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**Supplementary Material**

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Affinity and Cross-Reactivity Engineering of CTLA4-Ig To Modulate T Cell Costimulation

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CTLA4-Ig is an Fc fusion protein containing the extracellular domain of CTLA-4, a receptor known to deliver a negative signal to T cells. CTLA4-Ig modulates T cell costimulatory signals by blocking the CD80 and CD86 ligands from binding to CD28, which delivers a positive T cell costimulatory signal. To engineer CTLA4-Ig variants with altered binding affinity to CD80 and CD86, we employed a high-throughput protein engineering method to map the ligand binding surface of CTLA-4. The resulting mutagenesis map identified positions critical for the recognition of each ligand on the three CDR-like loops of CTLA-4, consistent with the published site-directed mutagenesis and x-ray crystal structures of the CTLA-4/CD80 and CTLA-4/CD86 complexes. A number of single amino acid substitutions were identified that equally affected the binding affinity of CTLA4-Ig for both ligands as well as those that differentially affected binding. All of the high-affinity variants showed improved off-rates, with the best one being a 17.5-fold improved off-rate over parental CTLA4-Ig binding to CD86. Allostimulation of human CD4+ T cells showed that improvement of CD80 and CD86 binding activity augmented inhibition of naive and primed T cell activation. In general, increased affinity for CD86 resulted in more potent inhibition of T cell response than did increased affinity for CD80. Optimization of the affinity balance to CD80 and CD86 to particular disease settings may lead to development of a CTLA4-Ig molecule with improved efficacy and safety profiles. The Journal of Immunology, 2012, 189: 4470–4477.

Cytoxic T lymphocyte-associated Ag-4 is a negative regulator that plays a critical role in terminating T cell activation in vivo (1). CTLA4-Ig, an Fc fusion protein containing the extracellular domain of CTLA-4, modulates T cell costimulatory signals by blocking two B7 molecules, CD80 and CD86, from binding to CD28, which delivers a positive T cell costimulatory signal (2). The Fc fusion protein of extracellular domain of CTLA-4 (CTLA4-Ig) is a potent inhibitor of immune responses in vitro and in vivo and is an approved agent for treatment of rheumatoid arthritis (abatacept) (3). CTLA4-Ig has also shown potential in other autoimmune diseases, such as systemic lupus erythematosus; however, it is not effective when used in transplant models of nonhuman primates where more complete block of the CD28 costimulatory signal is required (4, 5).

Recently, a second-generation CTLA4-Ig (belatacept) with improved avidity to CD80 and CD86 has been approved for the treatment of transplant rejection (6). Belatacept monotherapy showed superiority in maintaining renal allograft survival over abatacept monotherapy, demonstrating that the improved binding affinity translated into augmented immunosuppressive activity. However, belatacept required cotreatment with other immunosuppressants such as basiliximab, mycophenolate mofetil, or corticosteroids for long-term allograft survival (7), indicating that the potency in promoting tolerance is not strong enough to work as a single agent or that blocking pathways other than CD28 is necessary.

We developed a high-throughput mutation analysis method called point-by-point (PxP) in which next generation sequencing is used to determine the effect on affinity of every possible point mutation in a protein binding domain. Briefly, a DNA library comprising all possible single amino acid substitutions in the binding domain is constructed and expressed on the surface of mammalian cells via a transmembrane anchor. The library is sorted by flow cytometry based on the level of ligand bound, and episomal plasmid from the sorted subpopulations is recovered and sequenced using massively parallel pyrosequencing. The frequency of each mutant in each subpopulation is tabulated, allowing analysis of how the frequency of each mutant varies between the different subpopulations, giving an affinity ranking of the entire library. The method not only identifies variants with improved activity, but also neutral affinity mutations can be used to fix undesired characteristics of protein therapeutics, such as immunogenicity, heterogeneity, and stability. A similar method to map the protein sequence–function relationship using a display technology (e.g., phage display) with next generation sequencing (e.g., Illumina) has been described (8).

In this study, we employed the PxP method to map the ligand binding surface and engineered the activity of a non-Ab therapeutic protein, CTLA4-Ig. The CTLA4-Ig library containing 594 point mutations was generated and expressed on the surface of mammalian cells. Each mutation was ranked according to its affinity to CD80 or CD86. By combining these two data sets we have created a dual, integrated functional map of how CTLA-4 binds to both partners. By using the map, we identified a number of variants with improved binding to one or both ligands. The high-affinity variants showed improved activity in inhibiting T cell activation. Our study demonstrated the utility of the PxP analysis to create a map of the receptor–ligand interaction and the application of this method to engineer next generation biologics with improved function.
Materials and Methods

