

ORIGINAL ARTICLE

Mouse Cd59b but not Cd59a is upregulated to protect cells from complement attack in response to inflammatory stimulation

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Universally expressed CD59 is the sole membrane complement regulatory protein that protects host cells from complement damage by restricting membrane attack complex assembly. The human gene encodes a single CD59, whereas the mouse gene encodes a duplicated CD59, comprising mCd59a and mCd59b, with distinct tissue distribution. Recently, we revealed that Sp1 regulates constitutive CD59 transcription and that canonical nuclear factor kappa light chain enhancer of activated B cells (NF-κB) and cyclic AMP-responsive element-binding protein (CREB) regulate inducible CD59 transcription. However, the mechanisms that underlie mCd59 regulation remain unclear. Here we demonstrate that Sp1 controls broadly distributed mCd59a expression, whereas serum response factor (SRF) and canonical NF-κB regulate selectively expressed mCd59b. Tumor necrosis factor-α *in vitro* and lipopolysaccharide *in vivo* remarkably enhance the expression of mCd59b but not mCd59a by activating SRF and NF-κB, thus protecting cells from complement attack. In addition, cAMP analog treatment also dramatically increases mCd59b but not mCd59a expression in a manner independent of CREB, SRF and NF-κB. Therefore, mCd59b but not mCd59a may be the responder to external inflammatory stimuli and may have an important role in complement-mediated mouse models of disease.

Genes and Immunity (2015) 16, 437–445; doi:10.1038/gene.2015.29; published online 23 July 2015

INTRODUCTION

The complement system is essential to innate immunity and functions as a key modulator of adaptive immunity.¹ Complement activation, regardless of the initiated pathways, eliminates invading pathogens mainly via three major physiological effectors: (1) the potent pro-inflammatory anaphylatoxins C3a and particularly C5a, which modulate and prime the immune system; (2) C3b/iC3b deposited on targeted surfaces to opsonize the invaders in cooperation with immune cells; and (3) the membrane attack complex (MAC), that is, the C5b-9n complex, which is assembled on targeted surfaces for direct lysis.² MAC-mediated cytolysis is characterized by a rapid increase in intracellular Ca²⁺ accompanied by the loss of mitochondrial membrane potential and leakage of adenine nucleotide pools.³ Once enough MAC is assembled on the cell surface, it can lead to the direct lysis of targeted invaders.^{4,5} To protect autologous cells from bystander MAC-mediated damage, the sole membrane-bound complement regulator CD59 has a critical role in restricting MAC assembly.⁶

The human encodes a single *CD59* gene, whereas the mouse encodes duplicated CD59 comprising separated *mCd59a*⁷ and *mCd59b*⁸ genes that share 57.7% consensus in protein sequence. Human CD59 is ubiquitously expressed in most cells and tissues.⁹ mCd59a was identified as the primary regulator inhibiting MAC formation,¹⁰ because mCd59a is universally expressed and mCd59b is only selectively expressed in testis and on sperm.^{11,12} However, mCd59b demonstrates specific inhibitory efficacy similar to or even sixfold higher than mCd59a in restricting MAC assembly that may result from its different protein sequence.^{11,13}

In addition, mCd59b was suggested to have additional complement-independent roles in acrosome activation and motility.^{12,14} Deficient or reduced expression of mCd59a and/or mCd59b partly contributes to various immune and inflammatory processes and diseases such as transplant rejection, autoimmune hemocytopenia, systemic lupus erythematosus and atherosclerosis.^{15–18} Thus, further work is needed to shed light on the underlying mechanisms by which *CD59* is regulated in response to extracellular inflammatory stimulation. Recently, we comprehensively investigated the human CD59 transcriptional regulation, and revealed that the constitutive expression of human *CD59* is regulated by the widely expressed transcription factor trans-acting transcription factor 1 (Sp1), whereas canonical nuclear factor kappa light chain enhancer of activated B cells (NF-κB) and cyclic AMP-responsive element-binding protein (CREB, as an enhancer-binding protein) scaffolded by CBP/p300 are responsible for its inducible expression.¹⁹ However, although we previously mapped the promoter regions of *mCd59a* and *mCd59b*,²⁰ the details of their transcriptional regulation, and in particular the transcription factors involved, remain largely obscure.

Inflammation may induce the production of antibodies via the humoral immune response. Some are deleterious autoantibodies produced in autoimmune diseases that activate the classical complement pathway. Lipopolysaccharide (LPS), a major outer membrane component of gram-negative bacteria, may trigger the alternative complement pathway to accordingly initiate host defense.²¹ In addition, LPS promotes the secretion of pro-inflammatory cytokines represented by tumor necrosis factor-α

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(TNF- α), which further boosts inflammation. Therefore, we investigated the transcriptional regulation of *mCd59* in response to this inflammatory stimulus.

In this study, we found that *mCd59b* but not *mCd59a* is upregulated to protect cells from complement-mediated MAC damage. Furthermore, we revealed that both NF- κ B and serum response factor (SRF) cooperatively regulate the transcription of *mCd59b* under inflammatory stimulation, whereas the widely expressed *mCd59a* may be regulated by constitutive Sp1.

RESULTS

NF- κ B and SRF regulate *mCd59b* transcription

We recently demonstrated that Sp1 is responsible for human *CD59* constitutive expression, and canonical NF- κ B in conjunction with CREB (as an intron 1-located enhancer-binding protein) regulates human *CD59*-inducible transcription.¹⁹ Using MatInspector software (Genomatix Software GmbH, München, Germany), we first predicted the possible transcription factors for *mCd59a* (Gene accession no. NM_007652.5) and *mCd59b* (Gene accession no. NM_181858.1) expression in 2000-bp region upstream of transcriptional initiating site.¹⁹ Sp1- and NF- κ B-binding sites were exclusively located in the promoter regions of *mCd59a* and *mCd59b*, respectively. The NF- κ B response elements were located 151–163 bp upstream of *mCd59b* exon 1 (Figure 1a), whereas the Sp1 response elements were located 63–90 bp upstream of *mCd59a* exon 1 (Figure 2b). Next, we used a dual-luciferase reporter assay to identify the promoter region in mouse NIH/3T3 fibroblast cells. In agreement with our previous report,²⁰ the 150–200-bp and 200–237-bp elements upstream of *mCd59b* exon 1 may represent two separate promoter regions (Figure 1b). The 150–200-bp region contains the predicted NF- κ B response elements. Therefore, we mutated the critical nucleotides for NF- κ B binding in the 1–200-bp region upstream of *mCd59b* exon 1 (Supplementary Table 2). The results of the dual-luciferase reporter assay showed that promoter activity in the 1–200-bp region with nucleotides mutation was completely abolished to the level in the 1–150-bp region and was dramatically decreased compared with the 1–200-bp region without any nucleotide mutation (Figure 1c). We performed a ChIP assay to verify the physiological interaction of NF- κ B with this region using the specific primers indicated in Figure 1a. Antibodies directed against the canonical NF- κ B subunits p50, p65 or c-Rel captured this NF- κ B response element-containing region (Figure 1d). Therefore, these results strongly indicate that canonical NF- κ B may be a transcription factor for *mCd59b* expression.

