CD80 (B7-1) Binds Both CD28 and CTLA-4 with a Low Affinity and Very Fast Kinetics

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Summary

The structurally related T cell surface molecules CD28 and CTLA-4 interact with cell surface ligands CD80 (B7-1) and CD86 (B7-2) on antigen-presenting cells (APC) and modulate T cell antigen recognition. Preliminary reports have suggested that CD80 binds CTLA-4 and CD28 with affinities (K_d values $\sim$12 and $\sim$200 nM, respectively) that are high when compared with other molecular interactions that contribute to T cell–APC recognition. In the present study, we use surface plasmon resonance to measure the affinity and kinetics of CD80 binding to CD28 and CTLA-4. At 37°C, soluble recombinant CD80 bound to CTLA-4 and CD28 with K_d values of 0.42 and 4 μM, respectively. Kinetic analysis indicated that these low affinities were the result of very fast dissociation rate constants (k_{off}); sCD80 dissociated from CD28 and CTLA-4 with k_{off} values of $\geq$1.6 and $\geq$0.43 s^{-1}, respectively. Such rapid binding kinetics have also been reported for the T cell adhesion molecule CD2 and may be necessary to accommodate dynamic T cell–APC contacts and to facilitate scanning of APC for antigen.

CD28 (1, 2) and CTLA-4 (3) are structurally related cell surface molecules, largely restricted to the T cell lineage, which contribute to antigen recognition by T cells (for reviews see references 4–6). They do so by interacting with the structurally related ligands CD80 (B7-1, B7; 7, 8) and CD86 (B7-2, B70; 9–12), which are expressed on APC. Numerous reports (reviewed in references 4–6) have shown that, upon ligation, CD28 transmits activation signals that enhance T cell activation through the TCR. The role of CTLA-4 is more controversial (13), but recent in vivo studies (14–16) suggest that, unlike CD28, CTLA-4 ligation inhibits T cell activation, apparently by inhibiting signaling through the TCR (17).

Antigen recognition by T cells is unusual in that it occurs at the interface between two cells in direct physical contact, and CD28 is one of several T cell surface molecules that participate in this interaction (18, 19). It is becoming evident that T cells are sensitive to quantitative changes in the molecular interactions that contribute to T cell antigen recognition, and that quantitative differences in, for example, binding affinity, kinetics, or surface density, can lead to very different T cell responses (20–24). Clearly, our understanding of antigen recognition would be aided by knowledge of the affinity and binding kinetics of the individual molecular interactions. Recent studies have provided affinity and kinetic data on the interactions of the TCR with peptide–MHC (25) and CD2 with its ligands CD48 (26) and CD58 (27, 28). In contrast, the interactions of CD28 and CTLA-4 with their ligands are less well characterized. Estimates have been made of the relative avidity and kinetics of CD28 and CTLA-4 binding to CD80 and CD86 (8, 29, 30), but no definitive affinity or kinetic data are available. A soluble CD80 Ig fusion protein was reported to bind to CD28 with a dissociation constant (K_d) of $\sim$200 nM (29) and to CTLA-4 with a K_d of $\sim$12 nM (8), but there was evidence that this CD80 Ig was not monomeric in solution (29). More recent kinetic studies have relied entirely on dimeric Ig fusion proteins (30, 31). Although potentially informative for comparative purposes, such studies do not provide accurate affinity and kinetic data of the sort that can be used to develop quantitative models of Ag recognition by T cells.

In the present study, we undertook to obtain precise estimates of the solution affinity and kinetic constants for the interaction between CD80 and both CD28 and CTLA-4. Molecules involved in cell–cell recognition can have very low affinities and fast dissociation rate constants, leading to technical problems in their measurement (32, 33). Recent

studies (for reviews see 33, 34) have demonstrated that surface plasmon resonance (SPR)[1], as implemented in the BIAcore instrument (35), is well suited to analysis of such interactions. We use SPR to show that CD80 binds CD28 and CTLA-4 with affinities considerably lower than previously reported, and with very fast kinetics.

Materials and Methods

Expression of CTLA-4 Ig. The CTLA-4 Ig construct was very similar to that previously described (8) and was made by PCR, using as a template a cDNA library prepared from human peripheral blood acute lymphoblastic leukemia cells (Daenke, S., unpublished data). The 116 base 5′ primer CTAGCCACTGAAGCTTC-ACCAATGGGTGTACTGCTCACACAGAGGACGCTGCT-CAGTCTGGTCCTTGCACTCCTGTTTCCAAGCATGG-CGAGCATGGCAATGCACGTGGCCCAGCC encoded the oncostatin M leader (36) and 20 bases of CTLA-4 sequence and incorporated a HindIII restriction site. The encoded junction between the oncostatin leader (uppercase) and CTLA-4 (lowercase) was as follows: SMASMamhva. The 3′ primer ACGGATCC-TTATCAGAATCTGGGCACGGTTC encoded the CTLA-4 sequence immediately upstream of the transmembrane region followed by a splice donor site and a BamHI restriction site. The amplified product was subcloned into the pIg expression vector (37) using its BamHI and HindIII restriction sites. pIg incorporates, downstream of the cloning site, a genomic sequence encoding the constant region of human IgG γ_1 heavy chain. After splicing, the encoded junction between CTLA-4 (uppercase) and the γ_1 heavy chain (lowercase) would be as follows: CPDSDepsksc. The sequence was confirmed by dideoxy sequencing. Stable high level expression of CTLA-4 Ig in Chinese hamster ovary (CHO)–K1 cells was achieved by calcium phosphate cotransfection of the CTLA-4 Ig encoding plasmid together with the glutamine synthetase–encoding expression vector pEE14, as previously described (38–40). Clones expressing high levels of CTLA-4 Ig (~30–40 mg/L) were identified by inhibition ELISA using a mAb specific for human IgG (40) and grown up to confluence in bulk culture before switching to serum-free medium supplemented with 2 mM Na butyrate. CTLA-4 Ig was then purified by affinity chromatography on protein A–sepharose (Pharmacia Biotech AB, Uppsala, Sweden) followed by size-exclusion chromatography on a SUPERDEX S200 HR10/30 column (Pharmacia Biotech).

