Mutational Analysis and an Alternatively Spliced Product of B7 Defines Its CD28/CTLA4-binding Site on Immunoglobulin C-like Domain

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Summary

Costimulatory molecules B7 and B7-2 interact with T cell surface receptors CD28/CTLA4 and deliver a costimulatory signal essential for T cell growth. However, the structure basis of this interaction is not known. B7 and B7-2 are members of immunoglobulin (Ig) superfamily and their extracellular portion consists of an IgV- and IgC-like domain. Here we report that a naturally occurring, alternatively spliced form of B7 reveals that exon 3–encoded IgC domain is essential for CD28/CTLA4 binding. Mutational analysis of B7 demonstrates a critical role of several amino acids around loops between strands B and C and D and E, for binding CTLA4/CD28. These amino acids are clustered to form a single binding site centered at 201Y. A comparison of the effects of mutations on the binding of CD28 and CTLA4 reveals that CD28 and CTLA4 binds to the same site on B7. These results have important implications on the role of CTLA4 and CD28 in T cell costimulation. The structure of the CD28/CTLA4-binding site also provides valuable information for immune intervention targeted at the B7/B7-2–CD28/CTLA4 interactions.

Activation of T cells requires two types of biological signals: signal 1 is delivered by interaction between the TCR and MHC–peptide complex, whereas signal 2 is called the costimulatory signal (1–6). Recent studies from various laboratories indicate that signal 1 determines the specificity of T cell activation, whereas signal 2 determines the fate of T cells. T cells that receive both signals 1 and 2 clonally expand and differentiate into effector cells. In contrast, T cells that receive signal 1 alone are either functionally inactivated (anergized) and/or undergo programmed cell death (7–11).

Several interactions have been described that are involved in T cell costimulation (12–19). Accumulating evidence strongly suggests that the interaction between CD28/CTLA4 on T cells with their ligands B7 and/or B7-2 appears to be most important. First, anti-CD28 mAbs can augment T cell proliferation and prevent the induction of clonal anergy (20–23). Second, blocking B7 and B7-2 inhibits T cell proliferation and induces clonal anergy (11–24). APCs from mice with a targeted mutation of B7 gene show a significantly reduced costimulatory activity (25). Third, transfection of B7 or B7-2, the natural ligands for CD28 and CTLA4Ig confers costimulatory activity into the recipient cells (12, 15, 16, 26). In several tumor models tested, transfection with B7 increases tumor immunogenicity and leads a T cell–mediated rejection of tumors (27–30).

B7 and B7-2 are members of the Ig super family (15–17, 31). The extracellular portion consists of an IgV-like domain and an IgC-like domain. Murine B7 gene contains 5 exons encoding, respectively, signal peptide, IgV-like domain, IgC-like domain, transmembrane domain, and cytoplasmic domain. B7 and B7-2 bind to CD28 (32) and, with a higher affinity, to a less abundant receptor CTLA4 (33). It has been suggested that CD28 and CTL4 may transduce qualitatively different signals when engaged by B7 and/or B7-2 (34). As a first step to understand the mechanism of T cell costimulation mediated by B7/B7-2–CD28/CTLA4 interaction, we set out to determine the structural basis of these interactions. Here we report the B7-binding site for CD28 and CTLA4 as defined by a naturally occurring, alternatively spliced B7 and by site-directed mutagenesis.

Materials and Methods

Experimental Animals, Cell Lines, and mAbs

Male CBA/CaJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME) as donors of lymphocytes. B cell lymphoma CRCS-2, provided by Dr. Thorbecke (New York University Medical Center) (35), CH27, and M12 were cultured in RPMI medium containing 5% of FCS. Anti-B7 mAb 16.10A (36) was kindly

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provided by Dr. H. Reiser (Harvard Medical School, Boston, MA), mAb 7A5 was described fully (18).

Construction of the Fusion Proteins CD28Ig and CTLA4Ig

Fusion proteins CTLA4Ig and CD28Ig, comprised, respectively, the extracellular domains of murine either CTLA4 or CD28 and Fc portion of murine IgG2a, were generated according to a described procedure (33). Briefly, DNA encoding the extracellular region of murine CTLA-4 was amplified by PCR using the synthetic oligonucleotide CACAAGCTTGCCATGGCTTGTCTTGGACTC as forward primer, GGGCTCCTGATCAAGGTCAGAATCCGGGCATGGTTC as reverse primer, and cDNA from Con A–activated murine spleen T cells as template. The product of this PCR reaction was digested with HindIII and BclI and ligated together with a BclI/XbaI cleaved DNA fragment encoding the hinge, CH2 and CH3 domains of murine Ig Cγ2a. The latter fragment was amplified from cDNA prepared from a murine IgG2a hybridoma using the oligonucleotide GACCTGATCAGCGAGCCCAGAGGGCCCACA as forward primer, and GACCAGTCTAGATTTACCCGGAGTCCGGGAGAA as reverse primer. Ligation products were cloned into the CDM8 mammalian expression plasmid (Invitrogen, San Diego, CA) and the construction was verified by DNA sequence analysis. The encoded molecule comprises residues 1–161 of murine CTLA-4 fused to the hinge region of murine heavy chain Cγ2a. Similarly, the extracellular domain of CD28 (AA 1–150) was amplified from CD28 cDNA (37) kindly provided by Dr. J. Allison (University of California, Berkeley, CA), using CCCAAAGCTTCAATGACACTCAGGCTGCTG as forward primer and TTGTGGGCCCTCTGGGCTCGAGCTTAGGAGATGACTG as reverse primer. The PCR product was digested with ApaI. The IgG2a fragment was prepared by digesting CTLA4Ig construct with ApaI and XbaI. The CD28 fragment and the Ig fragment were ligated into pCDM8 vector digested with HindIII plus XbaI. The final construct was verified by DNA sequencing. The fusion proteins were prepared from CHO cells stably transfected with either CD28Ig or CTLA4Ig construct. The CHO cell supernatants were concentrated 10 times and used for this study. The supernatant contains 10 times the excess of fusion proteins required for saturating binding against wild-type B7 in flow cytometry.