DNA, proteins, and cell lines

Oligonucleotide primers were synthesized by Eurofins MWG Operon (Huntsville, AL) and Integrated DNA Technologies (Coralville, IA). Library gene synthesis was performed by Sloning BioTechnology (Puchheim, Germany, now Morphosys, Planegg, Germany). 293c18, 293T/17, CHO-K1, and Jurkat cells were from the American Type Culture Collection (Rockville, MD). Fusion proteins consisting of the extracellular domain of human CD80 or CD86 (aa 1–244 and 238, respectively) fused to human Cα constant domain containing a Cy,576 to Ser mutation for expression as a monomer were transiently expressed in 293T/17 cells. The production levels of the fusion proteins were determined by ELISA using goat anti-human λ chain Ab (BioSource International, Camarillo, CA) for capture and HRP-conjugated goat anti-human-κ chain Ab (SouthernBiotech, Birmingham, AL) for detection. The cdn.tentant transfected from transient transfection was used directly for FACS and AlphaLISA and the binding was detected by secondary reagents. Stable transfectants of CHO-K1-expressing CD80 or CD86 were generated as follows. Expression vectors containing genes encoding full-length human CD80 and CD86 were transfected into CHO-K1 by Lipofectamine 2000 (Invitrogen) and selected for stable transfection in growth media (45% DMEM, 45% F12K, 10% FBS) containing 0.5 mg/ml G418. The 1% of single colonies with the highest expression of CD80 or CD86 on the cell surface was sorted by FACS and cultured to establish stable cell lines. The expression levels between the two cell lines were confirmed to be roughly equivalent by FACS.

Mammalian display vector pYA205 to express Fc fusion protein

Mammalian cell surface display of the CTLA4-Ig library was performed using pYA205, a derivative of plasmid pYA104, an EBV-derived plasmid for display of full-length IgGl/k Abs on the surface of 293c18 cells via a GPI anchor. pYA104 was modified by 1) incorporating new flanking restriction sites for V region cloning (NotI for 5’ end of VL; ScaI for 3’ end of VH) to replace the entire L chain-IRE5-VH sequences with the gene encoding the CTLA-4 extracellular domain (aa 1–124), 2) replacing the human IgGl C region with an Fc region containing Cys to Ser mutations at positions 130, 136, and 139 as described in abatcept, and 3) replacing the GPI anchor with a transmembrane domain derived from human platelet-derived growth factor receptor.

Construction of synthetic genes and libraries

A synthetic DNA fragment encoding CTLA-4 was obtained by gene synthesis and subcloned into pYA205 to create a control surface expression plasmid, pYA205-CTLA4-Ig. Similarly, 33 degenerate DNA fragment pools containing mutations were cloned into pYA205-CTLA4-Ig to create 33 positional sublibraries. To ensure oversampling of each variant, ligations and Escherichia coli transformations were performed to ensure a minimum of 30-fold over-coverage for each of the 36 codons at each position with vector alone background <0.5%. Positional sublibraries were pooled in equimolar ratios before transfection.

Transfection of P XP libraries and cell sorting

The CTLA4-Ig library was transfected into 293c18 cells and maintained as described (9). To sort the library, ~2 × 10^5 cells were incubated with 8 mCD80-Cα or 5 nM CD86-Cα in PBS containing 0.5% BSA (FACS buffer) for 1 h on ice. Cells were washed and then incubated with a 1:100 dilution of goat anti-human λ chain-allophycocyanin (BioLegend) and a 1:100 dilution of goat anti-human IgG-FITC (Jackson ImmunoResearch Laboratories) for 30 min at 4˚C, washed three times with cold FACS buffer, and resuspended in PBS with 20 mM HEPES and 20 mM EDTA. Stained cells were sorted on a MoFlo MLD (DakoCytonation). Dead cells and debris were excluded by forward scatter and side scatter. At least 10^5 live cells were sorted from each gate and were cultured for several days before extracting plasmid DNA from 1 to 3 × 10^6 cells per population.

DNA recovery and pyrosequencing

Plasmid DNA was recovered from the sorted cells and treated with DpnI as described (9). Using the recovered plasmid as templates, amplicons for the CTLA-4 library were generated using PCR primers specific to CTLA-4 gene containing “A” and “B” adaptor sequences. Each amplicon was prepared in two versions, with the A and B adaptors in both orientations on the amplicons to allow sequencing from either end. Amplicons were pooled in equimolar amounts and pyrosequencing was performed on a PyroMark instrument using a modified sequencing protocol with the GS Titanium LV emPCR kit and GS Titanium Sequencing kit XL7R0 (454 Life Sciences/ Roche, Branford, CT).