Expression and Analysis of sCD80. The soluble CD80 construct was designed to encode the extracellular portion of CD80 up to lysine-209 followed by a carboxy-terminal oligo-histidine tag for purification (sCD80his). It was made by PCR using cDNA from MT-2 cells (HTLV-1-transformed human T cells) as template. The 5′ primer TAGTAGAAGCTTTCCCCATCC-GCTCAAGCAGGCCACCATGGGCCACACACGGAGG was complementary to the CD80 leader sequence but added an HindIII site and inserted, immediately upstream of the initiation codon, the 25 bases that precede the rat CD4 initiation codon (41). The 3′ primer TAGTAGTCTAGACTAATGATGATGA-TGATGATGCTTGGCTGTATTCCAGTTGAAGGT added six histidine residues and a stop codon after lysine 209, mutated thre-

onine 208 to alanine to remove a potential NH_2-linked glycosylation site, and added a XbaI site. The 10 carboxy-terminal amino acids of sCD80his were thus NTAKHHHHHH. The resulting PCR fragment was subcloned into the glutamine synthetase expression vector pEE14 (39) using its XbaI and HindIII restriction sites, and the sequence was confirmed by dideoxy sequencing. CHO-K1 cells were transfected as described (38, 39) with the sCD80his-encoding plasmid by calcium phosphate transfection. Clones expressing high levels of sCD80his (~40 mg/L) were identified by growth in the presence of [35S]methionine/[35S]cysteine (TRANS35S-LABEL; ICN Pharmaceuticals, Costa Mesa, CA), purification of labeled protein from the culture supernatant using Ni-NTA spin columns (Qiagen GmbH, Hilden, Federal Republic of Germany), and then SDS-PAGE of the protein followed by autoradiography. The best clone was grown up to confluence in bulk culture before switching to serum-free medium supplemented with 2 mM Na butyrate. sCD80his was purified by affinity chromatography using Ni-NTA resin (Qiagen GmbH) followed by size-exclusion chromatography on a SUPERDEX S200 HR10/30 column. The extinction coefficient (at 280 nm) of sCD80his was determined by amino acid analysis to be 1.41 ml.mg^{-1}. The carboxy-terminal his tag was cleaved off by incubating 2.5 mg of sCD80his in 1.5 ml Tris–saline buffer (140 mM NaCl, 10 mM Tris [pH 7.5]) with 1.2 U of carboxypeptidase A conjugated to agarose beads (Sigma Chemical, Poole, UK) for 16 h at 30°C with agitation. Amino acid analysis confirmed that ≥90% of the carboxy-terminal histidine residues (5.4 molecules per sCD80his molecule), but no other amino acids, were released during this incubation (data not shown). The carboxypeptidase A was removed by centrifugation and the digested sCD80his (sCD80) was repurified on a SUPERDEX S200 HR10/30 column (Fig. 1 *C*).

The proportion of sCD80 that possessed ligand binding activity was determined as follows. Protein A–sepharose beads (100 μl) were incubated with 0.4 mg of CTLA-4 Ig or (as a control) CD22 (d1-3) Ig (42) in 0.33 ml Tris–saline for 1 h at 4°C with rotation and then washed three times with 1.5 ml Tris–saline. CTLA-4 Ig or CD22 Ig beads (40 μl) were then added to 20 μl sCD80 (0.65 μg/μl in Tris–saline) and incubated for 4 h at 4°C with rotation. The beads were then pelleted and 10 μl of each supernant analyzed by SDS-PAGE, together with 3.3 μl of sCD80 that had not been exposed to beads (Fig. 1 *B*).

SPR Experiments. Binding experiments were performed by SPR on a BIAcore instrument (35) upgraded with the BIAcore Upgrade Kit (BIAcore AB, St Albans, UK). All experiments were performed at 37°C (except where otherwise indicated) using HBSS buffer (25 mM Hepes [pH 7.4], 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20) supplied by BIAcore. CTLA-4 Ig and CD28 Ig were covalently coupled by primary amine groups to the carboxymethylated dextran matrix on a research grade CM5 sensor chip (BIAcore) using the Amine Coupling Kit (BIAcore) as directed (43), with the following modifications. After an activation step of 15–240 s, CTLA-4 Ig (30 μg/ml in 10 mM Na acetate [pH 4.7]) or CD28 Ig (15 μg/ml in 10 mM Na acetate [pH 5]) were injected for 7 min. After coupling, CTLA-4 Ig and CD28 Ig were washed with a 3 min injection of NaOH (5 mM). NaOH (5 mM) was also used to regenerate immobilized CD28 Ig and CTLA-4 Ig. The anti-human IgG$_1$ mAb R10Z8E9 was used as previously described (44) to immobilize CD28 Ig and CTLA-4 Ig indirectly to the sensor surface via their Ig fragment.

Analysis of Affinity and Kinetic Data. Equilibrium binding analysis and analysis of the washout or dissociation phase was performed as previously described (27). Analysis of the injection or association phase was performed using the BIAevaluation software Version

