA-4 share the same binding site on their counterreceptors B7.1/B7.2. However, superposition of the CD28 crystal structure on the one of CTLA-4 in complex with the lipocalin gives rise to several steric clashes (Fig. S6), thus explaining the lack of measurable cross-reactivity.

Comparison of the CTLA-4 complex with the apo-structure of PRS-010#003 reveals significant changes in the conformation of its loops and adjacent parts of the β-barrel due to binding of the target protein (Fig. 4 A and B and Fig. S7). Interestingly, loop #3, which is partly disordered in the apo-protein, adopts a well-defined conformation upon complex formation, thus revealing an induced fit of this flexible segment. This structural phenomenon is well-known for antibodies (23). Compared with the apo-lipocalin, the whole upper part of the β-barrel widens in the complex with CTLA-4, because strands G and H together with loop #4 are bent outward. Consequently, there is also an induced fit for loop #4, albeit more subtle than for loop #3. In contrast, loop #1, which carries the largest number (altogether 7) of side-chain substitutions, does not show a significant conformational change upon complex formation, whereas the Cα positions 72–75 of loop #2 move slightly by up to 1.8 Å.
area even larger than the buried surface usually observed in vitro mutagenesis and selection has led here to an altered lipocalins cannot take advantage of such a genetic mechanism, segments, followed by somatic hypermutation (7). Although arises from the recombination of an inherited set of gene loops (CDRs) in the antibody combining site. There, diversity involves all 4 structurally variable loops of the engineered apo-lipocalin; second, a pronounced induced fit for one of the loops (#3, from disordered to ordered) as well as significant conformational rearrangements for 2 others (#2 and #4) upon complex formation with CTLA-4. Notably, this mechanism was not observed for binding of wild-type Lcn2 to its natural ligand FeIII-enterobactin (4).

The mode of interaction with the protein “antigen,” which involves all 4 structurally variable loops of the engineered lipocalin, is comparable with the role of the 6 hypervariable loops (CDRs) in the antibody combining site. There, diversity arises from the recombination of an inherited set of gene segments, followed by somatic hypermutation (7). Although lipocalins cannot take advantage of such a genetic mechanism, in vitro mutagenesis and selection has led here to an altered target specificity, yielding a molecular interface with an area even larger than the buried surface usually observed for antibody/antigen complexes (2,380 versus 1,550 Å² on average) (25).

The surprising resemblance regarding the mode of target binding between the engineered lipocalin and a typical Ig is illustrated by a structural comparison (Fig. 3) with the Fv region of the murine monoclonal antibody 8–18C5 (26). Its Fab fragment was crystallized in complex with the ectodomain of rat myelin oligodendrocyte glycoprotein (MOG), an Ig-type receptor whose extracellular domain has a fold homologous to the one of CTLA-4. In this antibody/antigen complex, the FG loop of MOG is similarly placed at the center of the combining site, in this case surrounded by CDRs H3 and L3 as well as H1. However, because of the generally shorter CDRs of this antibody—especially if compared with the extended loop #2 of the engineered lipocalin—its interface with the antigen is much smaller (1,664 Å²) than the one between PRS-010#003 and CTLA-4.

Discussion
The engineered lipocalin exhibits a mechanism of epitope recognition that strongly resembles the well-known interactions between antibodies and their antigens (24). In this respect, 2 characteristic phenomena are seen: first, high structural plasticity of the binding site, which comes apparent from the significant alterations in loop backbone conformation compared with the wild-type Lcn2 resulting from the amino acid exchanges in the engineered apo-lipocalin; second, a pronounced induced fit for one of the loops (#3, from disordered to ordered) as well as significant conformational rearrangements for 2 others (#2 and #4) upon complex formation with CTLA-4. Notably, this mechanism was not observed for binding of wild-type Lcn2 to its natural ligand FeIII-enterobactin (4).

The finding that the engineered lipocalin and a typical antibody can tightly bind the same kind of structural epitope demonstrates that the extraordinary functional properties of antibody CDRs for antigen recognition are less unique than previously assumed (7). In fact, the question arises why the immune system chose Igs for this task rather than lipocalins; possibly, this may be due to the large diversity of binding sites that can be generated in a rather simple biological mechanism via combinatorial pairing of Ig light and heavy chains.

Interestingly, the 2 CTLA-4 antagonistic antibodies that are currently under clinical investigation (27, 28) seem to recognize a slightly different epitope, because they do not show cross-reactivity between primates and mice. In contrast, PRS-010#003 binds murine CTLA-4 with just 4-fold-lower affinity than the human target, thus facilitating the use of corresponding animal models for in vivo studies as shown here for Leishmania infectivity. This species cross-reactivity of the engineered lipocalin is in line with the close structural relationship of CTLA-4 from both organisms (20, 21, 29) and the known fact that both human and mouse B7 receptors can bind to either human or mouse CTLA-4 (30, 31). Eighty-six percent of the interface between CTLA-4 and B7.1 is also buried in the complex with the engineered lipocalin. Of the altogether 41 aa that differ between the extracellular domains of human and murine CTLA-4 (20), only 4 residues occur in this region.

