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T Cell Proliferation-Augmenting Activities of the Gene 3 Protein Derived from a Phage Library Clone with CD80-Binding Activity

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We have isolated a phage clone, F2, by panning a phage library with a CTLA4-conformation recognizing mAb (anti-CTLA4 mAb). The unique sequence of 15 amino acids with an internal disulfide bond was inserted in the gene 3 proteins of F2 phage clone (F2-g3p). We show here that 1) F2-g3p was recognized with anti-CTLA4 mAb but not with anti-CD28 mAb, and 2) F2-g3p bound to CD80 but not to CD86. The surface plasmon resonance analysis showed that F2-g3p strongly bound CD80. F2-g3p inhibited the binding of CTLA4 to CD80 but not to CD86. In contrast, F2-g3p weakly inhibited the binding of CD28 with CD80. When hen egg lysozyme (HEL)-primed lymph node cells were stimulated with HEL in the presence of F2-g3p in vitro, cell proliferation was highly potentiated. In the absence of antigenic stimulation, F2-g3p induced no T cell proliferation, indicating the costimulatory nature of F2-g3p. The T cell-augmenting activity of the F2 clone was eliminated when the F2 clone was preincubated with CD80-Ig before the addition to the cultures, indicating the involvement of CD80-binding in the F2-g3p-mediated immunopotentiation. Thus, the F2 motif conferred CD80-binding activity and an immunoregulatory function to the g3p. The Journal of Immunology, 1998, 161: 6622–6628.
T cell augmenting activity of F2 clone or F2-g3p was abolished when they were preincubated with CD80-Ig before addition to cultures. These results suggested that F2-g3p preferentially blocked the negative signaling through the interaction of CTLA4 with CD80.

**Materials and Methods**

**Phage proteins**

The fd phage display library was constructed as described (30). The purification of phage proteins was performed as described previously (31). Briefly, the fd phages (7 mg/ml) were incubated with 1% SDS at 37°C for 20 min. The DNA and coat proteins were separated by size-exclusion chromatography on a HiLoad Superdex 200 (26/60) column (Pharmacia, Uppsala, Sweden). Elution was conducted by 50 mM Na2SO4 containing 5 mM citric acid and 0.1% SDS (pH 3.0) at the flow rate of 300 μl/min. The peaks were monitored at 280 nm by LC-10AD liquid chromatograph (Shimazu, Tokyo, Japan), equipped with SPD-10A UV-VIS detector (see Fig. 1A). SDS-PAGE was performed as described previously (24). The g3p fraction was resolved as a single band at 44 kDa, whereas the g8p fraction was resolved as a broad band by SDS-PAGE (see Fig. 1B). The g8p fraction might contain a minute amount of the other phage proteins. The fractions were extensively dialyzed with PBS (pH 7.3) before use.

**Fusion proteins**

The recombinant molecules were marine cosmopolitan molecules fused with human IgG Fc. The CD80-Ig, CD86-Ig, CD28-Ig, and CTLA4-Ig were prepared as described previously (24). These are divalent molecules.

**Antibodies**

The hamster anti-mouse CTLA4 mAb (UC10–4F10–11; PharMingen, San Diego, CA), the hamster anti-mouse CD28 mAb (37.51; PharMingen), human IgG Fc fragment (hFc; Jackson ImmunoResearch, West Grove, PA), AP-conjugated goat anti-human IgG (Zymed, San Francisco, CA), and AP-conjugated goat anti-hamster IgG (Zymed) were purchased. Anti-fd phage serum was prepared by hyperimmunizing BALB/c mice i.p. with 10 μg of K7 phage clone (control clone with the unrelated motif) in Freund’s incomplete adjuvant.

**ELISA**

ELISA was performed as described previously (24, 29). The binding activities of phage proteins were as follows: plastic plates (Nunc, Copenhagen, Denmark) were coated with the phage proteins (30 ng/60 μl/well) in 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl (TBS) containing 0.02% NaN3 at 4°C for 1 h. Blocking was done by using 350 μl of 1% BSA (Sigma, St. Louis, MO). The plates were washed five times with TBS containing 0.05% Tween-20 (TBS/Tween) and once with TBS. Varying doses of mAb or fusion proteins were added, followed by detection with AP-conjugated goat anti-hamster IgG or by AP-conjugated goat anti-human IgG (H+L) at a dilution of 1:250. The substrate (85 μl) consisted of 1 mg/ml p-nitrophenylphosphate (Wako, Osaka, Japan) and 10% diethanolamine (Wako) in TBS. Absorbance was read at 405 nm by microplate photometer (InterMed NJ-2011, Tokyo, Japan).