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[1]*Abbreviations used in this paper:* CHO, Chinese hamster ovary; FC, flow cell; k_{off}, dissociation rate constant; k_{on}, association rate constant; RU, response unit; sCD80his, soluble CD80 with carboxy-terminal oligo-histidine tag; SPR, surface plasmon resonance.
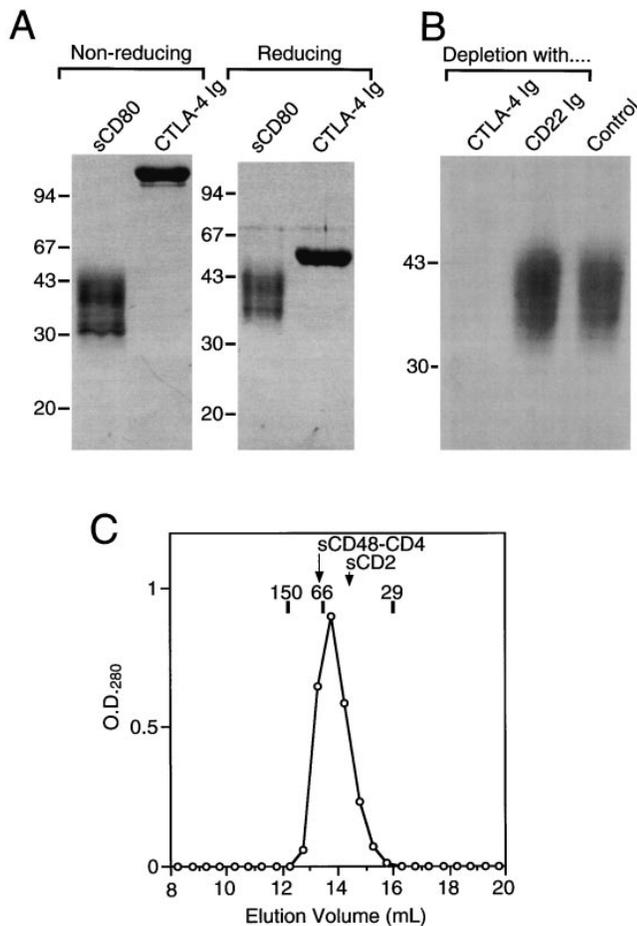
Figure 1. Biochemical analysis of sCD80 and CTLA-4 Ig. (*A*) sCD80 and CTLA-4 Ig (2.5 μg each) were analyzed by SDS-PAGE on 10% acrylamide under nonreducing and reducing (+β*ME*) conditions. The migration positions of the indicated (in kD) molecular mass markers are shown. (*B*) Measuring the binding activity of sCD80. Protein A–sepharose beads coated with CTLA-4 Ig or CD22 Ig were incubated with soluble sCD80, pelleted, and the supernatant analyzed for the presence of sCD80 by reducing SDS-PAGE on 12% acrylamide, together with sCD80 not exposed to beads (*Control*). (*C*) Analysis of sCD80 by size-exclusion chromatography. sCD80 (2.1 mg in 0.5 ml) was run on a SUPERDEX S200 HR10/30 column (Pharmacia) at 0.5 ml/min. The calibration markers shown (Sigma) were alcohol dehydrogenase (M_r 150,000), BSA (M_r 66,000), and carbonic anhydrase (M_r 29,000). sCD48–CD4 and sCD2 are described in the text.

2.1 (BIAcore). The k_{on} was determined by nonlinear curve fitting of the following equation to the injection phase.

$$R(t) = R_{eq} \cdot \left(1 - e^{-(k_{on} \cdot C + k_{off}) \cdot t} \right) \tag{1}$$

where $R(t)$ is the response (in response units) at time t, R_{eq} is the response at equilibrium, and C is the concentration of injected sCD80. The k_{off} value used for this fit was obtained by analysis of the washout phase of the same injection. For the CTLA-4–sCD80 interaction, the k_{on} was also estimated using an approach relying entirely on analysis of the injection phase, as described by Karrlson et al. (43). After injection at several different sCD80 concentrations,

plots of dR/dt versus R for each concentration (C) give straight lines with a slope $-k_s$ where

$$k_s = k_{on}C + k_{off} \tag{2}$$

Values for k_{on} and k_{off} were obtained by a linear fit of a plot of k_s versus C (see Fig. 4 *E*).

Results and Discussion

Expression and Characterization of Monomeric sCD80. A soluble form of CD80 with a carboxy-terminal oligo-his tag (sCD80his) was expressed in CHO-K1 cells. Preliminary experiments indicated that the oligo-histidine tag predisposed towards the formation of multimeric aggregates (van der Merwe, P.A., and S.J. Davis, unpublished data) and so it was removed by digestion with carboxypeptidase A (see Materials and Methods) to yield sCD80. Analysis of sCD80 by reducing SDS-PAGE indicated that it migrated as a broad band with a size range of M_r 35–45,000 (Fig. 1 *A*). This is consistent with the predicted size of the protein backbone (M_r 24,747) and variable glycosylation of the seven potential NH$_2$-linked glycosylation sites. sCD80 migrated slightly faster (M_r 30–43,000) under nonreducing conditions, indicating that the protein does not form disulphide-linked multimers, and consistent with the existence of two predicted disulphide bonds (45).

For valid affinity and kinetic analysis, it is critical that the interaction under study is monovalent (32), because increasing the binding valency leads to dramatic (but unpredictable) increases in the strength and stability of an interaction. The use of the term affinity is usually restricted to descriptions of the binding strength of monovalent interactions, whereas avidity is used to describe the binding strength of multivalent interactions. In the present study, monovalency was ensured by using a monomeric form of CD80 (sCD80). To check that sCD80 was indeed monomeric in solution, it was analyzed by size-exclusion chromatography (Fig. 1 *C*). sCD80 eluted at M_r ∼63,000 when using globular unglycosylated proteins to calibrate the column (Fig. 1 *C*). Although this is slightly higher than might be expected, sCD80 is heavily glycosylated and probably asymmetric. Therefore, we compared sCD80 with two similarly asymmetric glycosylated proteins, namely soluble rat CD2 (sCD2, M_r 26–34,000 on SDS-PAGE [46]) and soluble rat CD48–CD4 (sCD48–CD4, M_r 43–64,000 on SDS-PAGE [26, 47]), both of which are monomeric (26, 47). sCD80 eluted after sCD48–CD4 and ahead of sCD2 (Fig. 1 *C*), consistent with it existing as a monomer in solution.

sCD80 appeared to be folded correctly as it bound to all four mAbs tested (Table 1) and also bound both CD28 and CTLA-4 (see below). Accurate estimation of affinity and kinetic constants requires knowledge of the exact concentration of the soluble ligand, which in turn requires that the proportion of sCD80 that retains ligand binding activity is known. All of the sCD80 could not be assumed to be active because it had been purified using its oligo-histidine tag, and this would not discriminate between correctly and

Table 1. *mAbs that Bind the Recombinant Proteins Used in This Study*

Protein	mAb
sCD80*	2D10, mAb 104, L307, BB-1
CD28 Ig‡	CD28.1, CD28.2, CD28.3, CD28.5, CD28.6, CLB–CD28/1, KOLT-2
CTLA-4 Ig§	7F8, 10A8, 11D4

*2D10 (64) was from Dr. Daniel Olive (Inserm 119, Institut Paoli Calmettes, Marseilles, France). mAb 104 (65) was from Immunotech S.A. (Marseilles, France). L307 (66) was from Becton Dickinson (San Jose, CA). BB-1 (67) was from PharMingen (San Diego, CA).
‡CD28.1–CD28.6 (68) were from Dr. Daniel Olive. CLB–CD28/1(15E8 in [66]) was from Dr. René A. W. van Lier (Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands). KOLT-2 (69) was from Dr. Kimitaka Sagawa (Department of Immunology, Kurume University School of Medicine, Kurume, Japan).
§7F8, 10A8, 11D4 were produced as described (54).

