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# T Suppressor Lymphocytes Inhibit NF- $\kappa$ B-Mediated Transcription of CD86 Gene in APC<sup>1</sup>

Jianfeng Li,\* Zhuoru Liu,\* Shuiping Jiang,\* Raffaello Cortesini,<sup>‡</sup> Seth Lederman,<sup>†</sup> and Nicole Suciú-Foca<sup>2\*</sup>

CD8<sup>+</sup>CD28<sup>-</sup> human T suppressor cells (Ts) act on APC, inhibiting their ability to elicit Th activation and proliferation. This effect is due to inhibition of the CD40 pathway which normally leads to CD80 and CD86 up-regulation. To determine whether Ts inhibit expression of B7 molecules by blocking transcription, we cloned and characterized the CD86 promoter. Mutational analysis revealed that Ts inhibit transcription driven by the CD86 promoter. The NF- $\kappa$ B binding site, at -612 of the CD86 promoter, is essential for Th-induced transcription. In cultures containing Th and Ts, Ts inhibit Th-induced NF- $\kappa$ B activation in APC. Together, these findings indicate that Ts inhibition of NF- $\kappa$ B activation in APC is a means by which they regulate the activation and proliferation of Th. *The Journal of Immunology*, 1999, 163: 6386–6392.

Effective priming of T lymphocytes requires cell-to-cell interaction between APC and T cells. This interaction allows bidirectional costimulatory signals which activate the APC, presenting the processed Ag and those T cells with cognate-specific TCR. TCR triggering results in up-regulation of CD40L (CD154) expression (1, 2). CD40L interacts with CD40 on APC, inducing them to up-regulate B7 (CD80 and CD86) costimulatory molecules (3, 4). These B7 molecules interact with their counterreceptor (CD28), augmenting CD40L expression and inducing CD4 Th cell proliferation (5). T cell activation in the absence of B7 costimulation results in T cell paralysis or anergy (6–9). We have recently found that Ag-specific T suppressor cells (Ts)<sup>3</sup> render APC unable to up-regulate B7 molecules in response to Th signals (10–13). In addition, Ts-treated APC render Th paralyzed or anergic (13). These studies further showed that Ts inhibit the CD40-signaling pathway in APC (13). However, the mechanism underlying these effects remains unclear.

Human Ts derive from an oligoclonal population of CD8<sup>+</sup>CD28<sup>-</sup> T cells which recognize specific MHC-class I/Ag complexes on the surface of APC (10–12). Allospecific, xenospecific, and nominal Ag-specific suppressor T cell lines (TCL) can be generated in vitro by successive rounds of stimulating human T cells with allogeneic, xenogeneic, or Ag-pulsed autologous APC. CD8<sup>+</sup>CD28<sup>-</sup> Ts from these TCL inhibit proliferation of CD4<sup>+</sup> Th with cognate-Ag specificity. Ts activation via TCR interaction with class I MHC/Ag complexes requires cell-to-cell interaction between Ts and the APC used for Th priming (10, 11). In this system, APC act as a temporal bridge between Ts, which recognize

MHC-class I/peptide complexes, and Th, which recognize MHC-class II complexes. Ts render the APC unable to up-regulate B7 molecules or to provide efficient costimulation of Th. The level at which the expression of B7 molecules by APC is regulated by Ts is currently unknown.

## Materials and Methods

### Generation of alloreactive TCL

Alloreactive TCL were generated as previously described (10–13). Briefly, PBMC were separated from buffy coats by Ficoll-Hypaque centrifugation. Responding PBMC ( $1 \times 10^6$ /ml) were stimulated in 24-well plates with irradiated APC (3000 rad;  $0.5 \times 10^6$ /ml) obtained from allogeneic PBMC by depletion of CD2<sup>+</sup> cells. Cells were cocultured for 7 days in complete medium (RPMI 1640 supplemented with 10% human serum, 2 mM L-glutamine, and 50  $\mu$ g/ml gentamicin; Life Technologies, Grand Island, NY). After 7 days, responding cells were collected, washed, and rechallenge with the original stimulating cells. After 3 days, rIL-2 (Boehringer Mannheim, Indianapolis, IN) was added (10 U/ml) and the cultures were expanded for an additional 4 days. Th and Ts cells were then harvested from the TCL. CD4<sup>+</sup> Th cells were obtained by depleting CD8<sup>+</sup>, CD14<sup>+</sup>, and CD19<sup>+</sup> cells from the TCL. CD8<sup>+</sup>CD28<sup>-</sup> Ts cells were obtained by depleting CD4<sup>+</sup>, CD14<sup>+</sup>, CD19<sup>+</sup>, and CD28<sup>+</sup> cells, as previously described (10).

### Expression of CD86 on human B cells

The expression of CD86 on human B cells was analyzed by ELISA. Lysates prepared from purified human B cells were adhered to 96-well trays at 4°C for 24 h. CD86 Ag was detected using a mouse anti-human CD86 mAb (PharMingen, San Diego, CA).