**Inhibitory activities of phage proteins**

CTLA4-Ig or CD28-Ig was coated (10 ng/40 μl/well) on 96-well plastic plate. After blocking plates with BSA, biotinylated anti-CTLA4 mAb or biotinylated CD80-Ig (10 ng) premixed with varying concentrations of phage proteins at 4°C for 1 h was added to wells of plates. The binding was monitored by AP-conjugated streptavidin.

**SPR**

The kinetic studies of the binding between CTLA4-Ig and anti-CTLA4 mAb were done by SPR using BIAcore 2000 system (Pharmacia, Piscataway, NJ) at 25°C. CTLA4-Ig (10 μg/ml) was immobilized onto the sensor chip CM5 according to amine coupling protocol, and the unreacted sites were blocked with 1 M Tris-HCl (pH 8.5). The association reaction was initiated by injecting 5, 10, 20, or 30 mM anti-CTLA4 mAb. The analyte injection was performed at a flow rate of 5 μl/min. The dissociation reaction was done by washing with PBS containing 0.005% Tween-20. At the end of the cycle, the sensor chip surface was regenerated for 30 s with 0.1 M glycine-HCl buffer (pH 2.5). The association (kA km) and dissociation constants (kD km) were calculated using BIAcore system software (BIAevaluation, Version 2.1).

**T cell proliferation assay**

BALB/c mice were immunized s.c. with 50 μg HEL in Freund’s incomplete adjuvant 2 wk before or immunized i.p. with 10 μg of WT phage in PBS 4 wk before use. Cell cultures were conducted as described (24). Briefly, lymph node cells (1.5 × 106/0.2 ml/well) were stimulated with 5 μg/ml HEL in the presence or absence of varying doses of various phage clones or phage proteins (g3p or g8p) in round-bottom 96-well plates (Iwaki Glass, Tokyo, Japan). In the case of WT-primed mice, spleen cells (1.5 × 106/0.2 ml/well) were stimulated with or without 5 μg/ml of WT in the absence or presence of phage proteins in flat-bottom 96-well plates. Three days later, T cell proliferation was monitored by pulsing cells with 0.5 μCi [3H]thymidine (Amersham, Arlington Heights, IL) for the final 18 h.

**Results**

F2-g3p is recognized with anti-CTLA4 but not with anti-CD28 mAb

To characterize the CD80-binding activity of the F2 motif, phage proteins were purified by gel permeation chromatography (Fig. 1A). The second peak was the g3p fraction, which was resolved as a single band with m.w. of 44 kDa by SDS-PAGE (Fig. 1B). The third broad peak consisted of the g8p as a major protein containing a trace of other phage proteins (31).

The F2 motif of 15 amino acids was inserted in the g3p of the F2 phage clone. First, we tested the reactivity of the g3p and the g8p derived from the F2 phage clone (F2-g3p and F2-g8p) to anti-CTLA4 mAb (UC10–4F10–11) by ELISA. F2-g3p (30 ng/well)
was coated on the plates and tested on the reactivity to anti-CTLA4 mAb or anti-CD28 mAb. As shown in Fig. 2A, anti-CTLA4 mAb bound to F2-g3p. Neither anti-CD28 mAb nor normal hamster IgG bound to F2-g3p. The binding of anti-CTLA4 mAb to F2-g3p was dependent on the doses of F2-g3p (Fig. 2B). F2-g8p was not recognized with anti-CTLA4 mAb (Fig. 2C). The anti-CTLA4 mAb did not bind to the g3p or g8p derived from control phage (K7 or L4) that were randomly selected from the phage library (data not shown). These results indicated that F2-g3p bore the specific motif (F2 motif) that was recognized by anti-CTLA4 mAb.