Table 2. *Summary of Affinity Constants Measured by Equilibrium Binding*

Binding of sCD80 to	Immobilization*	Temperature	K_d (μM)‡
CTLA-4 Ig	Direct	37°C	0.42 (± 0.06, $n = 3$)
	Direct	25°C	0.26 (± 0.06, $n = 3$)
	Indirect	25°C	0.2 ($n = 1$)
CD28 Ig	Direct	37°C	4.0 (± 0.3, $n = 4$)
	Direct	25°C	2.5 (± 0.4, $n = 2$)
	Indirect	37°C	5.5 ($n = 1$)

*Direct immobilization was via primary amines on CTLA-4 Ig or CD28 Ig (see Materials and Methods). Indirect immobilization was via a directly coupled mAb that binds the Ig portion of CTLA-4 Ig and CD28 Ig, as previously described (44).
‡The values shown are the means of n determinations (± SD for $n \geqslant 3$ or ± range for $n = 2$).

incorrectly folded forms of sCD80. The activity of sCD80 therefore was estimated directly by depleting the sCD80 with protein A–sepharose beads coated with CTLA-4 Ig (Fig. 1 *B*). CTLA-4 Ig beads were able to deplete all the sCD80 (Fig. 1 *B*), whereas CD22 Ig beads did not deplete, indicating that close to 100% of the sCD80 retains CTLA-4-binding activity.

Expression and Analysis of CD28 Ig and CTLA-4 Ig. The recombinant CD28 used in the present study (CD28 Ig) was a homodimeric fusion protein incorporating the Fc portion of human γ_1 Ig heavy chain (29). We expressed and purified a similar CTLA-4 Ig fusion protein (see Materials and Methods). SDS-PAGE under reducing and nonreducing conditions indicated that CTLA-4 Ig was expressed as a disulphide-linked dimer (Fig. 1 *A*). For kinetic and affinity studies, CTLA-4 Ig and CD28 Ig were immobilized to a dextran matrix on the sensor surface either indirectly, via a mAb specific for human IgG$_1$, or by direct covalent coupling through primary amines (see Materials and Methods). CD28 and CTLA-4 mAbs (Table 1) bound to both directly and indirectly coupled forms of CD28 Ig and CTLA-4 Ig (data not shown). Furthermore, sCD80 bound with a similar affinity to both directly and indirectly coupled CD28 Ig and CTLA-4 Ig (see below; Table 2), indicating that direct covalent coupling did not substantially disrupt the native structure of these proteins. All subsequent measurements were made using directly coupled CD28 Ig and CTLA-4 Ig.

Affinity Measurements. Affinity and kinetic measurements were performed at 37°C except where otherwise indicated. The affinity of sCD80 binding to CTLA-4 and CD28 was measured directly by equilibrium binding analysis (Figs. 2 and 3), because this avoids the many potential pitfalls associated with kinetic measurements (see below; 48–50). Increasing concentrations of sCD80 were injected

over sensor surfaces to which CTLA-4 Ig or CD28 Ig had been immobilized (Figs. 2 *A* and 3 *A*, respectively). It is noteworthy that at all sCD80 concentrations binding reached equilibrium very rapidly (95% binding within 20 s; faster for CD28) and that, in the washout phase after the injection, bound sCD80 dissociated within 20 s (faster for CD28). These features are typical of interactions with very fast binding kinetics. The sCD80 samples were also injected over a control sensor surface (with no protein immobilized) to measure the background response (Figs. 2 *A* and 3 *A*, *right*). Larger background responses are seen in Fig. 3 *A* because a tenfold higher range of sCD80 concentrations was injected over CD28 Ig (Figs. 2 and 3, legends). For each sCD80 concentration the binding response (measured in arbitrary response units [RU]) at equilibrium was calculated by subtracting the response seen in the control flow cell from the response seen in the CTLA-4 (see Fig. 2 *B*) or CD28 (Fig. 3 *B*) flow cells. A plot of the binding response indicates saturable binding and direct fitting of a standard Langmuir binding isotherm to the data gave K_d values of 0.38 and 4.2 μM for sCD80 binding to CTLA-4 and CD28, respectively (Figs. 2 *B* and 3 *B*, *left*). Scatchard plots of the data gave very similar K_d values (Figs. 2 *B* and 3 *B*, *right*). The mean K_d (± SD) values from several independent determinations are shown in Table 2.

Kinetic Measurements. Measurements of binding kinetics are prone to error, particularly when the kinetics are very fast, as in the present study (48–50). One source of error is that part of the response is a background signal that does not represent true binding. To correct for this, the response obtained when sCD80 was injected through a control flow cell (containing no immobilized protein) was subtracted before analysis. Figs. 4 *A* and 5 *A* show typical responses obtained after injection of sCD80 through flow cells with two different levels of CTLA-4 Ig (or CD28 Ig) immobi-
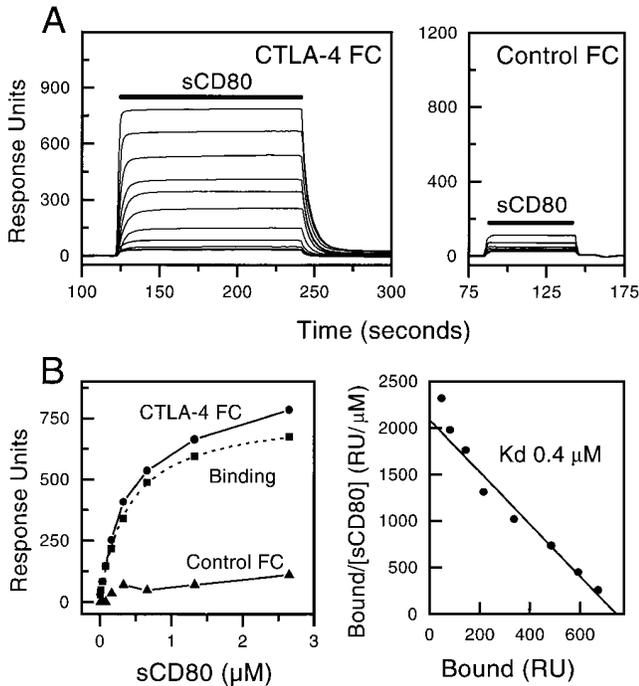
Figure 2. Measuring the affinity of sCD80 binding to CTLA-4 Ig by equilibrium binding. (*A*) A range of sCD80 concentrations (2.65 μM and nine twofold dilutions thereof) were injected sequentially (*solid bar*) for 2 min at 10 μl/min through a flow cell (FC) with either CTLA-4 Ig (1,500 RUs) or no protein (*Control*) immobilized. sCD80 (0.26 μM) bound to a very similar level (255 and 250 RUs) when injected at the beginning and end of the experiment (data not shown), indicating that the immobilized CTLA-4 Ig was stable. (*B*) The equilibrium responses in the CTLA-4 Ig (●) and Control (▲) FCs at each sCD80 concentration and the differences between these responses (representing actual binding, ■) are plotted. The dotted line represents a nonlinear fit of the Langmuir binding isotherm to the binding data and yields a K_d of 0.4 μM and a binding maximum of 774 RUs. A Scatchard plot of the same data is shown on the right. A linear regression fit yields a K_d of 0.4 μM and a binding maximum of 750 RUs.