### Cell cultures

Th cells ( $3 \times 10^6$ ) were incubated with CD2-depleted PBMC ( $3 \times 10^6$ ) from the stimulating cell donor in the presence or absence of Ts cells ( $1.5 \times 10^6$ ). After 6 or 48 h of incubation (as indicated), CD19<sup>+</sup> B cells were isolated from the cultures using CD19-coupled Dynal beads (Great Neck, NY).

### Northern blot analysis

Acid phenol-guanidinium thiocyanate procedure (Trizo, Molecular Research Center, Cincinnati, OH) was used to isolate total RNA from the human B cells. The concentration of total RNA was determined by absorption at OD<sub>260/280</sub>. Total RNA (20  $\mu$ g/lane) was subjected to electrophoresis. After separation on a formaldehyde gel (0.7%), RNA was transferred to a Gene Screen hybridization filter (NEN, Boston, MA) and cross-linked by a UV cross-linker (Stratagene, La Jolla, CA). The filter was then hybridized with the full-length human CD86 cDNA probe labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using the Random Primer Labeling System (Stratagene). After washing twice at room temperature (15 min/wash) with 2 $\times$  SSC

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<sup>3</sup> Abbreviations used in this paper: Ts, T suppressor cells; TCL, T cell lines.

containing SDS (0.1%) and once for 30 min at 60°C with 0.1× SSC containing SDS (0.1%), the filter was subjected to autoradiography overnight at -80°C. G3PDH mRNA was detected using G3PDH cDNA (Clontech Laboratories, Palo Alto, CA) to normalize samples for mRNA content.

#### Nuclear run-on transcription assay

Nuclear run-on transcription assays were performed as previously described (14). Human B cells, isolated as described above, were washed twice with cold PBS, pelleted, and resuspended in 10 ml of lysis buffer containing 0.01 M Tris (pH 7.4), 10 mM NaCl, 1 mM DTT, 0.5% (v/v) Nonidet P-40, 0.5 mM PMSF, and 100 µg/ml aprotinin. After 15 min of incubation on ice, the lysate was homogenized and then centrifuged at 500 × g for 5 min at 4°C. The nuclear pellet was washed once with 4 ml of lysis buffer and centrifuged at 500 × g for 5 min at 4°C. The supernatant was discarded and the nuclei were resuspended in 0.2 ml reaction buffer containing 10 mM Tris (pH 8.0), 5 mM MgCl<sub>2</sub>, 300 mM KCl, 1 mM DTT, 0.5 mM ATP, 0.5 mM cytidine 5'-triphosphate, 0.5 mM GTP, and 200 µCi of [ $\alpha$ -<sup>32</sup>P]UTP (3000 Ci/mM; Amersham, Arlington, Heights, IL). After 1 h of incubation at 30°C, RNA was extracted and resuspended in hybridization buffer containing 50 mM PIPES (pH 6.8), 10 mM EDTA, 600 mM NaCl, 0.2% SDS, and 100 µg/ml denatured salmon sperm DNA. After prehybridization at 80°C for 2 h in hybridization buffer containing 0.1% SDS, RNA was hybridized to denatured human CD86 cDNA and  $\beta$ -actin cDNA, and slot-blotted on nylon filters at 42°C for 72 h. After hybridization, filters were washed three times for 1 h in 2× SSC containing 0.1% SDS, then briefly air-dried and exposed to x-ray film for 4 days. Human CD86 cDNA and  $\beta$ -actin cDNA, denatured by incubation with 0.3 M NaOH for 30 min at 65°C, were spotted onto nylon filters and cross-linked with a UV cross-linker (Fisher, Pittsburgh, PA).

#### Isolation of a human CD86 genomic clone

A 1.4-kb genomic DNA clone, including the 5'-flanking fragment and part of the first exon of human CD86 gene, was isolated by screening a  $\lambda$  DH3 library (Stratagene) with a synthetic oligonucleotide probe that represents 120 nucleotides of the first exon of CD86 (15). The 1.4-kb insert was subcloned into pCR-Script vector (Stratagene) and sequenced in both directions. The 1.4-kb clone contained 77 nucleotides of the first exon and 1245 bp upstream of the transcription site of the CD86 gene.

#### Generation of 5'-deleted CD86 promoter constructs

CD86 promoter-reporter constructs representing either 5'-deleted CD86 upstream sequences or site-directed mutants of the -612 NF- $\kappa$ B or -238 NF- $\kappa$ B binding sites were generated by PCR then by subcloning into the pGL3-Basic vector (Promega, Madison, WI). In these PCRs, the CD86 genomic clone (described above) was used as a template. For 5'-deletion mutants, oligonucleotides used for priming were synthesized based on the sequence of the 5'-flanking region of human CD86 gene. In these reactions, the priming oligonucleotides represented overlapping sense and antisense sequences that amplified the entire pGL3 plasmid and insert. The 5'-deletion constructs represent -1179 to +48 (1.2 kb), -781 to +48 (0.8 kb), -407 to +48 (0.4 kb), and -212 to +48 (0.2 kb) of the CD86 gene. For the NF- $\kappa$ B binding site mutants, DNA primers were synthesized that contained the mutated NF- $\kappa$ B binding site sequences at -612 and -238. The constructs containing these mutated NF- $\kappa$ B binding sites represent nucleotides -1179 to +48 of the CD86 gene. DNA sequencing on both strands of the inserts confirmed the sequence of all constructs employed for transfection assays.