454 Data analysis

Data analysis was restricted to the CDR-like regions, which were the only regions where sequence variation was designed into the libraries. Regions of CDR-like sequences were identified from each read based on exact matches to 9 bp of wild-type flanking sequences. Sequences containing framshift mutations, nonsense mutations, or more than one mutation were eliminated from further analysis. After clean up of the data, each of the 1188 variant codons was read an average of 155 or 172 times each for CD80 and CD86, respectively (sum of H, M and L gates), with a reasonable distribution between the various codons and positions. Amino acids variants with <10 total occurrences were eliminated from analysis, resulting in the loss of 3 of 594 (0.5%) and 23 of 594 (3.8%) of the amino acid variants from the final data set for CD80 and CD86, respectively. Each variant was ranked for affinity on an H-L score, a +100 to −100 scale calculated as percentage frequency found in the high-affinity gate minus percentage frequency found in the low-affinity gate. Data for synonymous codons were averaged to generate a single score for each point mutation.

Expression and purification of CTLA4-Ig variants

The pYA205-CTLA4-Ig plasmid was converted to express the protein in secreted form as described (9). The resulting plasmid was transiently transfected into 293c18 and incubated in DMEM with 2% low IgG FBS (Hyclone). The supernatant was harvested on day 6, purified using protein G chromatography, and dialyzed against PBS. Final quantitation was performed by measuring OD280 and purity was evaluated by SDS-PAGE.

Competition AlphaLISA

Affinity of CTLA4-Ig variants to ligand was measured by an amplified luminescent proximity homogeneous assay. Abatcept (Bristol-Myers Squibb, Princeton, NJ) was biotinylated with sulfo-NHS-biotin using standard methods and dialogized against PBS. AlphaLISA acceptor beads were conjugated with goat anti-human Ca Ab by following the manufacturer’s instructions (“AlphaLISA assay development guide,” PerkinElmer, Waltham, MA). The binding assay was performed in a 384-well AlphaPlate (PerkinElmer) in assay buffer (1% BSA, 0.01% Tween 20 in PBS [pH 7.1]). For CD80 binding assays, 5 µl 1:4 serially diluted unlabeled CTLA4-Ig variant protein was added to each well followed by the addition of 5 µl 2.5 nM biotinylated abatcept. Then, a mixture of 5 µl 0.39 µg/ml CD80-Cα and 5 µl 25 µg/ml goat anti-human Ca-conjugated acceptor beads was added. Each data point of the dilution curve was duplicated in the assay plate. The plate was incubated in the dark for 1 h at room temperature. AlphaScreen streptavidin donor beads (PerkinElmer) were subsequently added to each well at 5 µg/ml to be 25 µl total reaction volume. The plate was incubated in the dark at room temperature for an additional 30 min. As a result, the fluorescence emission per each component was measured. The competitive variant protein to start with, 0.5 nM biotinylated abatcept, 0.078 µg/ml CD80-Cα, and 5 µg/ml each type of beads. For CD86 binding assays, the final concentration of each component per sample was 100 nM competitor variant protein to start with, 8 nM biotinylated abatcept, 0.1 µg/ml CD86-Cα, and 10 µg/ml each type of beads. Fluorescence of the plates was read on an EnVision multilabel reader (PerkinElmer). Data were fitted using nonlinear regression with the software GraphPad Prism (GraphPad Software, San Diego, CA) and reported as IC50 wild-type/IC50 mutant.

Surface plasmon resonance analysis

Affinity measurements were carried out on BIAcore model 2000 or T100 (Biacore/GE Healthcare, Piscataway, NJ) at 25˚C using HBS-EP+ with 0.1 mg/ml BSA as a running buffer. A CMS sensor chip was amine-coupled in all four flow cells at ~4000 resonance units with anti-6×His Ab (R&D Systems) to capture CD80 or CD86-Fc containing a 6×His tag (R&D Systems). CTLA4-Ig was injected in a 3-fold dilution series from 60 to 0.74 nM, and dissociation was monitored for 10 and 4 min for CD80 and CD86, respectively. Kinetic analysis was done by simultaneously fitting the association and dissociation phases of the sensorgram using the 1:1 System model (BIAcore). CTLA4-Ig was injected in a 3-fold dilution series from 60 to 0.74 nM, and dissociation was monitored for 10 and 4 min for CD80 and CD86, respectively. Kinetic analysis was done by simultaneously fitting the association and dissociation phases of the sensorgram using the 1:1 Langmuir binding model in BIAevaluation software (Biacore) as supplied by the manufacturer. Double referencing was applied in each analysis to eliminate background responses from the reference surface and buffer only control.