Figure 3. Measuring the affinity of sCD80 binding to CD28 Ig by equilibrium binding. (*A*) A range of sCD80 concentrations (26.5 μM and eight twofold dilutions thereof) were injected sequentially (*solid bar*) for 30 s at 10 μl/min through a FC with either CD28 Ig (immobilized at a level of 7800 RUs) or an irrelevant protein (*Control*) immobilized. sCD80 (0.82 μM) injected at the beginning and end of the experiment bound to a very similar level (160 and 157 RUs), indicating that the immobilized CD28 Ig was stable. (*B*) The equilibrium responses in the CD28 Ig (●) and Control (▲) FCs at each sCD80 concentration and the differences between these responses (representing actual binding, ■) are plotted. The dotted line represents a nonlinear fit of the Langmuir binding isotherm to the binding data and yields a K_d of 4.1 μM and a binding maximum of 939 RUs. A Scatchard plot of the same data is shown on the right. A linear regression fit yields a K_d of 4.3 μM and a binding maximum of 952 RUs.

lized, as well as through a control flow cell. Subtraction of the control flow cell response from the responses in the CD28 and CTLA-4 flow cells gives the actual binding response shown in Figs. 4 *B* and 5 *B* (*solid lines*), which was subjected to kinetic analysis. A second potential source of error is that binding and dissociation are limited by the rate at which protein is delivered to and removed from the sensor surface. This was addressed by using higher than normal flow rates (40 or 80 μl/min). These flow rates were judged to be optimal because halving them had little effect on the association (injection) or dissociation (washout) phases when sCD80 was injected over CTLA-4 (see Figs. 4 *B* and *C*) or CD28 (Figs. 5, *B* and *C*). It should be noted that even at very high flow rates, binding may still be limited by mass-transport within an unstirred layer close to the binding surface (48, 50). The effects of mass-transport limitations within this unstirred layer can be reduced by decreasing the level of immobilized ligand (see below). A third source of error is rebinding of protein during the dissociation (washout) phase, which will lead to underestimation of
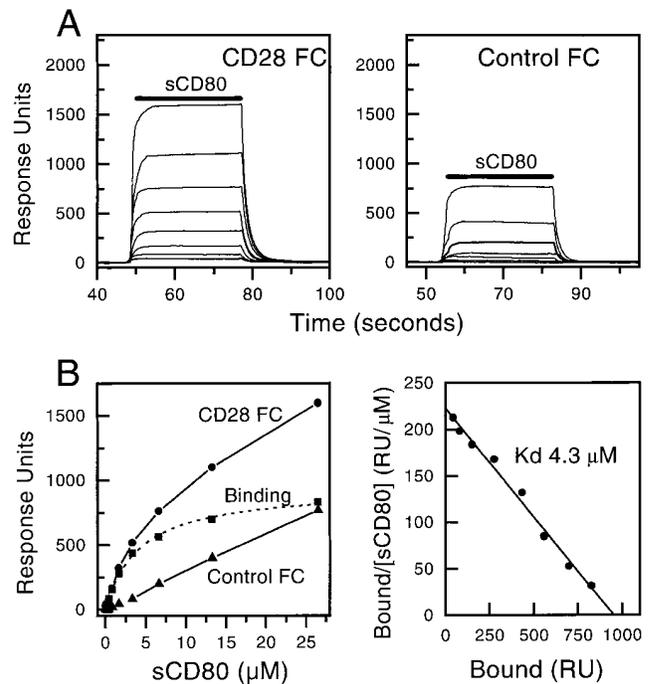
the dissociation rate. Rebinding can be decreased by immobilizing lower levels of ligand. This is illustrated in Figs. 4 *C* and 5 *C*, in which sCD80 dissociates more rapidly when the level of immobilized CTLA-4 or CD28 is decreased. sCD80 dissociated from sensor surfaces with high and low levels of CTLA-4 with apparent dissociation rate constants (k_{off}) of 0.24 s^{-1} and 0.43 s^{-1}, respectively (see Fig. 4 *C*; Table 3). Similarly, sCD80 dissociated from high and low levels of CD28 with k_{off} values of 1.1 s^{-1} and 1.6 s^{-1}, respectively (Fig. 5 *C*; Table 3). Although it is quite likely that some sCD80 rebinding is still occurring even at these lower CD28 Ig and CTLA-4 Ig levels, it proved difficult to collect data of satisfactory quality when CD28 Ig and CTLA-4 Ig levels were reduced further. Therefore, we assume that the actual k_{off} values for dissociation of sCD80 from CTLA-4 and CD28 are at least 0.43 s^{-1} and 1.6 s^{-1}, respectively.

