Abatacept Binds to the Fc Receptor CD64 But Does Not Mediate Complement-Dependent Cytotoxicity or Antibody-Dependent Cellular Cytotoxicity

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ABSTRACT. ***Objective.*** To assess the ability of abatacept to mediate complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC) of antigen-presenting cells, and to characterize the binding of abatacept to the 3 Fc receptor classes.

Methods. CDC was measured *in vitro* using rabbit, baby rabbit, guinea pig, or human complement with human B cell line PM-LCL as the target. ADCC was also measured with PM-LCL target cells, but with human peripheral blood mononuclear cells from 12 healthy blood donors as effectors. Fc receptor binding was analyzed *in vitro* by flow cytometry and surface plasmon resonance (SPR).

Results. In contrast to unmodified CTLA4-Ig, abatacept did not mediate CDC or ADCC of target B cells. While abatacept was found to bind its target receptor, CD80/86, it did not appreciably bind the low-affinity Fc receptors CD16 and CD32 as measured by flow cytometry and SPR. Abatacept was found to minimally bind the high-affinity Fc receptor CD69 as measured by flow cytometry and SPR with a K_d of 3×10^{-7} M as measured by SPR.

Conclusion. Abatacept does not mediate CDC or ADCC of target B cells *in vitro* and has limited Fc receptor binding. These data support the concept that abatacept therapeutic activity is primarily due to the binding to CD80/86 through the CTLA4 extracellular domain and not through activities mediated by the modified Fc domain. (First Release Sept 1 2007; J Rheumatol 2007;34:2204–10)

Key Indexing Terms:
ABATACEPT CYTOTOXICITY Fc RECEPTOR

Abatacept, a selective T cell costimulation modulator, is the first in a class of agents for the treatment of rheumatoid arthritis (RA) that selectively modulates the CD80/CD86:CD28 costimulatory signal required for full T cell activation[1]. Abatacept has demonstrated efficacy in patients with active RA and an inadequate response to methotrexate, and also in those patients with an inadequate response to anti-tumor necrosis factor therapy[1,2]. Recently, clinical trials have also demonstrated that abatacept therapy provides significant improvements in the health related quality of life scores in these same patient populations[2,3].

In studies in mice, abatacept or related T lymphocyte-associated antigen 4 immunoglobulin (CTLA4-Ig) molecules demonstrated safety and efficacy in murine models of lupus[4], suppression of a primary antibody response[5], cardiac allograft rejection[6], human pancreatic islet cell xenograph rejection[5], graft versus host disease[7], and skin allograft rejection[8].

Abatacept is a genetically engineered fusion protein that consists of the extracellular domain of human CTLA4 linked to the modified Fc (hinge, CH2, and CH3 domains) portion of human immunoglobulin G1 (IgG1)[9]. The addition of a modified Fc region from human IgG1 increases the *in vivo* stability of abatacept in this soluble form, which has a half-life comparable to native human IgG1[10].

The Fc region of human IgG binds to multiple receptors that function in the activation or inhibition of immune responses such as antibody-mediated inflammation, phagocytosis, maturation of dendritic cells, antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), regulation of cytokines, and B cell proliferation[11,12]. In abatacept, however, a series of directed, select mutations in the hinge region were introduced to improve protein production and reduce Fc-mediated binding to abrogate the effects normally associated with the binding of human IgG1 heavy chain, such as CDC and ADCC. The mutations in abatacept include the replacement of cysteine with serine at amino acid positions 130, 136, and 139 of the IgG1 constant region, and replacement of proline with serine at amino acid position 148 (Figure 1).

The Fc IgG receptors have been divided into 3 classes based on close relationships in their extracellular domains:

From Discovery Biology, Bristol-Myers Squibb, Princeton, New Jersey; and Protein Therapeutics Development, Bristol-Myers Squibb, Hopewell, New Jersey, USA.

Supported by Bristol-Myers Squibb.

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Accepted for publication July 5, 2007.