Considering that the NF- κ B-binding site is 150–200 bp upstream of *mCd59b* exon 1, the dual-luciferase reporter analysis results suggest that another potent transcription factor may bind the 200–237-bp region due to its high promoter activity (Figure 1b). In addition, based on the MatInspector prediction, SRF was hypothesized to be another transcription factor for *mCd59b* (Figure 1a). Accordingly, the dual-luciferase reporter assay was employed to detect promoter activity with core nucleotide mutations in the SRF binding site in NIH/3T3 cells (Supplementary Table 2). SRF core nucleotide mutations between 1–350 bp significantly decreased luciferase activity to a level similar to that of the 1–200-bp region, which contains an NF- κ B-binding site (Figure 1e). These results indicate that NF- κ B and SRF may regulate *mCd59b* transcription separately. In addition, the ChIP assay using specific primers (Figure 1a) and a specific antibody against SRF further demonstrated the direct interaction between SRF and the SRF response element-containing region (Figure 1f). Taken together, these results clearly suggest that both NF- κ B and SRF are the transcription factors regulating *mCd59b*.

We previously determined that in response to cAMP treatment, CREB is phosphorylated and binds to an enhancer region located

in the human *CD59* intron 1, thus upregulating *CD59* transcription together with NF- κ B.¹⁹ Here we further investigated whether CREB regulates *mCd59* transcription. Similarly, we treated mouse Hepa 1-6 cells with 8-Br-cAMP and found that *mCd59b* but not *mCd59a* dramatically increased at both the transcriptional and translational levels (Figures 3a and b). We accordingly reasoned that there may be an enhancer for CREB binding located in *mCd59b* intron 1, as in human *CD59* regulation. Thus, we cloned different fragments from *mCd59b* intron 1 and inserted them into the 3' terminus of the luciferase gene in a pGL3 plasmid that already contained the 1–500-bp region upstream of *mCd59b* exon 1 at the 5' terminus of the luciferase gene. As shown in Figure 3c, however, we failed to observe increased luciferase activity, even with the addition of 8-Br-cAMP, indicating that unlike human *CD59*, there is no enhancer region in *mCd59b* intron 1. In addition, we used 8-Br-cAMP to treat NIH/3T3 cells transfected with pGL3 plasmids that contained different fragments comprising up to 2000 bp upstream of *mCd59b* exon 1. There was no significant increase in luciferase activity (Figure 3d). We then cloned three distinct fragments comprising 2000–4000 bp and separately inserted them into a pGL3 plasmid containing the region 1–500 bp upstream of *mCd59b* exon 1. This dual-luciferase reporter assay exhibited no significantly increased promoter activity, regardless of additional treatment with 8-Br-cAMP (Figure 3e). These results revealed that no promoter/enhancer element is particularly able to respond to 8-Br-cAMP in the region located in *mCd59b* intron 1 and up to 4000 bp upstream of *mCd59b* exon 1. Importantly, cAMP-mediated *mCd59b* upregulation may be independent of SRF and NF- κ B activation. We induced CREB insufficiency by transfecting specific siRNA into Hepa 1–6 cells and observed remarkably increased *mCd59b* expression at the transcriptional and translational levels resulting from 8-Br-cAMP pretreatment (Figures 3f and g). Taking these data together, we concluded that unlike human *CD59*, *mCd59b* does not require CREB for its transcriptional regulation.

NF- κ B and SRF upregulate *mCd59b* but not *mCd59a* expression upon TNF- α treatment *in vitro*

TNF- α , an important pro-inflammatory cytokine, can activate both NF- κ B and SRF.^{22,23} We demonstrated that TNF- α considerably enhanced the nuclear accumulation of canonical NF- κ B and SRF in Hepa 1-6 cells (Figure 4a). Next, we employed TNF- α to determine whether activated NF- κ B and SRF upregulated the expression of *mCd59b*. NIH/3T3 cells were transfected with pGL3-related plasmids with or without TNF- α pretreatment. The inserted fragments were classified as 1–350 (containing both SRF- and NF- κ B-binding sites), 1–350 (SRF mut), 1–350 (NF- κ B mut), 1–200 (only NF- κ B-binding site) and 1–150 (no promoter activity; Figure 4b). TNF- α pretreatment significantly enhanced the transcriptional ability of each promoter(s)-containing pGL3 plasmid, as demonstrated by increased luciferase activity (Figure 4b). Accordingly, pretreatment with TNF- α for 8 h remarkably increased the mRNA levels of *mCd59b* but not *mCd59a*, as detected by quantitative real-time (qRT)-PCR in mouse Hepa 1-6 hepatoma cells (Figure 4c). Subsequently, *mCd59b* but not *mCd59a* was found to increase at the translational level by immunoblotting (Figure 4d). We noted that the luciferase activity shows no significant changes between 1–350 (containing both SRF- and NF- κ B-binding sites) and 1–350 (NF- κ B mut) regions in the absence and presence of TNF α treatment in NIH/3T3 cell. This result was most likely caused by the much weaker nuclear localization of NF- κ B and much stronger nuclear localization of SRF in NIH/3T3 cell than those in Hepa 1-6 cell nucleus (Supplementary Figure 2). We also performed dual-luciferase reporter assays to detect the transcriptional activities in regions of 1–350 (containing both SRF- and NF- κ B-binding sites) and 1–350 (NF- κ B mut) in Hepa 1–6 cells. The data showed that removing NF- κ B involvement by mutating the sequence displays significant