Association rate constants (k_{on}) for binding were estimated by nonlinear curve fitting to data from the injection phase (Figs. 4 *D* and 5 *D*) using k_{off} values obtained from
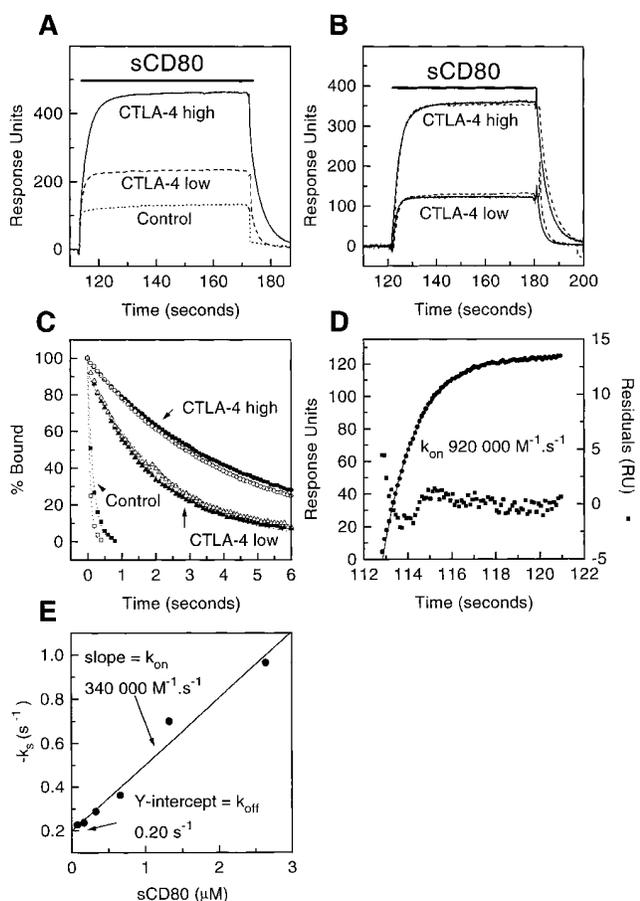
Figure 4. Estimating the k_{on} and k_{off} for sCD80 binding CTLA-4 Ig. (*A*) Example of primary data. sCD80 (265 nM) was injected (*solid bar*) at 40 μl/min through FCs with nothing immobilized (*Control*) or CTLA-4 immobilized at low (920 RUs) or high (2500 RUs) levels. (*B*) Effect of varying the flow rate. sCD80 (265 nM) was injected (*solid bar*) at 20 (*solid line*) or 40 (*stippled line*) μl/min through FCs with high or low levels of CTLA-4. Background responses (following injection through a control FC) have been subtracted. (*C*) Dissociation of sCD80 from FC with high (●, ○) or low (▲, △) levels of CTLA-4 Ig at flow rate of 20 (●, ▲, ■) or 40 (○, △, □) μl/min. Also shown is the fall in response in the same period following injection of sCD80 through control FC (■, □). The data fitted reasonably well to single exponential decay curves (*dotted lines*), yielding the following $t_{1/2}$ values: ●, 4.8 s; ○, 4.4 s; ▲, 2.2 s; △, 2.35 s; ■, 0.16 s; □, 0.075 s. (*D*) Estimating the k_{on}. Equation 1 (see Materials and Methods) was fitted (*solid line*) to data (●) from (*B*) (corresponding to binding of sCD80 to CTLA-4 [low level] at 40 μl/min), yielding the indicated residuals (■) and k_{on}. (*E*) Independent estimation of k_{on} and k_{off} by analysis of binding at different sCD80 concentrations. The rate at which the binding rate decreases ($-k_s$) during the injection phase was determined for a range of injected sCD80 concentrations (see Materials and Methods [43]) using data from Fig. 2 *A*. A linear regression fit of equation 2 to a plot of k_s versus sCD80 concentration yielded the k_{on} and k_{off} values.
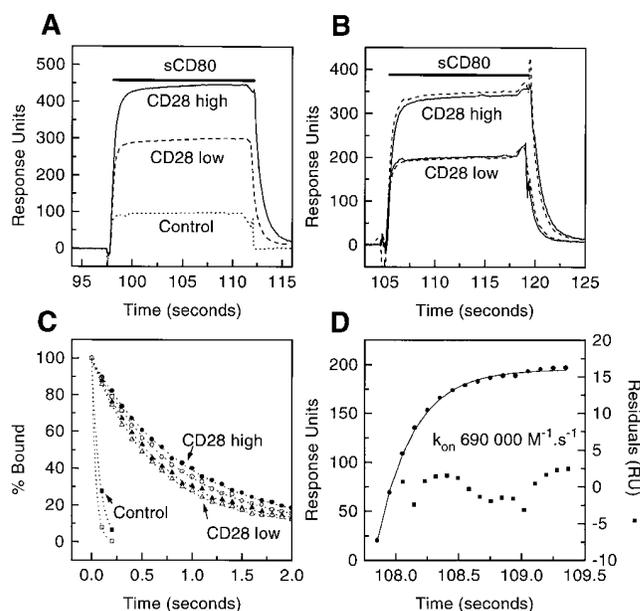
Figure 5. Estimating the k_{on} and k_{off} for sCD80 binding CD28 Ig. (*A*) Example of primary data. sCD80 (2.65 μM) was injected (*solid bar*) at 80 μl/min through FCs with nothing immobilized (*Control*) or CD28 Ig immobilized at low (3400 RUs) or high (6200 RUs) levels. (*B*) Effect of varying the flow rate. sCD80 (2.65 μM) was injected (*solid bar*) at 40 (*solid line*) or 80 (*stippled line*) μl/min through FCs with high or low levels of CD28 Ig. Background responses (following injection through a control FC) have been subtracted. (*C*) Dissociation of sCD80 from FC with high (●, ○) or low (▲, △) levels of CD28 Ig at flow rate of 40 (●, ▲, ■) or 80 (○, △, □) μl/min. Also shown is the fall in response in the same period following injection of sCD80 through a control FC (■, □). The data fitted well to single exponential decay curves (*dotted lines*), yielding the following $t_{1/2}$ values: ●, 0.93 s; ○, 0.84 s; ▲, 0.69 s; △, 0.64 s; ■, 0.075 s; □, 0.04 s. (*D*) Obtaining the k_{on} by nonlinear curve fitting. Eq. 1 (see Materials and Methods) was fitted (*solid line*) to data (●) from (*B*) (corresponding to binding of sCD80 to CD28 Ig [low level] at 80 μl/min), yielding the indicated residuals (■) and k_{on}.

analysis of the corresponding dissociation phase. Reasonably good fits were obtained with a simple one-to-one (Langmuir) binding model (Eq. 1). When CTLA-4 and CD28 were immobilized at the lower levels, sCD80 bound with mean k_{on} values of 9.4×10^5 M^{-1}s^{-1} and 6.6×10^5 M^{-1}s^{-1}, respectively (Table 3). Slightly slower k_{on} values were obtained with higher levels of immobilized CTLA-4

(6.2×10^5 M^{-1}s^{-1}) and CD28 (4.7×10^5 M^{-1}s^{-1}) (Table 3). As discussed above, this is likely to reflect underlying mass-transport limitations within the unstirred layer, which would be noticeable at higher levels of immobilized ligand (49, 50). Because it was not possible to eliminate completely the effect of mass transport limitations, we assume that the actual k_{on} values for sCD80 binding to CTLA-4 and CD28 are at least 9.4×10^5 M^{-1}s^{-1} and 6.6×10^5 M^{-1}s^{-1}, respectively.