#### CD86 reporter assays

Transfected B cells were cultured for 48 h in the presence of Th, Ts, mixtures of Th and Ts, or medium alone. B cell transfection was performed with DMRIE-C reagent (Life Technologies) according to the manufacturer's recommendations. Briefly,  $1 \times 10^6$  cells were cotransfected using 4 µg of CD86 promoter-GL3 reporter plasmid, and 1 µg of pSV  $\beta$ -galactosidase, which served as an internal control to normalize transfection efficiency, in 8 µl of DMRIE-C reagent. Twenty-four hours after transfection, Th, Ts, mixtures of Th and Ts cells at the indicated ratio, or control medium was added to B cell cultures. After an additional 24 h, cells were harvested, lysed, and assayed for luciferase activity by a luminometer (Promega). Statistical analysis of at least three separate experiments were performed using ANOVA.

#### EMSA

Oligonucleotide probes corresponding to the -612 NF- $\kappa$ B binding site sequence of human CD86 promoter were generated by annealing the synthetic oligonucleotides 5'-CTTAGGGACATCTATTAA-3' and 5'-TTA

ATAGATGTCCCTAAG-3'. The probe (250 pg), end labeled with [ $\gamma$ -<sup>32</sup>P]ATP, was incubated with 8 µg of B cell nuclear extract(s) at room temperature for 30 min. For competition assays, the nuclear extract was first incubated with 100-fold excess of unlabeled double-stranded oligonucleotides representing the -612 NF- $\kappa$ B site, NF- $\kappa$ B consensus site (5'-AGTTGAGGGGACTTCCAGGC-3'), or SP1 consensus site (5'-ATCGATCGGGGCGGGGCGAGC-3') for 15 min at room temperature and then incubated with the end-labeled -612 NF- $\kappa$ B probe for 30 min under the same conditions. For supershift assays, nuclear extracts were incubated with Abs to p50 or p65 (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h at 4°C before the addition of the labeled -612 NF- $\kappa$ B probe. Samples were separated in 8% polyacrylamide gels and the gels were subjected to autoradiography overnight at -80°C.

