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

Zhenghai Xu, Veronica Juan, Alexander Ivanov, Zhiyuan Ma, Dixie Polakoff, David B. Powers, Robert B. DuBridge, Keith Wilson and Yoshiko Akamatsu

*J Immunol* 2012; 189:4470-4477; Prepublished online 26 September 2012; doi: 10.4049/jimmunol.1201813
http://www.jimmunol.org/content/189/9/4470

**Supplementary Material**  
http://www.jimmunol.org/content/suppl/2012/09/26/jimmunol.1201813.DC1

**References**  
This article cites 29 articles, 8 of which you can access for free at:  
http://www.jimmunol.org/content/189/9/4470.full#ref-list-1

**Subscription**  
Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

**Permissions**  
Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  
Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts
Affinity and Cross-Reactivity Engineering of CTLA4-Ig To Modulate T Cell Costimulation

Zhenghai Xu, Veronica Juan, Alexander Ivanov, Zhiyuan Ma, Dixie Polakoff, David B. Powers, Robert B. DuBridge, Keith Wilson, and Yoshiko Akamatsu

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.

Copyright 2012 by The American Association of Immunologists, Inc. 0022-1767/12 $16.00

Abbott Biotherapeutics Corp., Redwood City, CA 94063; and Abbott Bioscience Center, Worcester, MA 01605

1Current address: AvidBiotics, South San Francisco, CA.
2Current address: Full Spectrum Genetics, Inc., South San Francisco, CA.

Received for publication June 29, 2012. Accepted for publication August 29, 2012.

Address correspondence and reprints request to Dr. Yoshiko Akamatsu, Abbott Biotherapeutics Corp., 1500 Seaport Boulevard, Redwood City, CA 94063. E-mail address: yoshiko.akamatsu@abbott.com

The online version of this article contains supplemental material.

Abbreviations used in this article: PxP, point-by-point; SPR, surface plasmon resonance.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12 $16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1201813
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 Slonking BioTechnology (Puchheim, Germany, now Morphosys, Planegg, Germany). 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 1–238, respectively) fused to human Cα constant domain containing a Cy5NH2 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 λ-chain Ab (SouthernBiotech, Birmingham, AL) for detection. The capture antibody was transiently transfected on an EnVision multilabel reader (PerkinElmer) at 25˚C using HBS-EP+ with α-CD80 or α-CD86 Ab (Biacore/GE Healthcare, Piscataway, NJ) to capture CD80 or CD86-Fc containing a 6xHis tag (R&D Systems) to capture CD80 or CD86-Fc containing a 6xHis tag (R&D Systems). CTLA4-Ig library was transfected into 293c18 cells and maintained as described (9). To sort the library, ∼1.188 variant codons were 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 %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 quantification 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. Abatacept (Bristol-Myers Squibb, Princeton, NJ) was biotinylated with sulfo-NHS-biotin using standard methods and dialedyzed 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 abatacept. 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 μl/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 signal per each well is proportional to the concentration of competitor variant protein to start with, 0.5 nM biotinylated abatacept, 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 abatacept, 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 core 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-6xHis Ab (R&D Systems) to capture CD80 or CD86-Fc containing a 6xHis 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 Langmuir binding model in BIACalculator 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 × 10^5 well) and CHO-K1 that stably expressed full-length CD80 or CD86 (1.0 × 10^5 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 Biosciences.
Biosciences (San Diego, CA). After 24 h, supernatants were assayed using an AlphaLISA 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 manufacturer’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 diluted in 50 µl media were first added to each well of round-bottom 96-well microtiter plates in triplicate. CD4+ T cells (5 x 10^5) were then mixed with freshly irradiated (10,000 rad) SU-DHL-6 cells (1 x 10^6) 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 harvester; PerkinElmer) 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 mutants. 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, 51TYMMGNETLF63, and 93KELMYPYPPYL104 as CDR1-like, CDR2-like, and CDR3-like, respectively, to introduce amino acid substitutions. The CDR-like loops we randomized overlap but differ from CDR-analogous regions predicted previously based on homology to V domain (10) (Fig. 1).

A CTLA4-Ig library comprising all possible single amino acid substitutions in the CDR-like loops was prepared by DNA synthesis. Instead of NNK/NNS degeneracy that results in varied frequency of each amino acid owing to redundancy in the genetic code, the library was generated using defined codons to allow for more equal representation of the amino acid variants. Each amino acid variant at 33 positions was represented by two codons (except Met and Trp with one codon each and cysteine, which was excluded) for a total complexity of 594 aa variants (Supplemental Table I). The libraries were cloned into an EBV-derived plasmid for mammalian cell surface display and transfected into 293c18 cells as described (9). The optimum staining conditions for cell sorting were determined to be 8 and 5 nM of CD80-C or CD86-C, respectively, using control cells displaying parental CTLA4-Ig. The library was first incubated with CD80-C or CD86-C followed by allophycocyanin-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. 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, respectively, within the green range. The high-affinity mutations employed in belatacept (LEA29Y) (7), A29Y and L104E, scored high for both CD80 and CD86, respectively, within the green range.

