NF-κB and Enhancer-binding CREB Protein Scaffolded by CREB-binding Protein (CBP)/p300 Proteins Regulate CD59 Protein Expression to Protect Cells from Complement Attack

Yiqun Du, Xiaoan Teng, Na Wang, Xin Zhang, Jianfeng Chen, Peipei Ding, Qian Qiao, Qingkai Wang, Long Zhang, Chaoqun Yang, Zhangmin Yang, Yiwei Chu, Xiang Du, Xuhui Zhou, and Weiguoh Hu

From the Cancer Institute, Collaborative Innovation Center of Cancer Medicine, Fudan University Shanghai Cancer Center and Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China, Institutes of Biomedical Sciences, Fudan University, Shanghai 200032, China, Department of Biochemistry and Molecular Biology, College of Life Sciences, Shaanxi Normal University, Xi’an 710062, Shaanxi, China, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China, Department of Immunology, Shanghai Medical College, Fudan University, Shanghai 200032, China, and Department of Pathology, Fudan University Shanghai Cancer Center, Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China

Background: CD59 is the sole membrane complement regulatory protein in restricting membrane attack complex assembly.

Results: CD59 gene produces eight transcripts that share three transcriptional initiation sites but the same open reading frame.

Conclusion: NF-κB and CREB (as an enhancer-binding protein) bridged by CBP/p300 are responsible for the inducible expression of CD59.

Significance: CD59 regulation mechanism suggests potential drug targets for controlling various complement-related human diseases.

The complement system can be activated spontaneously for immune surveillance or induced to clear invading pathogens, in which the membrane attack complex (MAC, C5b-9) plays a critical role. CD59 is the sole membrane complement regulatory protein (mCRP) that restricts MAC assembly. CD59, therefore, protects innocent host cells from attacks by the complement system, and host cells require the constitutive and inducible expression of CD59 to protect themselves from deleterious destruction by complement. However, the mechanisms that underlie CD59 regulation remain largely unknown. In this study we demonstrate that the widely expressed transcription factor Sp1 may regulate the constitutive expression of CD59, whereas CREB-binding protein (CBP)/p300 bridge NF-κB and CREB, which surprisingly functions as an enhancer-binding protein to induce the up-regulation of CD59 during in lipopolysaccharide (LPS)-triggered complement activation, thus conferring host defense against further MAC-mediated destruction. Moreover, individual treatment with LPS, TNF-α, and the complement activation products (sublytic MAC (SC5b-9) and C5a) could increase the expression of CD59 mainly by activating NF-κB and CREB signaling pathways. Together, our findings identify a novel gene regulation mechanism involving CBP/p300, NF-κB, and CREB; this mechanism suggests potential drug targets for controlling various complement-related human diseases.

The complement system is known as a major constituent of innate immunity and an important modulator of adaptive immunity; complement not only eliminates invading microbial pathogens, xenografts, and host debris but also orchestrates immunological and inflammatory processes (1, 2). The activation of the complement cascade leads to the direct lysis of invading pathogens by the membrane attack complex (MAC), phagocytosis opsonized by C3b/iC3b tagging, and the production of anaphylatoxins C3a/C5a; all these effects synergistically promote the clearance of foreign intruders. To prevent deleterious bystander effects on innocent host cells during this process, >10 circulating and membrane-bound complement regulatory proteins (mCRPs, including CD59) have evolved to restrict the activation of complement activation at diverse stages. The versatile functions of the complement system are

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1 Both authors contributed equally to this work.

2 To whom correspondence may be addressed. Tel: 86-21-61886999; Fax: 86-21-63520020; E-mail: weiguohu@fudan.edu.cn.

3 To whom correspondence may be addressed. Tel: 86-21-64175590; Fax: 86-21-64172585; E-mail: weiguohu@fudan.edu.cn.

The abbreviations used are: MAC, membrane attack complex; mCRP, membrane complement regulatory protein; CBP, cAMP-response element-binding protein (CREB)-binding protein; TF, transcription factor; RP, reverse primer; PI, propidium iodide; NHS, normal human serum; qRT, quantitative real-time.
able to be finely tuned to establish a delicate balance between activation and regulation but the tipping of this delicate balance has been attributed at least in part to various human disorders including immune, inflammatory, neurodegenerative, atherosclerosis, ischemic, and age-related diseases, the initiation, progression, drug resistance, and nonresponsiveness of cancer, and persistent pathogen infection (1). Therefore, it is crucial to understand how mCRPs respond to the extracellular inflammatory environment and complement activation under various conditions.

CD59 is a small, highly glycosylated and glycosylphosphatidylinositol-anchored membrane protein. It has been well defined as the sole mCRP in restricting MAC assembly and is widely expressed on all circulating cells and in almost all tissues; intriguingly, CD59 is weakly expressed in the central nervous system (3). Therefore, CD59 plays a crucial role in protecting autologous cells from destruction by complement. Deficient or reduced CD59 expression in pathogens or host cells may lead to the direct lysis of invading pathogens or autologous cells in various diseases, such as autoimmune hemolympenia and systemic lupus erythematous (4, 5). In contrast, high CD59 expression in normal host cells leads to the incapability of the complement system to destroy target cells and triggers comprehensive downstream pro-cell survival signaling (6). Therefore, these findings highlight the need to decipher the regulation of CD59 in human disorders. Some isolated studies have speculated that CD59 might be regulated by the transcription factors (TFs) Sp1 (7), TP53 (8), and ERK1/2/NF-κB (9) along with an enhancer in intron 1 (10); however, the underlying mechanisms remain largely obscure.

The ubiquitously expressed transcription factor Sp1 binds to GC-rich elements that are widely distributed in the promoters of housekeeping genes and regulates the expression of thousands of genes involved in diverse cellular processes, such as cell growth, differentiation, apoptosis, and immune responses (11); therefore, Sp1 has traditionally been regarded as a constitutive TF (12). However, NF-κB, which can be induced by both canonical and noncanonical signaling pathways, has critical regulatory functions in various processes including apoptosis, differentiation, and especially immunity (13). Additionally, CREB regulates the expression of a wide range of genes that are responsible for glucose homeostasis, survival, proliferation, memory, and learning (14) and is generally associated with the co-activator CREB-binding protein (CBP) and its close relative, p300 (15). Through their different binding sites, CBP and p300 are functionally essential for CREB and many other TFs, such as NF-κB, TP53, signal transducers and activators of transcription (STATs), and activator protein 1 (AP-1); CBP and p300 provide a protein scaffold to connect diverse TFs to the transcription apparatus, which places them at the center of various signaling pathways in cell growth, transformation, and development (16, 17). Nevertheless, few studies have revealed the functions of these trans-acting factors in protecting host cells from complement-mediated damage.