Kinetic constants for CTLA-4 binding were also estimated independently of the dissociation phase by analyzing the association phase after injection of different concentrations of sCD80 (see Materials and Methods [43]). Although less accurate and less stringent than a direct fit of equation 1 (48), this approach is useful for verification since it does not require prior measurement of the k_{off}, but instead provides its own independent estimate (see Fig. 4 *E*). Such an analysis was not possible for the lower affinity CD80–CD28 interaction because it has faster kinetics (compare Figs. 4 *D* and 5 *D*) and requires much higher sample concentrations. The k_{on} (3.2×10^5 M^{-1}s^{-1}) and k_{off} (0.2 s^{-1}) values mea-

Table 3. *Summary of Kinetic Measurements*

Binding of sCD80 to	Immobilization level*	k_{on} ($\times 10^{-5}$ M^{-1}s^{-1})	k_{off} (s^{-1})	$K_{d_{calc}}$ (k_{off}/k_{on}, µM)
CTLA-4 Ig	High	6.2 ± 1.8[§] ($n = 3$)	0.24 ± 0.03 ($n = 3$)	0.3
	High (linear regression)[‡]	3.4 ($n = 1$)	0.2 ($n = 1$)	0.63
	Low	9.4 ± 0.3 ($n = 2$)	0.43 ± 0.03 ($n = 3$)	0.46
CD28 Ig	High	4.7 ± 0.6 ($n = 4$)	1.1 ± 0.15 ($n = 3$)	2.3
	Low	6.6 ± 0.8 ($n = 4$)	1.6 ± 0.1 ($n = 4$)	2.4

*CTLA-4 Ig or CD28 Ig were immobilized directly. For CTLA-4 Ig, high and low levels were 2,500 and 900 RUs, respectively. For CD28 Ig, high and low levels were 6,200 and 3,400 RUs, respectively.

[‡]Data from Fig. 4 *E*.

[§]The values shown are the mean of n determinations ($\pm$ SD for $n \geqslant 3$ or $\pm$ range for $n = 2$).

sured in this manner for sCD80 binding to CTLA-4 Ig were only slightly slower than values obtained by the direct nonlinear curve fitting with similar levels of CTLA-4 (see Table 2).

The accuracy of the measured rate constants can be further assessed by comparing the ratio between the k_{off} and the k_{on} (which provides a calculated K_d or $K_{d_{calc}}$) with the K_d measured directly by equilibrium binding (Tables 2 and 3). For both CTLA-4 and CD28, the K_d and $K_{d_{calc}}$ were very similar (Tables 2 and 3), indicating consistency between the kinetic and equilibrium binding analyses. However, this internal consistency does not exclude the possibility that the true k_{on} and k_{off} values are both faster than the measured values. Therefore, the measured k_{on} and k_{off} values represent lower limits for the true k_{on} and k_{off}.

Comparisons with Other Studies. The affinities measured in the present study for CD80 binding CD28 (K_d 4 µM) and CTLA-4 (K_d 0.42 µM) are much lower than previously reported (8, 29). One possible difference is that these earlier measurements were made at 23°C (8, 29). However, we found that decreasing the temperature from 37°C to 25°C increased the affinity less than twofold (Table 2). A more significant difference is likely to be that the soluble CD80 Ig used in the earlier studies was not monomeric. This is supported by the observation that the CD80 Ig fusion protein used in these earlier studies, although monomeric on SDS-PAGE (M_r 70,000), eluted at M_r ~350,000 by size-exclusion chromatography (29), suggesting the presence of higher aggregates. Such aggregates could well account for the much higher affinities previously reported. However, this explanation does not account for 100- to 200-fold higher avidity observed for soluble CTLA-4 Ig compared with soluble CD28 Ig (for example see reference 30), a difference much larger than the 10-fold difference in affinity reported in the present study. This discrepancy is not due to differences in the recombinant proteins used in these studies, because we were able to reproduce the large difference in avidity by injecting CD28 Ig and CTLA4 Ig over a sensor surface onto which sCD80 had been immobilized (Fig. 6). CTLA-4 Ig injected at 0.1 and 1 µg/ml bound to higher levels than 100-fold higher concentrations of CD28 Ig (Fig. 6), indicating a >100-fold higher avidity. Although it is unclear why the avidity difference between CTLA-4 Ig and CD28 Ig is so much greater than the affinity difference, these results illustrate a major disadvantage of using multimeric proteins for comparative binding studies.

A similar affinity constant for the CTLA-4–CD80 interaction (K_d 0.21 µM at 25°C) has recently been obtained by SPR using a monomeric form of CTLA-4 (51) in solution and a CD80 Ig fusion protein (29) covalently immobilized onto the sensor surface (52). No monomeric form of CD28 was analyzed by Greene et al. (52) but, unexpectedly, an apparently dimeric form of CD28 (CD28tp) bound the immobilized CD80 Ig with an avidity (K_d 2.1–41 µM at 25°C) similar to the affinity that we obtained for monomeric sCD80 binding to immobilized CD28 (K_d 2.5 µM at 25°C). CD28tp also bound immobilized CD80 Ig with an
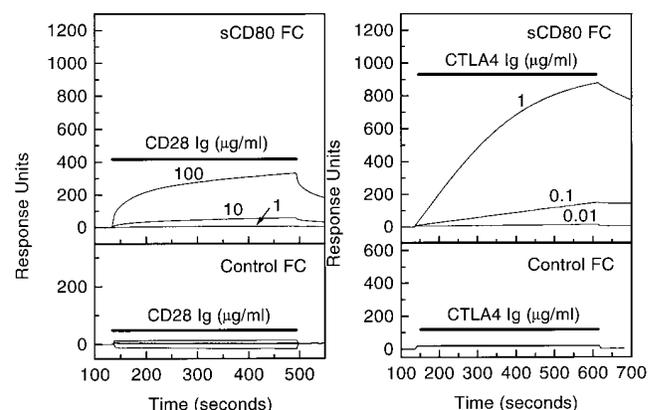

Figure 6. Comparison of avidity of CD28 Ig and CTLA-4 Ig binding to immobilized sCD80. CD28 Ig or CTLA-4 Ig were injected at 5 µl/ min (25°C) at the indicated concentration for 6–8 min through FC with either sCD80 (3300 RU) or no protein (*Control*) immobilized. The responses seen with injection of the three CTLA4 Ig concentrations through the Control FC were indistinguishable when superimposed.