CTLA4 Region:

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Aba   -MHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAA
C-Fc AMHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAA

Aba   TYMMGNELTFLDDSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPP
C-Fc TYMMGNELTFLDDSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPP

Aba   PYYLGIGNGTQIYVIDPEPCPDSD
C-Fc PYYLGIGNGTQIYVIDPEPCPDSD
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Fc Region:

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Aba   QEPKSSDKTHTSPPSPAPELLGGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
C-Fc --PKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
IgG1 KEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED

Aba   PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
C-Fc PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
IgG1 PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK

Aba   ALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES
C-Fc ALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES
IgG1 ALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES

Aba   NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS
C-Fc NGQPENNYKATPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS
IgG1 NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS

Aba   LSLSPGK------
C-Fc LSLSPGKHHHHHH
IgG1 LSLSPGK------
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Figure 1. Amino acid sequence comparison of abatacept (Aba), CTLA4-Ig (C-Fc), and the human IgG1 heavy chain constant region. IgG1 sequence begins at amino acid 125.

receptor I (here referred to as CD64), receptor II (here referred to as CD32), and receptor III (here referred to as CD16)[12]. The CD64 proteins are high-affinity receptors capable of binding monomeric IgG, whereas the CD16 and CD32 proteins bind IgG with lower affinities, usually restricted to recognizing IgG aggregates surrounding multivalent antigens[11].

This report describes studies that assess the Fc receptor binding of abatacept using surface plasmon resonance (SPR) binding to purified CD16, CD32, or CD64. Also assessed, by flow cytometry, was the binding of abatacept to cells expressing the Fc IgG receptors. Additionally, *in vitro* assays were used to determine the effector function of the Fc domain of abatacept using experiments to determine ADCC and CDC. The results of this study give further insight into the specificity of abatacept binding, functionality, and mechanism of action.

MATERIALS AND METHODS

Abatacept and CTLA4-Fc materials. Abatacept was manufactured at the facilities of Bristol-Myers Squibb in East Syracuse, New York. CTLA4-Fc was purchased from commercial suppliers (Biosource, Camarillo, CA, or R&D Systems, Minneapolis, MN, USA). Recombinant human CTLA4-Fc reagent is a fusion protein of CTLA4 fused to a nonmutated human IgG1 Fc tail. A comparison of the amino acid sequences between abatacept protein and CTLA4-Fc is provided in Figure 1.

Complement-dependent cytotoxicity. The effect of abatacept on CDC of target cells was measured *in vitro* using PM-LCL cells (an Epstein-Barr virus-transformed, CD80/86-positive human B cell line) as target cells. PM-LCL cells (1×10^6) were incubated at room temperature with antibody, abatacept at 30 µg/ml or 100 µg/ml, or CTLA4-Fc (Biosource) in 500 µl of RPMI medium containing 10% fetal calf serum (FCS). Positive controls used in the experiment were complement-fixing anti-human CD20 murine mAb as described[13], murine anti-CD86 mAb (BD Pharmingen), or CTLA4-Fc. After 30 min incubation, 500 µl of 2× concentration of various types of complement were added to the cells and incubated an additional 60 min at 37°C. PM-LCL cells were preincubated in RPMI medium plus 10% FCS (in the absence of abatacept, or control mAb) and then exposed to complement to determine the effect of complement alone. The types of complement used were low-tox-H rabbit complement at 1:32 dilution, baby rabbit complement at 1:16 dilution, guinea pig complement at 1:4 dilution (CedarLane Laboratories, Hornby, ON, Canada), and human complement at 1:8 dilution (Quidel Corp., Santa Clara, CA, USA). Each type of complement had been previously titrated to determine the optimal dilution for the detection of cell lysis. Following the incubation with complement, 50 µl propidium iodide (PI, 1 mg/ml) was added to detect dead cells. The PI-stained cells were subsequently analyzed by flow cytometry (Becton Dickinson FACScan) for uptake of PI. PI intercalates into the DNA of dead or dying cells; thus the percentage of cells that stain with PI indicates the extent of cell lysis.

Antibody-dependent cellular cytotoxicity. Target cells used in the assay were logarithmically growing PM-LCL cells labeled with sodium chromate (100 µCi/10^6 cells; NEN/Perkin Elmer) for 1 h at 37°C and washed extensively. Effector cells were peripheral blood mononuclear cells (PBMC) isolated by Ficoll-gradient separation from the whole blood of 12 different healthy blood

donors. Effector cells were combined with target cells in microwell plates at a ratio of 100:1 (optimized ratio), together with 30 µg/ml abatacept, CTLA4-Fc, ChiL6 control Ig (isotype-matched chimeric protein Ig), or buffer (effectors alone). The wild-type IgG1-Fc tail fused to CTLA4 is capable of facilitating complement fixation and serves as a positive control for these assays. Wells containing target cells with media only or with 1% Triton X-100 were assayed for spontaneous and maximal release of the target cells, respectively. All treatments were done in quadruplicate. Microplates were incubated 6 h at 37°C and 5% CO_2, then supernatants were removed and analyzed for chromium release by direct counting on a Cobra gamma counter. Percentage cytotoxicity was calculated according to the following formula: [(experimental release – spontaneous release)/(maximal release – spontaneous release)] × 100.