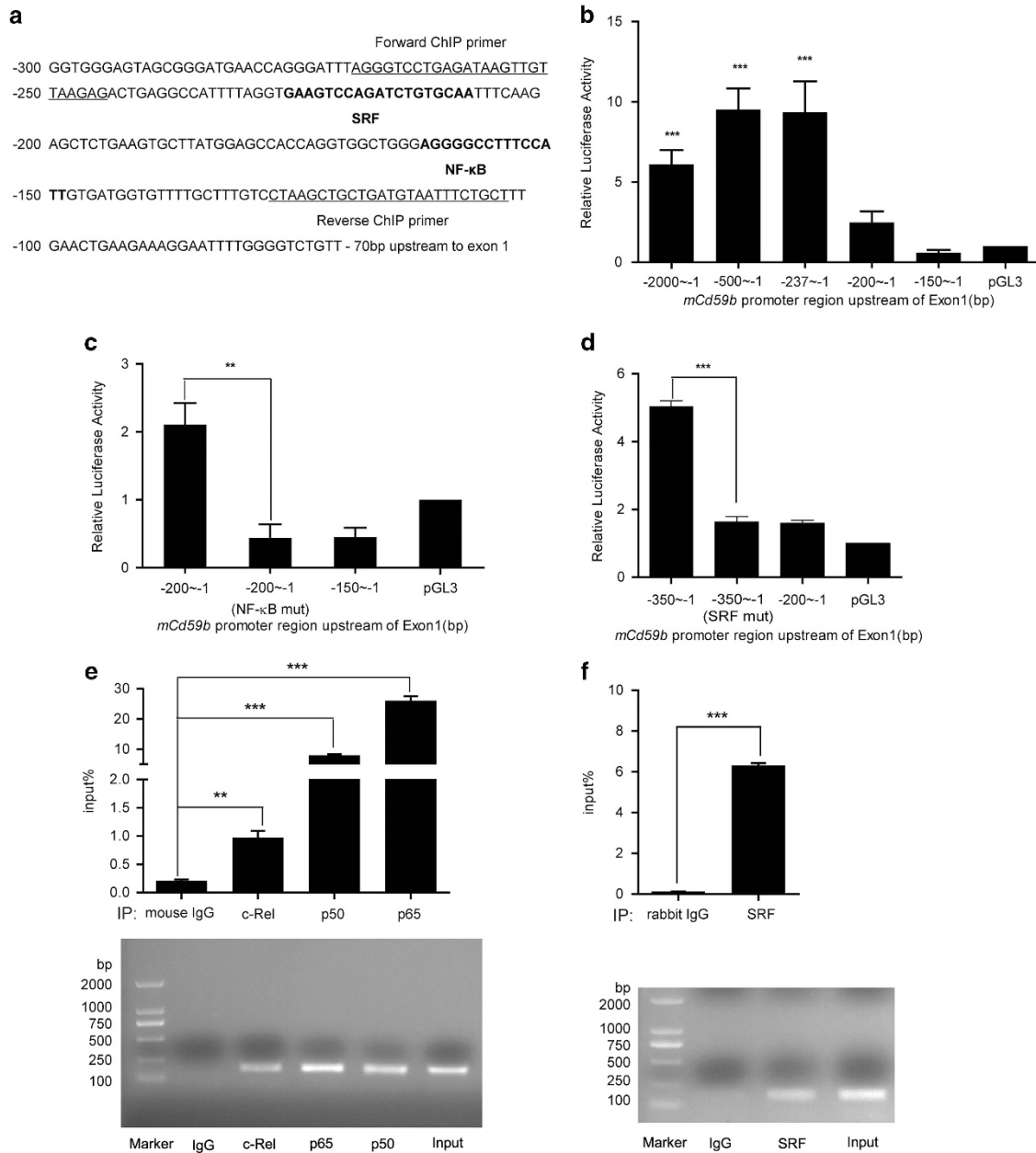


Figure 1. NF-κB and SRF regulate *mCd59b* transcription. **(a)** The sequence of the region from –300 to –70 bp upstream of *mCd59b* exon 1. The NF-κB and SRF response elements and the primer sequences for the CHIP assay are indicated. **(b)** Identification of *mCd59b* promoter regions using a dual-luciferase reporter assay. The significance was determined using one-way analysis of variance (ANOVA) with *post-hoc* testing, $***P < 0.001$ vs pGL3. The effects of NF-κB **(c)** or SRF **(d)** core nucleotide mutation on the activity of the *mCd59b* promoter in the –200 to –1 **(c)** or –350 to –1 **(d)**-bp regions. The significance was determined using one-way ANOVA with *post-hoc* testing, $**P < 0.01$, $***P < 0.001$ vs NF-κB or SRF mutants. **(e and f)** ChIP assay. Antibodies against c-Rel, p65 or p50 **(e)** or against SRF **(f)** were employed to capture the complex containing the relative promoter sequences, then the DNA fragments were determined by qRT-PCR. One-way ANOVA with *post-hoc* testing **(e)** and two-tailed Student's *t*-test **(f)** were used to determine the significance. $**P < 0.01$, $***P < 0.001$ vs mouse or rabbit IgG. All experiments were performed independently for three times in NIH/3T3 cells, data are presented as mean \pm s.d. of three independent experiments.

effect on *mCd59b* promoter activity in Hepa 1–6 cells (Supplementary Figure 3). These results clearly demonstrated that both SRF and NF-κB could regulate *mCd59b* transcription, and the difference of the two transcription factors in regulating transcription depends on their expression levels or activation status.

In addition, we functionally tested whether the upregulation of *mCd59b* upon TNF-α treatment in Hepa 1–6 cells protected the cells from complement attack. CK18 is commonly expressed in the mature hepatocyte membrane.²⁴ Therefore, an anti-CK18 monoclonal antibody was used to trigger the classical complement pathway in combination with normal human serum as a

complement resource. As expected, the results of the complement-dependent cytotoxicity assay showed that pretreatment with TNF-α in Hepa 1–6 cells significantly reduced the cytotoxicity of Hepa 1–6 cells due to upregulated *mCd59b* (Figure 4e). However, this protective capacity upon TNF-α pretreatment could be impaired by specific siRNA-induced insufficiency of SRF or NF-κB (Figure 4h). The efficacy of knockdown with specific siRNAs against SRF or NF-κB (p65, c-Rel) was confirmed by an immunoblotting assay (Figure 4f). The resulting modulation of TNF-α-induced *mCd59b* expression was also detected. Increased expression of *mCd59b* upon TNF-α treatment was significantly

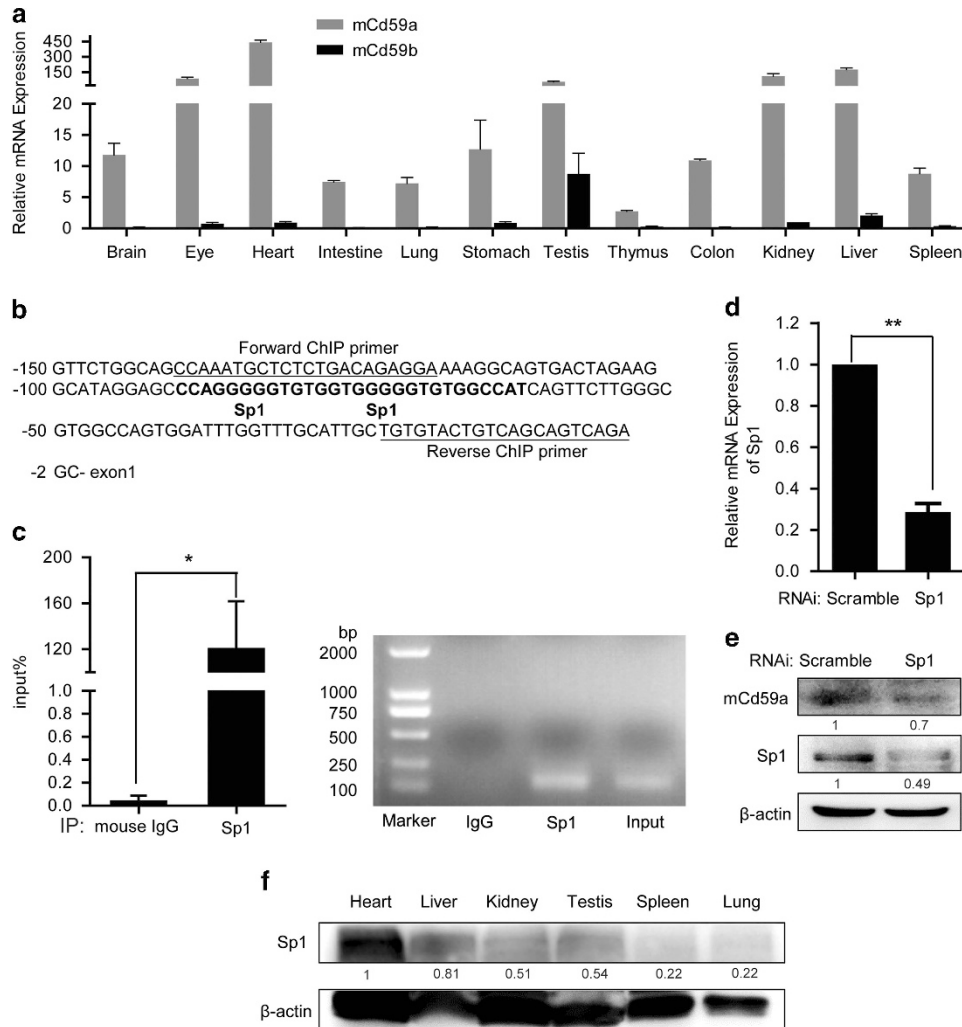


Figure 2. Sp1 regulates *mCd59a* transcription. **(a)** The tissue distribution of *mCd59a* and *mCd59b* as determined by qRT-PCR from six mice (half were male mice for testis detection). **(b)** The sequence of the -150 to -1 -bp region upstream of *mCd59a* exon 1. The predicted Sp1-binding site and the primers for the ChIP assay are indicated. **(c)** ChIP assay. An antibody against Sp1 was used to capture the complex containing the promoter sequence in NIH/3T3 cells. **(d and e)** The insufficiency of Sp1 induced by specific siRNA reduced *mCd59a* expression at both the mRNA **(d)** and protein **(e)** levels. The significance was determined using the two-tailed Student's *t*-test in **(c and d)**, $**P < 0.01$. **(f)** The expression level of Sp1 in the tested tissues pooled from three male mice. Data are presented as mean \pm s.d. of three independent experiments.