#### Western blotting assays

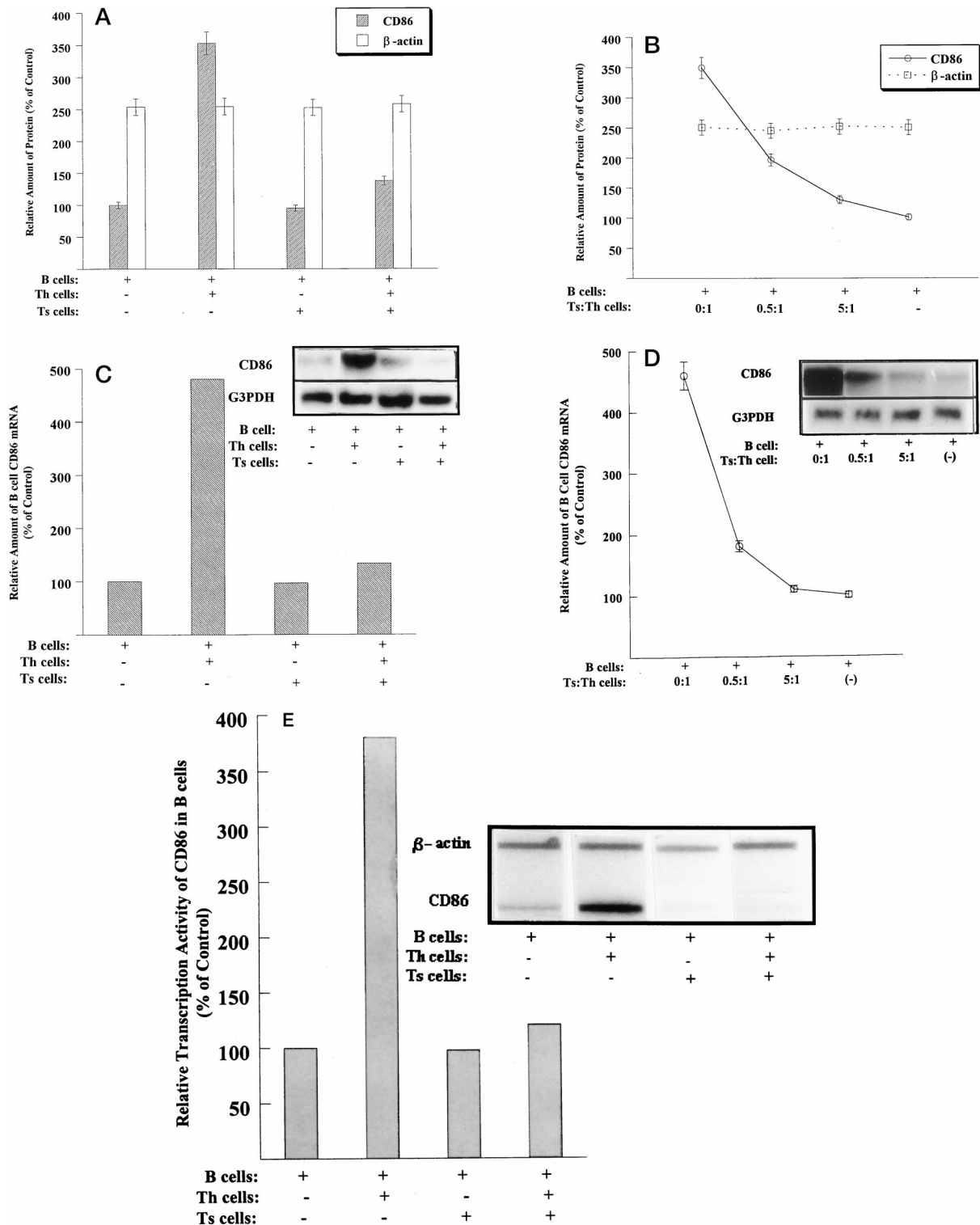
Ten micrograms of nuclear extracts from B cells, purified from 3- or 6-h cultures with Th, Ts, mixtures of Th and Ts, or medium, were separated in 12% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were briefly soaked in ddH<sub>2</sub>O, then blocked in Tris-buffered saline (Bio-Rad Laboratories, Hercules CA)/0.05% Tween 20 (TTBS) containing 5% BSA for 1 h at room temperature. Membranes were probed with either anti-p50 or anti-p65 (Santa Cruz Biotechnology) at 1:200 dilution in TTBS for 1 h, washed four times with TTBS, and then incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (Amersham) at 1:10,000 dilution in TTBS containing 5% goat serum. Membranes were washed four times with TTBS and developed using enhanced Hyperfilm-ECL (Amersham). Antiactin Ab (Santa Cruz Biotechnology) was used as control.

## Results and Discussion

In an effort to dissect the molecular events resulting in suppression, we have analyzed the regulation of CD86 expression in APC cocultured with Th and/or Ts. Based on flow cytometry studies, we previously have shown that Ts inhibit Th-induced APC up-regulation of CD86 expression (10–13). Because only extremely small numbers of dendritic cells can be procured from normal individuals, we used B cells which are also endowed with APC activity (16) and the capacity to stimulate Th in mixed lymphocyte culture. To determine whether this inhibition corresponds to decreased cellular CD86 protein in B cells, we used ELISA to measure CD86 protein in cell lysates. Th, Ts, or mixtures of Th and Ts were primed in cultures with CD2-depleted PBMC from an allogeneic donor. CD86 protein was measured in B cells purified from 48-h cultures with Th, Ts, mixtures of Th and Ts, or medium. A significant increase in the amount of CD86 protein was observed in B cells preactivated by Th relative to that of B cells in medium alone ( $p < 0.01$ ). When cultured alone with B cells, Ts cells had no effect on the amount of CD86 protein in B cells (Fig. 1A). However, Ts inhibit the Th-induced increase in B cell CD86 protein in a dose-dependent manner (Fig. 1B).

To characterize the effect of Th and Ts on the expression of steady-state CD86 mRNA levels, B cells that had been cultured for 48 h with Th, Ts, Th/Ts mixtures, or medium alone were purified and total RNA was prepared. Densitometry analysis of Northern blots for CD86 mRNA showed a 4.8-fold increase in CD86 mRNA in B cells preactivated by Th compared with control B cells (Fig. 1C). B cells cultured with Ts cells alone showed no significant change in CD86 mRNA (Fig. 1C). However, Ts inhibited the Th-induced up-regulation of B cell steady-state CD86 mRNA levels in a dose-dependent manner (Fig. 1D).