In conclusion, combinatorial engineering of a lipocalin that naturally binds a small molecule (FeIII-enterobactin, with a total buried surface of just ~800 Å²) has led to a dramatic alteration in the mode of ligand binding and a much extended interface with the prescribed protein target. Notably, this is an otherwise unknown example of a lipocalin from a higher organism that specifically recognizes another protein with high affinity, exhibiting an association constant similar or even better than for a monoclonal antibody (17). Compared with humanized antibodies that are currently under advanced clinical investigation, in particular for cancer therapy (32), such a CTLA-4-specific human lipocalin should provide several advantages with respect to improved tissue penetration, lack of Ig Fc-mediated receptor
interactions, and tunable blood clearance. In general, the class of binding proteins based on the lipocalin scaffold, dubbed anti-lipocalins, offers the benefits of small size and composition of a single polypeptide chain, efficient microbial production, and easy preparation of fusion proteins, thus opening many applications in biological research and medicine.

**Experimental Procedures**

A random library comprising $-2 \times 10^{10}$ mutants of Lcn2 was constructed via PCR assembly using degenerate oligodeoxynucleotides and applied for selection by phage display and panning against the immobilized extracellular domain of human CTLA-4 fused to an IgG1-Fc region. Enriched Lcn2 variants were expressed as soluble proteins in *E. coli* and screened in ELISA for binding to human CTLA-4-Fc in comparison with control proteins. Binding of biotinylated B7.1-Fc to CTLA-4-transfected CHO-K1 cells upon competition with Lcn2 variants was measured in a FACSCalibur instrument using a streptavidin–phycoerythrin conjugate. CTLA-4-attenuated T cell activation was tested in an MLR assay using human PBMCs. In vivo activity of a PEGylated Lcn2 variant was compared with that of an anti-CTLA-4 monoclonal antibody in a mouse model of *Leishmania donovani* infections.

For structural studies, the dimeric extracellular domain of human CTLA-4 and the Lcn2 variant PRS-010#003 were separately produced in *E. coli*. Purified protein preparations were mixed, and the resulting complex was isolated via gel filtration and crystallized in sitting drops with 1.4 M sodium malonate (pH 8.4) at 20 °C. The PRS-010#003 apo-protein was crystallized with 1.45 M ammonium sulfate, 0.1 M sodium cacodylate (pH 5.45), and 0.1% (vol/vol) Anapoe X-405 at 20 °C. Native synchrotron datasets were collected at the Berliner Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung. The crystal structure of the apo-protein was solved by molecular replacement using the coordinates of human wild-type Lcn2. The structure of the lipocalin/CTLA-4 complex was similarly solved by using also the coordinates of the extracellular region of human CTLA-4. Details of all methods are described in SI Text.

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**Fig. 4.** Structural variations in the binding site of the engineered lipocalin as a consequence of mutagenizing its loop region and of an induced fit upon target complexation. Crystal structures of (A) PRS-010#003 in complex with CTLA-4, (B) uncomplexed PRS-010#003 (chain B from the asymmetric unit), and (C) Lcn2 in complex with its natural ligand Fe$^{3+}$-enterobactin (PDB ID code 1L6M (4), chain A; ligand shown in a space-filling representation), together with their mutual superposition in 2 different orientations (D and E). The variable loop regions are colored, whereby the C$_\alpha$ positions of the 20 randomized amino acids are indicated as spheres. The structurally conserved β-barrel (2) is colored in shades of gray, whereas the loops at its closed end and the α-helix attached to its side are shown in light gray for all 3 structures. Loop #3 is partially disordered in the crystal structure of the apo-lipocalin as illustrated by a dotted line. The 3 Pro residues at the center of the CTLA-4 epitope are highlighted as stick representation.
Supporting Information

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SI Text

Bacterial Strains, Plasmids, and Recombinant DNA Methodology.

DNA manipulation and handling of transformed bacteria and phagemids followed standard procedures if not otherwise stated (1–3). E. coli K12 strains XL1-Blue (4), JM83 (5), and W3110 (6) were used for either phagemid production or soluble protein synthesis. The supE strain TG1/F (7) was used in the high-throughput (HT) ELISA screen. All vectors used in this study were based on the generic expression plasmid pASK75 (8). Cloning of the Lcn2/NGL dual DNA was described before (9).

Phage Display Selection of Lcn2 Variants. A random library of Lcn2 with ≈ 2 x 10^10 different combinatorial variants was constructed by PCR assembly with degenerate oligodeoxynucleotides as previously described (10–12). The Lcn2 natural gene used as PCR template was additionally modified by the amino acid exchanges R81A, K125A, and K134A. The library was subjected to phage display selection against a soluble human CTLA-4-Fc fusion protein (Chimerigen) captured on polystyrol plates (Maxisorp; Nunc) with an anti-human IgG-Fc γ fragment-specific antibody (Jackson ImmunoResearch). The wells coated with target were incubated with 8 x 10^12 phagemids from the Lcn2 random library, blocked with 2% wt/vol BSA, followed by 8 washing cycles and elution of adsorbed phagemids with 300 μL of 0.1 M glycine-HCl (pH 2.2) for 10 min and immediate neutralization with an appropriate amount of 0.5 M Tris. Five repeated selection cycles were carried out whereby phagebody amplification between each phanning cycle was performed as described (13).