**F2-g3p binds to CD80 but not CD86**

The anti-CTLA4 mAb recognized the CTLA4 conformation at the binding site to CD80 (24). This result suggested that F2-g3p might be a CTLA4 mimic with the binding activity to CD80 or CD86 molecules. Therefore, we tested this possibility by ELISA. First, the binding activity of the CD80-Ig to F2-g3p or F2-g8p was examined. The CD80-Ig (200 ng/well) were added to plastic wells that had been coated with varying doses of F2-g3p (Fig. 3A) or F2-g8p (Fig. 3B). Following the addition of AP-conjugated anti-human IgG and substrates, the absorbance of 405 nm was read at the indicated time after the onset of development.

**Discussion**

These results were summarized in Fig. 4D. The bindings of CTLA4-Ig/CD80-Ig and the anti-CTLA4 mAb/CTLA4-Ig showed the almost equal $K_d$ values (0.31 and 0.48 nM) respectively. In contrast to these reactions, the F2-g3p preparation associated to CD80-Ig at a slower rate ($k_{on} = 0.63$ vs $2.4 \times 10^7$ M$^{-1}$s$^{-1}$) and it dissociated from CD80-Ig at a faster rate ($k_{off} = 2.7$ vs $0.76 \times 10^{-4}$ s$^{-1}$), resulting in a lower avidity ($K_d = 4.29$ nM) in comparison with the CTLA4-Ig preparation.

**F2-g3p strongly inhibits the binding of CD80 to CTLA4 but only weakly to CD28**

To examine the inhibitory activities of F2-g3p on the interaction of costimulatory molecules, binding of CD80 to CTLA4 or to CD28 was assessed by ELISA in the presence or absence of F2-g3p or control phage molecules. The biotinylated CD80-Ig was preincubated with varying concentrations of F2-g3p, F2-g8p, or anti-CTLA4 mAb for 1 h at 4°C and then added to CTLA4-Ig-coated plates (Fig. 5A). Anti-CTLA4 mAb strongly inhibited the binding of CD80-Ig to CTLA4-Ig. F2-g3p also inhibited the binding of CD80-Ig to CTLA4-Ig (10 μg/ml) was immobilized onto the sensor chip CM5 (Fig. 4A). The association reaction was initiated by injecting the varying concentration of anti-CTLA4 mAb. The analyte injection was performed at a flow rate of 5 μl/min. The binding of CTLA4-Ig (Fig. 4B) or F2-g3p (Fig. 4C) to the immobilized CD80-Ig was also examined under identical experimental conditions. The gel permeation chromatography showed that the preparation of F2-g3p as well as CTLA4-Ig and CD80-Ig contained significant amounts of high m.w. aggregates. To demonstrate the binding activity of F2-g3p, we tentatively calculated the binding parameters using a simple one site-binding model from the response curve. Therefore, each binding parameter might be overestimated in terms of affinity (see Discussion). These results were summarized in Fig. 4D. The bindings of CTLA4-Ig/CD80-Ig and the anti-CTLA4 mAb/CTLA4-Ig showed the almost equal $K_d$ values (0.31 and 0.48 nM) respectively. In contrast to these reactions, the F2-g3p preparation associated to CD80-Ig at a slower rate ($k_{on} = 0.63$ vs $2.4 \times 10^7$ M$^{-1}$s$^{-1}$) and it dissociated from CD80-Ig at a faster rate ($k_{off} = 2.7$ vs $0.76 \times 10^{-4}$ s$^{-1}$), resulting in a lower avidity ($K_d = 4.29$ nM) in comparison with the CTLA4-Ig preparation.
CD80-Ig to CTLA4-Ig and showed complete inhibition at 50 ng/well, whereas F2-g8p showed no inhibitory activity on this binding. Neither F2-g3p nor F2-g8p showed any inhibition on the binding of CD86-Ig to CTLA4-Ig-coated plates while the anti-CTLA4 mAb completely inhibited this interaction (Fig. 5B). In contrast, F2-g3p showed only partial inhibitory activity on the binding of CD86-Ig to CTLA4-Ig-coated plates while the anti-CTLA4 mAb completely inhibited this interaction (Fig. 5B).