compromised by the insufficiency of either SRF or the canonical NF- κ B subunits c-Rel or p65 (Figure 4g). Therefore, we concluded that TNF- α may upregulate *mCd59b* but not *mCd59a* expression to protect cells from complement damage via the activation of NF- κ B and SRF signaling.

Sp1 is responsible for the constitutive expression of *mCd59a* in mouse tissues

We first investigated the mRNA expression profiles of *mCd59a* and *mCd59b* via qRT-PCR in various mouse tissues. *mCd59a* is universally expressed, with particularly high levels in the heart, liver, kidney, testis and eye. By contrast, *mCd59b* is only highly expressed in testis and is very weakly expressed in a small number of other tissues, including liver (Figure 2a).

We previously demonstrated that the *mCd59a* promoter region is located -122 to -36 bp upstream of exon 1 (ref. 20). However, the identity of the corresponding transcription factor remains unclear. As mentioned above, we predicted that this region contains two putative Sp1-binding sites (Figure 2b). Simultaneously, considering that human CD59 and *mCd59a*, unlike

mCd59b, are universally expressed and Sp1 is responsible for the constitutive expression of human CD59 (ref. 19) we hypothesized that Sp1 may regulate *mCd59a* transcription. Therefore, we employed a ChIP assay with a specific antibody directed against Sp1 to capture the region containing the hypothesized Sp1-binding sites. The result clearly revealed a physiological interaction between Sp1 and this region in NIH/3T3 cells (Figure 2c). The specific primers are shown in Figure 2b. Next, we knocked down Sp1 expression in NIH/3T3 cells using a specific siRNA (Figure 2d). The qRT-PCR and immunoblotting results demonstrated that *mCd59a* expression was remarkably reduced at both the transcriptional and translational levels (Figures 2d and e). Furthermore, we detected Sp1 expression in multiple mouse tissues with an immunoblotting assay. The results indicated that Sp1 is expressed at extraordinarily high levels in the heart, moderate levels in the liver, kidney and testis, and very low levels in the spleen and lung (Figure 2f). This is highly correlated with *mCd59a* transcription levels (Figure 2a). Together, these results clearly demonstrate that Sp1 may be responsible for the broad expression of *mCd59a* in mouse tissues.

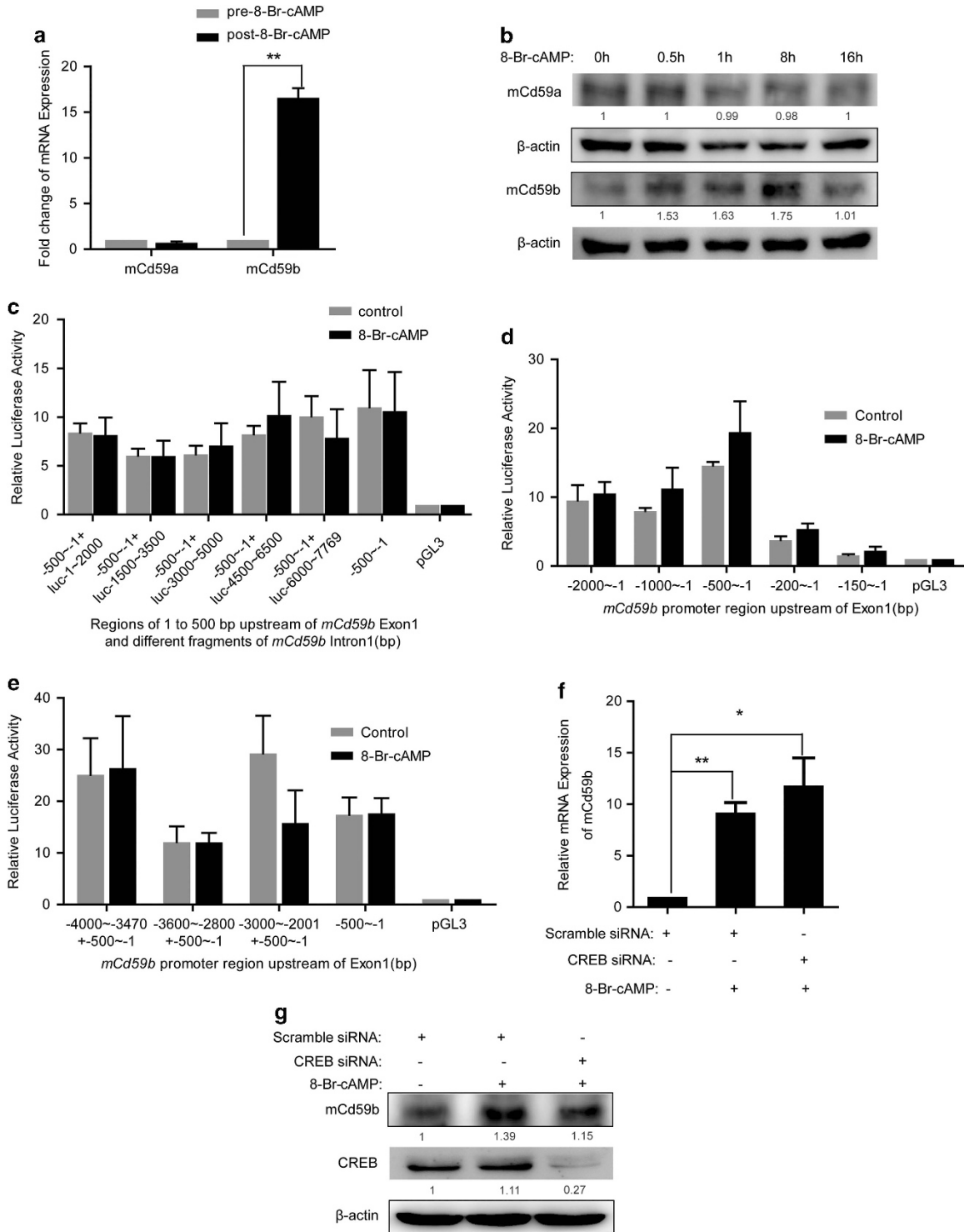


Figure 3. cAMP upregulation of mCd59b expression was not mediated by CREB. **(a and b)** 8-Br-cAMP treatment enhanced mCd59b but not mCd59a expression as determined by qRT-PCR **(a)** and an immunoblotting assay **(b)**. The significance was determined using the two-tailed Student's *t*-test, $***P < 0.01$ vs pre-8-Br-cAMP treatment. **(c)** *mCd59b* intron 1 had no effect in modulating the promoter activity in the -500 to -1 -bp region upstream of exon 1. **(d)** Treatment with 8-Br-cAMP failed to enhance the promoter activity of the -2000 to -1 -bp region upstream of *mCd59b* exon 1. **(e)** The addition of distinct regions in the -4000 to -2000 -bp region in exon 1 to the -500 to -1 -bp region upstream of *mCd59b* was unable to enhance promoter activity, regardless of 8-Br-cAMP treatment. There is no significance between control and 8-Br-cAMP treatment determined using two-tailed Student's *t*-test in **(c–e)**. **(f and g)** CREB insufficiency induced by specific siRNA did not impair the effects exerted by 8-Br-cAMP on increasing mCd59b expression, as evaluated by qRT-PCR **(f)** and an immunoblotting assay **(g)**. One-way analysis of variance with post-hoc testing was used to determine the significance in **(f)**, $*P < 0.05$, $**P < 0.01$ vs scramble siRNA. Data are presented as mean \pm s.d. of three independent experiments.