HT ELISA Screening. Anticalins were selected in a HT ELISA. Therein, Lcn2 variants equipped with an N-terminal T7 detection tag (Novagen) as well as a C-terminal Strep-tag II (IBA) were individually produced in a 96-well microtiter plate using the E. coli strain TG1/F-. Recombinant gene expression was induced overnight with addition of 1.2 μg/mL anhydrotretracycline to the bacterial cultures in 100 μL 2× YT medium containing 100 μg/mL ampicillin as soon as the culture reached an OD550 of 0.6 (shaking with 700 rpm at 22 °C and 60% relative humidity). Afterward, 40 μL of lysis buffer [400 mM sodium borate (pH 8.0), 320 mM NaCl, 4 mM EDTA, 0.3% wt/vol lysozyme] was added to each well, and the plate was further incubated for 1 h under agitation. To minimize nonspecific binding in the subsequent ELISA screen, the crude cell lysates were supplemented with 2% wt/vol BSA and 0.05% vol/vol Tween 20 and tested in ELISA for binding to human CTLA-4-Fc as well as several control proteins. The human CTLA-4-Fc target was captured on wells of black Fluotrac 600 ELISA plates (384 well; Greiner) by means of an immobilized anti-human IgG-Fc γ fragment-specific antibody (Jackson ImmunoResearch). The capture antibody and HSA, each at 5 μg/mL, were used as negative controls. Plates were blocked with PBST/0.05 containing 2% wt/vol BSA and subsequently incubated with 5 μg/mL hCTLA-4-Fc in PBS for 1 h. After incubation with the bacterial cell extract for 1 h, plates were washed 5 times, and bound Lcn2 variants were detected via an anti-T7 monoclonal antibody-HRP conjugate (Amersham), diluted 1:5,000 in PBST/0.05. QuantaBlu (Pierce) was used as fluorogenic HRP substrate. After 45 min of signal development at room temperature, fluorescence was excited at 320 nm (± 12.5 nm) and measured at 430 nm (± 17.5 nm) in a GENiosPlus plate reader (Tecan).

Preparation of Soluble Lcn2 Variants. Lcn2 variants were produced in the shake flask or at the bench top fermenter scale in the E. coli K12 strains JM83 or W3110 by using the expression vector pNGAL15 in the presence of LB-ampicillin medium according to published procedures (9, 13, 14). The Lcn2 variant was purified from the periplasmic fraction via chromatography with Strep-Tactin Superflow (IBA). After that, size exclusion chromatography was carried out with Superdex 75 (GE Life Sciences) employing PBS as a running buffer.

Affinity and Stability Maturation. The affinity and stability of the initial Lcn2 variant for human CTLA-4 was further improved in several cycles of in vitro evolution, by amplifying the central part of the coding region via error-prone PCR (15) by employing the nucleotides analogs 8-oxo-dGTP and dPTP (TEBU-Bio). The PCR product was purified by using the Wizard SV Gel and PCR Clean-Up System (Promega), cut with BstXI (Promega), and repurified to remove incompletely digested DNA fragments by using streptavidin-coated paramagnetic beads (Merck). The product was ligated with the appropriately prepared vector fragment, followed by phagemid library preparation. CTLA-4-specific Lcn2 variants with improved affinities were essentially selected as described above, with the following deviations: Phagemids and target were incubated in solution, and the selection stringency was increased by limiting the target concentration and incubation time to select on faster koff rates. The phagemids were eluted both under acidic and basic conditions, followed by bacterial infection with the combined eluate. The pool of selected CTLA-4-specific Lcn2 variants was ranked for affinity in a HT ELISA as described above. Binding activity was tested for hCTLA-4-Fc as well as the unrelated control proteins B7.1-Fc (R&D Systems), mCD28-Fc (R&D Systems), and hlgG (Jackson ImmunoResearch), respectively. To screen for Lcn2 variants with improved affinity, the bacterial cell extract was incubated with either 0.0 or 0.3 pmol of hCTLA-4-Fc in solution for 1 h at room temperature in a non-protein-binding polypyrrole plate (Greiner). The mixture was then transferred to plates coated with hlgG-Fc-specific antibody to capture the complexes between the Lcn2 variants and hCTLA-4-Fc. Bound Lcn2 variants were detected by using an anti-T7 monoclonal antibody-HRP conjugate. Selection of CTLA-4-specific Lcn2 variants with improved thermal stability was performed as described above except that the target concentration was set to 50 nM and the phagemids were heated to 60 °C or 70 °C, respectively, before incubation. Also, the bacterial cell extract was heated to 60 °C for 1 h before complex formation with the target during the HT ELISA to select for variants with higher thermal stability. To simultaneously screen for improved affinity, 20 nM soluble B7.1-Fc (R&D Systems) was added to the extracts to compete with the Lcn2 variants for binding to hCTLA-4-Fc, which was directly immobilized on the polystyrol plate at a concentration of 5 μg/mL in PBS.

Biacore Affinity Measurements. Real-time affinity analysis was performed at 25 °C using a Biacore T100 system (GE Healthcare). Anti-human IgG-Fc antibody (Jackson ImmunoResearch Laboratories) was immobilized on a CM5 sensor chip by using standard amine-coupling chemistry, resulting in a ligand density of 8,000 resonance units (RU). The reference channel was left blank (activated with EDC/NHS and subsequently blocked with ethanolamine). hCTLA-4-Fc (Chimerigen) or hCD28-Fc (R&D Systems) fusion proteins were captured on this surface to a
laboratory: the dissociation constant was calculated as
was used throughout. Kinetic data evaluation was performed by
of protein remained bound after the regeneration step. To
DNA was prepared by using the Endotoxin-free Maxiprep kit
Blue was transformed with the expression construct, and plasmid
RT-PCR using standard procedures. RNA was prepared from
Expression plasmid pCR-TGCCTCAGCTCTTGG were used for amplification. The PCR
CTLA-4 cDNA was then subcloned via XhoI/HindIII on the
by incubation with streptavidin–phycoerythrin for 30 min at
samples or samples and IFN-γ standard (Invitrogen), diluted in
in an appropriate dilution series in HBS-EP at a flow rate of 30
protein remained bound after the regeneration step. To
regenerated chip surface was