Construction of B7-HSA, B7IgV-HSA, and B7IgC-HSA

All fusion proteins were generated by three piece ligation including HindIII plus XhoI-digested B7-fragments, XhoI + XbaI-digested HSA fragment and HindIII + XbaI-digested pCDM8 vector. The B7 and HSA fragments are generated as follows:

B7. The extracellular portion of B7, including the signal peptide, IgV and IgC domain, were amplified by PCR, using GCTCGAAGCTTATGGCTTGCAATTGTCAG as forward primer, and GTCAGCCATCTCGAGTTTTTCCCAGGTGAAGTC as reverse primer. pCDM8-B7, which contains the entire reading frame of B7, was used as template. The product of this reaction encodes M1-K236 which is the entire extracellular portion of B7.

B7IgV. pCDM8-B7 was used as template and the GCTCGAAGCTTATGGCTTGCAATTGTCAG as the forward primer and GGGCTCGAGGTCTGCAGATGGGTTTCC as reverse primer. The product of this reaction encodes M1-D159, which contains the signal peptide, B7IgV domain plus 15 AA from B7IgC domain as spacer.

B7-IgC. First, the signal sequence of B7 (B7SP) was amplified with GCTCGAAGCTTATGGCTTGCAATTGTCAG as a forward primer and GAAGTCAGCATCTGAAGACACTTGTGAAAGACG as a reverse primer, and exon 3 sequence of B7 (B7E3) was amplified with GTGTCTTCAGATGCTGACTTCTCTACCCCCAAC as a forward primer and GTCAGCCATCTCGAGTTTTTCCCAGGTGAAGTC as a reverse primer. A mixture of B7SP and B7E3 fragments were used as template, GCTCGAAGCTTATGGCTTGCAATTGTCAG as a forward primer and GTCAGCCATCTCGAGTTTTTCCCAGGTGAAGTC as a reverse primer. The final PCR product encodes the signal peptide and exon 3 encoded B7 IgC domain (M1-D37 + A143-K236).

HSA. The HSA fragment that contains all HSA protein sequence except signal peptide was amplified using GAAAAACTCGAGATGAACCAAACATCTGTTGCA as forward primer and CACAAGTAAGGTTCCTTCACAAAG as reverse primer. pCDM8-HSA (13) was used as template. The final PCR product encodes LE as linker plus extracellular portion and GPI-signal motif of HSA sequence, N27-stop codon.

All PCR products were verified by DNA sequencing.

Analysis of B7 mRNA by PCR

mRNA were prepared from 5 × 10^6 of either CRCS, M12.4.1, CH27, or total spleen cells by the fast-tract poly(A)$^+$ RNA preparation kit (Clontech, Palo Alto, CA). First strand cDNA were prepared using 50 ng of hexamer random primers, 1 μg mRNA, 200 U Maloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, MD). Similar results were obtained when poly dT or B7 reverse primer were used to prepared the first strand cDNA. The B7 or GAPDH DNA were amplified from the cDNA by PCR reaction as has been described. The primers used were: B7 forward primer GAAGCTATGGCTTGCAATTGTCAG, B7 reverse primer AGAAGAACTAAAGGAAGACGGTCT, GAPDH forward primer ATGGTGAAGGTCGGTGTGAACGGATTTGGC, and GAPDH reverse primer CATCGAAGGTGGAAGAGTGGGAGTTGCTGT. The RT-PCR products were detected by Southern blot using ^{32}P-labeled murine B7 or GAPDH cDNA as probe.

Cloning of the RT-PCR Products

Two forms of the B7 RT-PCR products were purified from agarose gels and cloned into the pCDM8 vector. The DNA inserts were sequenced and confirmed to contain the full-length B7 open reading frame sequence or that of an alternatively spliced B7.

Site-directed Mutagenesis

Site-directed mutagenesis was carried out based on PCR. Briefly, oligonucleotide primers between 25 and 40 bp long were synthesized to contain the desired mutations. These primers were used to generate B7-fragments that were then used to replace the wild-type fragment to generate the B7 mutants. The DNA sequences of the regions that contain the mutation and the junction of ligations were verified by DNA sequencing. The mutations are listed in Table 1.

T Cell Proliferation

CD4 T cells were purified from CBA/CaJ mouse spleen cells by two rounds of treatment with complement plus an antibody cocktail, that consists of: anti-B220 mAb RA3-3/6.1, anti-HSA mAb J11d (38), anti-CD8 mAb M31(39), anti–Mac-1 mAb M1/70.15.11.5H (40), and anti-FcR mAb 2.4G2 (41), all hybridoma supernatants mixed in an equal proportion.

CD4 T cells were stimulated with anti-CD3 mAb 2C11.145 (1:40 supernatants) (42). COS cells transfected with either FcγRIIB2 (FcR), or FcR plus B7 or B7 mutants were treated with mitomycin C (100 μg/ml, 37°C for 1 h), and used as accessory cells. After

Table 1. *Summary of Amino Acid Substitutions and the Binding Characteristics of the B7 Mutants*

Mutants	Substitutions		Binding (MF)‡		
	Nucleic acid(s)	Amino acid*	Anti-B7	CD28Ig	CTLA4Ig
Experiment 1					
B7	TTGGAA>CTCGAG	No change	224	47(100)	649(100)
C165G	TGC>GGC	C^{165}>G	109	0(0)	30(10)
Loop BC					
P172A	CCA>GCT	P^{172}>A	113	2(7)	8(3)
K173E	AAG>GAG	K^{173}>E	271	44(77)	772(98)
P174A	CCT>GCT	P^{174}>A	191	0(0)	61(11)
R175S	CGC>AGC	R^{175}>S	276	15(26)	334(42)
S177A	TCT>GCT	S^{177}>A	294	40(63)	476(56)
L179V	TTG>GTC	L^{179}>V	234	12(18)	274(40)
E180D	GAA>GAC	E^{180}>D	259	38(68)	622(83)
LE/VD	TTGGAA>GTCGAC	$L^{179}E^{180}$>VD	188	16(40)	281(51)
Loop DE					
QD/LE	CAGGAT>CTCGAG	$Q^{194}D^{195}$>LE	132	3(11)	103(26)
QDP/LER	CAGGATCCT>CTCGAGCGT	$Q^{194}D^{195}P^{196}$>LER	102	1(3)	42(15)
QDP/LEH	CAGGATCCT>CTCGAGCAT	$Q^{194}D^{195}P^{196}$>LEH	93	1(3)	36(13)
LY/VD	TTGTAC>GTCGAC	$L^{200}Y^{201}$>VD	105	0(0)	0(0)
Experiment 2					
B7	No change	No change	134	73(100)	235(100)
Loop DE					
E199A	GAA>GCA	E199>A	97	16(31)	108(63)
L200A	TTG>GCG	L200>A	105	28(53)	142(85)
Y201A	TAC>GCC	Y201>A	50	1(3)	0(0)

* The amino acid sequences of murine B7 IgC domain are shown below with the conserved amino acid in bold. Underlined are the locations of β-sheet strands based on the Ig superfamily.