Inhibition of PHA-induced IL-2 production assay

Jurkat cells (3.0 × 10^3/well) and CHO-K1 that stably expressed full-length CD80 or CD86 (1.0 × 10^3/well) were incubated in RPMI 1640 (HyClone) supplemented with 10% FBS in the presence of 10 µg/ml PHA-P (Sigma-Aldrich, St. Louis, MO) with titrating amounts of CTLA4-Ig proteins. Control mouse Abs against CD80 and CD86 were purchased from BD
To set up the assays, varying concentrations of CTLA4-Ig or variants di-

The libraries were cloned into an EBV-derived plasmid for mam-

acid variant at 33 positions was represented by two codons (except 

frequency of each amino acid owing to redundancy in the genetic 

thesis. Instead of NNK/NNS degeneracy that results in varied 

lap but differ from CDR-analogous regions predicted previously 

targeted for PxP analysis are underlined, and the 

extracellular domain. The three CDR-like loops 

FIGURE 1. Amino acid sequence of CTLA4 

Biosciences (San Diego, CA). After 24 h, supernatants were assayed using an AnalphalISA human IL-2 kit (PerkinElmer) to measure the amount of 

secreted IL-2. In a typical 24-h assay without blocking agent, 2.1–3.5 ng/ml 

IL-2 was produced in the culture supernatant of Jurkat cells.

Inhibition of primary and secondary allostimulation assays 

SU-DHL-6, a human diffuse large B cell lymphoma cell line (CRL-2959; 

American Type Culture Collection) expressing both CD80 and CD86 on the cell surface, was maintained in RPMI 1640 containing 10% FBS. PBMCs were isolated from human whole blood or buffy coat by Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation following the manu-

facturer’s instructions of Leucosep (Greiner Bio-One, Frickenhausen, Germany). Human peripheral CD4+ T cells were then isolated from PBMCs using the EasySep negative selection human CD4+ T cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada). Resulting CD4+ T cells with purity >90% were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS, 50 μM 2-ME, and 100 U/ml penicillin-streptomycin. To set up the assays, varying concentrations of CTLA4-Ig or variants di-

luted in 50 μl media were first added to each well of round-bottom 96-well microtiter plates in triplicate. CD4+ T cells (5 × 10^5) were then mixed with freshly irradiated (10,000 rad) SU-DHL-6 cells (1 × 10^5) in a 5:1 responder/stimulator ratio in 150 μl culture media and then added to the wells containing CTLA4-Ig or variants, resulting in a final volume of 200 μl per well. The primary allogenic stimulation assays proceeded for 5 d before addition of 0.5 μCi/well [3H]thymidine for the final 18 h culture. Cells were harvested with a cell harvester (FilterMate OmniFilter 96-harvest; Perkin-

Elmer) using the manufacturer’s recommended conditions, and [3H]thymidine incorporation was measured using a scintillation counter (Wallac TriLux). For secondary allogenic stimulation assays, 6-d primary allostimulated T cells were harvested, isolated by Ficoll-Paque Plus density gradient centrifugation, and rested for 24 h. T cells were then restimulated with freshly irradiated B lymphoma cells at the same ratio as above in the presence of abatacept and its mutands. The secondary stimulation assays proceeded for 2 d before addition of 0.5 μCi/well [3H]thymidine for the final 18 h culture before harvesting as above. Cell proliferation data were analyzed with GraphPad Prism 5 software using a nonlinear regression curve fit method.

Results Construction, expression, and fluorescence-activated cell sorting of CTLA4-Ig library

CTLA-4 is a member of the Ig superfamily, comprising a single Ig-fold extracellular domain with three CDR-like loops initially defined by structural prediction (10). To identify regions that might impact ligand binding, we examined the published x-ray crystal structures of the CTLA-4/CD80 and CTLA-4/CD86 complexes (11, 12). We identified three regions, 26PGKATEVR33, 51TYMMGNETLFDLD63, and 99KELMYPYPPPYL1034 as CDR1-like, CDR2-like, and CDR3-like loops, respectively, to introduce amino acid substitutions. The CDR-like loops we randomized over-

malian cell surface display and transinfected into 293e18 cells as described (9). The optimum staining conditions for cell sorting were determined to be 8 and 5 nM of CD80-Ca or CD86-Ca, respectively, using control cells displaying parental CTLA4-Ig. The library was first incubated with CD80-Ca or CD86-Ca followed by alloglycosaminyl-labeled anti-human λ chain Ab for detection. To normalize surface expression levels, the library was also stained using FITC-labeled anti-human IgG Ab. Cells expressing parental CTLA4-Ig represent a diagonal line in a two-color staining for ligand binding and Fc expression (Fig. 2A, 2C). However, the staining pattern of the library was substantially broadened, indicating the presence of variants with both higher and lower affinities (Fig. 2B, 2D). Cells were collected from three gates drawn to enrich for variants with higher, lower, and neutral or medium affinity using the parental FACS pattern as a guide.