This study shows that the CD59 gene produces eight distinct transcripts, seven of which are newly identified, that share three different transcriptional initiation sites but the same open reading frame. Furthermore, we found that Sp1 mainly regulates the constitutive expression of CD59, whereas NF-κB and CREB connected by CBP/p300 are responsible for the inducible expression of CD59. Furthermore and surprisingly, CREB is identified as an enhancer-binding protein for the first time. However, the potential TF roles of TP53 and CREB are negligible due to the very low abundance levels of their regulated transcripts. Finally, we investigated the signaling pathways that are responsible for up-regulating the transcription of CD59 in lipopolysaccharide (LPS)-triggered complement activation that subsequently confers cell protection from complement-induced and MAC-mediated destruction. Our findings clearly delineate a novel interaction between CBP/p300, NF-κB, and CREB in gene transcriptional regulation; this interaction is important for the defense of host cells against complement attack via the up-regulation of CD59 transcription.

**EXPERIMENTAL PROCEDURES**

**Cells Culture**—HeLa, A549, H1299, and U937 cells (Type Culture Collection Cell Bank, Chinese Academy of Sciences) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics; the IMR32 cell line (American Type Culture Collection) was grown in minimum Eagle’s medium supplemented with 10% FBS and 1% antibiotics.

**Reagents**—Recombinant human TNF-α was purchased from PROSPEC (East Brunswick, NJ); LPS and 8-Br-cAMP were obtained from Sigma. C5a, C5b-6, C7, C8, C9, and the rabbit anti-human C5b-9 antibody were obtained from CompTech (Tyler, Texas). FITC-conjugated anti-mouse IgG, anti-β-actin (C4), anti-TF1IB (D-3), anti-CDS9 (H-7), anti-CDS46 (M177), anti-CDS55 (H-7), anti-phospho-ERK1/2 (E-4), anti-p65 (F-6), anti-p50 (E-10), anti-cRel (B-6), anti-RelB (D-4), anti-Sp1 (E-3), anti-CREB-1 (24H4B), anti-phosphorylated CREB-1 (Ser-133) (sc-101663), anti-TF53 (DO-2), anti-CBP (451), and anti-p300 (N-15) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX), Acetyl-TP53 (Lys-382) (2525), anti-phospho-TF53 (Ser-15) (16G8), anti-phospho-TF53 (Ser-20) (9287), anti-Akt (40D4), and anti-phospho-Akt (Ser-473) (193H12) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-NF-κB p100/p52 (ab7972) and anti-phosphorylated Sp1 (Thr-453) (ab59257) antibodies were obtained from Abcam (Cambridge, MA). Anti-p65 (17-10060), anti-c-Rel (09-040), anti-RelB (EP613Y), anti-CREB (AB3006), and anti-phospho-CREB (Ser-133) (17-1013) antibodies, all used only for chromatin immunoprecipitation (ChIP) assays, and the anti-acetyl-TF53 (Lys-373) (06-916) antibody were obtained from Millipore (Billerica, MA). The FITC-conjugated mouse anti-human CDS9 mAb (p282/H19) was obtained from BD Pharmingen. The FITC-conjugated AffiniPure goat anti-rabbit IgG (H + L) (305-095-003) was obtained from Jackson ImmunoResearch (West Grove, PA), Propidium iodide (PI) was obtained from Invitrogen. Normal human serum (NHS) was pooled from 12 healthy persons and aliquoted, then stored at −80 °C until use.

**GeneRacer PCR**—GeneRacer PCRs were performed according to the manufacturer’s protocol (Invitrogen). The CD59-specific reverse primer (RP; shown in Fig. 1 A) was synthesized as 5’-TCTGTTAAGTTACACAGGTCTTC-3’. The first PCR was run with the GeneRacer first primer and RP. 1 μl of the
first-round PCR product was used to perform semi-nested PCR with the GeneRacer semi-nested primer and RP. The second PCR product was analyzed on a 12% PAGE gel, and the DNA was stained with etidium bromide. Finally, the bands were excised from the PAGE gel, cloned into an appropriate T-vector, and sequenced.

Quantitative RT-PCR—Total RNA was extracted using TRIzol reagent (Invitrogen) and was reverse-transcribed to cDNA using a Reverse Transcription System (Promega). The input cDNA was standardized and then amplified for 45 cycles with SYBR Green Master Mix and gene-specific primers on an ABI Prism 7900HT machine (Applied Biosystems); endogenous β-actin was regarded as an internal control, and samples were analyzed in triplicate. The primers for amplifying β-actin were 5’-TGGGACGACATGGAGAAAAT-3’ (F) and 5’-GCCGACGTCATAGGAGAAAT-3’ (R). The primers for amplifying CD59 transcripts T1-T8 were 5’-GATGCGTGTCTCATACAA-3’ (F) and 5’-AAGGATGCTCCACATTTC-3’ (R). The primers for amplifying T1-T4 were 5’-AGGCTGGAAGAGATCTTGGG-3’ (F) and 5’-AGGCTATGACCTGAATGGCC-3’ (R). The primers for amplifying T5 were 5’-CTTACGTGAGGAGCCAGAGG-3’ (F) and 5’-AATGAGACACGCCATCAAAAT-3’ (R). The primers for amplifying T6-T8 were 5’-TGACTCACTGACCTGATGGG-3’ (F) and 5’-CTATGACCTGAATGGCAGAGG-3’ (R).

Dual-luciferase Reporter Assay—The fragments upstream of exons 1, 1’, and 1” were cloned and inserted upstream of the luciferase gene in the pGL3 Basic Vector (Promega). The fragment from −888 to −1155 bp upstream of exon 2 was inserted into the 3’ end of the luciferase gene in the relative pGL3 plasmid for enhancer activity identification, and the promoter-containing region was inserted at the 5’ end of the luciferase gene. Furthermore, the critical nucleotides were mutated using a QuickChange site-directed mutagenesis kit (Stratagene) directly in the pGL3 plasmids containing the predicted trans-acting factor binding sites as described in the text (see Figs. 2A, 2A, 4A, and 5A). The primer sequences for constructing the pGL3 plasmids with the above fragments inserted are not listed due to space considerations; the primer sequences used to generate the trans-acting factor binding site mutations are shown in supplemental Table 1.