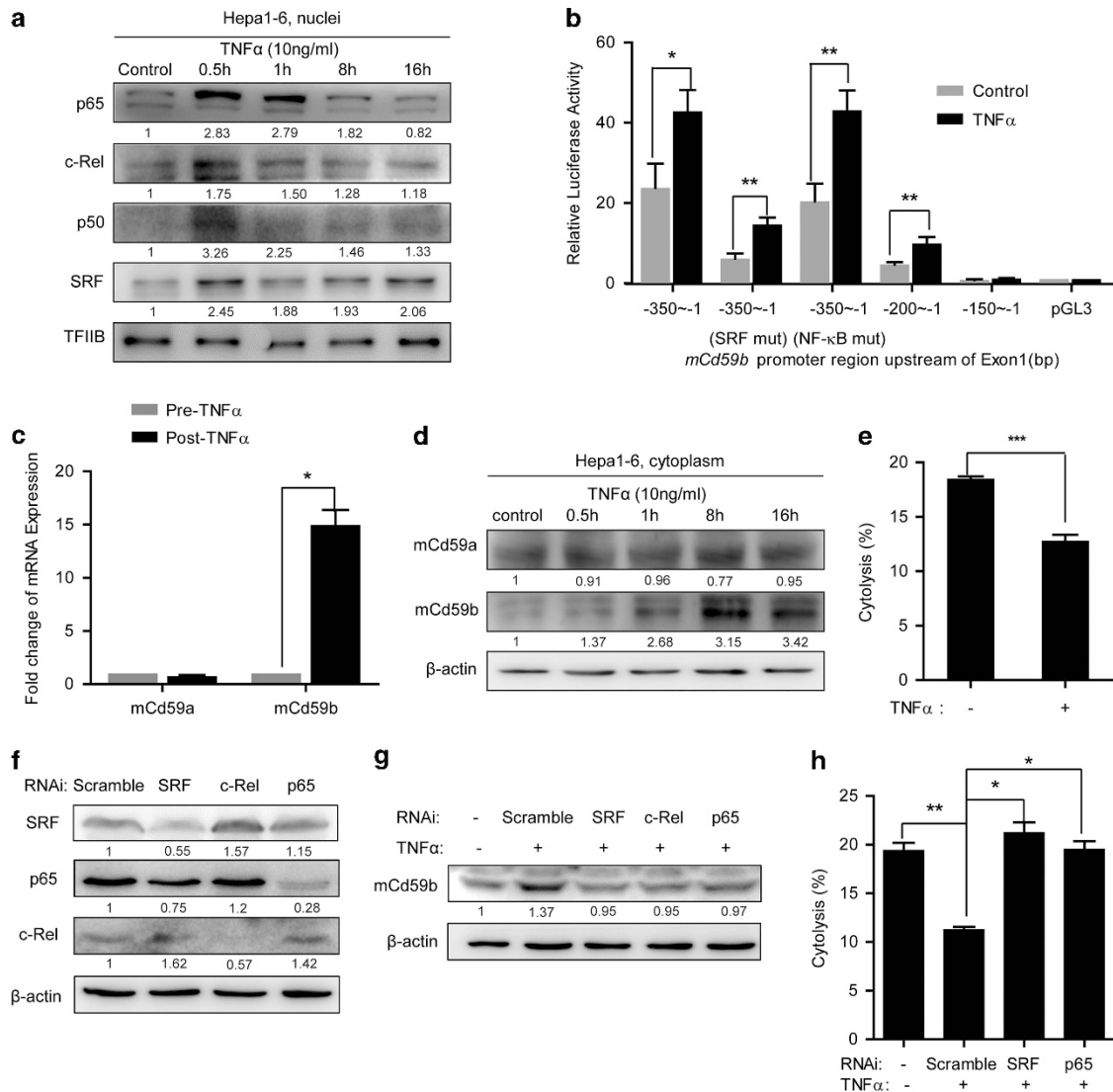


Figure 4. TNF- α treatment increased mCd59b expression by activating canonical NF- κ B and SRF. **(a)** TNF- α treatment enhanced the nuclear accumulation of canonical NF- κ B and SRF. **(b)** TNF- α treatment enhanced the promoter activity of the -350 to -1-bp region upstream of *mCd59b* exon 1, which contains NF- κ B and SRF response elements. **(c and d)** TNF- α treatment increased mCd59b but not mCd59a expression as determined by qRT-PCR **(c)** and an immunoblotting assay **(d)**. **(e)** Pretreatment with TNF- α significantly reduced complement-mediated cytolysis in Hepa 1-6 cells. The significance was determined using two-tailed Student's *t*-test, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. **(f)** Specific siRNAs against SRF, c-Rel or p65 inhibited target protein synthesis. **(g and h)** The insufficiency of SRF, c-Rel or p65 induced by specific siRNAs abrogated the increased mCd59b expression resulting from TNF- α treatment **(g)** and subsequently restored sensitivity to complement-mediated cytolysis **(h)**. The significance was determined using one-way analysis of variance with *post-hoc* testing in **(h)**, * $P < 0.05$ and ** $P < 0.01$ vs scrambled siRNA transfection and TNF- α pretreatment. Data are presented as mean \pm s.d. of three independent experiments.

Mouse Cd59b but not Cd59a is upregulated upon LPS treatment *in vivo*

We demonstrated above that mCd59b but not mCd59a is upregulated upon TNF- α treatment *in vitro* via activated SRF and canonical NF- κ B signaling, thus conferring cellular protection from complement attack. LPS is an important bacterial virulence factor that not only promotes the production of potent pro-inflammatory TNF- α but also triggers the alternative complement pathway. Thus, we injected LPS intraperitoneally to mimic inflammation in the mice and then collected mouse tissues to measure mCd59a and mCd59b expression levels, which may help us to understand the host response to complement attack; mice injected with phosphate-buffered saline (PBS) intraperitoneally were used as control group. Using qRT-PCR, we found that mCd59b rather than mCd59a transcription increased twofold to sixfold in the colon, kidney, liver and spleen (Figure 5a).

The immunoblotting results further revealed that mCd59b but not mCd59a was accordingly elevated at the protein level (Figure 5b). Therefore, these results strongly suggest that mCd59b but not mCd59a is upregulated to protect cells from potential complement attack during infection or inflammation *in vivo*.

