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Original article

SM22α inhibits vascular inflammation via stabilization of IκBα in vascular smooth muscle cells

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ABSTRACT

Smooth muscle (SM) 22α, an actin-binding protein, is down-regulated in atherosclerotic arteries. Disruption of SM22α promotes arterial inflammation through activation of reactive oxygen species (ROS)-mediated nuclear factor (NF)-κB pathways. This study aimed to investigate the mechanisms by which SM22α regulates vascular inflammatory response. The ligation injury model of SM22α$^{-/-}$ mice displayed up-regulation of inflammatory molecules MCP-1, VCAM-1, and ICAM-1 in the carotid arteries. Similar results were discovered in human atherosclerotic samples. *In vitro* studies, overexpression of SM22α attenuated TNF-α-induced IκBα phosphorylation and degradation, accompanied by decreased NF-κB activity and reduced inflammatory molecule expression. Using coimmunoprecipitation, we found that SM22α interacted with and stabilized IκBα in quiescent VSMCs. Upon TNF-α stimulation, SM22α was phosphorylated by casein kinase (CK) II at Thr139, leading to dissociation of SM22α from IκBα, followed by IκBα degradation and NF-κB activation. Our findings demonstrate that SM22α is a phosphorylation-regulated suppressor of IKK–IκBα–NF-κB signaling cascades. SM22α may be a novel therapeutic target for human vascular diseases and other inflammatory conditions.

1. Introduction

Inflammation has been considered as an important etiological factor of cardiovascular diseases, such as atherosclerosis and restenosis [1]. The transcription factor NF-κB plays a central role in the regulation of genes involved in inflammatory responses [2,3]. Activation of NF-κB is controlled by inhibitor of NF-κB proteins (IκBα), which sequester the majority of NF-κB in the cytoplasm as complexes by masking their nuclear localization signals. Upon pro-inflammatory stimulation, IκBα is phosphorylated by IκB kinases (IKK), and degraded via ubiquitin-proteasome pathway, thus permitting nuclear translocation of the NF-κB subunits and inducing inflammatory gene activation [4]. The inhibitory activity of IκBα can be modulated by the cytoskeleton-associated protein through physical interaction with IκBα rather than phosphorylation by IKKβ [5]. However, little is known about the adapter and regulation of targeting IκBα in inflammatory responses.

Smooth muscle (SM) 22α, also known as transgelin, is a 22 kDa protein abundant in the smooth muscle cells (SMCs) of vertebrates [6]. The DNA and protein sequences of SM22α are highly conserved across species, which shares 97% identity among mouse, rat, and human [7]. We and others have demonstrated that SM22α binds to actin, and facilitates actin filament assembly into bundles, which may enhance the contractility and mobility of VSMCs [8]. The activity of SM22α is necessary for maintaining the differentiated phenotype of VSMCs [8]. Otherwise, it contributes to the stabilization of cellular structure via interaction with actin [9,10]. Although SM22α$^{-/-}$ mice displayed normal vasculature development and function state, such as blood pressure and heart rate, the role of SM22α may not be compensated under pathological conditions [11]. Emerging evidence suggests an important role for SM22α in pathogenesis of a variety of human diseases. The expression of SM22α is downregulated in inflammation-related diseases, such as atherosclerotic plaques, abdominal aortic aneurysms, and several types of cancer [12]. Loss of SM22α in apolipoprotein E knockout mice led to enlarged atherosclerotic lesions with prominent macrophage infiltration, a sign of enhanced inflammation [13]. Our previous studies revealed that overexpression of SM22α inhibited VSMC proliferation and neointimal formation induced by balloon injury via blockade of the Ras-ERK1/2 signaling pathway [14]. Furthermore, SM22α, as a PKCδ-regulating and PKCδ-regulated adaptor protein, modulated vascular oxidative stress *in vitro* and *in vivo* through activation of PKCδ-p47^{phox} axis via modulation of actin dynamics [15]. Our findings suggest that SM22α may act as an adapter or scaffold protein to modulate signaling. More recently, the aortic transcriptomes of SM22α$^{-/-}$ mice

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reveal that loss of SM22α can actively contribute to the pathogenesis of atherosclerosis, because the knockout of *Sm22α* results in an increased expression of pro-inflammatory genes in the aortas which are controlled by NF-κB (RelA) [16]. However, the mechanism by which disruption of SM22α activates NF-κB is not fully understood.