Table 4. *Comparison of the Binding Constants for the Interactions of the TCR, CD2, CD4, and CD28 with Their Respective Ligands*

Interaction	k_{on} (M^{-1}s^{-1})	k_{off} (s^{-1})	K_d* (μM)	References
TCR binding peptide–MHC				
2B4 TCR–MCC peptide plus I-E^k	900–1,700	0.057–0.09	5–50	(70, 71)
2C TCR–p2C peptide plus L^d	11,000–210,000	0.006–0.026	0.1–0.5	(72, 73)
42.12 TCR–OVA peptide plus K^b	3,100	0.02	7–19	(22)
CD2–CD58	$\geqslant$400,000	$\geqslant$4	9–22	(27)
CD4–MHC class II	–	–	$\geqslant$3	(74, 75)
CD28–CD80	$\geqslant$660,000	$\geqslant$1.6	4	Present study

*K_d values shown were obtained by equilibrium binding, but the range of K_d values shown for TCR interactions includes some values calculated from the k_{off} and k_{on}.

unusually slow apparent k_{on} (7,000 M^{-1}s^{-1}). One explanation for this discrepency, which is consistent with the slow apparent k_{on}, is that only a small proportion of the CD28tp was active. An alternative explanation is that covalent immobilization of the CD80 Ig alters preferentially its interaction with CD28 but not CTLA-4.

Functional Implications. To understand the functional implications of the solution affinity and kinetic constants reported in the present study, it is necessary to extrapolate these values to interactions between the membrane-tethered forms of these molecules (28, 53). Like CD28, CD2 binds its ligand CD58 in solution with a very low affinity and rapid binding kinetics (27). Dustin et al. (28) have shown that the interaction between membrane-tethered CD2 and CD58 also exhibits rapid binding kinetics. This suggests that the fast solution-binding kinetics observed between these molecules is a reflection of rapid binding kinetics when they are attached to membrane. Theoretical considerations (53) suggest that the difference in the affinity of soluble CD80 for CD28 versus CTLA-4 is likely to be conserved for membrane-attached forms of these molecules. If correct, this implies that CTLA-4 will engage ~10-fold lower surface densities of CD80 than CD28 because of its ~10 fold higher affinity. Unlike CD28, CTLA-4 is not expressed on resting T cells (4) and, although expression is induced on T cell activation, CTLA-4 is always present at a ~30- to 50-fold lower surface density than CD28 (54). Under these circumstances CTLA-4 is unlikely to compete effectively for CD80 and displace it from CD28, despite its higher affinity. Instead, both CD28 and CTLA-4 are likely to engage CD80 when they are expressed simultaneously. In this regard, it is noteworthy that inhibitory signals transmitted through CTLA-4 appear to be dominant over the activation signals transmitted through CD28 (55, 56).

The affinity constants reported in the present study and previous studies of CD2 and CD4 suggest that accessory T cell molecules, including those with signaling functions, have affinities comparable to or lower than the reported affinities of TCRs for their specific peptide–MHC ligands (Table 4). Even more striking are the 10- to 100-fold faster k_{off} values seen for the CD2–CD58 and CD28–CD80 interactions compared with TCR/peptide–MHC interactions (Table 4). While it is possible that future studies will identify TCR/peptide–MHC interactions with similarly fast k_{off} values, these results suggest that the accessory molecule interactions are generally more unstable than TCR interactions. Indeed, the binding of CD4 and CD8 to MHC class II and MHC class I is likely to stabilize further TCR/peptide–MHC complexes relative to accessory molecule interactions (57).

These observations suggest that within the contact area between T cells and APC or target cells (18, 58), TCR/peptide–MHC complexes will generally be longer lived than CD28–CD80 or CD2–CD58 complexes. This raises the question as to whether the rapid binding kinetics of CD2 and CD28 interactions are functionally significant. One crucial feature of T cell antigen recognition is the capacity of T cells to detect as few as 10 specific peptide–MHC ligands on cells (59–61). Furthermore, Valitutti et al. (62) recently reported that, even when presented with very low levels of peptide–MHC (~100 complexes per cell), a substantial proportion of TCRs on the T cell surface (~18,000) appeared to be ligated in the process of T cell activation. Two important implications of these results are the following: (*a*) many peptide–MHC molecules will need to diffuse through the zone of contact between a T cell and an APC for the TCR to encounter specific peptide–MHC molecules; and (*b*) each specific peptide–MHC molecule will need to engage serially many TCRs (62). In turn, this implies that peptide–MHC and TCR molecules will need to diffuse rapidly into and out of a stable zone of contact between the T cells and APC and/or that new contacts will continually need to be formed between these cells (63). It seems likely that the very fast binding kinetics of CD2 and CD28 interactions will facilitate these processes by contributing to a highly dynamic contact zone between T cells and APC.

Since CD28 and CTLA-4 are clearly signaling molecules, able, like the TCR, to transmit positive and negative cells to T cells, the very fast k_{off} values reported in the present study indicate that signaling between cells can be transmitted by very transient molecular interactions. Because in the case of the TCR, the nature of the signal (positive or negative) appears to be highly dependent on the kinetics of its interaction (22), it is possible that signaling through CD28 and CTLA-4 will be similarly sensitive to binding kinetics. Preliminary data suggest that CTLA-4 binds CD86 with a lower affinity and faster dissociation rate constant than CD80 (52), which may contribute to the reported functional differences between CD80 and CD86 (6).

In this study, we show that CD28 and CTLA-4 bind CD80 with far lower affinities than previously reported and that these low affinities are the result of very fast dissociation rate constants. These kinetic parameters are similar to those measured for CD2–ligand interactions but very different to reported values for TCR–ligand interactions. We propose that the fast binding kinetics of CD28 (and CD2) serve to optimize T cell antigen recognition by (*a*) allowing rapid formation and disruption of contacts between T cells and APC and (*b*) facilitating lateral diffusion of TCRs and peptide–MHC molecules through T cell–APC contact zones.

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