^{143}**A**DF**S**T**P**N**I**T**E**SGNPSADTK**R**I**T**C**F**A**S**GG**F****P**K**P**R**F****S**W**L**E**N**GRELPG**I**N**T**T**I****S****Q**D**P**E**S**E**L**Y**T**I****S****S**QLDFNTTRNH**T**I**K**C**L**I**K**YGDAH
A B C D E F

VSEDFTWEKPPED^{240}.
G

‡ The data shown are MF of transfected COS cells after being stained with either anti-B7 mAb 10.16A.1 or chimeric molecules CD28Ig or CTLA4Ig, with that of controls (staining in the absence of first step-reagents) substracted. Numbers in the parentheses are % wild-type B7 binding after normalizing the cell surface expression of B7 mutants as described in experimental procedure. Each staining has been repeated for at least three times.

66 h of culture, the cells were pulsed with 1 μCi/well of [^{3}H]TdR for additional 6 h. T cell proliferation was measured by the cpm of the incorporated [^{3}H]TdR. The data shown were means of duplicates with variation <15%.

Flow Cytometry

Mock-transfected COS cells, or COS cells transfected with either wild-type or mutant B7 were used between 72 and 96 h after transfection. The antibodies used were: anti-B7 mAbs 10.16A.1, 3A12, 7A5 (18), anti-HSA antibody M1/69 (43), fusion protein CD28Ig, and CTLA4Ig. The second step reagents used were FITC-labeled, goat anti-mouse IgG, goat anti-hamster IgG, and mouse anti-rat IgG. All of these second step reagents give essentially identical background staining in the absence of primary antibody, therefore only one control is shown in each experiment. Details of flow cytometry have been described (44). Data shown in Table 1 are mean fluorescence (MF)[1] obtained by flow cytometry with that of the controls (in the absence of first step reagents) subtracted.

[1] *Abbreviation used in this paper:* MF, mean fluorescence.

The numbers in parentheses are the percent wild-type B7 binding after normalizing the cell surface expression of individual mutant B7 based on binding of an anti-B7 mAb 10.16A.1, that binds the IgV domain regardless of mutation in the IgC domain. Wild-type B7 binding percentage was calculated by the following formula: % wild-type B7 binding = $100 \times x/y$, where $x = (MF_{CD28Ig \text{ or } CTLA4Ig})/(MF_{anti\text{-}B7 \text{ mAb}10.16A.1})$ when mutant molecules are used, and y is the same ratio when the wild-type B7 molecule is used.

Results

A Naturally Occurring, Alternatively Spliced B7 Reveals a Critical Role of B7IgC Domain in Binding CD28 and CTLA4. In the process of analyzing the B7 expression in a number of different cell lines by PCR, we have observed two major forms of B7 cDNA: a longer form of 930 bp and a shorter form of ~650 bp (Fig. 1 *a*). Cloning and cDNA sequencing revealed that the larger band is B7, whereas the shorter band is a truncated B7 with a deletion from base 428 to base 699 (number begins at the start codon), which is the entire sequence of exon 3 (Fig. 1 *b*). Thus this shorter form of B7 is a product from an alternative splicing of B7. As the only extracellular portion of the protein encoded by this alternatively spliced B7 gene is the IgV domain, we call it B7IgV. To test whether B7IgV contains CD28/CTLA4-binding sites,