Stable Transfection of CHO Cells with Human CTLA-4 cDNA. The full-length cDNAs coding for human
CHO-K1 cells (400,000) (DSMZ no. ACC 110) were
with a CTLA-4-specific monoclonal antibody (8H5; Ancell). High levels of CTLA-4 cell surface expression were detected
expanded in zeocin-supplemented media for FACS analysis. The unconjugated secondary antibody (mouse anti-

Preparation of a PEGylated CTLA-4-Specific Lcn2 Variant for in Vivo Studies. A version of PRS-010#004a comprising a free cysteine
were lysed by

FACS Competition Assay. Different concentrations of the Lcn2 variant or
as a positive control, of the antibody BN13 (BD Bioscience) were mixed with recombinant biotinylated human
B7.1-Fc (Aancell) at 10 nM final concentration and added to
100,000 of the CTLA-4 transected CHO-K1 cells. Samples were
incubated at 4 °C for 2 h, washed twice in PBS containing 2%
w/vol BSA, and detection of bound hB7.1-Fc was accomplished by
incubation with streptavidin–phyceroerythrin for 30 min at
4 °C. Mean fluorescence intensities were determined by flow
cytometry and fitted to a sigmoidal dose–response model by
using Prism (GraphPad) to determine EC50 values. Wild-type
Lcn2 or isotype control antibody did not lead to measurable
inhibition of B7.1 binding to the CTLA-4 expressing CHO cells.

Mixed Lymphocyte Reaction (MLR) Assay. Human peripheral blood
mononuclear cells (PBMC) were isolated as buffy coat by density
centrifugation on Lymphoprep (Nycomed), followed by
washes, and resuspended in IMDM (BioWhittaker) supplemented
with 5% human serum and 80 μg/mL gentamycin. PBMCs were cultured for 72 h at 1 × 10^6 cells per mL in the

Tissue Cross-Reactivity Studies. The CTLA4-specific Lcn2 variants and the wild-type lipocalin were applied to cryosections of
normal human tissues that had been obtained via autopsy or
surgical biopsy (1 donor per tissue, where available) at 2
concentrations (40 μg/mL and 5 μg/mL). Additionally, each tissue was stained with a murine anti-CTLA-4 monoclonal antibody
(BN13; BD Bioscience) and an isotype-matched negative control
antibody. Tissues were embedded in Tissue-Tek O.C.T. medium,
frozen on dry ice, and stored in sealed plastic bags below –70 °C.
Tissues were sectioned at ~5 μm, fixed in acetone for 10 min.,
and rinsed twice in PBS. Endogenous peroxidase was blocked by
incubating the slides with the peroxidase solution provided in the
EnVision+ Kit (Dako) for 5 min, followed by rinsing twice in
PBS. Next, the slides were blocked for 20 min in PBS containing
0.5% wt/vol casein; 1% wt/vol BSA, and 1.5% normal goat
serum. Then, the Lcn2 variant or the antibody was applied at
room temperature for 1 h, followed by rinsing twice with PBS.
The unconjugated secondary antibody (mouse anti-Strep-tag;
Qiagen) was applied for 30 min. Finally, the slides were rinsed
twice with PBS and treated with the peroxidase-labeled goat
ant-mouse IgG polymer supplied in the EnVision+ kit for 30
min. After again rinsing twice with PBS, the signals were
developed with the DAB+ solution for 8 min. Slides were rinsed
with tap water, counterstained with hematoxylin, blued in
saturated lithium carbonate, dehydrated with alcohol, cleared in
yxene, and coverslipped for interpretation. All slides were read
by a certified pathologist to identify the tissue or cell type stained
and the intensity of staining.
the pellet with the inclusion bodies was resuspended in washing buffer [20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 1.5 M urea], incubated at ambient temperature for 60 min, homogenized again, and centrifuged as before. The pellet was treated 1:20 (wt/vol) with solubilization buffer [20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 6 M Gdn-HCl, 1.5 mM 2-mercaptoethanol] and incubated for 1 h on ice. The denatured protein solution was refolded by quick dilution (at 4 °C) at a ratio 1:20 into refolding buffer [50 mM Tris-HCl (pH 8.2), 20 mM NaCl, 1 mM KCl, 0.5 M arginine, 1 mM GSH, 0.1 mM GSSG]. After 18 h, the solution was concentrated 5-fold by ultrafiltration and dialyzed [20 mM Tris-HCl (pH 7.4), 1 M NaCl]. The refolded Lcn2 variant was purified via chromatography on Butyl-Sepharose (GE Healthcare) by using a step gradient of 1,000–10 mM NaCl for elution.