The activity of the CD59 promoter was measured using a dual-luciferase reporter assay in HeLa, IMR32, A549, and H1299 cells. The indicated cells were transfected in 96-well plates with basic or modified pGL3 and pRL (Promega) using Lipofectamine 2000 (Invitrogen) without or with 1 mM 8-Br-cAMP pretreatment. After 24 h, the dual-luciferase activities were measured with the luciferase reporter assay system (Promega) using a Bio-Tek synergy HT microplate reader, and the firefly luciferase activities were normalized to the Renilla luciferase activities. These assays were repeated three or more times.

To study the effects of Sp1, p60, p50, c-Rel, CREB, CBP, and p300 deficiency on CD59 expression, we transfected siRNAs-specific for these factors (GenePharma, Shanghai, China) into HeLa cells. The TP53 shRNA (Santa Cruz) was transfected into A549 cells. The siRNA sequences were as follows: 5’-UCUCUGGACCGUGUCAGG-3’ (scrambled), 5’-GCCATATCCCTTACGTA-3’ (p65), 5’-TTTTTAGACACCTAACATT-3’ (p50), 5’-GTTGAAACCTGAAC-3’ (c-Rel), 5’-GGUGGAAUGACCCCGTTT-3’ (CREB), 5’-GGAGAAAATGCTGCT-3’ (Sp1), 5’-A-ACATGGAAGCCTGTTCA-3’ (CBP), and 5’-AATGGACGACTAACCATTGTT-3’ (p300). Forty-eight h after transfection, the cells were collected for dual-luciferase reporter assays and flow cytometry as described below.

To further demonstrate the regulation of TP53 on T5, A459 cells were co-transfected with gradually increasing amounts of a wild type TP53 expression vector (a kind gift from Dr. J. C. Bourdon, University of Dundee, UK), a pGL3 plasmid containing a −1 to −200-bp fragment upstream of CD59 exon 1’ and pRL; the dual-luciferase activity was measured at 48 h after transfection.

Electrophoretic Mobility Shift Assay (EMSA)—HeLa cells were treated with or without 10 ng/ml TNF-α or 1 mM 8-Br-cAMP for 1 h. The nuclear fraction was isolated with the nuclear extract kit (Active Motif), and its concentration was quantified using the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). Aliquots of each nuclear extract were stored at −80 °C until use.

The single-stranded complementary oligonucleotide fragments corresponding to different CD59 promoter and enhancer regions (supplemental Table 2) were synthesized (Invitrogen) and biotinylated using a biotin 3’-end DNA labeling kit (Thermo Fisher Scientific). The biotinylated complementary oligonucleotide pairs were annealed to make double-stranded biotin-labeled probes by boiling for 5 min and cooling slowly overnight. Unlabeled complementary oligonucleotide pairs and unlabeled mutated complementary oligonucleotide pairs were also annealed to make double-stranded competitor probes and non-competitor probes.

EMSAs were performed according to the instructions provided for the LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific). The migration of biotin-labeled probes was detected using an ImageQuant™ LAS 4000 biomolecular imager (GE Healthcare) with streptavidin-horseradish peroxidase conjugates that bind to biotin and a chemiluminescent substrate.

ChIP—Chromatin was immunoprecipitated according to the user guide with the EZ-Magna G Chromatin Immunoprecipitation kit (Millipore). Occupancy was assessed by quantitative PCR for samples precipitated with a specific antibody versus samples precipitated with the control immunoglobulin G (12−371, Millipore) using the specific primers indicated in the related figures.

Immunoblotting—Mammalian cell lysis/extraction reagent (Sigma) was used to generate total cell lysates. A nuclear protein extraction kit (Active Motif) was used to isolate nuclear and cytoplasmic proteins. After electrophoresis, the proteins were transblotted to 0.2-μm polyvinylidene difluoride membranes (Millipore), and nonspecific binding sites were blocked with 5% BSA/TBS-Tween followed by incubation in primary antibodies overnight at 4 °C. The blots were incubated with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) and were then developed using SuperSignal West Femto Chemiluminescence Substrate (Thermo Fisher Scientific).
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Scientific). The blots were visualized using an ImageQuant™LAS 4000 biomolecular imager (GE Healthcare).

To study the effects of exogenous Sp1 and NF-κB (p65/p50) on CD59 expression, we transfected their expressing plasmids pcDNA-Sp1 or pcDNA-p50/pcDNA-p65 (kindly gifted by Dr. Bing Sun, Institut Pasteur of Shanghai, Chinese Academy of Sciences) into IMR32 cells for 24 h. In the p65/p50 co-transfected conditions, the cells also received further TNF-α treatment for 4 h (for detection of p65/p50 translocation into nucleus) and for 16 h (for detection of CD59 expression) before harvest. The cells were then harvested for immunoblotting assays.

Additionally, to study the effects of siRNA against CREB on 8-Br-cAMP-induced CD59 up-regulation, HeLa cells were transfected with CREB siRNA for 48 h. Then the cells were further treated with 1 mM 8-Br-cAMP and harvest at 1 or 16 h for testing CREB/pCREB levels and CD59 expression, respectively.

Streptavidin-Dynabeads Pulldown Assay—The double-stranded biotinylated CD59 enhancer probe covering −842 to −1160 bp upstream of exon 2 was PCR-amplified by PCR using a HeLa cell DNA template and the following primers: forward primer, 5′-biotin/GGAGCCCTCAATTGTGAA-3′; reverse primer, 5′-biotin/-GAGTCATCTACCCCT-3′ (Invitrogen). To prepare the mutated CREB enhancer probe, we first cloned the above enhancer region into a T-vector and then mutated the critical CREB-binding site (supplemental Table 1) using the QuikChange site-directed mutagenesis kit (Stratagene). The binding assay took advantage of the high binding affinity between Dynabeads® M-280 Streptavidin (Invitrogen) and biotinylated DNA; this high affinity allows for the precipitation of DNA-bound proteins using a magnet. Briefly, the beads were washed with 1× binding and washing buffer; after washing, 150 μl of a HeLa nuclear extract (500 μg) was incubated with 160 μl of 2× binding and washing buffer and 10 μl of biotinylated probe (10 μg) at room temperature for 30 min. The coated beads were washed with 1× binding and washing buffer on the magnet, and the beads were then resuspended in 50 μl of elution buffer at 90 °C for 5 min to release the immobilized biotinylated probe. The supernatant was collected on the magnet, and the levels of the indicated proteins were analyzed by immunoblotting.