DISCUSSION

The complement system, a major component of innate immunity, is an important first-line defense against invading pathogens. It can be subtly tuned physiologically to establish a delicate balance between activation and regulation. However, the tipping of this balance resulting from hyperactivation and/or hyporegulation may contribute in part to various human diseases. CD59 is the sole membrane complement regulator for restricting MAC assembly. Human CD59 and mCd59a are universally expressed in almost all

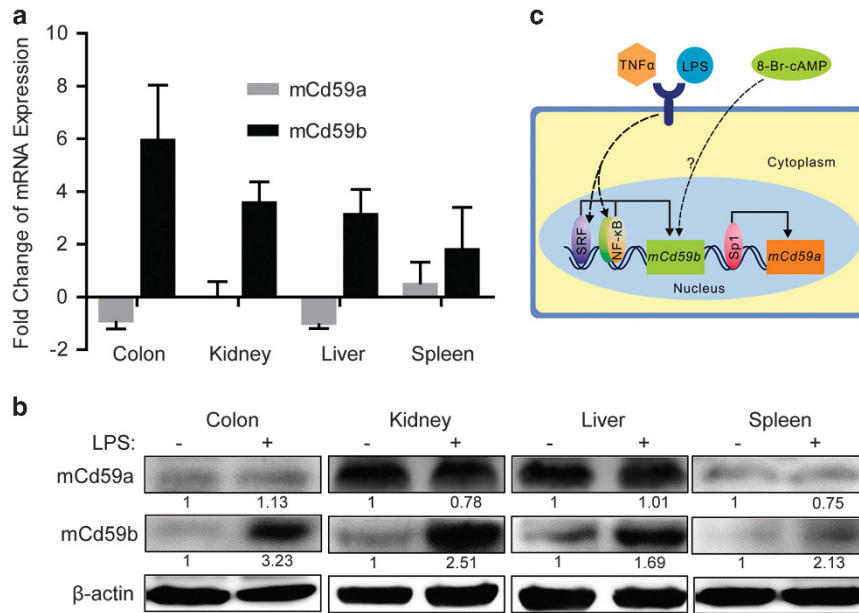


Figure 5. LPS treatment increased mCd59b but not mCd59a expression *in vivo*. The mRNA (a) and protein (b) levels of mCd59a and mCd59b were determined by qRT-PCR and immunoblotting assay. Samples of control and the experimental group were pooled, respectively, from three adult female mice that received PBS or LPS pretreatment for 48 h. (c) Schematic diagram of the transcriptional regulation of *mCd59a* and *mCd59b*. Data are presented as mean \pm s.d. of three independent experiments.

tissues, whereas mCd59b is selectively expressed in testis.¹² Therefore, it is critical to discover how CD59 responds to the extracellular inflammatory environment in the context of various disorders. We previously revealed that TNF- α is responsible for the constitutive expression of human *CD59*, whereas NF- κ B and CREB regulate the inducible expression of this gene. In this study, we further determined that Sp1 is responsible for mCd59a expression, whereas SRF and NF- κ B are responsible for mCd59b regulation in response to TNF- α *in vitro* and LPS *in vivo* stimulation (Figure 5c).

NF- κ B has a critical role in apoptosis, differentiation and, in particular, immunity.²⁵ The NF- κ B proteins comprise five distinct proteins: RelA (p65), RelB, c-Rel, p105 (p50 when processed) and p100 (p52 when processed). These proteins form homo- or heterodimers with distinct functions.^{26–28} Normally, these NF- κ B proteins are sequestered in the cytoplasm by inhibitors of kappa B. Following the phosphorylation and subsequent ubiquitination and degradation of inhibitors of kappa B, NF- κ B proteins are released and translocated into the nucleus to activate the transcription of a variety of genes encoding proteins for inflammation and survival.^{25,29} SRF, a transcription factor associated with cell proliferation and differentiation, neuronal transmission, and muscle development and function, has important regulator functions in tissue injury and wound healing.³⁰ Genetic deficiency of murine *SRF* induces early lethality before mesoderm formation.³¹ Recent studies have also demonstrated a role for SRF in the regulation of inflammation and immunity. SRF is essential for neutrophil migration in response to inflammation³² and it indirectly modulates type I interferon signaling in macrophages, without interfering in the classic JAK/STAT/ISGF3 pathway.³³ Interestingly, complement-mediated sublytic MAC can cause *c-fos* transcriptional activation in myotubes by inducing the binding of SRF along with ternary complex factor Elk1 and Sap1a to the *c-fos* serum response element, which may subsequently contribute to the pathogenesis of autoimmune diseases, such as myasthenia gravis.³⁴ Sublytic MAC could not only enhance the assembly of the SRF/Elk1/Sap1a ternary transcription complex via the activation of ERK1 and thus Elk1 (ref. 34) but could also activate NF- κ B signaling by promoting NF- κ B nuclear translocation.^{19,35} TNF- α exhibits similar functions in activating

SRF²³ and NF- κ B,²⁵ which was further demonstrated in this study (Figure 4a). Therefore, the activation of SRF and NF- κ B by inflammatory sequelae, including complement activation, in turn induces the upregulation of mCd59b to protect host cells against potential MAC-mediated damage, which suggests a feedback loop for host defense. In addition, 8-Br-cAMP markedly increased the expression of mCd59b but not mCd59a, similar to its role in human CD59 regulation. However, this cAMP-induced signaling to upregulate mCd59b is independent of CREB, NF- κ B and SRF, and the underlying mechanism requires future investigation.

The transcription factor Sp1 is ubiquitously expressed to regulate the expression of thousands of genes involved in diverse cellular processes such as cell growth, differentiation, apoptosis, angiogenesis and the immune response.³⁶ Sp1 binds GC-rich elements that are widely distributed in the promoters of housekeeping genes³⁷ and is a constitutive transcription factor that regulates basal promoter activity.^{38,39} The broad distribution of mCd59a in almost all mouse tissues as a primary complement regulator at the terminal phase^{10,10} may be explained by our finding that Sp1 regulates mCd59a transcription. In addition, the differences in transcriptional regulation between mCd59a and mCd59b may explain their differential distributions.

Similar with human CD59 distribution and regulation, mCd59a is widely distributed and regulated by the housekeeping transcription factor Sp1, and mCd59b is partially regulated by NF- κ B. However, *mCd59a* and *mCd59b* merges into one *CD59* gene in humans. Further, in response to external inflammatory stimuli, mCd59b-inducible expression regulated by NF- κ B and SRF has evolved into human CD59 regulated only by NF- κ B. Although 8-Br-cAMP treatment upregulates both mCd59b and human CD59 expression, the downstream molecule CREB, interestingly as an enhancer-binding protein, regulates only human CD59 but not mCd59b expression.¹⁹ The differences in copy number and transcriptional regulation of *CD59* between mouse and human may result from evolutionary selection pressure. Therefore, the differences and similarities among human CD59, mCd59a and mCd59b should be taken into account when interpreting mouse model data and translating it to human physiology and diseases.

MATERIALS AND METHODS

Cell culture and reagents

The mouse cell lines Hepa 1-6 and NIH/3T3 (Type Culture Collection Cell Bank, Chinese Academy of Sciences) were maintained in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Recombinant human TNF- α was purchased from PeproTech (Rocky Hill, NJ, USA, Catalog #300-01 A). LPS and 8-Br-cAMP were purchased from Sigma (St Louis, MO, USA). Rabbit anti-mCd59a polyclonal antibody (FL-123); mouse anti-p65 (F-6), anti-p50 (E-10), anti-c-Rel (B-6), anti-Sp1 (E-3), anti-TFIIIB (D3) and anti- β -actin (C4) antibodies; goat anti-mouse IgG-HRP; and goat anti-rabbit IgG-HRP were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). A rabbit anti-SRF (D71A9) XP monoclonal antibody was obtained from Cell Signaling Technology (Danvers, MA, USA). Propidium iodide was obtained from Invitrogen (Waltham, MA, USA). Normal human serum as a complement resource was pooled from 10 healthy persons and aliquoted, then stored at -80°C until use.