Fc receptor binding assayed by flow cytometry. Before evaluation of abatacept binding, the relevant expression of CD80, CD86, and Fc IgG receptors CD16, CD32, and CD64 on Raji and U937 cell lines was evaluated using fluorescence flow cytometry. Logarithmically growing Raji and U937 cells were washed in staining buffer [1% bovine serum albumin (BSA) in phosphate buffered saline (PBS)] and stained (1×10^6 cells/sample in 0.1 ml) with FITC-labeled murine IgG1 isotype control; with FITC-labeled murine anti-human CD80 and CD86 antibodies or phycoerythrin (PE)-labeled murine IgG1 isotype control (BD Biosciences, San Jose, CA, USA); and with PE-labeled murine anti-human CD16, CD32, and CD64 (Ancell Corp., Bayport, MN, USA) at 4°C for 30 min. Cells were washed and analyzed on a FACScan flow cytometer.

The specific binding of abatacept to CD80, to CD86, and to the Fc IgG receptors on U937 and Raji cell lines was examined. Logarithmically growing Raji and U937 cells were washed in staining buffer (1% BSA in PBS) and preincubated (1×10^6 cells/sample in 0.1 ml) with 750 µg/ml of whole mouse IgG (Sigma, St. Louis, MO, USA) to block Fc IgG receptor binding, or with saturating levels (300 µg/ml each) of purified murine anti-human CD80 and CD86 antibodies (BD Biosciences) to detect blocking of CD80/CD86 binding, or with no added antibodies for 30 min at 4°C. Following this preincubation, 30 µg/ml abatacept, or a ChiL6 control immunoglobulin (an irrelevant isotype-matched chimeric immunoglobulin), or no immunoglobulin was added, and then incubated an additional 30 min at 4°C. Cell samples were washed and incubated with FITC-conjugated donkey anti-human IgG $F(ab)'_2$ (Jackson Immunoresearch, West Grove, PA, USA) for 30 min at 4°C. Cells were washed and analyzed on a FACScan flow cytometer.

Surface plasmon resonance. Specific binding of abatacept or CTLA4-Fc (R&D Systems) to the 3 recombinant human Fc IgG receptor surfaces (CD16, CD32, and CD64) was compared by SPR detection using a BIAcore 3000 instrument. Recombinant human CD16b (Fcγ RIII B, NA2 allele), CD32b/c (Fcγ RII B/C), and CD64 (Fcγ RI) receptors (R&D Systems; catalog numbers 1597-FC, 1875-CD, 1257-FC, respectively) were immobilized covalently at low densities onto the surfaces of 3 flow cells on a CM5 sensorchip. Abatacept and CTLA4-Fc at a concentration of 1000 nM and a negative control were injected simultaneously over the 3 human Fc receptor surfaces at a flow rate of 25 µl/min, allowing for a 10 min association time and a 2 h dissociation time. Additionally, equilibrium binding of abatacept to the immobilized CD64 fusion protein was evaluated by SPR. CD64 was immobilized covalently via primary amino groups at a low density (~1000 resonance units, RU) on the surface of the flow cells of CM5 sensor chips. Abatacept was diluted to concentrations ranging from 25 nM to 25 µM and then injected over the human Fc receptor surface to generate binding sensorgrams. Equilibrium binding of these concentrations in RU was measured and plotted against sample concentration. These plots were fitted with a 4-parameter curve-fit model using XL Fit software (Excel). The dissociation constant K_d for the abatacept/Fc receptor interaction was determined at the inflection points of the individual fitted curves. The following experimental parameters and techniques were used to perform and evaluate the SPR experiments; diluent and running buffer: HBS-EP (BIAcore, HEPES-buffered saline with 0.05% P20 surfactant and 0.01% EDTA), flow rate 25 µl/min, temperature 25°C; abatacept concentrations: 25, 100, 250, 1000, 2500, 10,000, 25,000 nM; equilibrium binding measurements: BIAcore 3000 control software, report points set at 595 seconds after injection start; 4-parameter curve-fit: XL Fit (Excel, version 2 build 30).