Flow Cytometry—HeLa cells were individually transfected with scrambled siRNA, p65 siRNA, or CREB siRNA for 48 h or treated with 10 ng/ml TNF-α, 15 μg/ml C5b-6/7/8/9, 50 ng/ml LPS, or 50 ng/ml LPS plus 20% NHS for 30 min. U937 cells were individually treated with 50 ng/ml C5a or 50 ng/ml LPS plus 20% NHS for 30 min, then washed with PBS and cultured for 16 h in drug-free medium. The challenged cells were then treated without (to detect CD59 expression after knocking down p65 or CREB) or with 15 μg/ml C5b-6/7/8/9 for 30 min (for the other treatments) and harvested for flow cytometry analysis. Briefly, the collected cells were washed twice with PBS, then incubated with FITC mouse anti-human CD59 in siRNAs, LPS plus NHS or C5b-6/7/8/9 treatment, or rabbit anti-human C5b-9 followed by FITC goat anti-rabbit IgG in individual treatments with LPS, TNF-α, or C5a, then stained with 2 μg/ml PI for 15 min before running in a Cytomix FC 500 MXP (Beckman Coulter).

Statistical Analysis—The data are presented as the means ± S.D. The significant differences between two groups were determined using the one-tailed Student’s t test for unpaired data. p values less than 0.05 were considered statistically significant.

RESULTS

Identification of CD59 Transcripts and Their Initiation Sites—To identify all of the transcription initiation sites in the human CD59 gene, we performed 5′ GeneRacer PCR that is designed to amplify only capped transcripts. Given that all transcripts of CD59 share a common sequence at the end of the open reading frame in exon 4, we designed an RP complementary to exon 4 to amplify all of the transcripts (Fig. 1A). The PCR products labeled a and b are nonspecific, but the strong product band labeled d is specific and is termed transcript 1 (T1) (Fig. 1, A and C); T1 was the only CD59 transcript that had been identified previously. In addition, the remaining faint bands (the area labeled c) were also determined to be specific. These transcripts were called T2-T8, respectively, except for T7, which was identified in silico, likely due to its trace abundance (Fig. 1C). T5 may initiate from another transcription start site (Fig. 1D), and T6-T8 may be produced by another transcription mechanism and by distinct alternative splicing, such as alternative 5′ donor site and intron retention (Fig. 1E). It is important to note that the mechanisms of alternative splicing are beyond the scope of this study because all of the transcripts share the same protein sequence.

Next, we compared the expression abundance of three groups of CD59 transcripts using qRT-PCR. T1-T4 are markedly more abundant than the other transcripts in HeLa, IMR32, A549, and H1299 cells, with the exception of T6-T8, whose levels are comparable to T1-T4 in IMR32 cells (Fig. 1F). Furthermore, all of the cell lines express comparable levels of the transcripts except IMR32 cells, most likely due to the low T1-T4 abundance (Fig. 1G). These findings demonstrate that T1-T4 are the predominant CD59 transcripts; therefore, the transcriptional regulation of T1-T4 may be critical for CD59 expression.

Sp1 and NF-κB Are the Major TFs for CD59 Transcription—CD59 is constitutively expressed in most tissues. The widely expressed Sp1 was hypothesized as its TF because the region of −35 to −70 bp upstream of exon 1 contains two Sp1 conserved binding sites (7) (Fig. 2A). Therefore, we employed a dual-luciferase reporter assay to identify the promoter region upstream of exon 1 in HeLa and IMR32 cells; these two cell lines were selected because IMR32 cells express much lower levels of CD59 than HeLa cells (Fig. 2B, inset). The −35 to −70-bp promoter fragment displays promoter activity in both cell lines; the promoter activity of the −103 to −135 bp fragment appears to be even stronger but only in HeLa cells. Therefore, we con-
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Supplemental Fig. S1, JOURNAL OF BIOLOGICAL CHEMISTRY

In addition, the results of binding site mutation assays in the conserved NF-κB promoter region located in the transcriptional promoter region of HeLa cells (Fig. 2B) indicate that inactivating mutations in the NF-κB-binding sites completely abrogated the promoter activity (Fig. 2C). Furthermore, the regions of −35- to −70-bp and −70- to −135-bp in HeLa cells, indicating no synergistic interaction between the TFs binding to these two regions. Furthermore, the regions of −35- to −70-bp and −70- to −135-bp exhibit similar promoter activity (Fig. 2B), suggesting that there is no additional promoter region located in the −100- to −1000-bp fragment. In addition, the results of binding site mutation assays in HeLa cells (Fig. 2A) indicate that inactivating mutations in the conserved NF-κB- and/or Sp1-binding sites led to an overwhelming decrease in promoter activity. The promoter activity in the −103- to −168-bp fragment disappeared completely when the conserved NF-κB-binding site was mutated to be inactive; simultaneously mutating the NF-κB- and Sp1-binding sites completely abrogated the promoter activity (Fig. 2C). These findings suggest that Sp1 and NF-κB may be the binding proteins for these promoter regions.

Using a ChIP assay, we further demonstrated that Sp1 and NF-κB can indeed bind to these promoter regions. The antibodies against Sp1 and phosphorylated Sp1, but not an isotype control antibody, could amplify a target sequence containing the putative Sp1-binding sites in the −35- to −70-bp fragment with specific primers (Fig. 2A and D). Similarly, the antibodies against p65, p50, and c-Rel, but not against RelB, p52, or an isotype control antibody, could amplify the specific band in the −103- to −135-bp fragment (Fig. 2E). The results of the EMSA further confirmed this observation. The biotin-labeled probe containing these Sp1-binding sites could form a specific band that could be quenched by biotin-unlabeled (cold) probe but not by a mutated probe. Furthermore, this specific band shifted after adding antibodies against Sp1 or phosphorylated Sp1 (Fig. 2F). A similar result was obtained for the NF-κB test; the specific band disappeared or shifted after adding cold probe or antibodies against p65, p50, and c-Rel but not after adding antibodies against RelB and p52 (Fig. 2G). Therefore, these findings demonstrate that canonical NF-κB and Sp1 most likely regulate CD59 transcription separately.