To discriminate between mCd59b and mCd59a, we entrusted Shanghai Genomics, Inc. to generate an anti-mCd59b polyclonal antibody by immunizing two New Zealand rabbits using the keyhole limpet hemocyanin-coupled mCd59b-specific epitope NKNLDGLEEP (from 70 to 79 aa). After three courses of immunization, the rabbits were killed and the sera were collected. The applicability and specificity of the rabbit antisera were further verified by immunoblotting in mCd59b-overexpressing Hepa 1-6 cells (Supplementary Figure 1). The mCd59b-overexpressing Hepa 1-6 cells were generated as follows: the coding sequence of mCd59b was cloned using PCR with specific primers (forward: 5'-GGAATT CATGAGAGCTCAGAGGGGAC-3'; reverse: 5'-CGGGATCCCCGAAGCAAAA CTTCAAATGG-3') and inserted into pEGFP-N1 via the *EcoRI* and *BamHI* sites. After verification by sequencing, the mCd59b-pEGFP-N1 plasmid or control vector plasmid was transfected into Hepa 1-6 cells using Lipofectamine 2000 (Invitrogen). After 48 h, the positive cells were selected for with G418 ($1000\text{ ng }\mu\text{l}^{-1}$, Sigma) for three rounds.

Dual-luciferase reporter assay

As described previously,¹⁹ we used a dual-luciferase reporter assay to identify the regions with promoter or enhancer activity in *mCd59b*. The fragments upstream of *mCd59b* exon 1 and of *mCd59b* intron 1 were cloned and inserted into the pGL3 Basic Vector (Promega, Madison, WI, USA). Details of the primers used to clone these fragments are given in Supplementary Table 1. Double-strand DNA fragments with critical site mutations for NF- κ B or SRF (Supplementary Table 2) were synthesized by SBS Genetech Co., Ltd (Beijing, China). Then, the pGL3-derived plasmids together with the pRL-TK plasmid were transfected into NIH/3T3 cells using Lipofectamine 2000 (Invitrogen) with or without TNF- α pretreatment (10 ng ml^{-1}) or 8-Br-cAMP (1 mM). After 48 h, dual-luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) on a Bio-Tek synergy HT microplate reader. Firefly luciferase activity was normalized to *Renilla* luciferase activity. These assays were repeated three times.

ChIP assay

We performed the ChIP assay as described previously,¹⁹ and the related primers are shown in Figures 1a and 2b.

Quantitative real-time-PCR

Total RNA from cells or mouse tissues was extracted with TRIzol reagent (Invitrogen) and transcribed into cDNA using a Reverse Transcription System (Promega). The input cDNA was standardized and then amplified for 40 cycles with SYBR Green Master Mix (Invitrogen) and gene-specific primers on an ABI Prism 7900HT machine (Applied Biosystems, Waltham, MA, USA). The housekeeping gene *ACTB* (gene encoding β -actin) was used as an endogenous control, and samples were analyzed in triplicate. The qRT-PCR primers are listed in Supplementary Table 3.

To study the effects of Sp1, SRF, c-Rel, p65 or CREB on *mCd59* expression, Hepa 1-6 cells were transfected with specific siRNAs (GenePharma Co., Ltd, Shanghai, China) using Lipofectamine 2000 (Invitrogen). The siRNA sequences are indicated in Supplementary Table 4. After 48 h, the cells were harvested to evaluate *mCd59b* and/or *mCd59a* expression at the transcriptional level by qRT-PCR and at the translational level by immunoblotting (see below).

Immunoblotting assay

Immunoblotting assay was performed according to a standard protocol as described previously.¹⁹ The bands of immunoblotting were quantified by ImageJ (Bethesda, MD, USA) and corrected for a loading control.

Complement-dependent cytotoxicity assay

The complement-dependent cytotoxicity assay was performed as described in our previous report.⁴⁰ In brief, Hepa 1-6 cells were treated with TNF- α (10 ng ml^{-1}) for 16 h before the assay, followed by treatment with anti-CK18 antibody and normal human serum for 1.5 h and incubation with propidium iodide ($2\text{ }\mu\text{g ml}^{-1}$) at room temperature for 15 min; samples were immediately analyzed on a Cytomics FC 500 MPL (Beckman Coulter, Brea, CA, USA).

Animal studies

Animal studies were approved by the Animal Ethics Committee at Shanghai Medical School, Fudan University. Eight-week-old Balb/C mice were purchased from Slac Laboratory Animal (Shanghai, China). To detect the physiological expression of mCd59a and mCd59b, the tissues indicated in Figure 2a were collected from three male and three female mice after complete perfusion with PBS as described previously.¹²

To investigate the *in vivo* effects of LPS on mCd59a and mCd59b expression, six female mice were equally divided into LPS-treated (i.p., $10\text{ }\mu\text{g kg}^{-1}$ body weight) or PBS-treated (i.p., equal volume PBS) groups. After 48 h, mice were sacrificed and perfused. Liver, kidney, spleen and colon were then collected for analysis.

Statistical analysis

The data are presented as the means \pm s.d. The significant difference between two groups was determined using the two-tailed Student's *t*-test for unpaired data. *P*-values < 0.05 were considered statistically significant. All qRT-PCR were performed in triplicates. One-way analysis of variance was employed to analyze multiple comparisons, and specific significant differences were determined with *post-hoc* testing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This research was supported by grants to WH from the National Natural Science Foundation of China (81171910, 81372258), the Major State Basic Research Development Program of China (2013CB910802) and the Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning.

REFERENCES

- Morgan BP, Marchbank KJ, Longhi MP, Harris CL, Gallimore AM. Complement: central to innate immunity and bridging to adaptive responses. *Immunol Lett* 2005; **97**: 171–179.
- Dunkelberger JR, Song WC. Complement and its role in innate and adaptive immune responses. *Cell Res* 2010; **20**: 34–50.
- Papadimitriou JC, Ramm LE, Drachenberg CB, Trump BF, Shin ML. Quantitative analysis of adenine nucleotides during the prelytic phase of cell death mediated by C5b-9. *J Immunol* 1991; **147**: 212–217.
- Esser AF. The membrane attack complex of complement. Assembly, structure and cytotoxic activity. *Toxicology* 1994; **87**: 229–247.
- Ollert MW, Kadlec JV, David K, Petrella EC, Bredehorst R, Vogel CW. Antibody-mediated complement activation on nucleated cells. A quantitative analysis of the individual reaction steps. *J Immunol* 1994; **153**: 2213–2221.
- Zhou X, Hu W, Qin X. The role of complement in the mechanism of action of rituximab for B-cell lymphoma: implications for therapy. *Oncologist* 2008; **13**: 954–966.
- Powell MB, Marchbank KJ, Rushmere NK, van den Berg CW, Morgan BP. Molecular cloning, chromosomal localization, expression, and functional characterization of the mouse analogue of human CD59. *J Immunol* 1997; **158**: 1692–1702.
- Qian YM, Qin X, Miwa T, Sun X, Halperin JA, Song WC. Identification and functional characterization of a new gene encoding the mouse terminal complement inhibitor CD59. *J Immunol* 2000; **165**: 2528–2534.