**FIGURE 4.** SPR analysis on the binding of F2-g3p to CD80. Analysis was performed by BIAcore 2000 system. A, The binding of anti-CTLA4 mAb to CTLA4-Ig. CTLA4-Ig (10 μg/ml) was immobilized onto the sensor chip CM5. Anti-CTLA4 mAb was injected at the indicated concentrations ranging from 1.85 to 100 nM. B, The binding of CTLA4 to CD80. CD80-Ig was immobilized onto the sensor chip by the same protocol as A. CTLA4-Ig was injected at the indicated concentrations ranging from 3.4 to 61.6 nM. C, The binding of F2-g3p to CD80. CD80-Ig was immobilized onto the sensor chip by the same protocol as A. F2-g3p or CTLA4-Ig was injected at the indicated concentrations. D, Summary of SPR analysis. The binding parameters of CTLA4-Ig to CD80-Ig were calculated using a simple one site-binding model. The association ($k_{on}$) and dissociation ($k_{off}$) rate constants were estimated from the response curve and the dissociation equilibrium constant ($K_d$) was calculated from the equation of $K_d = k_{off}/k_{on}$.

**FIGURE 5.** F2-g3p inhibits the binding of CD80 to CTLA4 completely but partially to CD28. A, F2-g3p inhibits the binding of CTLA4 to CD80. The biotinylated CD80-Ig (10 ng/well) dissolved in TBS containing 100 μg/ml of BSA was preincubated with varying concentrations of F2-g3p, F2-g8p, or anti-CTLA4 mAb for 1 h at 4°C and then added to CTLA4-Ig (10 ng/well)-coated plastic plates. Experimental conditions are identical in A–D. B, F2-g3p does not inhibit the binding of CTLA4 to CD86. The biotinylated CD86-Ig was used in lieu of the biotinylated CD80-Ig. C, F2-g3p partially inhibits the binding of CD28 to CD80. The biotinylated CD86-Ig was used in lieu of the biotinylated CD80-Ig of C.
CD80-Ig to CD28-Ig-coated plates (Fig. 5C). At 16 ng/well of F2-g3p, CD80/CTLA4 interaction was strongly inhibited, but CD80/CD28 interaction was the least affected. In CD28 binding systems (Fig. 5, C and D), anti-CD28 mAb (37.51) did not exhibit the complete inhibition even at 50 ng/well. It is uncertain whether this result may be due to the characteristics of the 37.51 clone or CD28-Ig. F2-g8p showed no blocking effect. CD86-Ig to CD28-Ig binding was not inhibited by either F2-g3p or F2-g8p, but it was inhibited with anti-CD28 mAb (Fig. 5D). These results suggested a distinct structural requirement for CD80 binding domain to either CTLA4 or CD28, in which F2-g3p could distinguish each other.

**The augmenting activity of F2-g3p on T cell proliferation**

The inhibitory activity of F2-g3p on the interaction of CD80 with CTLA4 suggested that F2-g3p might affect the T cell activation stimulated with Ags. We have previously shown that the F2 phage clone highly augmented the proliferative response of WT-primed spleen cells in comparison with the responses induced with WT or control phage clones (24). To examine whether the augmenting activity of the F2 phage is due to costimulatory nature, the HEL-primed lymph node cells were stimulated with or without 5 µg/ml of HEL in the absence or presence of F2-g3p, F2-g8p, K7-g3p, or K7-g8p at 0.1, 0.3, 1.0, 3.0, or 10 µg/ml, respectively. The T cell proliferation was augmented by the simultaneous stimulation of Ag (HEL) and F2 on day 0. HEL-primed lymph node cells were stimulated with 3.0 µg/ml of HEL. Various phages (3 µg) were added on day 0, 1, or 2 into cultures.

To test the timing of the F2 addition relative to antigenic stimulation, various phage clones were added on day 0, 1, or 2 after the HEL stimulation (Fig. 6C). The augmentation was induced only by the simultaneous addition of both HEL and F2 phage clone. This effect was not induced by the addition of L4 or WT phage clone. The experiments using F2-g3p also gave the same results (data not shown). Thus, the F2 motif inserted in g3p molecules showed an immunoregulatory activity which is characteristic for the responses regulated with the T cell costimulatory molecules.