To further confirm that Sp1 and NF-κB are the regulators for T1-T4, we knocked down the expression of these factors using specific siRNAs in HeLa cells. Insufficiency of Sp1 or the NF-κB subunits p65, p50, and c-Rel (Fig. 2H) resulted in significantly reduced expression of T1-T4 and consequently the total transcript levels (T1-T8) but not of T5 or T6-T8 (supplemental Fig. S1, A–D). Using immunoblotting and flow cytometry, we found that the translational level of CD59 was decreased by knocking down either Sp1 or NF-κB subunits p65, p50, or c-Rel (Fig. 2H and supplemental Fig. S1, E and F). In addition, both of the exogenous Sp1 and p65/p50 by transfecting their expressing...
plasmids could significantly up-regulate CD59 expression (Fig. 2, I and J). Moreover, the treatment of TNF-α could further increase CD59 expression in p65/p50 co-transfected condition (Fig. 2I). Taken together, we conclude that Sp1 and NF-κB are sufficient to activate CD59 transcription.

Given that Sp1 is ubiquitously expressed and the canonical NF-κB dimer is translocated into the nucleus upon upstream signaling activation, our findings unambiguously demonstrate that Sp1 is responsible for the constitutive expression of CD59 and that canonical NF-κB signaling is responsible for the inducible expression of CD59.

TP53 Is Not the Main TF for CD59 Transcription—TP53 has been indicated to confer cancer cell resistance to complement destruction by enhancing CD59 expression (8), but it remains unclear whether TP53 functions as a TF or not. One of the putative TP53-binding sites (18) is coincidentally located in the region of 159 to 189 bp upstream of CD59 exon 1 (Fig. 3A), which led us to hypothesize that TP53 may be the TF for T5. Similarly, we used a dual-luciferase reporter assay to determine the promoter region for T1-T4 transcription (Fig. 3B), in which the expression levels of Sp1, p65, p50, and c-Rel in the nucleus and the expression level of CD59 in total lysates, n = 3. C, analysis of the effects of mutations in potential NF-κB and Sp1 binding sequences on the activity of the CD59 promoter in HeLa cells. n = 3. D and E, ChIP assay. Antibodies against Sp1 or phosphorylated-Sp1 (D) and against p65, p50, or c-Rel (E) were used to capture the complex containing the relative promoter sequences in HeLa cell nuclei. F–G, EMSA. Specific oligonucleotides containing Sp1 (F) or NF-κB (G) binding sites could interact with the relative TFs; the specificity of this interaction was determined using a cold probe, a mutated probe, and super-shifted bands (indicated by an arrow or bracket). H, the effects of siRNAs against Sp1, p65, p50, and c-Rel on CD59 expression in HeLa cells. The efficacy of siRNAs is shown by the expression of TFs prepared from the nuclear extracts, and the CD59 expression level was detected in total cell lysates. NS, non-silencing. J, the exogenous Sp1 by transfecting its expressing plasmids could up-regulate CD59 expression, which was enhanced by adding further TNF-α.

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FIGURE 2. NF-κB and Sp1 are the TFs for T1-T4. A, partial sequences upstream of exon 1. The predicted NF-κB-binding site, two predicted Sp1-binding sites, and the primer sequences for the ChIP assay are indicated. B, identification of the promoter region for T1-T4 transcription using the dual-luciferase reporter assay. Inset, the expression levels of Sp1, p65, p50, and c-Rel in the nucleus and the expression level of CD59 in total lysates, n = 3. C, analysis of the effects of mutations in potential NF-κB and Sp1 binding sequences on the activity of the CD59 promoter in HeLa cells. n = 3. D and E, ChIP assay. Antibodies against Sp1 or phosphorylated-Sp1 (D) and against p65, p50, or c-Rel (E) were used to capture the complex containing the relative promoter sequences in HeLa cell nuclei. F–G, EMSA. Specific oligonucleotides containing Sp1 (F) or NF-κB (G) binding sites could interact with the relative TFs; the specificity of this interaction was determined using a cold probe, a mutated probe, and super-shifted bands (indicated by an arrow or bracket). H, the effects of siRNAs against Sp1, p65, p50, and c-Rel on CD59 expression in HeLa cells. The efficacy of siRNAs is shown by the expression of TFs prepared from the nuclear extracts, and the CD59 expression level was detected in total cell lysates. NS, non-silencing. J, the exogenous Sp1 by transfecting its expressing plasmids could up-regulate CD59 expression, which was enhanced by adding further TNF-α.
observed that its promoter activity was abolished in A549 cells (Fig. 3B). These results suggest that TP53 may be the TF for T5.

Next, we used EMSA and ChIP assays to examine the physical binding of TP53 to the above region. The biotin-labeled probe containing TP53 binding sites could produce a specific band in an EMSA; this specific band disappeared after adding biotin-unlabeled probe but not after adding a mutated probe. Moreover, this specific band shifted after adding antibodies against TP53 or phosphorylated or acetylated TP53 at different sites, especially acetylated TP53 at Lys-373 (Fig. 3C). Furthermore, the specific TP53 antibody, but not a control isotype antibody, could pull down this TP53-binding site-containing fragment in a ChIP assay using the specific primers (Fig. 3, A and D). These results demonstrate the specific interaction between TP53 and the −150- to −189-bp region upstream of CDS9 exon 1′.

Moreover, we used siRNA to knock down the expression of TP53 and then measured its effect on CDS9 expression in A549 cells. As we expected, only T5 was significantly reduced, but not T1-T4, T6-T8, or whole transcripts T1-T8 (Fig. 3E). Accordingly, there is no obvious change in the CD59 expression level (Fig. 3F). However, we over-expressed TP53 by transfecting H1299 cells with a vector containing the wild type TP53 gene, and immunoblotting revealed that the level of CDS9 protein (G) but did increase the levels of transcript T5 significantly (H) in H1299 cells, n = 3, *P < 0.001 relative to the vehicle control.