Pyrosequencing and data analysis

Cells collected from the FACS gates were expanded for several days and plasmids were recovered. PCR amplicons generated from plasmids recovered from each gate were pooled and pyrosequenced on a single run of a 454 GS FLX instrument. Each variant was ranked on a %H-L score, as described in Materials and Methods. Higher affinity variants should be enriched in the high gate and occur with less frequency in the low gate, whereas lower affinity variants should be enriched in the low gate and depleted in the high gate. Thus, a variant found exclusively in the high-affinity gate would be scored +100 and a variant that was found exclusively in the lower affinity gate would receive a score of ~100.

%H-L scores for each variant are presented as heat maps for the interactions with CD80 and CD86 (Fig. 3A, 3B). Warm colors represent negative scores for mutations with reduced binding. Green represents scores near 0, indicating neutral binding. Cool colors represent positive scores indicating improved binding. Average scores of wild-type synonymous codons were −11.4 and −4.6 for CD80 and CD86, respectively, within the green range. The high-

affinity mutations employed in belatacept (LEA29Y) (7), A29Y and L104E, scored high for both CD80 and CD86 in the blue–purple range (for A29Y, +50.5 and +63.4; for L104E, +99.5 and +76.8, respectively).

The average scores for each position were mapped onto the crystal structures of the CTLA-4/CD80 and CD86 complexes (11, 12), giving an overall depiction of tolerability to mutation in the CTLA4-ligand complexes (Fig. 3C, 3E). The map identified key interactions of CTLA-4 with each ligand consistent with results from interactions of the x-ray structures and mutational analysis, such as the MYPPPY motif as well as E31 and Y103, which are known to be critical for interaction with the ligands (10–12). The tolerability map of CD80 binding generated by PxP analysis (Fig. 3C) agrees with the map created from alanine scanning mutagenesis for this motif (Fig. 3D, data derived from Ref. 10) but with greater resolution by averaging the effects from 18 substitutions per position. In analogy to the analysis of an Ab binding surface, the CDR3-

like loop located at the center of the binding surface has sig-

nificant interactions with the ligand; thus, these residues had lower tolerance for substitution, resulting in negative average scores. In contrast, noncontact, solvent-exposed residues on the periphery

1 1 1 1 1 1
2 0 0 0 0 0
3 0 0 0 0 0
4 0 0 0 0 0
5 0 0 0 0 0
6 0 0 0 0 0
7 0 0 0 0 0
8 SGGNQVNLTIQGGLRAMDTGLYICKVELMYPPPYLYLGIGNGTQIYVDDPECPDSD
9 0 0 0 0 0
10 0 0 0 0 0
11 0 0 0 0 0
12 MHWVAQPAVVLASSRQIASFSVCEYASPGKATEVRVTVRQADSOQVTECAATYMMGNETLFDLDQISICTGT

FIGURE 1. Amino acid sequence of CTLA4 extracellular domain. The three CDR-like loops targeted for PxP analysis are underlined, and the CDR-analogous regions originally defined by Peach et al. (11) are in boldface.
were much more tolerant to substitution, resulting in positive average scores. A generally lower tolerance to mutations for CD80 over CD86 suggests that affinity to CD80 is more optimized as it is, whereas affinity to CD86 has more room for improvement.

**Binding characterization of CTLA4-Ig variants**

We defined the neutral range of %H-L score as the mean ± 2 SD of that observed for the wild-type synonymous codons. Thus, variants that scored greater than the neutral range are predicted to have higher affinity and those with lower scores than the neutral range are predicted to have lower affinity. For binding to CD80 and CD86, the neutral range was defined as −35.1 to +12.8 and −53.4 to +44.2, respectively. Variants with scores greater than this range are predicted to have higher affinity, which will be the top ∼36 or ∼42 variants from the highest scores for CD80 and CD86, respectively (i.e., the top 7 or 6% of total variants). Because some variants appeared as high-affinity candidates for both ligands, we selected a total of 37 variants for characterization in soluble form, based on data quality (i.e., a large number of reads plus good synonymous codon agreement), as well as to span a diverse set of positions. The 37 variants were purified and confirmed by competition AlphaLISA (Fig. 4). There was no difference in binding activity between wild-type CTLA4-Ig and commercial abacetus (Fig. 4B, first and last set of bars). All but three variants (V32D, V32P, and T51P) showed at least 1.5-fold improvement to either ligand in this assay, indicating that 92% of expressed variants predicted to be high binders actually showed improved binding. V32P and T51P, with average scores of +44.4 and +67.6 to CD86, respectively, expressed poorly. It is possible that there may have been folding issues due to structural alteration associated with the proline residue. Affinity increases >11-fold (K28H and K93Q) and >14-fold (A29H) for CD80 and CD86, respectively, were observed. Examples of variants preferentially affecting binding to either one (e.g., L96K) or both ligands (e.g., K28H) were found. The %H-L scores of each codon for these 37 variants and an additional set of 10 neutral and low-affinity binders showed good correlation with affinity measured by AlphaLISA competition, demonstrating considerable accuracy in predicting affinities (Supplemental Fig. 1).