RESULTS

Abatacept does not mediate complement-dependent cytotoxicity. A human B cell line, PM-LCL, was exposed to complement alone or in combination with anti-CD20, anti-CD86, CTLA4-Fc or abatacept at 30 and 100 µg/ml. Each component was tested for activity with rabbit, baby rabbit, guinea pig, and human complement. The background level of B cell lysis in the presence of complement alone ranged from 0% with rabbit complement to 4.2% with guinea pig complement (Figure 2). As positive controls, CTLA4-Fc, anti-CD86, and anti-CD20 were tested, and induced significant CDC in the presence of both rabbit complement (24.2%, 22.7%, and 34.6%, respectively) and baby rabbit complement (43.5%, 56.7%, and 61.9%). Only anti-CD20 demonstrated significant activity in the presence of guinea pig and human complement (67.4% and 53.8%). In contrast, abatacept had minimal effect on CDC, with a range of activity from 0.2% to 3.5% with rabbit complement, 0.3%–4.3% with baby rabbit complement, 3.9%–5.8% with guinea pig, and 0–4.1% with human complement. These effects were similar to complement alone and demonstrated no dose-dependence (Figure 2).

Abatacept does not induce antibody-dependent cellular toxicity. The ability of abatacept to induce ADCC was evaluated in separate PBMC preparations derived from 12 healthy blood donors. Figure 3 shows ADCC data expressed as percentage cytotoxicity (± SD) above the value of effectors alone. CTLA4-Fc-mediated ADCC was observed in all 12 donors with a range of 7.4% to 38.7%. In contrast, abatacept at 30 µg/ml demonstrated no significant ADCC in any donor, with values similar to those of control Ig fusion protein.

Fc IgG receptor and CD80/86 expression on U937 and Raji cell lines. Analysis of the U937 monocyte cell line by flow cytometry revealed that these cells express significant levels of the Fc IgG receptors CD32 and CD64, but insignificant levels of detectable CD80 and CD86 (Figure 4).

In contrast, flow cytometry analysis of the Raji B lymphoblastoid cell line revealed significant expression only of Fc IgG receptor CD32, as well as both CD80 and CD86 (Figure 4C, 4D). Neither cell line expressed measurable levels of Fc IgG receptor CD16 (Figure 4A, 4C). The cell-surface expression of CD64 and CD32 in the presence or absence of CD80 and CD86 expression, and the ability to compete for binding with anti-CD80/86 or murine IgG, enabled a direct comparison of the relative binding of abatacept to 2 of the 3 receptor subclasses.

Abatacept binding to U937 and Raji cell lines. Binding of control Ig to U937 cell lines was blocked with excess murine IgG, but not with anti-CD80/86, thus demonstrating that the binding of control Ig was specific for the Fc IgG receptor CD64 on the surface of the U937 cell line (Figure 5A). In this