FIGURE 3. TP53 regulates the transcription of T5. A, partial sequences upstream of exon 1′. The predicted TP53-binding site and primer sequences used for the ChIP assay are indicated. B, identification of the promoter region for T5 transcription using a dual-luciferase reporter assay. Inset, TP53 and CDS9 expression levels in total cell lysate. C, EMSA. TP53, particularly acetylated TP53 at Lys-373, can bind to the promoter region upstream of exon 1′. D, ChIP assay. The antibody against TP53 can precipitate the complex of TP53 and its binding promoter sequences in A549 cell nuclei, as determined by PCR with specific primers. E and F, knockdown of TP53 significantly reduced the expression of transcript T5 (E) but not CDS9 protein expression (F) in A549 cells. n = 5, G and H, overexpression of TP53 did not increase the expression levels of CDS9 protein (G) but did increase the levels of transcript T5 significantly (H) in H1299 cells. n = 3, *P < 0.001.
luciferase activity increased in correlation with gradually increasing amounts of transfected TP53 plasmids (Fig. 3).

These results clearly demonstrate that TP53 appears to be a negligible TF for CD59 regulation.

CREB Is Another TF for CD59 Transcription

To identify the TF regulating T6-T8, we first searched the region for promoter activity upstream of exon 1 using a dual-luciferase reporter assay. The promoter fragment from -250 to -375 bp had high luciferase activity and an inactivating mutation in the predicted CREB-binding site abolished the promoter activity (Fig. 4A).

Furthermore, we also observed that the promoter activity of this fragment increased radically with an additional enhancer region in the -500- to -1000-bp fragment, which will be discussed later.

ChIP and EMSA results further demonstrated that this promoter region could bind to CREB. A CREB antibody, particularly a phosphorylated CREB antibody, could pull down the DNA sequence containing the putative CREB-binding site using specific primers (Fig. 4C).

It has been reported that cAMP results in the activation of protein kinase A, in turn phosphorylating CREB and consequently inducing transcription. Here, we used 8-Br-cAMP, a modified form of cAMP that can easily penetrate the cell membrane, to clarify whether phosphorylated CREB could enhance the promoter activity in this region. Indeed, treating HeLa cells that were transfected with a pGL3 plasmid containing the CD59 -1 to -375-bp fragment with 8-Br-cAMP significantly increased the relative luciferase activity compared with untreated HeLa cells (Fig. 4E). These results further indicate...

**FIGURE 4.** CREB is the TF for T6-T8. A, sequences upstream of exon 1. The predicted CREB-binding site and the primer sequences used for the ChIP assay are indicated. B, identification of the promoter region for T6-T8 transcription using a dual-luciferase reporter assay. Mut, mutant. Inset, CREB and phosphorylated-CREB expression levels in the nucleus and the CD59 expression levels in the cytoplasm. C, ChIP assay. In a PCR with specific primers, a single specific band could be amplified from the complex captured by antibodies against CREB and phosphorylated-CREB in HeLa cell nuclei. D, EMSA. The specific oligonucleotide containing the CREB-binding site could produce a specific band from HeLa cell nuclear extracts. E, relative luciferase activity was increased by pretreatment with 1 mM 8-Br-cAMP in HeLa cells transfected with pGL3 containing the -1 to -375-bp fragment upstream of exon 1. n = 4. F, qRT-PCR. Stimulation of HeLa cells with 8-Br-cAMP induced elevated levels of all CD59 transcripts, especially T6-T8. n = 3. G, immunoblotting. Treatment with 8-Br-cAMP led to phosphorylation of CREB and accordingly increased CD59 expression in HeLa cells. *, p < 0.05 relative to cells treated with no 8-Br-cAMP.
that CREB binds to this promoter region and exhibits transcription activity.

Accordingly, treatment with 8-Br-cAMP led to a 6-fold increase in the T6-T8 transcription level in HeLa cells; unexpectedly, we still observed that the expression levels of T1-T4 and T5 (and thus the total transcript levels T1-T8) also increased 2-fold, although this change was not statistically significant for T5 (Fig. 4F). Consistent with this observation, CD59 protein expression increased gradually 1 h after the administration of 8-Br-cAMP, which may have resulted from the overwhelming amount of phosphorylated CREB starting at 0.5 h after treatment (Fig. 4G). Therefore, these results indicate that CREB is the TF that regulates the expression of T6-T8 and that CREB plays other, more important roles in CD59 transcription.

**CREB Regulates CD59 Transcription as an Enhancer-binding Protein Together with Other TFs Scaffolded by CBP/p300**

It was previously reported that an enhancer sequence in intron 1 between 888 and 1155 bp upstream of exon 2 is required for a high level of CD59 transcription (Fig. 5A) (10), but the precise location and the relevant binding protein remain unknown. As described earlier, we identified CREB as the TF for T6-T8 and unexpectedly found that CREB may also regulate T1-T4 and T5. Using MatInspector (Genomatix Software GmbH), we found that this enhancer region contains a CREB binding half-site, TGACG, on the opposite strand (14)(Fig. 5A). These findings led us to hypothesize that CREB may be the elusive enhancer-binding factor. We cloned this enhancer sequence and inserted it into the 3' end of the luciferase gene in various pGL3 plasmids containing different promoter regions (Fig. 5B).
NF-κB and CREB Bridged by CBP/p300 Regulate CD59 Transcription

We then used a dual-luciferase reporter assay to study the effect of the inserted enhancer and observed that the relative luciferase activity increased >2-fold in all constructs except those pGL3 plasmids containing Sf1 (−1 to −70 bp upstream of exon 1) and TP53-binding sites (−1 to −200 bp upstream of exon 1) (Fig. 5B). We presume that Sf1 might not interact with enhancer-binding CREB and that the enhancer region is required to be upstream of the promoter for TP53 binding. Furthermore, we mutated critical nucleotides in the putative CREB binding region (Fig. 5A) and observed that the relative luciferase activities decreased markedly in all constructions (Fig. 5B). Moreover, co-infection with an siRNA against CREB with the pGL3 plasmids containing both the promoter and enhancer regions could significantly reduce the relative luciferase activity (data not shown), thereby leading to considerable reductions in CD59 transcript and protein levels (Fig. 5, C and D, and supplemental Fig. S1, A–F). Moreover, the increased CD59 expression by 8-Br-cAMP administration was dramatically reduced by specific CREB siRNA pretreatment (Fig. 5D). Together, these results strongly suggest that CREB is the enhancer-binding protein that induces the high level of CD59 transcription.

CBP and p300 have been shown to interact with phosphorylated CREB and to enhance transcription synergistically (16). Indeed, the reduced expression of CBP or p300 due to siRNA treatment could decrease the relative luciferase activities in the enhancer-containing pGL3 plasmids (data not shown), further reducing the expression levels of all CD59 transcripts and protein, as detected by qRT-PCR and immunoblotting (Fig. 5, C and D).