Eight variants were selected to evaluate binding kinetics to each ligand using surface plasmon resonance (SPR). Under the conditions employed, the binding $K_D$ values for parental CTLA4-Ig were determined to be 0.29 and 3.6 nM for CD80 and CD86, respectively. The improvement in $K_D$ of each variant normalized by parental value (fold $K_D$, Table I) was roughly consistent with the results obtained from competition AlphaLISA. Affinity improvements (fold $K_D$) of up to 6.1-fold (K93Q) for CD80 and 4.4-fold (A29H) for CD86 were observed by SPR. Two variants (T51N and L96K) that showed reduced binding against CD80 in AlphaLISA showed affinity loss in SPR as well. The affinity improvements were mostly due to reduced off-rate, with the best one showing 17.5-fold improvement in off-rate over parental CTLA4-Ig (A29H for CD86).

**Biological activity of CTLA4-Ig variants**

To evaluate whether affinity increases translated to improved biological potency, we first employed an IL-2 production assay using Jurkat T cells as a responder and CHO-K1 expressing either CD80 or CD86 as a stimulator cell. CTLA4-Ig can block PHA-induced IL-2 production by Jurkat cells in the presence of co-stimulatory signals provided by a cell line expressing either CD80 or CD86 (13) (Supplemental Fig. 2A). Inhibition curves of wild-type CTLA4-Ig and commercial abacetus closely overlapped, indicating equivalent potency in inhibition of IL-2 production from Jurkat cells (Supplemental Fig. 2B). The average IC$_{50}$ values of parental CTLA4-Ig were determined to be 1.2 and 17.2 nM for CD80 and CD86, respectively. The double mutation employed in belatacept (A29Y + L104E) showed improvements of IC$_{50}$ values in CD80- and CD86-dependent T cell blocking activity of 2.0- and 11.5-fold, respectively. Using this assay, the potencies of CTLA4-Ig variants in inhibition of CD80- or CD86-mediated T cell activation were compared (Table I, Supplemental Fig. 2C). A general trend was observed where higher affinity to ligand resulted in improved potency in inhibition of T cell activation, although the trend was stronger for increased affinity to CD86 than to CD80. For instance, K28H and K93Q with 5- to 6-fold improvement in $K_D$ against CD80 showed only ∼2-fold improvement in potency. Alternatively, A29H with 4.4-fold improvement in $K_D$ to CD86 resulted in as much as 6.2-fold improvement in potency (Table I). A similar trend was observed throughout the variants tested, indicating that improved affinity to CD86 had a greater impact on bioactivity than improved affinity to CD80.

We next evaluated the potency of CTLA4-Ig variants in inhibiting alloantigen-stimulated T cell proliferation. Purified CD4$^+$ T cells were stimulated with an allogenic B cell line, SU-DHL-6, positive for both CD80 and CD86 (data not shown). Inhibition curves of wild-type CTLA4-Ig and commercial abacetus closely overlapped, indicating equivalent potency in inhibition of proliferative response in CD4$^+$ T cells. All variants with improved binding to CD80 and/or CD86 showed greater inhibition of proliferative responses of CD4$^+$ T cells in both primary and secondary responses. Improvements in IC$_{50}$ were observed in a range of 1.5- to 20-fold and 1.5- to 10-fold for primary and secondary responses, respectively (Fig. 5).

Overall, affinity improvement to CD80 and/or CD86 resulted in inhibition of primary proliferative responses of CD4$^+$ T cells greater than secondary responses, reflecting the reduced dependency of memory T cells on the CD28 pathway for secondary responses (14). Among the variants tested, the A29H mutation resulted in the maximum potency for inhibition of both primary and secondary proliferative responses, ∼20- and 10-fold, respectively. K28H was
second most potent with ~12- and 8-fold improvement in inhibiting primary and secondary responses, respectively. Interestingly, the L96K and T51N mutations with reduced affinity for CD80 resulted in loss of potency to inhibit primary or secondary responses, indicating that loss of binding toward CD80 up to 10-fold seems to be well compensated by improved binding to CD86. In conclusion, higher affinity to either ligand resulted in improved potency in inhibition of proliferative response in alloantigenic stimulation, consistent with the results from a 24-h assay using Jurkat and CHO-K1 cell lines expressing CD80 or CD86 (Table I, Supplemental Fig. 2C).