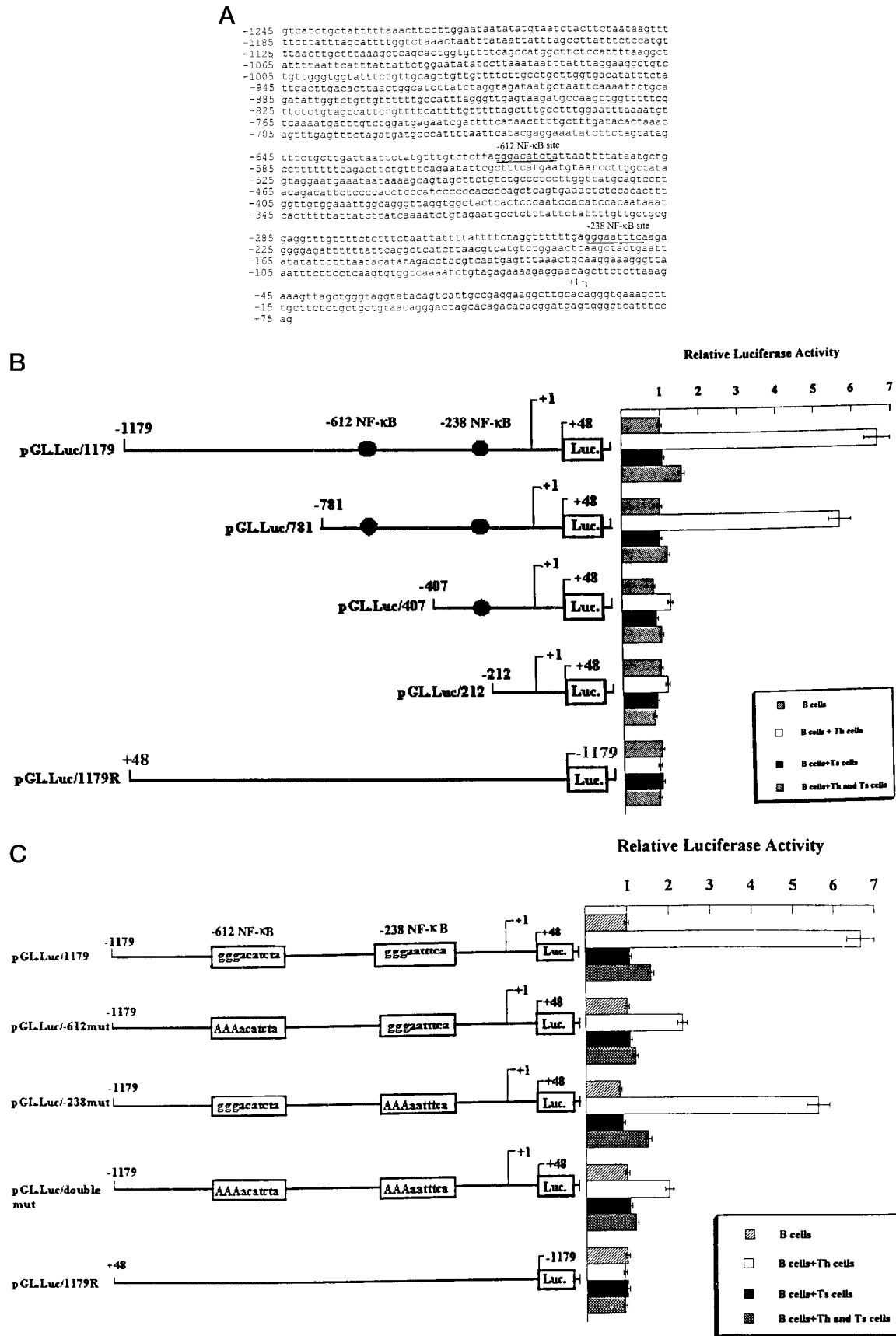
To determine whether the Ts effect on CD86 expression occurs at the level of transcription, we performed nuclear run-on assays (14) on B cells purified from cultures containing Th, Ts, Th/Ts mixtures, or medium alone. Th induced a 3.7-fold increase in B cell CD86 transcription compared with that in control B cells (Fig. 1E). Ts cells did not alter B cell CD86 transcription. However, Ts inhibited the Th-induced increase in B cell CD86 transcription. These data indicate that Ts suppress the Th-induced up-regulation of B cell CD86 expression at the level of transcription.



**FIGURE 1.** Effect of Ts on Th-induced B cell CD86 protein and mRNA expression and transcription. ELISA for protein (A and B), Northern Blots for mRNA (C and D), and nuclear run-on assays (E) were performed on B cells purified from 48-h cultures containing CD2-depleted APC and Th, Ts, mixtures of Th and Ts, or medium. For dose-response studies (B and D), increasing numbers of Ts were added to cultures containing a fixed amount of Th and APC. Error bars in A and B represent SEM of triplicate wells. Experimental OD values for  $\beta$ -actin control were  $2.606 \pm 0.212$ ,  $2.552 \pm 0.297$ ,  $2.567 \pm 0.271$ , and  $2.667 \pm 0.310$  for B cells alone, B cells plus Th cells, B cells plus Ts, and B cells plus Th and Ts cells, respectively. Experimental OD values for CD86 were  $0.821 \pm 0.071$  for B cells alone. Insets (C–E) show hybridization data and figure shows results of densitometry analysis, normalized by G3PDH signals (C and D) or  $\beta$ -actin (E).