Moreover, we determined the interaction between CBP/p300, enhancer-binding protein CREB, and the transcription factors NF-κB, TP53, and CREB. First, we determined the direct and specific binding of CREB to this enhancer region by EMSA (Fig. 5E). Next, we used a ChIP assay to examine this interaction. An antibody against CREB or phosphorylated CREB could pull down oligonucleotides containing the CREB binding enhancer region (left section in Fig. 5F) and the promoter regions of T1–T4, T5 (middle section in Fig. 5F), and T6–T8 (Fig. 4C) detected with individual specific primers (Figs. 2A, 3A, 4A, and 5A). However, antibodies against p50 and p65 cloud also pull down oligonucleotides containing the enhancer region (right section in Fig. 5F) detected with specific primers (Fig. 5A). Finally, a Streptavidin-Dynabeads pulldown assay was employed to further demonstrate this interaction. The biotin-labeled enhancer probes (−842 to −1160 bp upstream of exon 2) with or without a critical CREB-binding site mutation were preincubated with Streptavidin-Dynabeads. We found that beads conjugated to the original enhancer but not beads conjugated to the mutated enhancer could pull down CREB, CBP, p300, p50, p65, and TP53 but not Sf1 (Fig. 5G). Together, our findings unequivocally demonstrate that CBP/p300 can scaffold the CD59 enhancer-binding CREB and promoter binding NF-κB, TP53, or CREB to regulate CD59 transcription.

Subsequently, we functionally tested whether reduced CD59 expression, e.g. induced by knocking down p65 or CREB, is able to sensitize human cells to complement destruction. HeLa cells were transfected with specific siRNAs against p65 or CREB for 48 h and then complement components C5b-6, C7, C8, and C9 (C5b-6/7/8/9) were sequentially added, in which C5b-6/7/8/9 served as the complement damage to the cells; flow cytometric analysis revealed significantly higher C5b-9 deposition and PI staining in the p65 or CREB siRNA-treated cells compared with scrambled siRNA-treated cells (supplemental Fig. S1, G and H). These results further establish that both NF-κB and CREB are important regulators for host defense against complement damage via the regulation of CD59 expression.

LPS-triggered Complement Activation Increases CD59 Expression and Confers Cell Protection from Complement Destruction—LPS is a major component of the outer membranes of Gram-negative bacterial cells. LPS binding to the TLR4–CD14–MD2 receptor complex can trigger many signaling pathways and can activate downstream transcription factors, including NF-κB, promoting the secretion of proinflammatory cytokines, such as TNF-α. Meanwhile, LPS can activate the alternative complement pathway, thereby causing local C5a production and inducing the deposition of C3b/iC3b and MAC in bacterial and possibly host cells.

HeLa and U937 cells were treated with LPS and NHS for 30 min and were then challenged with C5b-6/7/8/9 16 h later. The CD59 expression level increased significantly, leading to a reduction in PI staining as measured by flow cytometry (Fig. 6, A and B). LPS–triggered complement activation could induce the production of TNF-α, SC5b-9, and C5a; therefore, we detected the individual effects of these factors on protection from MAC-mediated damage. After HeLa cells were treated with C5b-6/7/8/9, we similarly observed an increase in CD59 expression and a reduction in PI staining with C5b-6/7/8/9 treatment (Fig. 6A) in which C5b-6/7/8/9 most likely functions to activate the NF-κB pathway as shown in Fig. 7A. We did not detect C5b-9 deposition instead of CD59 expression in the pre-treatment of LPS plus NHS or C5b-6/7/8/9 conditions due to the interference of prior C5b-9 deposition (data not shown). Furthermore, if HeLa cells were treated with LPS or TNF-α or if C5a receptor-expressing U937 cells were treated with C5a, we found that less C5b-9 was deposited in cells and fewer cells were stained by PI after the administration of C5b-6/7/8/9 (Fig. 6, C and D). These findings suggest that LPS-induced complement activation, LPS itself, TNF-α, C5a, and SC5b-9 are able to promote host defense against further MAC attack by increasing CD59 expression. Furthermore, the diverse functions of C5b-6/7/8/9 in the experiments above were switched by the expression level of CD59.

The results of immunoblot analysis further demonstrate that LPS–induced complement activation, LPS, TNF-α, or individual stimulation with C5b-6/7/8/9 could markedly increase the CD59 expression level in HeLa cells, accompanied by slight, if any, increases in CD55 and CD46 expression (Fig. 6, E and F). Moreover, when U937 cells were challenged by C5a, we observed that the CD59 expression level increased markedly and that the CD55 expression level increased slightly; the CD46 expression level was unchanged (Fig. 6G).

Notably, the stimulation of these cells by LPS–induced complement activation, LPS, TNF-α, C5b-6/7/8/9, or C5a was relatively weak at the indicated dosages and durations. If the stimulation level was enhanced, the challenged cells underwent sustained apoptosis and experienced more C5b-9 deposition.
despite the higher CD59 expression level (data not shown). This observation is consistent with previous descriptions that strong stimulation with LPS, TNF-α/H9251, or C5a can induce apoptosis (19) and that early apoptotic cells are more sensitive to complement-induced and MAC-mediated cytolysis (20). Therefore, the LPS-induced weak complement activation could up-regulate CD59 expression, protecting host cells from complement destruction, but a more strongly activated complement system would instead induce the clearing of the invading pathogens regardless of the CD59 up-regulation in host cells.

Because T1-T4 are the predominant CD59 transcripts, we further investigated the response of their trans-acting factors Sp1, NF-κB, and CREB to the above stimuli. LPS, TNF-α, or C5b-6/7/8/9 could induce the nuclear translocation of NF-κB and especially p65/p50 and the slight phosphorylation of Sp1 in HeLa cells. Furthermore, either LPS or TNF-α but not C5b-6/7/8/9 treatment could promote CREB phosphorylation (Fig. 7A). Additionally, although the nuclear translocation of NF-κB and Sp1 phosphorylation were not observed in U937 cells after C5a treatment, we noted marked CREB phosphorylation (Fig. 7B), which likely resulted from the enhanced phosphorylation of upstream ERK1/2 (Fig. 6G). Therefore, we successfully unraveled the molecular mechanisms that underlie the up-regulation of CD59 expression by the LPS-induced inflammatory environment.