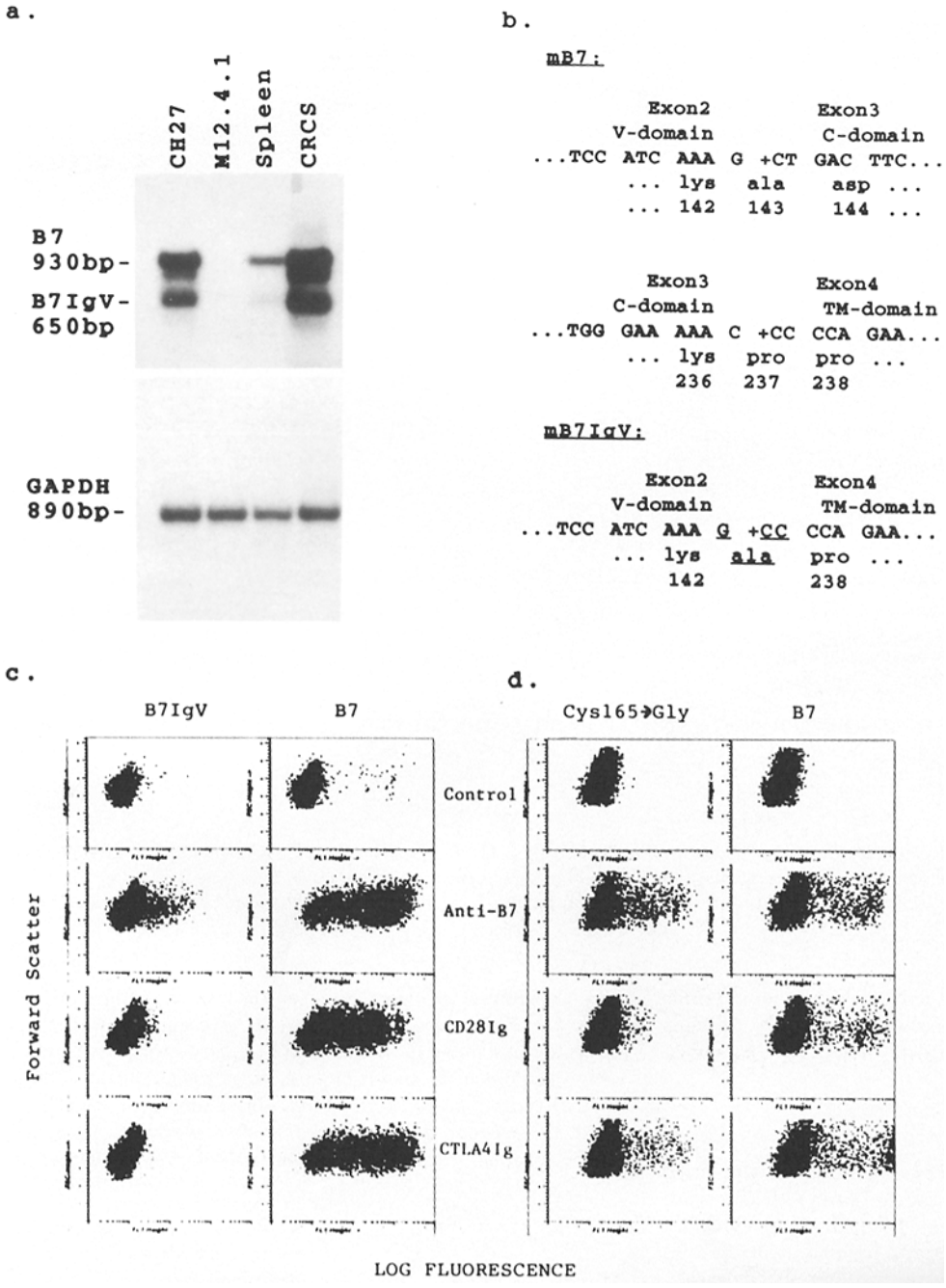

Figure 1. A naturally occurring, alternatively spliced B7 maps the CD28/CTLA4-binding site into the exon 3–encoded region. (*a*) Amplification of various forms of B7 mRNA by RT-PCR. First strand cDNA prepared with random hexamer primers from RNA of either spleen or B leukemic cell lines CH27, CRCS, or M12 were amplified using B7 of GAPDH forward and reverse primers that should amplify the open reading frame of the full-length B7 (12). The PCR products were analyzed using either a full-length B7 open reading frame probe or a GAPDH probe (12). (*b*) The short B7 cDNA is an alternatively spliced form of B7. (*Top*) The junctional sequence of the full-length B7 that is identical to the sequence of the DNA in the higher molecular weight band; (*bottom*) a predicted junctional sequence of an alternatively spliced B7, B7IgV, that is identical to the sequence of the DNA in the lower molecular weight band. (*c*) Analysis of the binding of B7IgV, B7 to anti-B7 mAb 7A5, and fusion proteins CD28Ig and CTLA-4Ig. Comparable expression of B7IgV is detected with two other anti-B7 mAbs 3A12 and 10.16A.1 (data not shown). (*d*) Mutation 165C>G eliminates CD28 binding and reduces CTLA4Ig binding by ~10-fold.

we generated fusion proteins that consist of murine IgG2a Fc portion and extracellular domains of either murine CD28 (CD28Ig) or murine CTLA4 (CTLA4Ig). We then used these fusion proteins and a panel of anti-B7 mAbs to monitor the cell surface expression of B7 and B7IgV on COS cells transiently transfected with these genes. Whereas the B7 binds anti-B7 mAbs (Fig. 1 *c* and data not shown) CD28Ig and CTLA4Ig, B7IgV binds anti-B7 mAbs but not CTLA4Ig and CD28Ig (Fig. 1 *c*). The lack of binding to CTLA4 was not due to poor cell surface expression of the B7IgV, because CTLA4Ig binding to wild-type B7 is better than the anti-B7 mAbs used. Thus the structure encoded by exon 3, which is the B7IgC domain, is involved in binding CD28/CTLA4. Consistent with this notion, a mutation of 165C>G that destroys the disulfate chain in the IgC domain, eliminates B7 binding to CD28Ig and reduces its binding to CTLA4Ig by 10-fold (Fig. 1 *d*, Table 1).

Site-directed Mutagenesis Defines the Roles of the Conserved Residues within the B7IgC Domains for CD28/CTLA4 Binding. So far, four molecules that bind CD28/CTLA4 have been identified, namely human B7, human B7-2, murine B7, and murine B7-2 (15–17, 26, 31). As these four molecules cross-react with both human and murine CD28/CTLA4, we reasoned that the structure involved in binding CD28/CTLA4 should be conserved among these four molecules. Previous analysis of B7 sequence homology (15–17, 26, 31) revealed that 17 of the 98 amino acids in the IgC domain are conserved. Among them, two are cysteines at positions 165 and 219. All 11 conserved amino acids between two cysteines are clustered at or near two loops, if we use the structure of IgC-domain as a reference (Table 1 legend). One is the loop between strands B and C (loop B-C), and the other between strand D and E (loop D-E). This analysis suggests that these two regions are involved in binding to CD28/CTLA4. To further define the CD28/CTLA4-binding sites, we generated a series of B7 mutants by site-directed mutagenesis and tested their binding to CD28Ig and CTLA4Ig. Binding to anti-B7 mAbs was used as an indicator for cell surface expression of B7 mutants. As shown in Table 1 and Fig. 2, mutants 172P>A and 174P>A fail to bind CD28Ig, and their binding to CTLA4Ig is reduced by 10-fold. In contrast, mutations of 173K>E, 177S>R, and 180E>D have little effect on B7 binding to CD28Ig/CTLA4Ig. Mutations 175R>S, 179L>V reduced CD28Ig binding by four- to fivefold, and CTLA4Ig binding by twofold. These results indicate that amino acids at the B-C loop are involved in binding CTLA4/CD28.

Similarly, amino acids in the D-E loop are critical for CD28/CTLA4 binding. As shown in Fig. 3 and Table 1, mutation 194Q>L195D>E reduces CD28 binding by 10-fold and CTLA4Ig binding by fivefold. An additional mutation in this region 194Q>L195D>E196P>H, or 194Q>L195D>E196P>R eliminates CD28-binding site and reduces CTLA-4Ig binding by 10-fold. The most drastic effect is caused by mutation 200L>V201Y>D, that leads to elimination of binding to both CTLA4Ig and CD28Ig. This result demonstrates that one or both of these amino acids is essential for binding CD28/CTLA4Ig. To test this, we replaced, one by one, three amino acids 199E, 200L, and 201Y with A and tested the effect of these mutations on B7 binding to CD28 and CTLA4. As shown in Fig. 4, replacement of either 199E or 200L by A does not significantly reduce the binding of B7 to CD28 and CTLA4, suggesting that these two amino acids are not critical for CD28/CTLA4Ig binding. In contrast, mutation 201Y>A eliminated the binding of both CD28 and CTLA4, suggesting that the 201Y plays a critical role in binding CD28 and CTLA4.