The protein was further purified by size-exclusion chromatography (Superdex75 16/60; GE Healthcare) in PBS (pH 7.4). The protein quality in each fraction was assessed by SDS/PAGE, and those fractions with the highest content of monomer were pooled and concentrated to ~1 mg/mL. The free cysteine residue was activated by addition of 50 μM TCEP (Tris(2-carboxyethyl)phosphine; Sigma) under stirring for 3 h at ambient temperature. Then, activated solid linear 30k PEG (Sunbright ME-300MA; NOF) was added at a 2:1 (mole/mole) ratio and the solution was stirred for 2 h. After adding solid NaCl up to a conductivity of 140 mS/cm the protein was bound to Butyl-Sepharose and eluted by using a NaCl step gradient. The PEGylated lipocalin was finally purified by size-exclusion chromatography as above.

Investigation of Immunostimulatory Activity in a Leishmania Infection Model. BALB/c mice were obtained from Charles River, housed under specific pathogen-free conditions and used at 6–10 weeks of age. All experiments were approved by the University of York Animal Procedures and Ethics Committee and performed under U.K. Home Office license. L. donovani (LV9) was maintained by passage in RAG-2−/− mice. Mice were infected by injecting 2−3×107 amastigotes i.v. via the lateral tail vein. Groups of infected mice were then (i) untreated, (ii) treated daily from day 1 to day 14 with the PEGylated Lcn2 variant (PRS-010#004a; 30 mg/kg i.p. each injection) or control (wild-type Lcn2 as fusion partner dimerization (17), was amplified from a human lymphocyte cDNA preparation via PCR using phosphothioate primers 5′-GGAGTTACCATATGCAATGGCCAGCCATCTG3′ and 5′-CAGTACGATTTAATAGCCAGTCTGATGTTT3′ (18). The amplification product was digested with NdeI and HindIII, purified by agarose gel electrophoresis, and inserted into the appropriately cut expression vector pSA1 (19). The paired extracellular domain of dimeric human CTLA-4 was produced as inclusion bodies in E. coli BL21 (DE3) harboring the resulting expression vector as well as pLysE (20). Cultures (2 L) were grown in LB medium supplemented with 100 mg/L ampicillin and 30 mg/L chloramphenicol at 37 °C. Gene expression was induced at a cell density OD600 = 0.6 by adding 0.5 mM IPTG and shaking was continued for 16 h overnight. The cells were harvested by centrifugation (4,225 × g, 15 min, 4 °C), resuspended in 20 mL of ice-cold 50 mM Tris-HCl (pH 7.5), 25 mM NaCl, 1 mM DTT, and stored in the freezer. After thawing, they were passed 3 times through a French pressure cell (AMINCO) at 16,000 psi. The homogenate was centrifuged (26,900 × g, 20 min, 4 °C) to sediment the inclusion bodies. After 3 cycles of resuspending in the buffer mentioned above and centrifugation, the inclusion bodies were solubilized in 6 M Gdn-HCl, 50 mM Tris-HCl (pH 8.5), 6 mM DTT by stirring overnight at 4 °C. After removal of the undissolved residue by centrifugation (26,900 × g, 15 min, 4 °C) oxidative refolding of the denatured protein solution was achieved by rapid dilution (1:10) into 0.6 M Gdn-HCl, 50 mM Tris-HCl (pH 8.5), 0.3 mM DTT, followed by stirring for 3 days at 4 °C (21). The protein solution was dialyzed twice against 20 mM Tris-HCl (pH 7.5), and precipitated protein was removed by centrifugation (26,900 × g, 15 min, 4 °C). After filtration through a 0.45-μm membrane (Schleicher & Schuell), the solution was applied to a 1-mL Resource Q anion exchange column (Amersham Pharma
cia) equilibrated with 20 mM Tris-HCl (pH 7.0). Bound protein was eluted with 20 column volumes in a linear gradient containing finally 50% of 20 mM Tris-HCl (pH 8.0), 1 M NaCl. One major peak appeared at a concentration of ~300 mM NaCl and corresponded to the covalently linked homodimeric CTLA-4 domain, which was verified via reducing and nonreducing SDS/ PAGE. This species was finally purified by gel filtration using a Superdex 75 16/60 column (Amersham Pharmacia) equilibrated with PBS.

Preparation of Lipocalin/Target Complex and Crystallization. The engineered lipocalin PRS-010#003 was produced by periplasmic secretion in the E. coli K12 strain W3110 harboring the expression vector pNGAL62 via fermentation in a 10-L bioreactor (Biostat B; B. Braun) at 25 °C based on a published protocol (14). The protein was purified from the periplasmic cell fraction via hydrophobic interaction chromatography (Butyl-Sepharose; Amersham Pharmacia), followed by anion exchange chromatography (Q-Sepharose; Amersham Pharmacia) and size exclusion chromatography (Superdex 75 HiLoad 16/60 prep grade; Amersham Pharmacia). Finally, the purified protein was dialyzed against 10 mM Tris-HCl (pH 7.5), 100 mM NaCl. After mixing the engineered lipocalin and the recombinant dimeric CTLA-4 domain (dCTLA4) in 2:1 molar ratio, the solution was kept rolling at 4 °C for 1 h, then concentrated to ~20 mg/mL by ultrafiltration (10,000 MWCO; 15 mL; Millipore), and directly applied to a Superdex 200 16/60 chromatography column (Amersham Pharmacia) equilibrated with 10 mM Tris-HCl (pH 7.5), 100 mM NaCl. Elution fractions corresponding to the protein complex were collected and analyzed via reducing and nonreducing SDS/PAGE. The fractions were pooled and concentrated to 9 mg/mL by ultrafiltration. Dynamic light scattering analysis (Zetasizer Nano-S; Malvern Instruments) revealed a monodisperse solution with apparent molecular size for globular proteins of 62.2 kDa (Mwcalc for the dimeric complex: 66.9 kDa). Crystals of the engineered lipocalin alone were grown at 20 °C using the same technique by mixing each 1 μL of the protein solution (10 mg/mL) and of the precipitant solution containing 1.4 M ammonium sulfate, 0.1 M sodium cacodylate (pH 5.45),
0.1% (vol/vol) Anapoe X-405 (Hampton Research), followed by 1-day equilibration against 0.4 M of the same solution. All crystals were harvested directly from the drop by using Nylon loops (Hampton Research), cryoprotected by transfer through the precipitant solution supplemented with 25% (vol/vol) glycerol, and frozen in a 100 K nitrogen stream (Oxford Cryosystems).