To further characterize the mechanism by which CD86 transcription in B cells is regulated by Th and Ts, we cloned a 1.4-kb genomic fragment that contains 1245 bp of the 5'-flanking region

upstream of the human CD86 gene (15) (Fig. 2A). We searched for known transcription-factor binding-consensus sequences in the 5'-flanking sequence using the Transfast program (Gesellschaft für



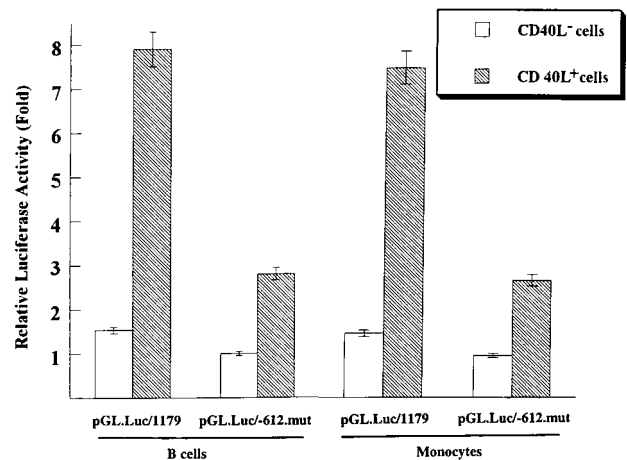
**FIGURE 2.** Generation and functional analysis of luciferase chimeras from the 5'-flanking region of the human CD86 gene. A, Nucleotide sequence of the 5'-flanking region and 76 nucleotides of the first exon of the human CD86 gene. The transcription start site determined by primer extension analysis (data not shown) is denoted as +1. Two potential NF-κB binding sites are boxed. B and C, Luciferase assays were performed on B cells purified from 48-h cultures containing CD2-depleted APC and Th, Ts, mixtures of Th and Ts, or medium alone. B, The properties of the 5'-flanking region of the CD86 gene luciferase reporter gene constructs are shown. C, The properties of the wild-type and PCR-generated mutated constructs are shown.

Biotechnologische, Braunschweig, Germany). This search revealed multiple potential transcription-factor binding sequences, including at least two canonical NF- $\kappa$ B binding sites, one at  $-612$  and another at  $-238$  (Fig. 2A).

To delineate elements that regulate CD86 transcription and which may be responsive to signals from Th and/or Ts cells, a series of luciferase reporter gene plasmids were generated in a pGL3-Basic vector that represents 5' deletions of the 5'-flanking region of the CD86 gene (Fig. 2B). The resulting constructs (termed pGL.Luc/1179, pGL.Luc/781, pGL.Luc/407, and pGL.Luc/212) were transfected into B cells to measure their transcriptional activities. The control construct contained the 1179-bp DNA element cloned into pGL3-Basic vector in the opposite orientation (pGL.Luc/1179R). When exposed to Th cells, B cells transfected with reporters driven by the 1179- and 781-bp DNA elements showed significant increases in luciferase activity (6.8- and 5.7-fold, respectively) compared with B cells transfected with the control construct ( $p < 0.01$ ). In contrast, Th cells did not induce luciferase activity in B cells transfected with the 407- and 212-bp DNA elements. Ts inhibited the Th-induced luciferase activity in B cells transfected with the 1179- or 781-bp DNA elements ( $p < 0.01$ ) (Fig. 2B). Hence, Th cells trigger transcription driven by the 1179- and 781-bp elements in B cells, and Ts inhibit this effect. In addition, these data indicate that Th and Ts control CD86 transcription by exerting effects on a segment of the 5'-flanking DNA located between  $-1179$  and  $-407$  upstream of the transcription start site of CD86. Because the transcription factor NF- $\kappa$ B is known to play an important role in many of the biological effects exerted by CD40, we focused on the NF- $\kappa$ B binding site at  $-612$  (17).

To determine whether the  $-612$  binding site is functionally important in the B cell CD86 response to Th and Ts signals, we generated a reporter construct driven by the 1179-bp DNA segment that contained a mutated  $-612$  NF- $\kappa$ B binding site (Fig. 2C). As controls, reporter constructs that contained either a mutated NF- $\kappa$ B consensus binding site at  $-238$  or the mutated NF- $\kappa$ B binding sites at  $-612$  and  $-238$  were used. In the absence of Th, B cells transfected with the  $-612$ -mutated 1179-bp construct (pGL.Luc/ $-612$  mut) showed levels of background luciferase activity similar to those of B cells transfected with the wild-type 1179-bp construct (pGL.Luc/1179). In the presence of Th, B cells transfected with the wild-type construct or the mutated  $-238$  NF- $\kappa$ B consensus binding site (pGL.Luc/ $-238$  mut) showed enhanced luciferase activity (6.7- and 5.8-fold, respectively), whereas Th-activated B cells transfected with the  $-612$ -mutated construct showed substantially less activity (78% less than the wild-type construct) ( $p < 0.01$ ) (Fig. 2C). The data suggest that the  $-612$  NF- $\kappa$ B consensus binding site within the CD86 promoter plays an important role in the transcriptional activation of CD86 expression induced by T helper cells in B cells.