The results of the site-directed mutagenesis reveal that all mutations that affect CD28Ig binding affect CTLA4Ig binding. Interestingly, the effects of mutations on CD28 and CTLA4 binding follow a grossly similar hierarchy (Table 1), although CTLA4Ig binding is generally more resistant to mutations. These results strongly suggest that CTLA4 and CD28 have the same binding site on B7.

An important issue is whether IgC domain contains all

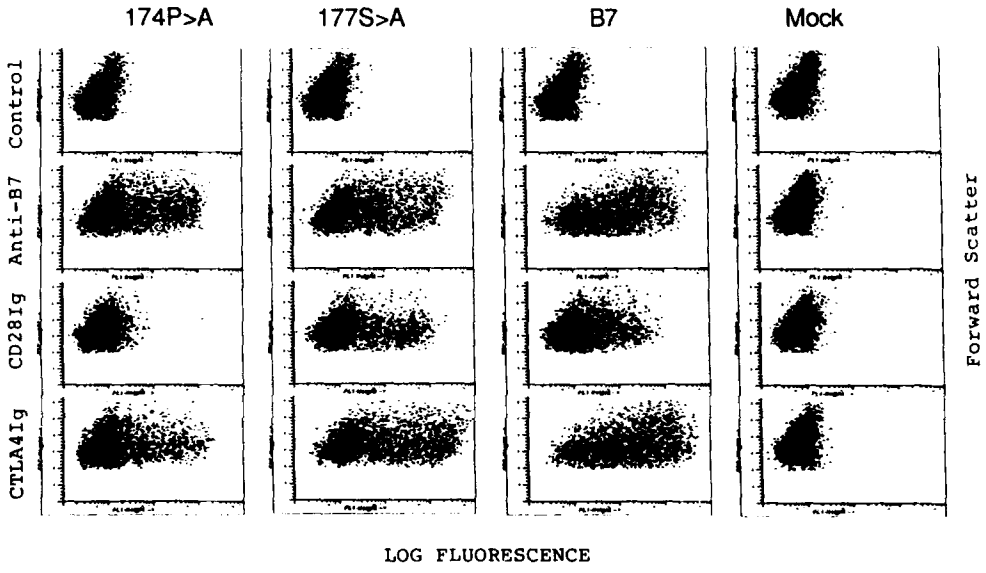

Figure 2. Effects of two representative mutations in BC loop on the CD28Ig/CTLA4Ig binding. As described in the Fig. 1 legend, mock-transfected COS cells or COS cells transfected with either wild-type B7 or two B7 mutants as indicated, were incubated with either anti-B7 mAb 7A5 or fusion protein CD28Ig/CTLA4Ig. The binding of the first step reagents were detected by FITC-labeled second step reagents. Data shown were FACS profiles.

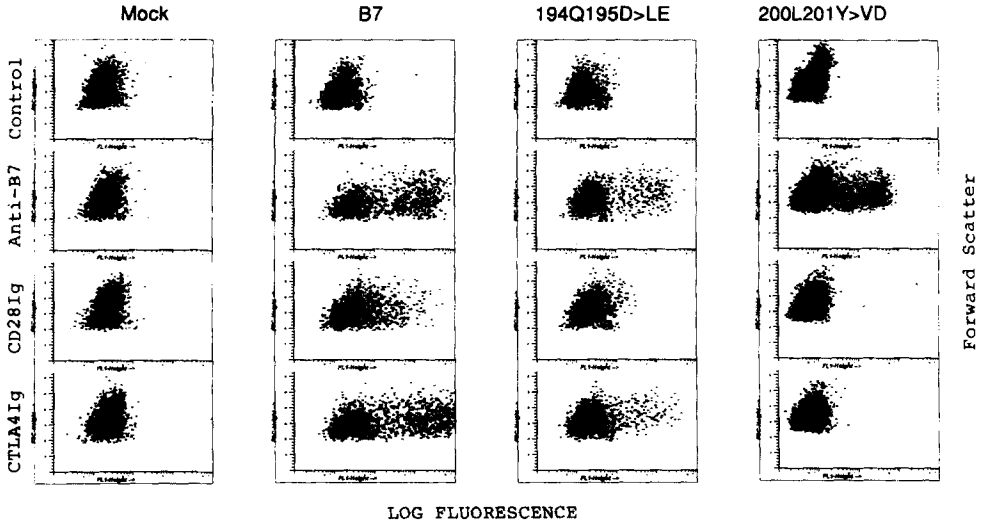

Figure 3. Effects of two representative mutations in DE loop on CD28/CTLA4Ig binding. See Fig. 2 legend for details.

the necessary information for binding CD28/CTLA4. To address this issue, we deleted the IgV domain and transfected the B7IgC into COS cells. No binding is detected in COS cells transfected with the B7IgC (data not shown). However, as no antibody against this domain is available, it is equally possible that this domain is not stable enough to give cell surface expression. We therefore constructed a chimeric molecule that consists of the B7IgC domain and the heat-stable antigen extracellular domain. As shown in Fig. 5, whereas the chimeric molecules are expressed on the cell surface as demonstrated by its binding to anti-HSA mAb, they do not bind CD28Ig and CTLA4Ig. These results can be interpreted as evidence that B7IgV domain may play a role in the CTLA4/CD28 binding. However, it should be stressed that as the overall structure of the IgC domain cannot be ascertained at this stage, it is equally possible that the role of B7IgV is to allow correct folding of the CD28/CTLA4 binding sites.