In this study, we demonstrated that SM22α interacted with and stabilized IκBα to inhibit NF-κB p65 nuclear translocation. CKII-mediated phosphorylation of SM22α promoted the dissociation of the IκBα–SM22α complex, which led to IκBα phosphorylation and degradation. These findings reveal a critical role for SM22α in controlling NF-κB activity and vascular inflammation, and suggest that SM22α may serve as a new target to design therapeutic drug and to prevent further organ damage in vascular inflammatory diseases.

2. Methods

2.1. Animals

The SM22α-knockout mice line (B6.129S6-*Tagln*$^{tm2(cre)Yec}$/J) which has a Cre-recombinase gene inserted into the endogenous *SM22α* locus was purchased from the Jackson Laboratory [17]. Sprague–Dawley rats were obtained from the Experimental Animal Center of Hebei Medical University. Only male mice, at least 12 weeks of age, were used for experiments. All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011), and were approved by the Institutional Animal Care and Use Committee of Hebei Medical University.

2.2. Adventitial gene transfer and carotid artery ligation injury

The carotid artery injury of the mice was induced by complete ligation of the left common carotid artery as previously described [18]. Anesthesia was maintained by mask inhalation of isoflurane. The common carotid artery was dissected free of the surrounding connective tissue and Ad-GFP-SM22α (5×10^9 pfu/mL) or Ad-GFP (5×10^9 pfu/mL) was suspended together in 50 μL pluronic F127 gel (25% wt/vol) and applied around the carotid artery. Being euthanized using intraperitoneal overdose anesthesia with sodium pentobarbital (200–250 mg/kg). Carotid arteries were harvested 2 weeks after ligation.

2.3. Human subjects

The study was carried out in accordance with the ethical principles for medical research involving human subjects set out in the Helsinki Declaration, and was approved by the ethical committee at our institution. Carotid arteries with atherosclerotic plaque were obtained from 8 autopsied patients with carotid atherosclerotic plaques (males, average age, 75 ± 4.7 years). As controls, nonatherosclerotic carotid arteries of similar size were obtained from 8 autopsied patients (males, average age, 74 ± 5.2 years) who died of nonatherosclerotic diseases. Each specimen was immediately frozen in liquid nitrogen for RNA isolation. All patients or their relatives gave informed consent prior to their participation in the study.

2.4. Cell culture and treatment

Rat VSMCs were isolated from aortas of 80–100 g male Sprague–Dawley rats anesthetized intraperitoneally with urethane (1.5 g/kg body weight) as described previously [19]. Mice were sacrificed by cervical dislocation in order to obtain tissues to harvest VSMCs. The VSMCs of wild-type and SM22α$^{-/-}$ mice were isolated from aortas with 1% collagenase. The VSMCs were cultured in low glucose DMEM supplemented with 20% FBS. Fetal bovine serum was obtained from PAA Laboratories (Canadian Origin, NO. A15-751). Human embryonic kidney 293 cells were purchased from ATCC and maintained in high glucose Dulbecco's modified Eagle's medium supplemented with 10% FBS. Before stimulation with TNF-α (10 ng/mL, PeproTech) and infection with adenoviruses, VSMCs were incubated in serum-free medium for 24 h. For inhibitor studies, cells were pretreated for 4 h with the CKII inhibitor CX-4945 (10 μmol/L, Selleckchem) or vehicle control (0.1% DMSO, Sigma), before the addition of TNF-α. To block the degradation of ubiquitin, cells were pretreated with MG132 (10 μmol/L, Sigma) or vehicle control (0.1% DMSO) for 4 h, and then stimulated by TNF-α.

2.5. Small interfering RNA (siRNA) transfection

The siRNA duplexes targeting rat SM22α mRNA (siSM22α), 5′-GCUAGUGGAGUGGAUUGUATT-3′ and 5′-UACAAUCCACUCCACUAG CTT-3′, and CKII siRNA (siCKII) 5′-CAAACUAUAAUCGUACAUC-3′ and 5′-GAUGUACGAUUAUAGUUUG-3′, were obtained from Invitrogen. Scrambled siRNA (siCon) 5′-GCUAGAGUAGCGGUGAAUUCGTT-3′ and 5′-CGAAUUCAC CGCUACUCUAGCTT-3′ served as a negative control. The siRNAs were transiently transfected into VSMCs using RNAi-MAX reagent (Invitrogen).