Data Collection, Model Building, and Structural Refinement. Native synchrotron X-ray diffraction data sets for the apo-protein and the target complex (Table S2) were collected at BESSY (Berlin, Germany) beamline 14.1. Data were processed by using HKL2000 (22), and the space group was determined as P1 for the apo-protein, with 8 molecules in the asymmetric unit, and P321 for the complex, with 1 lipocalin/CTLA-4 monomer in the asymmetric unit. Structure determination of the apo-protein was accomplished by molecular replacement with Phaser (23) using the X-ray structure of human wild-type Lcn2 (PDB ID code: 1DFV, chain B). Model building was performed with O (24) and Coot (25). Energy-restrained crystallographic refinement was carried out with maximum-likelihood algorithms implemented in CNS (26), using published parameters (27). The initial model was subjected to a rigid body and positional refinement. Bulk solvent, overall anisotropic B factor corrections, and noncrystallographic restraints were introduced depending on the behavior of the R_free index. Refinement proceeded in several cycles, which were interrupted for manual rebuilding, always aiming at the maximum-likelihood target (see Table S2). The structure of the protein complex was similarly solved by molecular replacement using 1 molecule of the refined crystal structure of the apo-lipocalin and 1 molecule of the X-ray structure of the extracellular domain of human CTLA-4 (PDB-ID code: 1I85, chain C). Model building and refinement was carried out in a similar fashion as above (see Table S2). All graphical representations were prepared by using PyMOL (28). Surfaces were calculated with AREAIMOL (29) by using a probe radius of 1.4 Å. For hydrophobic surface representation, residues were colored according to increasing hydrophobicity from green (hydrophilic) over white (neutral, including the polypeptide backbone) to brown using a groupwise parameter set (30). The coordinate error estimate was calculated with SFCHK (29). The structural models for the apo-protein and the complex have been deposited at the RCSB Protein Data Bank under accession codes 3BX8 and 3BX7, respectively.

Detailed Description and Comparison of Molecular Interactions in the Crystal Structures. The bound FG loop of CTLA-4, which contains the 90MYP PPP Y105 motif, is directly centered at the lipocalin cavity, leading to contacts with several core residues in the β-barrel. In particular, Y106 (Lcn) forms a hydrogen bond to the backbone oxygen of P102 (CTLA-4), and the phenyl ring of F123 (Lcn) interacts with the cyclic side chain of P103 (CTLA-4). The substitutions K125L and K134A in the lipocalin provides the necessary space and lead to hydrophobic interactions with P101 and P103. The 2 flanking residues Y100 and Y104 (CTLA-4) are fixed by hydrogen bonding to N96 (Lcn) and by a hydrophobic contact with I70 (Lcn), respectively. Y52 (Lcn) forms a hydrogen bond to the backbone nitrogen of Y104 (CTLA-4). The adjacent epitope residue Y105 (CTLA-4) is connected to the backbone nitrogen of L42 on the other side of the long loop #1 in the engineered lipocalin. There, the new side chain D44 stabilizes a backbone oxygen of P28 and the side-chain hydroxyl group of T32 (CTLA-4). Both contacts seem to cause a conformational change in the BC loop of CTLA-4 compared with its complex with B7.1.

Looking at the interaction of secondary structure elements in the protein–protein complex, the twisted β-sheet (CC’FG) of CTLA-4 is complemented by a similar twist of the β-strands C and D in the β-barrel of the engineered lipocalin, which facilitates specific contacts by the mutated region of loop #2. There, the new residue E77 leads to a double hydrogen bond and salt bridge with R35 (CTLA-4), and K75 (Lcn) forms another salt bridge with E48 (CTLA-4). Residue K74 (Lcn) and the substituted side chain H73 (Lcn) both form hydrogen bonds with the backbone oxygen of V46 (CTLA-4), whereas the new residue S72 (Lcn) contacts E97 (CTLA-4) via a water molecule.

Analysis of the molecular surface properties of the engineered lipocalin indicates a rather hydrophilic interface with several nonpolar side chains at the bottom of the cavity, resulting in pairwise hydrophobic contacts between Y100 (CTLA-4) and Y101 (Lcn), P101 (CTLA-4) and L125 (Lcn), P103 (CTLA-4) and A134/F123 (Lcn), Y105 (CTLA-4) and L56/141 (Lcn), and Y104 (CTLA-4) and Y52/170 (Lcn).