Discussion
We used PxP, a high-throughput mutation analysis method to efficiently and comprehensively determine the effect on affinity of every possible point mutation in the binding surface of CTLA-4 for two ligands, CD80 and CD86. The method has been successfully used to functionally map and engineer the affinity of mAbs (C. Forsyth, V. Juan, Y. Akamatsu, R. DuBridge, M. Doan, A. Ivanov, Z. Ma, D. Polakoff, J. Razo, K. Wilson, and D. Powers, submitted for publication). Unlike Abs, where Ag binding occurs through the well-known CDR loops of the H and L chains, we used additional information from the crystal structures of CTLA-4 in complex with CD80 and CD86 (11, 12) to define and target equivalent CDR-like regions of CTLA-4 for inclusion in the mutant library. The crystal structures show that direct interactions of CTLA-4 with either ligand occur primarily through a conserved hexapeptide motif (97MYPPPY102) within the CDR3-like region, whereas direct contacts to the CDR1- and CDR2-like regions are limited to only a few positions. The structure of the CDR1-like region was not completely resolved in the CTLA4/CD86 complex (lacking 25SPGK28) owing to the flexible nature of this loop (11). Interestingly, we identified several high-affinity mutations to CD86 in the CDR1-like loop (K28H, K28T, A29H, A29T, A29Y, A29W, and T30G), suggesting that these mutations may improve overall affinity by stabilizing the local structure of this loop. The CDR2-like loop contributes relatively little to ligand recognition, with the exception of T51, which is in the binding interface with CD86. From the 13 aa mutated in the CDR2-like region, we discovered mutations not only at T51 but also at M53, M54, L58, and L61, which favorably impact binding to one or both ligands. Within the 12-aa
CDR3-like loop, we found the $^{97}$MYPPPY$^{102}$ motif to be strikingly intolerant to mutations, especially for interaction with CD80, whereas beneficial mutations that alter the affinity balance for CD80 and CD86 clustered adjacent to this conserved motif (K93, L96, and L104).

A general trend was observed where increased affinity for CD86 resulted in more potent inhibition of T cell response than increased affinity for CD80. For example, the two high-affinity mutations employed in belatacept resulted in 25-fold improved binding to CD80 (by AlphaLISA; 8.1-fold by BiAcore), but this

Table I. Summary of characterization of CTLA4-Ig variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>$k_a$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
<th>$K_D$ (M)</th>
<th>$k_a$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
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Equilibrium binding constants ($K_D$) were calculated by SPR of wild-type CTLA4-Ig variants and CD80 or CD86 were measured as described in Materials and Methods. $K_D$ values of variants measured by SPR were normalized against that of CTLA4-Ig (wild-type) to represent the improvement in $K_D$ (Fold $K_D$). Similarly, improvement in binding measured by competition AlphaLISA (Fig. 4) and inhibition in IL-2 production (Supplemental Fig. 2C) were normalized against IC$_{50}$ of control and are shown as “Fold Comp.” and “Fold IL-2,” respectively. A29Y + L104E is the mutation employed in belatacept (7).
translated to only 2.0-fold improvement in potency in a CD80-mediated T cell activation assay. Alternatively, the 31-fold improvement in affinity for CD86 for these mutants (by AlphaLISA; 7.1-fold by BLACore) translated to as much as an 11.5-fold improvement in potency in inhibiting CD86-mediated T cell activation (Table I). It is known that CTLA4-Ig is ~100-fold less potent at inhibiting CD86-mediated immune responses when compared with CD80-mediated responses (15). This difference can be explained by the faster dissociation rate of CD86 over CD80 (7) (Table I) and by the difference in avidity between CD80 and CD86. Avidity is the combined strength of multiple interactions whereas the affinity measured by SPR is the strength of a single interaction. It is known that the avidity of the molecule is especially important for biological activity. CD80 is known to form dimers, creating the possibility of an ordered latticework of molecules at the site of T cell/APC contact with CTLA-4 dimers (16). On the contrary, analysis of CD86 in the crystal structure and in solution indicated that CD86 is unlikely to form stable dimers (17, 18). The binding affinity of native CTLA4-Ig to CD80 may already be sufficient to induce clusters on the membrane through avidity, and the additional impact of potency solely by improving affinity may be small. The contribution of avidity to CD80-dependent costimulation may also explain the results for the L96K mutation, which shows significant reduction in CD80 affinity (10-fold less) whereas the loss in potency was only 2.4-fold in the Jurkat assay (Table I, Supplemental Fig. 2C). The loss of potency seen for L96K in inhibiting CD80-mediated activation can easily be compensated for by its improvement in CD86 binding under conditions where costimulation from CD86 is available (Fig. 5). Whereas both CD80 and CD86 can contribute to primary and secondary T cell responses, it is possible they contribute at different levels under conditions where differential levels of ligand expression are present and other costimulatory/adhesion molecules are involved.