**Involvement of CD80-binding activity in the F2-mediated augmentation of T cell response**

HEL-primed lymph node cells showed the augmented T cell response when stimulated with HEL in the presence of F2-g3p or CD80-Ig alone in vitro (Fig. 7A). K7-g3p or human IgG did not induce this effect. To determine the involvement of CD80-binding activity of F2 motif in the augmentation of T cell response, HEL-primed cells were stimulated with HEL in the absence or presence of F2 phages (0.5 µg/ml) preincubated with concentrations of CD80-Ig or hFcγ ranging from 0.1 to 1.0 µg/ml. CD80-Ig strongly inhibited the F2-mediated augmentation of T cell response, whereas control hFcγ or CD86-Ig (data not shown) had no effects (Fig. 7B). This effect was also shown using human CD80-Ig (data not shown). These results indicated that the binding of F2-g3p to CD80-Ig neutralized each other’s augmenting activity and suggested the involvement of CD80-binding activity of F2 in the augmentation of T cell response.

**Discussion**

The F2 phage clone was isolated by anti-CTLA4 mAb from a phage display library (29). We demonstrated that F2 phage strongly augmented the T cell proliferation of spleen cells derived from WT-immunized BALB/c mice (24). As the peptide motif is inserted in the g3p molecule of phage, we studied here whether the
g3p molecule derived from the F2 phage clone was responsible for the augmenting effect through the regulation of T cell costimulatory signals. In accordance with the characteristics of F2 phage clone, F2-g3p bound to CD80 but not to CD86 (Fig. 3). The SPR analysis also showed the strong binding of F2-g3p to CD80. The binding valency of the molecule affects the strength and stability of the binding interaction (15, 32, 33). Greene et al. (32) reported that the CTLA4-Ig as a divalent molecule showed the complex kinetics with the high ($K_d = 1\text{ nM}$) and low avidity ($K_d = 150\text{ nM}$) phases to the immobilized CD80-Ig in comparison with the monovalent CTLA4 ($K_d = 211\text{ nM}$), which might be due to the different valencies of the complex. They demonstrated that the association reaction of the monovalent or divalent CTLA4-Ig to CD80-Ig was very rapid with $k_{on}$ value of $4.4 \times 10^5$ or $8.9 \times 10^5 \text{ M}^{-1}\text{S}^{-1}$, respectively. This value was similar to the $k_{on}$ value ($2.4 \times 10^5 \text{ M}^{-1}\text{S}^{-1}$) of CTLA4-Ig preparation used here (Fig. 4). Morton et al. (15) reported that the $k_{on}$ value was estimated to be $1.4 \times 10^6 \text{ M}^{-1}\text{S}^{-1}$ in our experimental condition. F2-g3p associated to CD80 at a slower rate than CTLA4-Ig. Greene et al. (32) also determined the dissociation reaction of monovalent or divalent CTLA4-Ig from the immobilized CD80-Ig as $9 \times 10^{-3}$ or $4 \times 10^{-3} \text{ S}^{-1}$ of $k_{off}$, respectively. In our results, the $k_{off}$ of the CTLA4-Ig preparation from CD80-Ig was calculated to be $7.6 \times 10^{-5} \text{ S}^{-1}$ (Fig. 4). This slow rate may be due to the aggregated form of the CTLA4-Ig preparation and rebinding in dissociation reaction (32). Morton et al. (15) reported that the $k_{off}$ value was estimated to be $5.8 \times 10^{-4} \text{ S}^{-1}$. The $K_d$ values on the binding of divalent CTLA4-Ig to CD80-Ig were estimated to be comparable between Greene’s (1 nM), Morton’s (0.4 nM), and ours (0.3 nM). The $k_{off}$ of the F2-g3p preparation from CD80-Ig was calculated to be $2.7 \times 10^{-4} \text{ S}^{-1}$, indicating that F2-g3p dissociated from CD80-Ig at a faster rate than the CTLA4-Ig preparation used here. The $K_d$ value on the binding to CD80 was eventually one order lower than that of the binding of CTLA4-Ig to CD80-Ig or CTLA4-Ig to anti-CTLA4 mAb (Fig. 4D).