Binding to CD28/CTLA4 Is Essential for the Costimulatory Activity of B7. To determine whether binding to CD28/CTLA4 was necessary for the costimulatory activity of B7, we compared the costimulatory activity of wild-type B7 that binds CD28/CTLA4 with the B7 mutants that have lost the ability to bind CD28/CTLA4. As shown in Fig. 6 *a*, wild-type B7 costimulates T cell proliferative responses to anti-CD3 mAb, whereas the alternatively spliced form of B7 (B7IgV) fails to do so. Because anti-B7 mAb 7A5 binds B7IgV rather

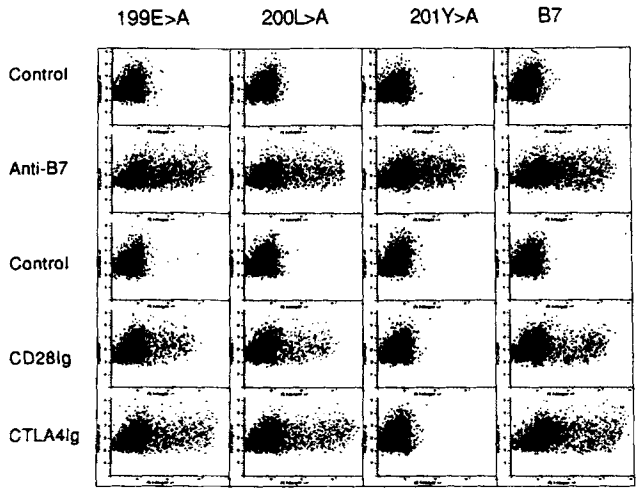

Figure 4. The central role of 201Y in CD28/CTLA4 binding. COS cells transfected with either wild-type B7 or B7-201Y>A mutant were compared for their binding to anti-B7 mAb 7A5 or fusion proteins.

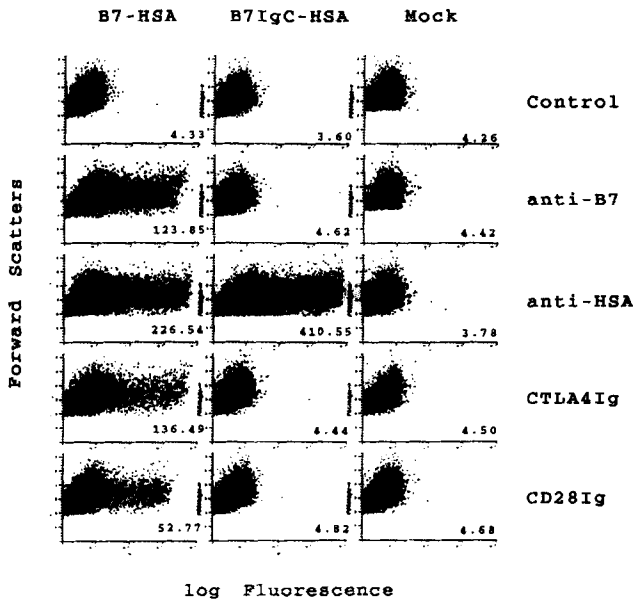

Figure 5. B7IgC-HSA fails to bind CTLA4Ig and CD28Ig. B7IgC-HSA were constructed as described in Materials and Methods. COS cells were transiently transfected with either B7-HSA of B7IgC-HSA. 3 d after transfection, the transfected COS cells were analyzed for the expression of the HSA (M1/69), B7 (7A5) epitopes or CD28/CTLA4Ig-binding sites.

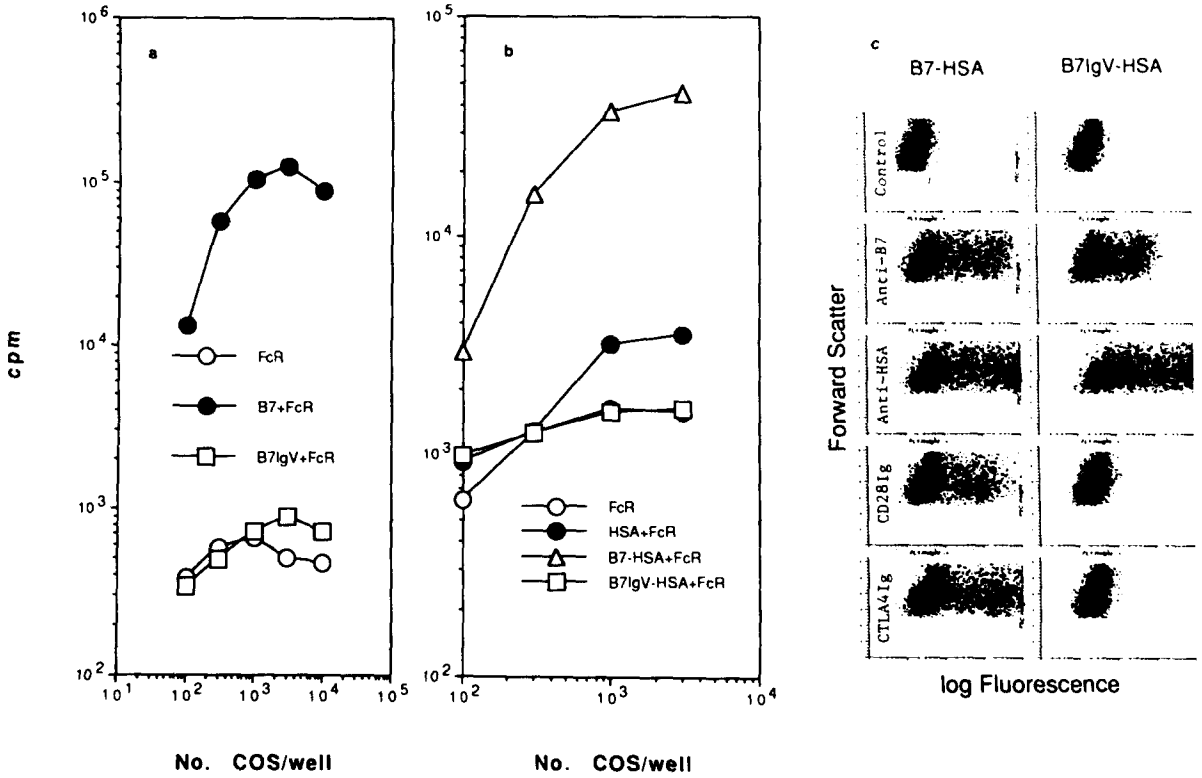

Figure 6. B7IgV cannot costimulate proliferation of CD4 T cells. COS cells were transfected with either FcR or with FcR plus either wild-type or mutant B7 molecules and used to stimulate proliferation of CD4 T cells in the presence of anti-CD3 for 72 h. (*a*) Proliferation of CD4 T cells using COS cells transfected with FcR or FcR plus either wild-type B7 or B7IgV. (*b*) Costimulatory activity of B7-HSA and B7IgV-HSA for CD4 T cells. COS cells transfected with either FcR or FcR in conjunction with either B7-HSA or B7IgV-HSA were treated with mitomycin C and used as accessory cells. T cell proliferation has been repeated for at least three times. (*c*) Expression and binding characteristics of the B7-HSA or B7IgV-HSA.