In the crystal structure of the uncomplexed lipocalin variant PRS-010#103 significant changes in the conformation of the loops and adjacent parts of the β-barrel are seen in comparison with the CTLA-4 complex. For example, in the case of loop #4 1 strand of the β-hairpin is bent into the direction of the β-barrel axis and the other away from it, thus resulting in a twist of the entire loop. In particular, there is a large shift (by >8 Å concerning the most remote atom positions) for the mutated Phe residue (Y132F), which now directly points into the lipocalin cavity and makes a contact to a solvent molecule. This shift is partly caused by steric repulsion with the exchanged bulky Arg side chain in loop #1 (A40R), which now fills the space that was occupied by Tyr-132 in the wild-type lipocalin.

Furthermore, in the case of loop #3, no electron density was observed for residues 97–103 at its tip, indicating that this flexible segment is disordered in the engineered apo-lipocalin. The same loop attains a well-defined conformation upon complex formation, thus revealing an induced fit of this flexible segment. Loop #3 interacts with the BC loop of CTLA-4 via hydrophobic contact between its residue Y100 and A31 (CTLA-4) and with the FG loop via a hydrogen bond between N96 (Lcn) and Y100 (CTLA-4). In addition, β-strand F of the lipocalin, on the C-terminal side of loop #3, is fixed via a contact between Y106 and the epitope residue P102 (CTLA-4).

In the upper part of its cavity, additional electron density was observed for the apo-lipocalin, which perfectly matches the polyethylene glycol moiety of the detergent Anapoe X-405 that was present during protein crystallization. This finding is in line with the first published crystal structure for human Lcn2, wherein a bound fatty acid was detected (31). In fact, some of the interacting hydrophobic residues are similar (Y52, T34, Y56, F123, T136, and Y138). In contrast, the cognate ligand enterobactin is bound to Lcn2 mainly via the positively charged side chain R81, K125, and K134, leading to electrostatic and π interactions (32).

The structure of the binding pocket is significantly altered by exchange of Trp-79 with the small Thr side chain, which explains the observed loss of binding ability for enterobactin.

Compared with the apo-lipocalin, the whole upper part of the β-barrel opens in the complex with CTLA-4, as strands G and H together with loop #4 are bent outward. This movement seems to be in concert with the displacement observed for the BC-loop in the bound CTLA-4. The flexibility of β-strand H
and the flip of the substituted side chain F132 of the engineered lipocalin in the complex back to the position that Y132 assumes in the wild-type Lcn2 structure (see above) provide space to accommodate the BC loop of the target protein. The reconstitution of the original side chain conformation for the aromatic residue at position 132 is facilitated by the fact that the spatially neighboring residue R40 in loop #1 forms 2 hydrogen bonds to the BC-loop of the bound CTLA-4 and adopts a new conformation.

Fig. S1. Sensorgrams for the Biacore affinity analysis of the Lcn2 variants. A fusion protein of the extracellular receptor domain with an Ig Fc region was captured onto a sensorchip (~300 RU), and the purified soluble Lcn2 variant was applied at varying concentrations. Parameters deduced from these measurements are listed in Table S1. (A) PRS-010#001 (at 3,000, 1,500, 750, 375, 187.5, and 93.75 nM) versus human CTLA-4. (B) PRS-010#002 (at 3,000, 1,500, 750, 375, 187.5, and 93.75 nM) versus human CTLA-4. (C) PRS-010#003 (at 3,000, 1,500, 750, 375, 187.5, and 93.75 nM) versus human CTLA-4. (D) PRS-010#004 (at 600, 300, 150, 75, 37.5, and 18.75 nM) versus human CTLA-4. (E) PRS-010#005 (at 100, 50, 25, 12.5, and 6.25 nM) versus human CTLA-4. (F) PRS-010#003 (at concentrations as in C) versus human and murine CTLA-4 in comparison with human CD28.
C) Lcn2 variant #003 versus human CTLA-4-Fc

D) Lcn2 variant #004 versus human CTLA-4-Fc

Fig. S1. (continued).
E) Lcn2 variant #005 versus human CTLA-4-Fc

![Graph showing response over time for E) Lcn2 variant #005 versus human CTLA-4-Fc](image)

F) Lcn2 variant #003 versus human and murine CTLA-4-Fc as well as human CD28-Fc

![Graph showing response over time for F) Lcn2 variant #003 versus human and murine CTLA-4-Fc as well as human CD28-Fc](image)

Fig. S1. (continued).
Fig. S2. Histochemical analysis of acetone-fixed cryo-sections of normal human tissues (tonsil and spleen) with the engineered CTLA-4 specific lipocalin PRS-010#003 or, as negative control, wild-type Lcn2. Bound lipocalin was detected via an antibody against the Strep-tag II, followed by a peroxidase-labeled goat anti-mouse IgG polymer and hematoxylin counter-staining. Note membrane staining of mononuclear cells present in tonsils and spleen with the engineered lipocalin (with a pattern comparable to the hCTLA-4 specific monoclonal antibody BN13) whereas no specific staining was observed for the control. There were no signs of cross-reactivity of PRS-010#003 (or BN13) with CTLA-4 negative cells or any other human tissue component.
**Fig. S3.** The disulfide bridge at the beginning of the stalk region of the CTLA-4 membrane receptor that links the 2 subunits in the homodimer of the recombinant ectodomain. 2F_o-F_c electron density contoured at 1.0 σ is shown around Cys-122 and the preceding Pro residue. The disulfide bond runs across a 2-fold crystallographic symmetry axis that links the 2 identical monomers from neighboring asymmetric units.
Specific recognition of the FG-loop of CTLA-4 with the YPPPY sequence motif. View is into the binding site of the engineered lipocalin (pink) in complex with the extracellular domain of human CTLA-4 (gray) together with 2Fo-Fc electron density contoured at 1.0 o. The central sequence motif at the tip of the FG-loop of CTLA-4 contains 2 Pro residues in cis configuration, which are clearly defined in the electron density. This loop is deeply buried in the cavity of the engineered lipocalin and involved in several nonpolar contacts with aromatic side chains (see Fig. S5). The resulting structural blockade of the CTLA-4 epitope, which is otherwise engaged in the contact with the natural receptor B7.1, results in the strong antagonistic activity of the engineered lipocalin.