To determine whether the  $-612$  NF- $\kappa$ B consensus site also plays a role in CD40 triggering of CD86 transcription in APC (18), the following experiments were performed. CD2-depleted PBMC were transfected with the 1179-bp wild-type (pGL.Luc/1179) and  $-612$  mutant (pGL.Luc/ $-612$  mut) reporter constructs and incubated with 293 cells expressing CD40L (CD40L<sup>+</sup> 293 cells) or with control CD40L<sup>-</sup> 293 cells (19). After 48 h, CD19<sup>+</sup> B cells and CD14<sup>+</sup> monocytes were isolated from the cultures and luciferase activity was measured. CD40 triggering of B cells resulted in a 7.6-fold increase in activity driven by the wild-type promoter element and only a 2.3-fold increase in activity driven by the  $-612$  mutant reporter (Fig. 3). Similarly, CD40 triggering of monocytes induced a 7.8-fold increase, whereas the  $-612$  mutant induced only a 2.2-fold increase of luciferase activity (Fig. 3). These data



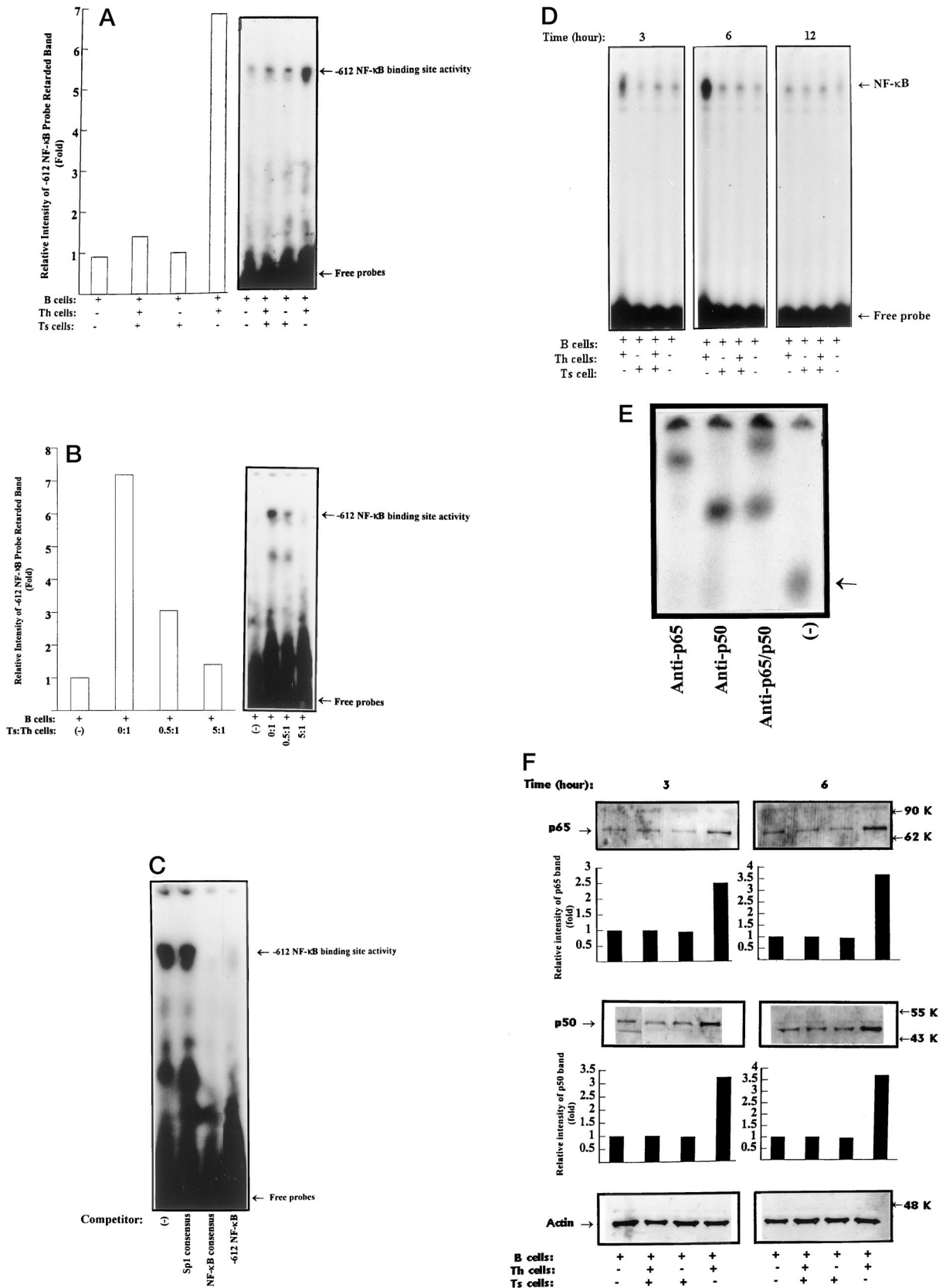
**FIGURE 3.** Effect of CD40 triggering on CD86 transcription in B cells and monocytes. CD2-depleted PBMC were transfected with the wild-type (pGL.Luc/1179) or mutant (pGL.Luc/ $-612$  mut) constructs and cocultured with CD40L<sup>+</sup> 293 cells or CD40L<sup>-</sup> 293 cells. Luciferase activity was measured on purified B cells or monocytes, as indicated. The CD40-induced APC luciferase activity is shown relative to the activity of APC cultured with CD40L<sup>-</sup> 293 cells. Error bars represent SEM of triplicate wells.