poorly (Fig. 1), this lack of costimulation could be attributed to poor cell surface expression of B7IgV. To overcome this problem, we generated two fusion proteins. One consists of the extracellular domain of B7 and that of the HSA, the other is made of HSA and B7 fragments AA 1–159 (that consists of B7IgV domain plus 15 amino acids from IgC domain as spacer between B7IgV and the HSA). This truncated molecule does not contain the structures necessary for CD28/CTLA4 binding. Again, B7-HSA but not B7IgV-HSA costimulates T cell proliferation (Fig. 6 *b*). These results demonstrate that the B7IgV domain does not costimulate T cell proliferation. COS cells transfected with HSA failed to costimulate T cell proliferation as opposed to HSA-transfected CHO cells (13, 45), presumably because of differential glycosylation of HSA (6). As measured by anti-HSA antibody M1/69, the amount of fusion proteins expressed on the cell surface is comparable to B7-HSA and B7IgV transfected COS cells, although the anti-B7 mAb 7A5 binds B7-HSA fusion protein ~10-fold better than the B7IgV-HSA (Fig. 6 *c*). The HSA portion does not interfere with the conformation of wild-type B7 and B7IgV as their binding to a panel of three anti-B7 mAbs as well as CTLA4/CD28 is similar to that of B7 and B7IgV (Fig. 1 and Fig. 6 *c*, and data not shown). More importantly, mutation 201Y>A, which eliminates CD28/CTLA4 binding, also eliminates the costimulatory activity of B7; whereas B7 mutants 199E>A and 200L>A, which retain the CTLA4/CD28-binding site, have costimulatory activity (Fig. 7). Thus binding to CD28/CTLA4 is critical for the costimulatory activity of B7, consistent with a previous study that showed that the Fab fragment of anti-CD28 blocks the costimulatory activity of B7 (26).

Discussion

We have reported here that a naturally occurring, alternatively spliced B7 that lacks IgC domain has lost the CD28/CTLA4Ig-binding sites. Site-directed mutagenesis based on homology analysis revealed several conserved amino acids within the IgC-like domains are involved in binding CD28 and CTLA4. To determine whether these amino acids can be clustered in the three-dimensional structure, we have mapped the residues of our mutagenesis study to an available Fab C-domain structure (3FAB, reference 46). There are a few x-ray protein structures available from the Brookhaven Protein Data Bank that contain IgC-like domains. Supposition of these structures shows that the loops B-C and D-E and these four strands from all known Fab IgC domains align very well structurally (data not shown). Residues which upon mutation significantly reduce CD28/CTLA4 binding around loops B-C and D-E form a localized region towards one end

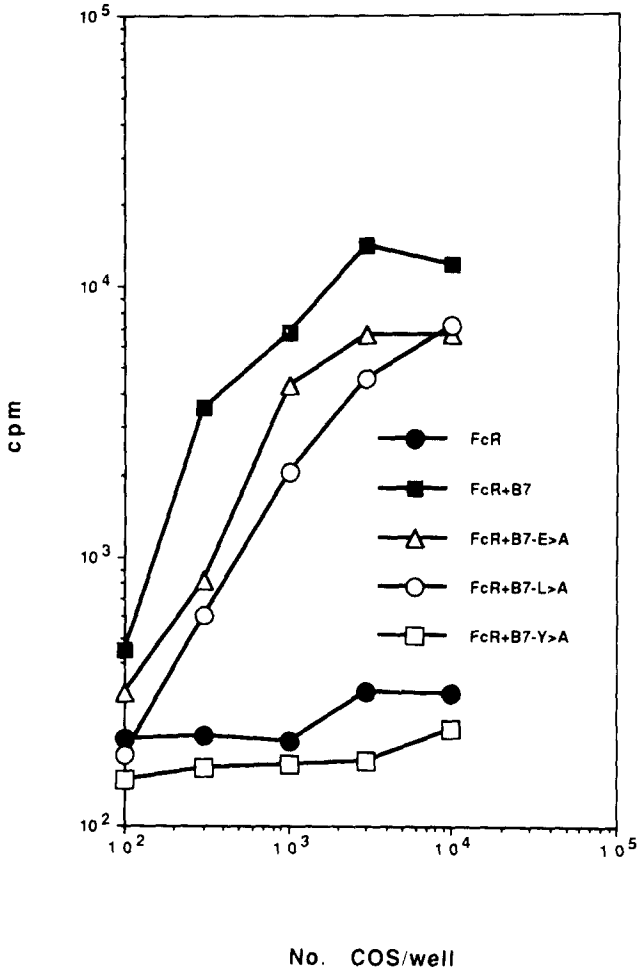

Figure 7. Costimulatory activity of B7 requires CD28/CTLA4-binding site. COS cells were transfected with either FcR alone or FcR plus wild-type B7 or B7 mutants. The costimulatory activity of the COS cells were determined by proliferation of CD4 T cells to anti-CD3 mAb. Data shown are representative of two independent experiments.

of the molecule even though they come from different locations of the sequence. This indicates that B7 has a localized CD28/CTLA4-binding site (Fig. 8). Although the backbone of 201Y is on the other side of the molecule from this region, its side chain is located approximately at the center of this region, sandwiched between loop B-C and loop D-E, consistent with our results that mutation of this residue has the most drastic effect on CD28/CTLA4 binding. This tyrosine is conserved among human B7, B7-2, and murine B7, and in murine B7-2, it has been conservatively replaced by phenylalanine.