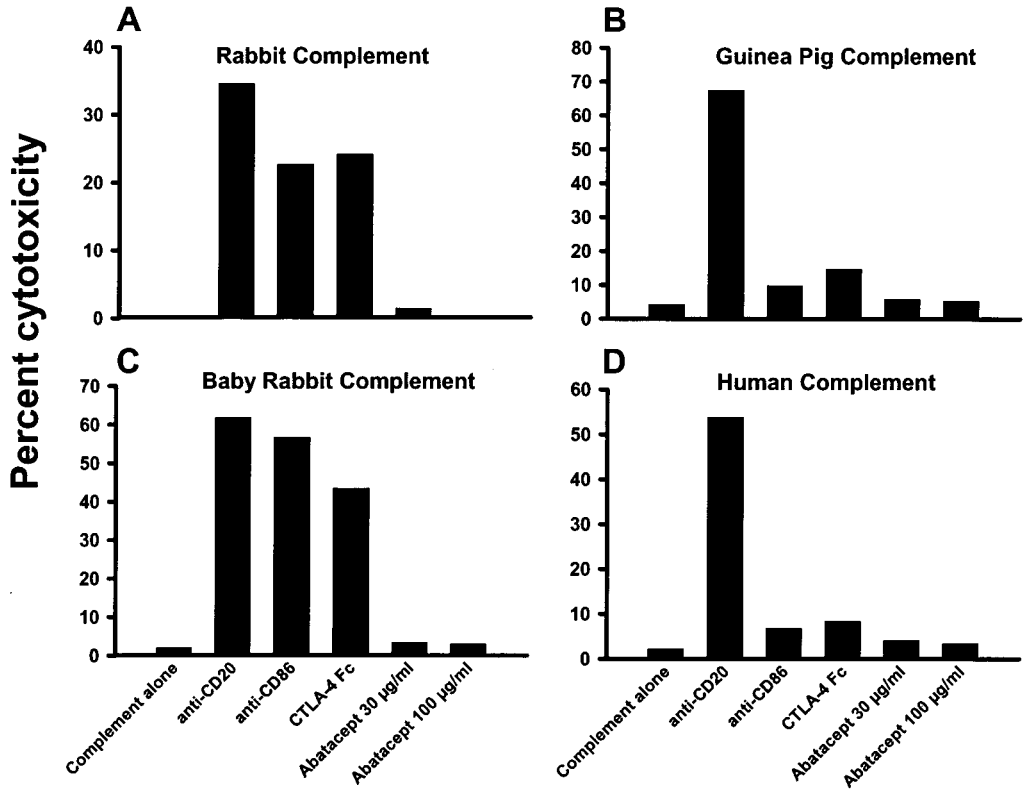

Figure 2. Abatacept does not mediate CDC of target B cells *in vitro.* Percentage toxicity was evaluated with rabbit, baby rabbit, guinea pig, and human complement.

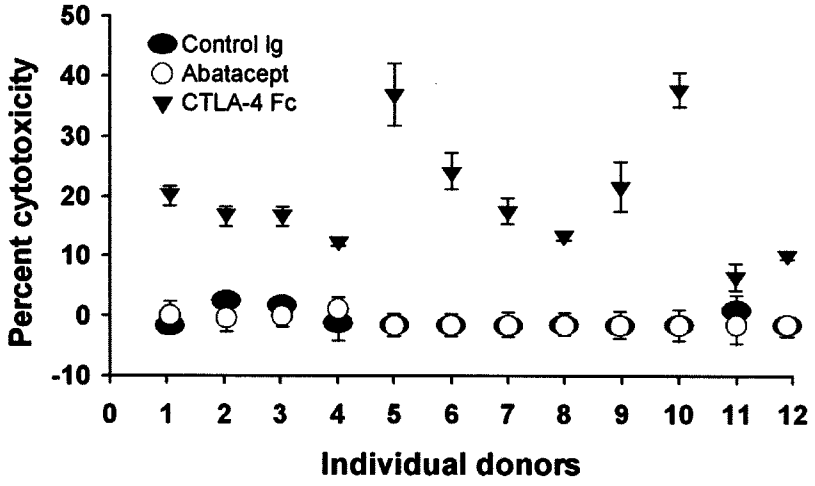

Figure 3. Abatacept does not induce target cell ADCC *in vitro*. Percentage cytotoxicity of PBMC preparations from 12 normal donors (± standard deviation) using abatacept, CTLA-4 Fc, or control Ig is expressed as the value above effectors alone.

same cell line, decreased Fc IgG receptor binding was observed with abatacept (Figure 5B). Similar to Ig control, the lower level of abatacept binding to these cells was ablated by excess murine IgG, but not anti-CD80/86, demonstrating that abatacept was binding specifically to the Fc IgG receptor CD64 on these cells.

In contrast, there was much less binding of control Ig to the CD32-expressing Raji cell lines (Figure 5C). Abatacept demonstrated strong binding to Raji cells, consistent with the high level of CD80 and CD86 on these cells (Figure 5D). Anti-CD80 and anti-CD86 blocked the binding of abatacept, whereas murine IgG had little effect, thus demonstrating that