2.6. Luciferase reporter assay

HEK293 cells were transfected by using Lipofectamine2000 reagent (Invitrogen). The NF-κB luciferase reporter vector is designed to measure the binding of transcription factors to the κ enhancer. It contains six tandem repeats of NF-κB binding site (TGGGGACTTTCCGC) as promoters upstream of the luciferase transcription start site in the vector. Total concentration of transfected DNA was corrected by addition of pCDNA3.1 vector (Invitrogen). Luciferase activities were measured using a Dual Luciferase Assay Kit (Promega). Specific promoter activity was expressed as the relative activity ratio of firefly luciferase to Renilla luciferase.

2.7. Oligonucleotide pull-down assay

The oligonucleotides containing NF-κB consensus sequences biotin-5′-AGTTGAGGGGACTTTCCCAGG-3′ were used. Nuclear protein extracts (100 μg) were precleared with ImmunoPure streptavidin-agarose beads (20 μL/sample; Promega) for 1 h at 4 °C. After centrifugation for 2 min at 12,000 rpm, the supernatant was incubated with 100 pmol of biotinylated double-stranded oligonucleotides and 10 μg of poly (dI-dC) overnight at 4 °C with gentle rocking. Then 30 μL of streptavidin-agarose beads was added, followed by a further 1 h of incubation at 4 °C. The protein–DNA–streptavidin–agarose complex was washed 4 times with lysis buffer, separated on a 10% SDS-PAGE, and subjected to Western blotting with different antibodies.

2.8. CKII activity assay

VSMCs were treated with TNF-α for different times, and then harvested. CKII activity was detected by using the CKII Kinase Assay Kit (CycLex, Cat# CY-1170) according to the manufacturer's manual.

2.9. Statistics

Data analysis was performed by using SPSS version 16.0. Data are presented as the means ± SEM. Paired or unpaired data were compared by Student's *t* tests. Differences among groups were analyzed with one-way or two-way analysis of variance (ANOVA). For all statistical comparisons, $P < 0.05$ was considered significant.

An expanded Materials and methods section is available in the Online Supplemental material.