show that CD86 transcription induced by CD40 ligation in APC depends on the  $-612$  NF- $\kappa$ B consensus site.

To further characterize the functional significance of the  $-612$  NF- $\kappa$ B binding site and the transcription factors which bind to it, EMSAs were performed on nuclear extracts from B cells purified from cultures containing Th, Ts, Th/Ts mixtures, or medium. For these studies, a synthetic 18-mer probe derived from the  $-612$  NF- $\kappa$ B-consensus binding-site sequence in the CD86 promoter was used. Th induced a 6.9-fold increase in the intensity of a band corresponding to retardation of the  $-612$  NF- $\kappa$ B probe (Fig. 4A) over the level seen in B cells cultured without Th. Ts inhibited the Th-induced effect in a dose-dependent manner (Fig. 4B). Control experiments using unlabeled, double-stranded oligonucleotides confirmed the specificity of the  $-612$  NF- $\kappa$ B probe because the signal was inhibited by preincubation with either unlabeled  $-612$  NF- $\kappa$ B or consensus NF- $\kappa$ B oligonucleotides, but not by preincubation with an Sp-1 oligonucleotides (Fig. 4C). These data suggest that the  $-612$  NF- $\kappa$ B probe binds specifically to activated NF- $\kappa$ B. Kinetic analysis of NF- $\kappa$ B induction showed activation at 3 h, maximal activation at 6 h, and a return to baseline at 12 h (Fig. 4D).

To further characterize the transcription factors that bind to the  $-612$  NF- $\kappa$ B binding site, we performed supershift experiments using Abs specific for p65 and p50. The <sup>32</sup>P-labeled  $-612$  NF- $\kappa$ B probe was added to nuclear extracts isolated from B cells that had been preactivated by Th cells and preincubated with Abs as indicated above. The anti-p65 and anti-p50 Abs retarded the mobility of the  $-612$  NF- $\kappa$ B probe/protein complex (Fig. 4E). Kinetic studies using Western blotting showed that Th increased the levels of nuclear p65 and p50, and that Ts inhibited this effect (Fig. 4F). These data indicate that the  $-612$  NF- $\kappa$ B probe binds specifically to NF- $\kappa$ B and most likely interacts with p65/p50 heterodimers. Hence, cell-to-cell interactions between Th and APC induce APC to activate NF- $\kappa$ B, which plays an important role in driving the transcription of CD86.

Similar to the role that NF- $\kappa$ B plays in CD86 transcription, NF- $\kappa$ B activation previously has been shown to drive the transcription of CD80, the other B7 molecule (20). Both of these molecules, CD80 and CD86, are up-regulated following ligation of CD40, which has been shown to induce NF- $\kappa$ B activation (21–23).



**FIGURE 4.** EMSA analysis of transcription factor binding to the -612 NF-κB site. *A*, Nuclear extracts from B cells purified from 6-h cultures with Th, Ts, mixtures of Th and Ts, or medium were incubated with a probe representing the -612 NF-κB site. *B*, Increasing numbers of Ts were incubated with a fixed amount of Th and CD2-depleted APC. Nuclear extracts from B cells purified from these cultures were tested as above. *C*, Before addition of <sup>32</sup>P-labeled -612 NF-κB probe, the nuclear extracts were preincubated with unlabeled double-stranded oligonucleotides representing the -612 NF-κB site, a consensus NF-κB site, or an SP-1 binding site, as indicated. *D*, Kinetic analysis of NF-κB induction in B cells cocultured with Th and/or Ts for 3, 6, or 12 h. *E*, Supershift analysis of nuclear extracts from Th-stimulated B cells using anti-p50, anti-p65, or a mixture of anti-p50 and anti-p65 Abs. Arrow indicates non-supershifted NF-κB DNA complex. *F*, Western blot analysis of p65 and p50 in nuclei of B cells cultured for 3 and 6 h with Th and/or Ts. Actin was used as an internal control for normalizing signals.

Taken together with our previous finding that Ts cells inhibit CD40 signaling in APC, the present study indicates that Ts modulate Th reactivity in vitro by regulating NF- $\kappa$ B activation in APC. This finding may have importance for the development of new immunomodulatory strategies for the treatment of autoimmune diseases and transplant rejection.

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