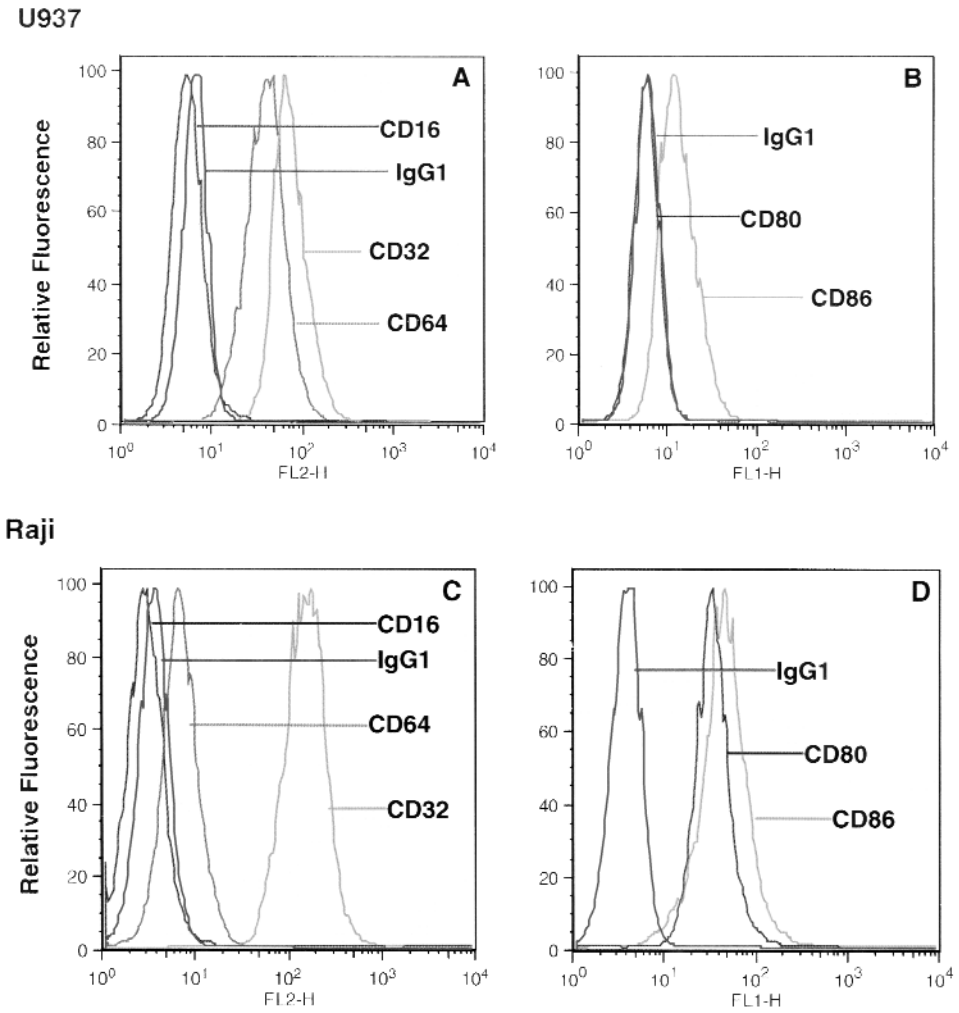

Figure 4. IgG Receptor and CD 80/86 expression on the U937 and Raji cell lines as measured by flow cytometry. A. U937 cells express CD32 and CD64 and not CD16 FcγR. B. U937 cells express insignificant levels of CD80/86. C. Raji cells express CD32 and not CD64 or CD16 FcγR. D. Raji cells express significant levels of CD80/86.

abatacept was specifically binding to CD80/86 on the surface of these cells mediated by the CTLA4 domain. The combined binding results from the U937 and Raji cell lines also demonstrate that abatacept specifically binds through the modified Fc region to the high-affinity Fc receptor CD64, but not to the low-affinity Fc receptor CD32 on the surface of these cells.

Surface plasmon resonance. Abatacept or CTLA4-Fc-positive control was injected at a concentration of 1 µM over an immobilized CD64 surface (described above). Figure 6A displays the combined sensorgrams for binding and dissociation of abatacept and CTLA4-Fc. Consistent with results from flow cytometric analysis, both molecules bound to CD64; however, CTLA4-Fc bound with higher affinity relative to abatacept. Additionally, abatacept demonstrated complete dissociation from the CD64 receptor approximately 2000 seconds after binding, while CTLA4-Fc did not completely dissociate from the CD64 receptor after binding. Table 1 summarizes the SPR binding results for abatacept and CTLA4-Fc to the 3 Fc receptors, CD64, CD32, and CD16. The data confirm the results from the cell-surface binding assays, and demonstrate that abatacept binds specifically only to CD64, and not to CD16 or CD32. Further, the binding of abatacept to CD64 was lower than the binding observed with CTLA4-Fc.

Because abatacept did not require the use of surface regeneration reagents (which caused loss of binding capacity with all 3 Fc IgG receptor surfaces) due to complete dissociation from CD64 after binding, it was possible to evaluate the equilibrium binding and dissociation constant (K_d) of abatacept to the CD64 high-affinity Fc receptor. A 4-parameter curve-fit of abatacept binding in concentrations from 25 nM to 25 µM is shown in Figure 6B. The K_d for abatacept binding to CD64 was calculated to be 3×10^{-7} M.