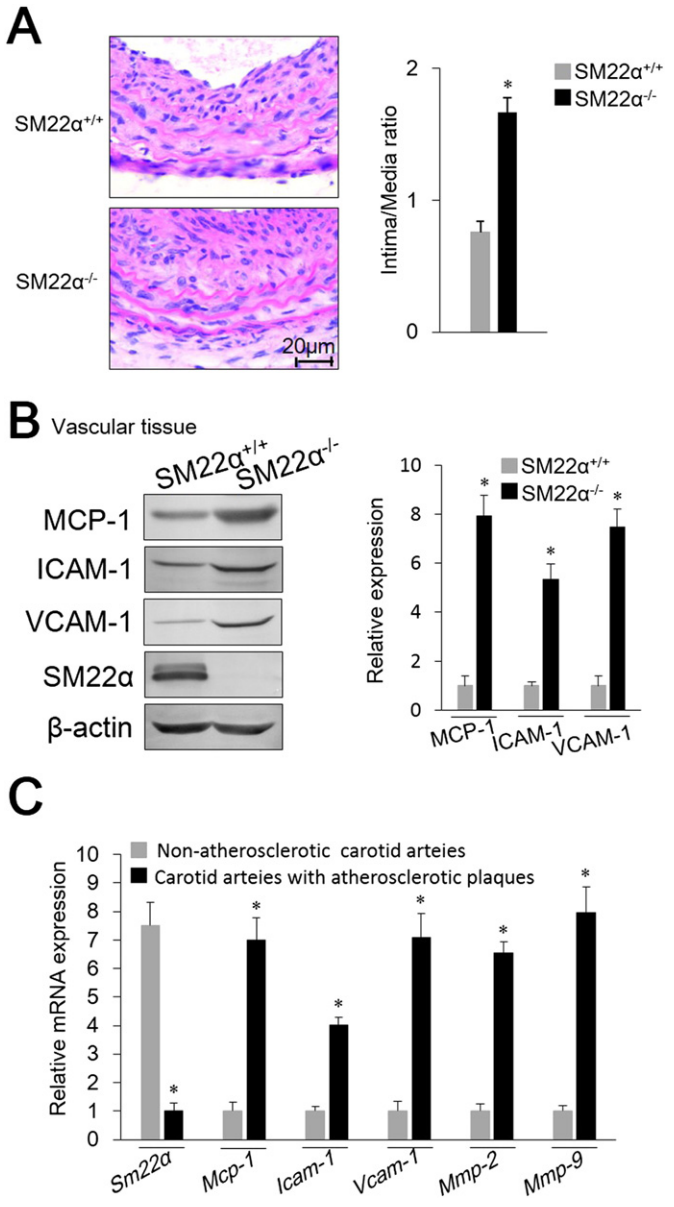

Fig. 1. Disruption of SM22α aggravates vascular inflammation. A, Photomicrographs of representative cross sections from carotid arteries of SM22α$^{-/-}$ and SM22α$^{+/+}$ mice 14 days after ligation. Bars: 20 μm. The intimal areas and intima/media ratios were quantified. B, Western blot analysis (left) and quantification (right) of the MCP-1, ICAM-1 and VCAM-1 protein levels in arteries from SM22α$^{-/-}$ and SM22α$^{+/+}$ mice with 14-day ligation injury. A–B, Data are presented as the mean ± SEM, $^{*}P < 0.01$ versus SM22α$^{+/+}$ mice ($n = 6$ per group). C, Real-time RT-PCR of Sm22α and pro-inflammatory molecules mRNA in human carotid arteries with plaques and nonatherosclerotic carotid arteries. The relative amount of mRNA was normalized to the 18s rRNA content in each sample. Normalized expression values are presented. Data are presented as the mean ± SEM, $^{*}P < 0.05$ versus nonatherosclerotic carotid arteries ($n = 8$ per group).

3. Results

3.1. Disruption of SM22α aggravates vascular inflammation

Previous studies found that SM22α$^{-/-}$ mice developed higher inflammatory responses after artery injury [20]. In the present study, to determine the roles of SM22α under pathological conditions, we performed left carotid artery ligation in SM22α$^{-/-}$ mice and their SM22α$^{+/+}$ littermates. After 14 day ligation, the intima-to-media (I/M) ratio was increased in the injured carotids from SM22α$^{-/-}$ mice compared with arteries from SM22α$^{+/+}$ controls (Fig. 1A). Using Western blot, we confirmed that expression of inflammatory molecules, including MCP-1, VCAM-1, and ICAM-1, was significantly increased in carotid arteries of SM22α$^{-/-}$ mice compared with their SM22α$^{+/+}$ mice littermates (Fig. 1B). We then looked for evidence of SM22α involvement in human vascular inflammation, and found that the expression of SM22α significantly decreased in the carotid arteries with atherosclerotic plaque, accompanied by increased expression of these inflammatory molecules, compared with nonatherosclerotic carotid arteries (Fig. 1C). These findings suggest that there is a strong correlation between decreased SM22α expression and vascular inflammation. To determine whether SM22α inhibits the vascular inflammation *in vivo*, the carotid artery ligation model was prepared using SM22α$^{-/-}$ mice, and transduced with either Ad-GFP-SM22α or Ad-GFP. The results showed that the expression of inflammatory molecules was reduced in the carotid artery rescuing SM22α expression after vascular injury for 14 days, compared with the vehicle control (Fig. S1). The data suggest that overexpression of SM22α improved injury-induced vascular inflammation in SM22α$^{-/-}$ mice *in vivo*.