Blockade of CD80 or CD86 can have divergent effects in disease outcome depending on the stage of disease or the tissues involved (19). CTLA4-Ig variants with biased specificity toward CD80 over CD86 may be preferable for treatment of multiple sclerosis, as CD80 is preferentially expressed in the CNS (20, 21). CTLA4-Ig variants with higher affinity for both CD80 and CD86 may be more effective treatments for transplant rejection where blockade of both CD80 and CD86 is important (22, 23). CTLA4-Ig variants with biased specificity toward CD86 over CD80 may have an advantage in treatment of diabetes and other autoimmune disease where CD86 is the predominant CD28 costimulator (24, 25). Native CTLA-4 has an affinity bias toward CD80 over CD86. When the function of endogenous CTLA-4 is critical for immune homeostasis, reducing affinity toward CD80 may improve safety. For example, blockade of CD80 has been shown to exacerbate disease progression in a model of type 1 diabetes (26). The mechanism of exacerbation is uncertain, but it may be due to the inhibitory function of CTLA-4 on activated T cells or an effect on regulatory T cells. The expression of CTLA-4 on regulatory T cells is thought to be critical for their activity (27). CTLA4-Ig treatment can result in a reduction of the number of regulatory T cells and in some cases exacerbation of autoimmunity (28). In addition to the ability of CTLA4-Ig to inhibit costimulatory signals through CD28, it may trigger APC production of IDO, an intracellular enzyme that breaks down tryptophan and suppresses T cells through tryptophan starvation (29). The CTLA4-Ig variants described in this study would be useful tools to evaluate the effect of affinity alteration on reverse signaling through CD80 and CD86.

In conclusion, we identified point mutations that differentially alter the affinity of CTLA4-Ig for CD80 and CD86 from the detailed maps of binding surface generated by PxP technology. The data can serve as the basis for the design of next generation CTLA4-Ig molecules with improved efficacy and safety profiles in selected disease indications.
Acknowledgments
We thank Fiona Harding and Gary Starling for helpful discussions and advice regarding T cell functional assays, Susan Rhodes and Rick Powers for molecular biology and protein chemistry support, Peter Lambert for statistical analysis and advice, Steve Hartman for assistance with the AlphaLisa assay, Melanie Wong for help with BIAcore analysis, Charles Forsyth for assistance with graphic figures, and Don Halbert, Stan Falkow, and Paul Hinton for critical reading and comments on the manuscript.

Disclosures

References
**Supplementary Table I.** Codons used in CTLA4-Ig library.

<table>
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Two codons were used to encode each amino acid except Met and Trp. Substitution to Cys was excluded from the library. A total of 36 codons were incorporated per position.
Supplementary FIGURE 1. Correlation of %H-L scores with binding affinity measured by competition AlphaLISA. Total of 47 amino acid substitutions including those predicted to be low and neutral in affinity were measured by competition AlphaLISA. Reduction in IC$_{50}$ value is shown in y-axis as fold improvement in relative binding to CD80 (left) or CD86 (right). %H-L scores from total of 126 codons including 38 WT synonymous codons (solid circles) and 88 codons encoding mutants (open circles) are shown in x-axis.
Supplementary FIGURE 2. The ability of CTLA4-Ig variants to inhibit IL-2 production by Jurkat T cells upon stimulation. IL-2 production from Jurkat upon stimulation with PHA and CHO-K1 expressing CD80 or CD86 in the presence or absence of different agents was compared. CTLA4-Ig (CTLA-4, 20nM or 500nM) or blocking antibodies to CD80 or CD86 (anti-80/86, 5nM) were able to substantially inhibit the production of IL-2. No IL-2 is produced from Jurkat in the absence of PHA or CHO-K1 negative in CD80 or CD86 expression. Similarly, no detectable IL-2 was produced from culture supernatant of CHO-K1 without Jurkat (A). Full inhibition curves with titrating amount of abatacept (Bristol Myers Squibb), wild-type CTLA4-Ig, or CTLA4-Ig with A29Y L104E double mutation. The double mutation resulted in improvement in IC$_{50}$ for 2-fold and 11.5-fold, respectively (B). Relative potencies of selected novel CTLA4-Ig variants in inhibition of IL-2 production normalized with an IC$_{50}$ value obtained from wild-type CTLA4-Ig are shown. Error bars represent range of two independent sets of experiments performed in duplication (C).