Fig. S4.
Fig. S5. Hydrophobic surface representation of the engineered lipocalin (Bottom) and the ectodomain of human CTLA-4 (Top) crystallized as complex. The separated molecules are shown with view onto the epitope of the CTLA-4 ectodomain (FG loop) and into the cavity of the lipocalin, respectively, whereby amino acid contact positions are labeled. A 180° rotation of one of the partners around a horizontal axis leads to the protein complex. The contact surface indicates a predominantly hydrophilic interface with several specific hydrophobic contacts at its center. In particular, there are pairwise contacts between Y100 (CTLA-4) and Y100 (Lcn), P101 (CTLA-4) and L125 (Lcn), P103 (CTLA-4) and A134/F123 (Lcn), Y105 (CTLA-4) and L36/I41 (Lcn), and Y104 (CTLA-4) and Y52/I70 (Lcn). Surfaces are colored according to the calculatory octanol/water partition coefficient logP of amino acid building blocks as a measure of their hydrophobicity (30, 33). Coloring is approximately as follows: green, −3.0, highly polar or charged; brown, 1.0, hydrophobic; white, neutral and polypeptide backbone.
Fig. S6. Superposition of the CD28 crystal structure (PDB ID code 1YJD) with the one of the CTLA-4/lipocalin complex. Superposition was performed by using 82 homologous Cα positions (CD28: 3–13, 17–25, 31–39, 46–52, 68–106, 110–116; CTLA-4: 3–13, 18–26, 32–40, 48–54, 68–106, 109–115) according to a previously described structure-based alignment (34), resulting in an r.m.s.d. of 1.053 Å. The engineered lipocalin is shown with the loop region colored red, whereas CTLA-4 is depicted in light blue with a translucent surface. CD28 is colored green with the 3 N-linked glycans (at Asn residues 19, 53, and 87) shown in yellow. Side chains that stick out of the volume confined by CTLA-4 and which would lead to clashes with the engineered lipocalin are labeled. The 2 views represent orientations rotated by 150°. From this analysis, formation of a stable complex between the engineered lipocalin and CD28 seems highly unlikely because residues N1, K2, L4, Y26, R31, E108, K109 would sterically interfere with loop #1 to a considerable extent. Furthermore, Y54 would lead to a clash with loop #3, whereas the glycosylation at N53, which is specific for CD28, might be compatible.
Fig. S7. Superposition of wild-type Lcn2 (PDB ID code 1L6M) with the uncomplexed engineered lipocalin. The latter contains an artificial ligand from the crystallization solution, Anapoe X-405, bound to the central cavity, whose polyethylene glycol moiety can be traced in the electron density. Native Lcn2 is depicted in light blue, whereas the engineered lipocalin is colored green. Specific contact residues of the latter with the artificial ligand are highlighted as sticks colored olive. The side-chain conformation of aromatic residue 132 (light blue) observed for the wild-type protein is strongly changed after substitution to Phe in the engineered apo-lipocalin (see dashed line) but almost restored in its complex with CTLA-4 (see Results).
Table S1. Affinity parameters for the interaction between Lcn2 variants and human CTLA-4 from the Biacore analysis

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<th>Lcn2 variant</th>
<th>$k_{on}$ M$^{-1}$ s$^{-1}$</th>
<th>$k_{off}$ s$^{-1}$</th>
<th>$K_D$ M$^{-1}$</th>
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<td>$5.21 \times 10^{03} \pm 5.4$</td>
<td>$4.16 \times 10^{-04} \pm 3.6 \times 10^{-07}$</td>
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<td>PRS-010#002</td>
<td>$9.24 \times 10^{03} \pm 1.4$</td>
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<td>$2.38 \times 10^{-08} \pm 2.09 \times 10^{-11}$</td>
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<td>$9.00 \times 10^{-09} \pm 2.32 \times 10^{-11}$</td>
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<tr>
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<td>$9.68 \times 10^{05} \pm 760$</td>
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<td>$2.41 \times 10^{-10} \pm 3.28 \times 10^{-13}$</td>
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<tr>
<td>PRS-010#003 vs. murine CTLA-4</td>
<td>$1.40 \times 10^{04} \pm 9.0$</td>
<td>$5.78 \times 10^{-04} \pm 3.6 \times 10^{-07}$</td>
<td>$4.14 \times 10^{-08} \pm 3.89 \times 10^{-11}$</td>
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### Table S2. Crystallographic data collection and refinement statistics

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<td>(a = 61.482, b = 92.861, c = 98.825, \alpha = 110.8^\circ, \beta = 90^\circ, \gamma = 109.4^\circ)</td>
<td>(a = b = 120.706, c = 82.957, \alpha = \beta = 90^\circ, \gamma = 120^\circ)</td>
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<tr>
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<tr>
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Values of the highest resolution shell are given in parentheses.