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-Ig ligand complexes (Fig. 3C, 3E). The map identified key interactions of CTLA-4 with each ligand consistent with results from 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 significant 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 of the ligand were scored higher, corresponding to the higher average scores of the variants at these positions.
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 codon 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 abatacept (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 costimulatory signals provided by a cell line expressing either CD80 or CD86 (13) (Supplemental Fig. 2A). Inhibition curves of wild-type CTLA4-Ig and commercial abatacept 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 abatacept 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 response 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 (lacking25SPGK28) 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></th>
<th>CD80</th>
<th></th>
<th>CD86</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_\text{a}$ (M$^{-1}$s$^{-1}$)</td>
<td>$k_\text{d}$ (s$^{-1}$)</td>
<td>$K_D$ (M)</td>
<td>Fold $K_D$</td>
<td>Fold Comp.</td>
</tr>
<tr>
<td>CTLA4-Ig (wild-type)</td>
<td>3.0 × 10$^6$</td>
<td>8.7 × 10$^{-4}$</td>
<td>2.9 × 10$^{-10}$</td>
<td>1.0</td>
</tr>
<tr>
<td>K28H</td>
<td>2.8 × 10$^6$</td>
<td>1.6 × 10$^{-4}$</td>
<td>5.7 × 10$^{-11}$</td>
<td>5.1</td>
</tr>
<tr>
<td>A29H</td>
<td>2.9 × 10$^6$</td>
<td>4.1 × 10$^{-4}$</td>
<td>1.4 × 10$^{-10}$</td>
<td>2.1</td>
</tr>
<tr>
<td>T51N</td>
<td>4.3 × 10$^6$</td>
<td>2.5 × 10$^{-3}$</td>
<td>5.9 × 10$^{-10}$</td>
<td>0.5</td>
</tr>
<tr>
<td>T51Y</td>
<td>2.2 × 10$^6$</td>
<td>1.6 × 10$^{-4}$</td>
<td>7.4 × 10$^{-11}$</td>
<td>4.0</td>
</tr>
<tr>
<td>L58G</td>
<td>1.7 × 10$^6$</td>
<td>1.6 × 10$^{-4}$</td>
<td>9.3 × 10$^{-11}$</td>
<td>3.2</td>
</tr>
<tr>
<td>K93M</td>
<td>2.6 × 10$^6$</td>
<td>2.1 × 10$^{-4}$</td>
<td>8.0 × 10$^{-11}$</td>
<td>3.7</td>
</tr>
<tr>
<td>K93Q</td>
<td>3.2 × 10$^6$</td>
<td>1.6 × 10$^{-4}$</td>
<td>4.8 × 10$^{-11}$</td>
<td>6.1</td>
</tr>
<tr>
<td>L96K</td>
<td>7.5 × 10$^6$</td>
<td>1.5 × 10$^{-4}$</td>
<td>2.1 × 10$^{-10}$</td>
<td>0.1</td>
</tr>
<tr>
<td>A29Y + L104E</td>
<td>2.9 × 10$^6$</td>
<td>1.1 × 10$^{-4}$</td>
<td>3.6 × 10$^{-11}$</td>
<td>8.1</td>
</tr>
</tbody>
</table>

Equilibrium binding constants ($K_D$) calculated by $k_a/k_d$ of 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 BIAcore) 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 CTLA4-Ig 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 CD80 over CD86 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 CD86 over CD80. 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.

FIGURE 5. Inhibition of primary and secondary allostimulated T cells by CTLA4-Ig variants. Inhibition of primary (A) and secondary (B) alloresponse by CTLA4-Ig and its variants are shown. In primary alloresponse assays, purified human CD4+ T cells were incubated with a irradiated allogenic B cell line, SU-DHL-6, for 6 d in the presence of increasing concentrations (x-axis) of CTLA4-Ig or its variants (A). In secondary alloresponse assays, T cells were first incubated with SU-DHL-6 for 6 d in the absence of fusion protein. T cells were then purified over Ficoll, rested for 24 h, and then restimulated with SU-DHL-6 in the presence of CTLA4-Ig or its variants for a further 3 d (B). Proliferative responses of T cells were measured by [3H]thymidine incorporation (y-axis). Symbols represent means of triplicate determinations. Data are representative of at least three independent experiments.
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
26. Forsyth for assistance with graphic figures, and Don Halbert, Stan Falkow, and Paul Hinton for critical reading and comments on the manuscript.