DISCUSSION

The results from these studies demonstrate that abatacept containing a modified Fc domain was determined to have no cyto-

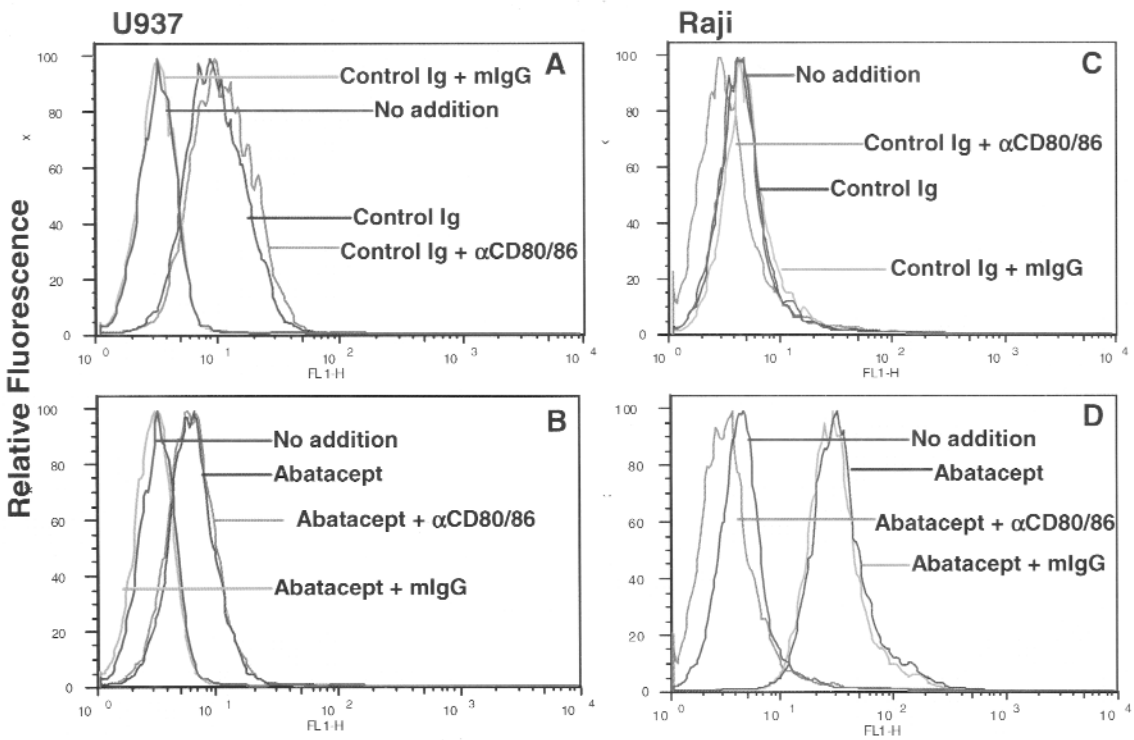

Figure 5. Abatacept binding to U937 and Raji cell lines as measured by flow cytometry. A. Background control binding to U937 cells. B. Abatacept binding to U937 cells. C. Background control binding to Raji cells. D. Abatacept binding to Raji cells.