F2-g3p inhibited the binding of CTLA4 to CD80 but not to CD86. It is to be noted that F2-g3p inhibited the interaction of CD80 with CTLA4 completely, but with CD28 only partially (Fig. 5). Furthermore, F2-g3p did not inhibit the interaction of CD86 with CTLA4 or with CD28. Site-directed mutagenesis analysis demonstrated that the exon 3-encoded IgC domain of CD80 is essential for CD28/CTLA4 binding and suggested that CD28 and CTLA4 bind to the same site on CD80 (10). In this context, our results suggested that F2-g3p finely distinguished the conformational structural differences of the binding site of CD80 to CTLA4 from that of CD80 to CD28. Furthermore, it was reported that the IgV domain of human CD86 is sufficient to costimulate T lymphocytes (16). Although our results were obtained from murine molecules, it is conceivable that the binding site of F2-g3p to CD80 may be located to IgC domain but not IgV domain because F2-g3p showed no interference on the binding of CD80 to CTLA4/CD28.

F2-g3p as well as the F2 phage clone showed remarkable augmenting activity on the T cell proliferation in the presence of antigenic stimulation (Fig. 6). The augmentation was induced only by the simultaneous addition on day 0 of both HEL and F2 phage in vitro (Fig. 6C). F2-g3p or F2 phage did not induce T cell proliferation without antigenic stimulation, indicating their T cell costimulatory characteristics. CTLA4-Ig inhibits T cell activation because CTLA4-Ig binds CD80 as well as CD86. On the other hand, F2-g3p binds CD80 but not CD86. It is conceivable that F2-g3p directly stimulates T cells through their CD80 molecules. However, it is unlikely because CTLA4-Ig as well as F2-g3p should augment the T cell proliferation if the direct stimulation through CD80 molecules is possible. We prefer an alternative hypothesis that F2-g3p may selectively block the negative signal through CTLA4/CD80 interaction but not through CTLA4/CD86. The binding of CD80 to CD28 was slightly blocked at $16\text{ nM}$ of F2-g3p (Fig. 5, A and C) while the binding of CD80 to CTLA4 was strongly inhibited, indicating that F2-g3p interfered only partially with the interaction of CD80 to CD28. Furthermore, F2-g3p did not inhibit the interaction of CD86 to CD28 (Fig. 5D). Thus, the positive signal via CD80/CD28 and CD86/CD28 might become dominant in comparison with the negative signal, eventually resulting in the augmentation of T cell proliferation. Not only F2-g3p but also CD80-Ig (Fig. 7) and CD86-Ig (data not shown) enhanced the T cell proliferation induced with HEL in vitro. These effects may also be attributed to the inhibition of CTLA4 binding for negative regulation. F2-g3p was relatively more effective in the
enhancing activity than CD80-Ig. This might be due to the concentration of natural counter receptor molecules (CD28) in the microenvironment of early response phase, i.e., high concentration of CD28+ cells competitively reduce the chance for the binding of cell surface CTLA4 to CD80-Ig. In contrast, CD80 expression is very low at early response phase and could not work as an effective competitor for the binding of F2-g3p to CTLA4.

The blockade of CTLA4 engagement with its natural ligands by Fab fragments of anti-CTLA4 mAbs enhanced T cell proliferation both in vivo and in vitro (8, 18, 20). The magnitude is 3 to 4 times higher than the response without the anti-CTLA4 mAb (20). In the T cell proliferation system induced with suboptimal dose (0.6 μg/ml) of anti-CD3 mAb, F2-g3p enhanced the response at a comparable degree with the response induced with anti-CTLA4 mAb (data not shown). In the case of F2-g3p inhibition but not anti-CTLA4 mAb inhibition, CD86 could interact with CTLA4 to deliver the negative signal even in the presence of F2-g3p. Thus, these results suggested that the negative signal through CD86/CTLA4 seemed to be less effective.

Phage library has been used to determine the continuous epitope (sequential determinant) mapping, and the synthetic peptides of these motifs were examined for their biological activities (22, 26). Yayon, A., D. Aviezer, M. Safran, J. L. Gross, Y. Heldman, S. Cabilly, D. Givol, and E. Katchalski-Katzir. 1997. Isolation of peptides that inhibit binding of basic fibroblast growth factor to its receptor from a random phage-epitope library. Science 273:435.

This study suggests that the F2 sequence may be useful in developing molecules and influence the elicited immune response (34). Truneh, A., M. Reddy, P. Ryan, S. D. Lyn, C. E. L. Myidents, R. P. Sekaly, and J. P. Allison. 1995. Differential effects of CTLA4 substitutions on the binding of human CD80 (B7-1) and CD86 (B7-2). J. Immunol. 156:1047.


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