In addition, it has been thoroughly investigated before that 8-Br-cAMP, LPS, TNF-α, or C5b-6/7/8/9 could activate NF-κB and/or CREB. Therefore, we further performed the EMSA assay to identify whether these stimuli can enhance the bindings of the TFs to cis-acting elements. As shown in Fig. 7C, the biotin-labeled probe containing the CREB-binding enhancer site could form a stronger specific band after 8-Br-cAMP treatment compared to no 8-Br-cAMP treatment. Moreover, we also found that TNF-α treatment produced similar results. The biotin-labeled probes containing CREB-binding enhancer site or NF-κB binding promoter site developed a stronger specific band after TNF-α treatment compared to no TNF-α treatment (Fig. 7D and E). All of these specific bands could be partially super-shifted by anti-pCREB or anti-NF-κB and completely quenched by cold probe. Therefore, these results demonstrated that NF-κB and CREB regulate CD59 expression through directly binding to the related promoter and enhancer sites, respectively.

**DISCUSSION**

In this study we have comprehensively investigated the molecular mechanisms of CD59 transcriptional regulation. Sp1 regulates the constitutive expression of CD59 and NF-κB and enhancer-binding CREB bridged by CBP/p300 mainly regulate the inducible expression of CD59 (Fig. 7F). This finding may provide a unique mechanism for the transcriptional regulation of other genes, where TFs not limited to NF-κB, TP53, and CREB, enhancer-binding CREB, CBP/p300, and the basal transcription apparatus interplay cooperatively. Finally, we used
LPS as an example to investigate how host cells survive MAC-mediated complement damage in the pathogen-induced inflammatory environment by up-regulating CD59 transcription. Notably, CREB is much more important as an enhancer-binding protein than as a TF for CD59 transcriptional regulation. This novel function of CREB may further explain why a genome-wide analysis revealed the number of putative target genes for CREB to be as high as \( \frac{H11011}{H5000} \) (17).

The spontaneously weak complement activation can be physiologically promoted by C1-tickover in the classical pathway or by C3-tickover in the alternative pathway for immune surveillance (21). The sufficient MAC assembled in the cell membrane is able to induce direct cytolysis; however, a sublytic MAC concentration may activate extensive downstream cell-survival signaling via such mechanisms as \( \text{Ca}^{2+} \) influx and reactive oxygen and nitrogen species production (6). Therefore, host cell survival from potential MAC-mediated cytolysis requires the constitutive expression of CD59. However, the complement system can be intensively activated by various antibodies and pathogenic components, which necessitates higher and inducible levels of CD59 expression for host defense. Therefore, the dysregulation of NF-\( \kappa B \), CREB, CBP/p300, and likely Sp1 may be involved in substantial human disorders, such as neurodegenerative diseases, autoimmunity, atherosclerosis, chronic infection, and cancer, at least in part through the aberrant regulation of CD59.

The deficiency of CD59 on clones of circulating cells in paroxysmal nocturnal hemoglobinuria due to a somatic mutation in the \( \text{PIG-A} \) gene may render erythrocytes and white blood cells susceptible to MAC-mediated cytolysis (22). Recently, it was reported that the deficiency of CD59 in the cell membrane due to the C89Y mutation in CD59 is associated with childhood familial chronic Coombs-negative hemolysis and relapsing immune-mediated polyneuropathy (23). However, the down-regulation of CD59 is also involved in the pathogenesis of many human diseases to different extents. Complement activation,
such as by β-amyloid, plays an important role in the pathogenesis of neurodegenerative diseases such as Alzheimer disease (24), and the degree of neuron damage has been positively correlated with the intensity of deposition of complement-activating products in plaques or tangles, especially Scs5b-9; this phenomenon is most likely due to the undetectable expression of mCRPs, including CD59 (25, 26). Therefore, it is reasonable to hypothesize that a CD59 deficiency may contribute to the pathogenesis of Alzheimer disease (27). Numerous findings have demonstrated that NF-κB and CREB play critical roles in synaptic plasticity and long-term memory formation by regulating the expression of various genes involved in cell survival, differentiation, and development (28, 29); however, few studies have detected their functions in regulating the expression of mCRP genes to protect nerve cells from complement attack.

Here, we clarify that the neuroblastoma cell line IMR32 expresses very low levels of CD59, mostly due to the absence of p65 and p50 but not the other NF-κB subunits, the slightly phosphorylated CREB, and possibly the low expression level of Sp1 (Fig. 2B, inset). Meanwhile, it has been suggested that the deficient expression of mCRPs, especially CD59, is associated with autologous cell destruction by complement attack in autoimmune disorders, such as autoimmune hemolytic anemia, systemic lupus erythematosus, autoimmune thrombocytopenia, and aplastic anemia (4, 5, 30), possibly resulting from NF-κB dysfunction due to genetic mutation (31). In addition, mouse CD59 genetic deficiency promotes atherogenesis in animal models (32); similarly, this study revealed the pathogenic roles of CD59 in atherosclerosis, a chronic inflammatory and immune vascular disease (33). Understandably, the up-regulation of CD59 expression may provide a benefit in the prevention and treatment of these diseases.

In contrast, the acquired overexpression of CD59 confers resistance to complement-mediated cytolyis in cells infected with hepatitis B virus (9), hepatitis C virus (34), and human immunodeficiency virus (35), contributing to the occurrence of persistent infections. Furthermore, chronic inflammation, especially when induced by pathogens, has been implicated in tumorigenesis, where many proinflammatory and pathogenic virulence factors are produced in local tumor sites (36). These factors, such as TNF-α, IL-1β, IL-6, IL-10, and LPS, in turn up-regulate CD59 expression, as demonstrated here and previously (37, 38), thereby allowing nascent tumor cells or cancer stem cells to escape from complement surveillance (39, 40). Moreover, CD59 also has diverse complement-independent roles, including anti-apoptotic (41) and tumor angiogenic functions (42). Indeed, CD59 is overexpressed in most tumors and is thought to be the most effective mCRP in protecting tumor cells from complement attack (43). Based on our findings in this study, it can be speculated that the actively stimulated NF-κB (44), CREB (45), and possibly Sp1 (46) signaling pathways may be responsible for the up-regulation of CD59 expression, thus triggering tumor cells to escape from complement attack. Given that the NF-κB and CREB signaling axis has already been proposed as a drug target in cancer therapy (47, 48), the down-regulation of CD59 would be an unexpected secondary effect that further supports this concept.

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