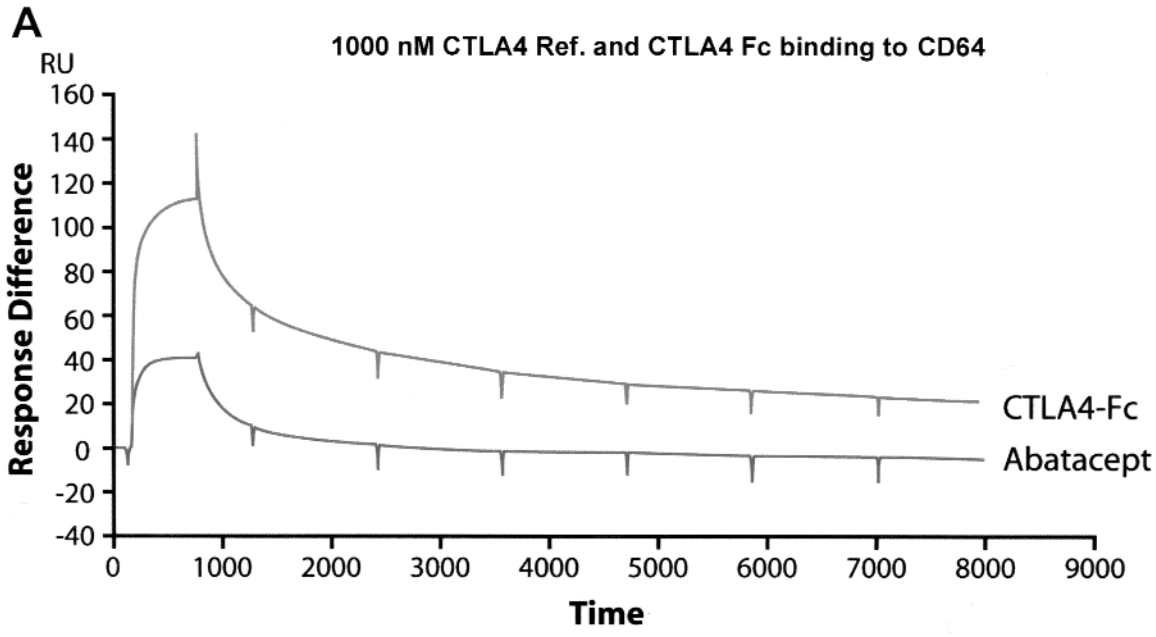

Figure 6. A. Sensorgram of abatacept and CTLA4-Fc binding to immobilized CD64 as measured by surface plasmon resonance (SPR).

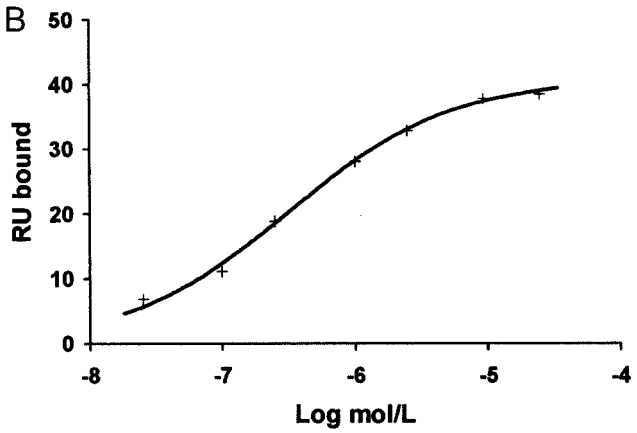

Figure 6. B. Abatacept binding to immobilized human CD64 as measured by SPR. Results are plotted using a 4-parameter curve-fit as described in Materials and Methods.

Table 1. Binding of abatacept and CTLA4-Fc fusion proteins as measured by surface plasmon resonance. Results are expressed in resonance units.

	CD16	CD32	CD64
Fc receptor density	1722	856	1563
Abatacept	–3	–2	40
CTLA4-Fc	219	55	113

toxicity associated with either CDC or ADCC mechanisms *in vitro*. In contrast, CTLA4-Fc, which differs from abatacept by containing a non-engineered Fc domain, was fully functional for both CDC and ADCC. These results indicate that directed mutations in the abatacept molecule appear to have yielded the intended function of diminishing cytotoxicity of CD80/CD86-expressing target cells.

To understand the mechanistic rationale for these results, *in vitro* binding studies using immobilized Fc receptors were conducted using SPR. The results demonstrate that abatacept bound only to the high-affinity Fc IgG receptor I (CD64). Although binding to additional allelic variants of the Fc receptors was not specifically investigated, there is a high degree of sequence homology in the extracellular domains of the 3 identified human CD32 genes and both CD16 alleles. As expected, CTLA4-Fc bound to all 3 immobilized Fc IgG receptors studied (CD16, CD32, and CD64). The binding of CTLA4-Fc to CD64 also occurred with higher affinity as compared to the binding observed for abatacept.

Cell-surface binding studies using Raji and U937 cell lines with flow cytometry analysis examined Fc binding in the context of cell-surface presentation of the Fc receptors. These studies confirmed that abatacept bound specifically via the Fc region to cells expressing CD64; however, in agreement with results from SPR, the observed binding for abatacept in cell-surface binding experiments was lower relative to CTLA4-Fc. Also in agreement with SPR, no specific binding of abatacept to CD32 was observed in the cell-surface binding experiments. These studies demonstrate that abatacept retains some level of Fc binding for the high-affinity Fc IgG1 receptor (CD64), but not the lower affinity Fc IgG receptors CD32 and CD16. Further, this binding is not functional *in vitro* for either CDC or ADCC-mediated activity. CD4-Ig fusion proteins that bind to CD64 but do not fix complement have been described[14]. The reduced binding of abatacept to CD64, undetectable binding to CD16 and CD32, and the lack of ADCC and complement fixation could be the result of steric hindrance or altered glycosylation, among other possibilities. For instance, the deletion of hinge cysteines makes conformational changes in the IgG portion of abatacept probable, and introduction of multiple serine residues makes altered glycosylation a possibility. For example, one of the 3 serines that replaced cysteines in the Fc region (serine 139) was found to be glycosylated. Together, these results support the concept that abatacept therapeutic activity is mediated through the binding of CD80/86 by the CTLA4 extracellular domain and not through activities mediated by the modified Fc domain.

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