3.2. SM22α inhibits NF-κB nuclear translocation and DNA binding activity in VSMCs

We then define the causal relationship between the SM22α expression and the cell inflammatory responses. We showed that knockdown of SM22α increased TNF-α-induced expression of ICAM-1, VCAM-1 and iNOS at the mRNA and protein levels in VSMCs (Figs. S2A–B), whereas overexpression of SM22α decreased the expression of pro-inflammatory cytokines (Figs. S2C–D). NF-κB pathway promotes vascular inflammation by inducing pro-inflammatory molecules and adhesion proteins in vascular cells in an NF-κB nuclear translocation-dependent fashion [21,22]. We found that the knockdown of endogenous SM22α using specific siSM22α significantly enhanced TNF-α-induced nuclear translocation of RelA/p65 (Fig. 2A). The similar results were discovered in VSMCs isolated from SM22α$^{-/-}$ mice (Figs. 2B, S3). Conversely, overexpression of SM22α inhibited it (Figs. 2C, S4). To further address the inhibition of NF-κB activation by SM22α, DNA binding and transcriptional activity of NF-κB were analyzed in VSMCs stimulated with TNF-α. The results from agarose oligonucleotide pull-down assay showed that overexpression of SM22α reduced DNA binding activity of NF-κB (Fig. 2D). The inhibitory effect of SM22α on NF-κB activity was further determined by transiently co-expressing SM22α with a luciferase reporter driven by a 6-tandem-repeat NF-κB element (Fig. 2E). These results suggest that SM22α inhibits VSMC inflammatory responses through inhibition of NF-κB activation, and may be a novel target for anti-inflammatory intervention.

3.3. SM22α inhibits TNF-α-induced IκBα phosphorylation and degradation

It has been known that the IKK-mediated phosphorylation of IκBα is a key early step of the nuclear translocation and activation of NF-κB, which lead to its subsequent dissociation from NF-κB [4]. To determine whether SM22α inhibiting NF-κB activation involves IKK repression, VSMCs were transfected with siSM22α to knockdown or infected with Ad-GFP-SM22α to overexpress SM22α. The overexpression of SM22α markedly reduced TNF-α-induced IκBα phosphorylation and degradation compared with VSMCs infected with vehicle Ad-GFP (Fig. 3A). Conversely, knockdown of endogenous SM22α expression resulted in excessive phosphorylation and degradation of IκBα, which displayed decreasing or even disappearing of IκBα compared with siCon (Fig. 3B). Similarly, TNF-α-induced IκBα phosphorylation and degradation in VSMCs of SM22α$^{-/-}$ mice were much more significant than that in WT mice (Fig. 3C). These results strongly suggest that SM22α may have ability to stabilize IκBα in VSMCs.