- 9 Meri S, Waldmann H, Lachmann PJ. Distribution of protectin (CD59), a complement membrane attack inhibitor, in normal human tissues. *Lab Invest* 1991; **65**: 532–537.
- 10 Baalasubramanian S, Harris CL, Donev RM, Mizuno M, Omidvar N, Song WC *et al*. CD59a is the primary regulator of membrane attack complex assembly in the mouse. *J Immunol* 2004; **173**: 3684–3692.
- 11 Harris CL, Hanna SM, Mizuno M, Holt DS, Marchbank KJ, Morgan BP. Characterization of the mouse analogues of CD59 using novel monoclonal antibodies: tissue distribution and functional comparison. *Immunology* 2003; **109**: 117–126.
- 12 Donev RM, Sivasankar B, Mizuno M, Morgan BP. The mouse complement regulator CD59b is significantly expressed only in testis and plays roles in sperm acrosome activation and motility. *Mol Immunol* 2008; **45**: 534–542.
- 13 Qin X, Miwa T, Aktas H, Gao M, Lee C, Qian YM *et al*. Genomic structure, functional comparison, and tissue distribution of mouse Cd59a and Cd59b. *Mamm Genome* 2001; **12**: 582–589.
- 14 Qin X, Dobarro M, Bedford SJ, Ferris S, Miranda PV, Song W *et al*. Further characterization of reproductive abnormalities in mCd59b knockout mice: a potential new function of mCd59 in male reproduction. *J Immunol* 2005; **175**: 6294–6302.
- 15 Zhang J, Hu W, Xing W, You T, Xu J, Qin X *et al*. The protective role of CD59 and pathogenic role of complement in hepatic ischemia and reperfusion injury. *Am J Pathol* 2011; **179**: 2876–2884.
- 16 Wu G, Hu W, Shahsafaie A, Song W, Dobarro M, Sukhova GK *et al*. Complement regulator CD59 protects against atherosclerosis by restricting the formation of complement membrane attack complex. *Circ Res* 2009; **104**: 550–558.
- 17 Ruiz-Arguelles A, Llorente L. The role of complement regulatory proteins (CD55 and CD59) in the pathogenesis of autoimmune hemocytopenias. *Autoimmun Rev* 2007; **6**: 155–161.
- 18 Krus U, King BC, Nagaraj V, Gandasi NR, Sjolander J, Buda P *et al*. The complement inhibitor CD59 regulates insulin secretion by modulating exocytotic events. *Cell Metab* 2014; **19**: 883–890.
- 19 Du Y, Teng X, Wang N, Zhang X, Chen J, Ding P *et al*. NF-kappaB and enhancer-binding CREB protein scaffolded by CREB-binding protein (CBP)/p300 proteins regulate CD59 protein expression to protect cells from complement attack. *J Biol Chem* 2014; **289**: 2711–2724.
- 20 Qin X, Ferris S, Hu W, Guo F, Ziegeler G, Halperin JA. Analysis of the promoters and 5'-UTR of mouse Cd59 genes, and of their functional activity in erythrocytes. *Genes Immun* 2006; **7**: 287–297.
- 21 Pangburn MK, Morrison DC, Schreiber RD, Muller-Eberhard HJ. Activation of the alternative complement pathway: recognition of surface structures on activators by bound C3b. *J Immunol* 1980; **124**: 977–982.
- 22 Madonna R, Geng YJ, Bolli R, Rokosh G, Ferdinandy P, Patterson C *et al*. Co-Activation of nuclear factor-kappaB and myocardin/serum response factor conveys the hypertrophy signal of high insulin levels in cardiac myoblasts. *J Biol Chem* 2014; **289**: 19585–19598.
- 23 Li YP, Schwartz RJ. TNF-alpha regulates early differentiation of C2C12 myoblasts in an autocrine fashion. *FASEB J* 2001; **15**: 1413–1415.
- 24 Wells MJ, Hattton MW, Hewlett B, Podor TJ, Sheffield WP, Blajchman MA. Cytokeratin 18 is expressed on the hepatocyte plasma membrane surface and interacts with thrombin-antithrombin complexes. *J Biol Chem* 1997; **272**: 28574–28581.
- 25 Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* 2004; **25**: 280–288.
- 26 Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF-kappaB signaling pathways. *Nat Immunol* 2011; **12**: 695–708.
- 27 Hayden MS, Ghosh S. NF-kappaB in immunobiology. *Cell Res* 2011; **21**: 223–244.
- 28 Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. *Annu Rev Immunol* 1994; **12**: 141–179.
- 29 Kanarek N, London N, Schueler-Furman O, Ben-Neriah Y. Ubiquitination and degradation of the inhibitors of NF-kappaB. *Cold Spring Harb Perspect Biol* 2010; **2**: a000166.
- 30 Chai J, Tarnawski AS. Serum response factor: discovery, biochemistry, biological roles and implications for tissue injury healing. *J Physiol Pharmacol* 2002; **53**: 147–157.
- 31 Arsenian S, Weinhold B, Oelgeschlager M, Ruther U, Nordheim A. Serum response factor is essential for mesoderm formation during mouse embryogenesis. *EMBO J* 1998; **17**: 6289–6299.
- 32 Taylor A, Tang W, Bruscia EM, Zhang PX, Lin A, Gaines P *et al*. SRF is required for neutrophil migration in response to inflammation. *Blood* 2014; **123**: 3027–3036.
- 33 Xie L, Sullivan AL, Collier JG, Glass CK. Serum response factor indirectly regulates type I interferon-signaling in macrophages. *J Interferon Cytokine Res* 2013; **33**: 588–596.
- 34 Badea TD, Park JH, Soane L, Niculescu T, Niculescu F, Rus H *et al*. Sublytic terminal complement attack induces c-fos transcriptional activation in myotubes. *J Neuroimmunol* 2003; **142**: 58–66.
- 35 Kilgore KS, Schmid E, Shanley TP, Flory CM, Maheswari V, Tramontini NL *et al*. Sublytic concentrations of the membrane attack complex of complement induce endothelial interleukin-8 and monocyte chemoattractant protein-1 through nuclear factor-kappa B activation. *Am J Pathol* 1997; **150**: 2019–2031.
- 36 Tan NY, Khachigian LM. Sp1 phosphorylation and its regulation of gene transcription. *Mol Cell Biol* 2009; **29**: 2483–2488.
- 37 Philipsen S, Suske G. A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. *Nucleic Acids Res* 1999; **27**: 2991–3000.
- 38 Bouwman P, Philipsen S. Regulation of the activity of Sp1-related transcription factors. *Mol Cell Endocrinol* 2002; **195**: 27–38.
- 39 Chuang JY, Wang YT, Yeh SH, Liu YW, Chang WC, Hung JJ. Phosphorylation by c-Jun NH2-terminal kinase 1 regulates the stability of transcription factor Sp1 during mitosis. *Mol Biol Cell* 2008; **19**: 1139–1151.
- 40 Hu W, Ge X, You T, Xu T, Zhang J, Wu G *et al*. Human CD59 inhibitor sensitizes rituximab-resistant lymphoma cells to complement-mediated cytotoxicity. *Cancer Res* 2011; **71**: 2298–2307.

Supplementary Information accompanies this paper on Genes and Immunity website (<http://www.nature.com/gene>)