Whereas it is formally possible that mutations of the amino acids in B-C and D-E loops affect the CD28/CTLA4 binding by causing a general distortion of the three-dimensional structure of B7, the fact that several mutations in such a localized area selectively affect CD28/CTLA4 binding indicates that this is highly unlikely. Using three anti-B7 mAbs (7A5, 3A12, and 10.16A.1, which bind three independent epitopes in B7IgV domain as determined by cross-blocking studies, data not shown) we found that the conformation of B7 is not significantly affected by the mutations we generated, although some mutations have a minor effect on the strength of 7A5 binding (data not shown). It is of interest to note that 172P and 174P are conserved among a large number of IgC-like domains, thus raising a possibility that these two amino acids are important for the overall structure of Ig. However, it is equally possible that the CD28/CTLA4-binding site evolves from a conserved structure of IgC-like domain.

An unresolved question is the contribution of the B7IgV domain in CD28/CTLA4 binding. Several lines of evidence can be interpreted in light of the possibility that B7IgV may be involved in binding CTLA4/CD28. First, our present study reveals that several anti-B7 mAbs (18, 36) that blocked CTLA4Ig binding and B7-mediated T cell costimulation bind to the IgV domain. It should be emphasized that because the amino acids involved in binding CD28/CTLA4 defined by the site-directed mutagenesis are located in an area close

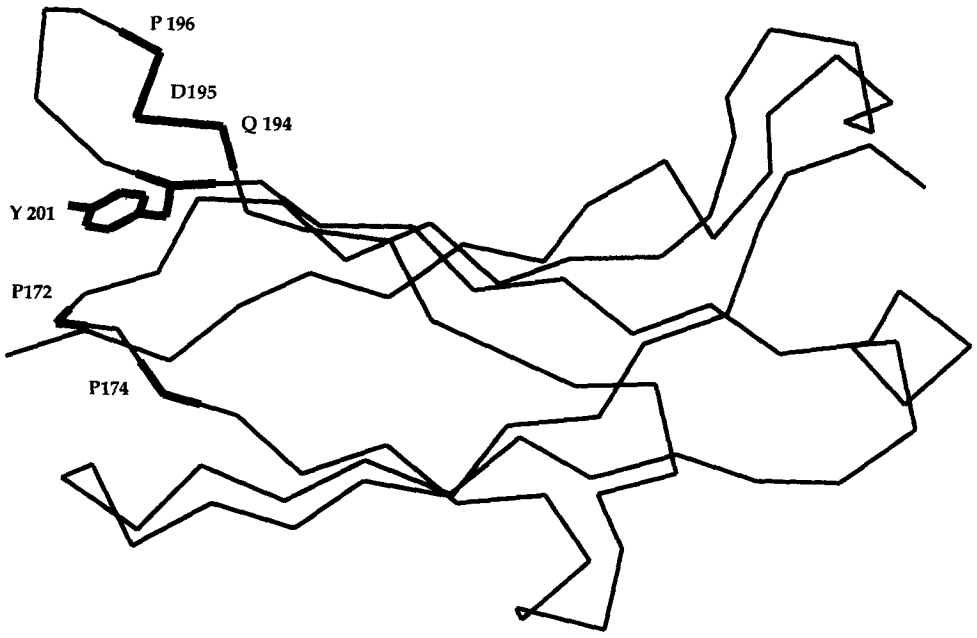

Figure 8. Three-dimensional model of B7IgC domain based on the x-ray structure of Ig Fab fragment. Only the 201Y side chain is shown explicitly, the rest of the molecule is represented by Cα trace. Residues that upon mutation significantly reduce CD28/CTLA4 are labeled (■), all of them are within 7Å of 201Y.

to the IgV domains, the effect of several anti-IgV antibodies can be explained by steric hindrance. Second, the IgC domains have not been shown to bind CD28/CTLA4 in the absence of IgV domain. Third, Inobe et al. (47) reported that a stable cell line thought to be transfected with the B7IgV domain can bind CD28 and CTLA4. However, as no characterization of the stable transfectant has been done to verify the gene product, it is hard to reconcile the results with our study.

An important conclusion that can be derived from the results of our mutational analysis is that CD28 and CTLA4 bind to the same site on the B7IgC domain. Thus, all mutations that affect CD28 binding affect CTLA4 binding. More strikingly, the effects of mutations on CD28 and CTLA4 binding follow a grossly similar hierarchy (Table 1), although CTLA4Ig binding is generally more resistant to mutations, consistent with an earlier finding that CTLA4 has a ~17-fold higher affinity for B7 (33). This conclusion has an important implication on the mechanism of costimulation by B7. As CD28 and CTLA4 have different cytoplasmic domains, they may transduce qualitatively different signals (34). As CD28 and CTLA4 do not form heterodimers (33), and because they are located at distinct patches on the T cell surface as detected by confocal microscopy (34), the simplest mechanism that allows direct interaction of CD28-mediated signaling machinery with that mediated by CTLA4 would be a cross-linking of the two molecules by B7 or B7-2. By necessity, this model implies that CTLA4 and CD28 have distinct binding sites on B7 to allow cross-linking by a single B7 molecule. Our results that CTLA4 and CD28 bind B7 at the same sites do not support this model. We favor an alternative hypothesis that CTLA4 and CD28 bind different B7 molecules and transduce distinct biological signals. The facts that CTLA4 is expressed after T cell activation and that CTLA4 has a higher affinity for B7 or B7-2 suggests that CD28–B7/B7-2 interaction is likely to dominate at the induction phase of immune responses, whereas CTLA4–B7/B7-2 interaction is likely to dominate effector phase of immune responses. Our previous study (30) demonstrated that B7 can play an important role at both the induction and effector phases of T cell responses, thus raising an interesting possibility that CD28–B7 interaction may deliver a signal necessary for induction of T cell responses, whereas CTLA4–B7 interaction may deliver a signal for effector phase of T cell responses. Experiments are underway to test this hypothesis.

Whereas it is known that antibody stimulation through CD28 transduces the costimulatory signal for T cells, and that B7 binds CD28 and delivers the costimulatory activity, it is still formally possible that B7 costimulate T cells via other yet unidentified receptors on T cells. Our results demonstrate that B7 mutants that lose their binding to CD28 and CTLA4 also lose their costimulatory activity. These results demonstrate that B7 costimulate T cells by binding CD28/CTLA4.

Finally, recent studies have demonstrated that immune intervention targeted at the CD28/CTLA4–B7/B7-2 interaction have a vast potential in transplantation (48, 49) and tumor therapy (27–30). The structure of B7-binding sites for CD28/CTLA4 defined in this study provides valuable information for immunotherapy.

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