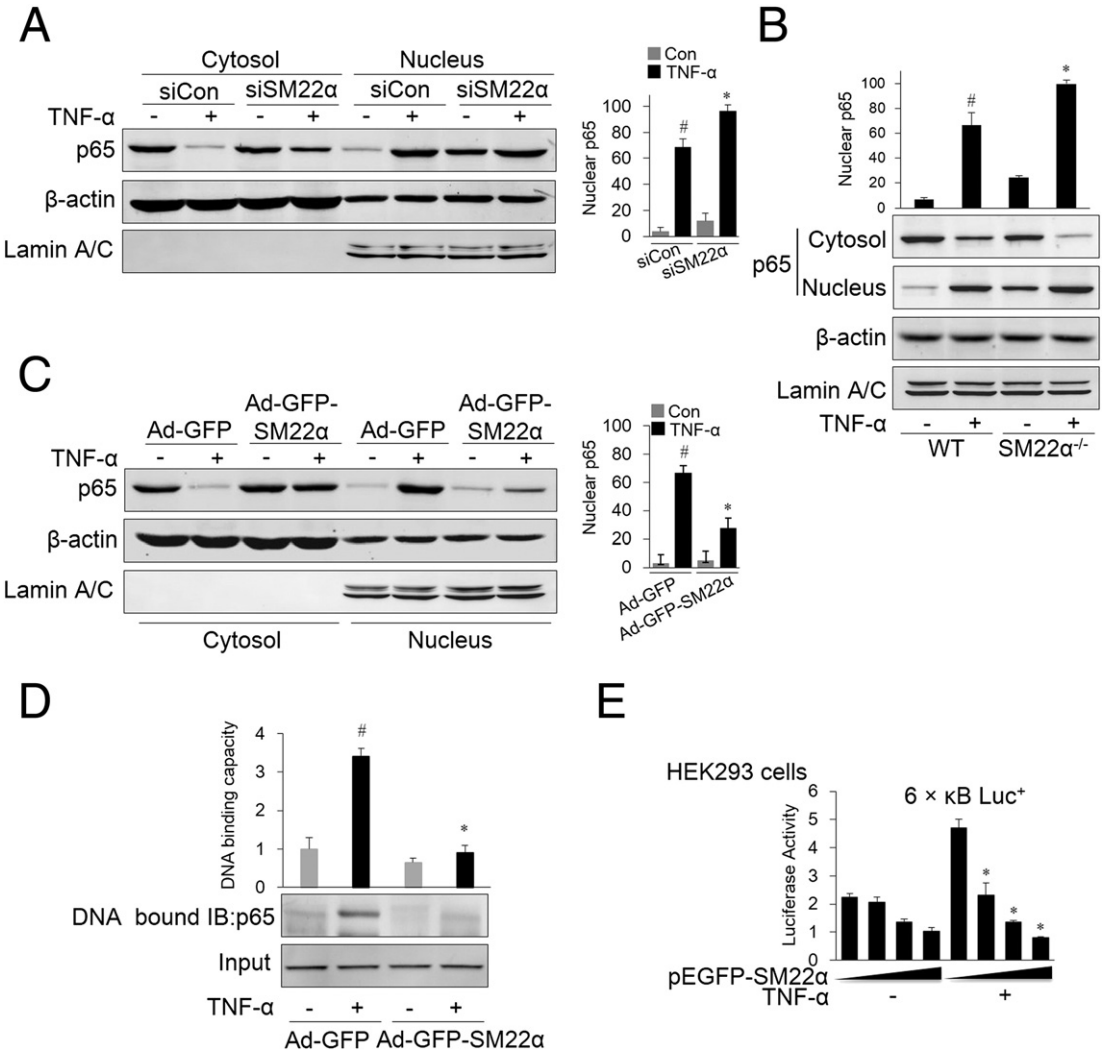

Fig. 2. SM22α inhibits NF-κB nuclear translocation and DNA binding activity in VSMCs. A, Western blot for NF-κB p65. VSMCs were transfected with siCon or siSM22α, and then treated with (+) or without (−) TNF-α (10 ng/mL) for 30 min. LaminA/C was measured as a loading control for nuclear fractions. Data are presented as the mean ± SEM, $^{\#}P < 0.01$ versus siCon (−) and $^{*}P < 0.01$ versus siCon (+). B, Western blot for NF-κB p65. SM22α$^{-/-}$ mice VSMCs were treated with (+) or without (−) TNF-α. C, Western blot for NF-κB p65. VSMCs were infected with Ad-GFP or Ad-GFP-SM22α, and then treated with (+) or without (−) TNF-α. B–C, Data are presented as the mean ± SEM, $^{\#}P < 0.01$ versus WT (−) or Ad-GFP (−) and $^{*}P < 0.01$ versus WT (+) or Ad-GFP (+). D, Agarose oligonucleotide pull-down assay of NF-κB DNA-binding activity. VSMCs were infected with Ad-GFP or Ad-GFP-SM22α, and then treated with (+) or without (−) TNF-α for 1 h. Input lanes correspond to the original cell extracts used for the oligonucleotide pull-down assay. Data are presented as the mean ± SEM, $^{\#}P < 0.01$ versus Ad-GFP (−) and $^{*}P < 0.01$ versus Ad-GFP (+). E, Luciferase assay in HEK293 cells transfected with the 6 × κB-luc, pEGFPC$_2$ and pEGFPC$_2$-SM22α followed by exposure to TNF-α for 6 h. Ratios of transfected 6 × κB-luc and pEGFPC$_2$-SM22α were 16:1, 4:1, 2:1 and 1:1. Data are presented as the mean ± SEM, $^{*}P < 0.01$ versus TNF-α treated (6 × κB-luc and pEGFPC$_2$-SM22α were 16:1).

3.4. SM22α interacts with and stabilizes IκBα

SM22α is an actin-binding protein. Previous study indicated that IκBα physically interacted with a cytoskeleton-associated protein through its signal response domain to modulate NF-κB activation [5]. These let us to hypothesize that SM22α interacts with IκBα to prevent it from phosphorylation and degradation. To test this hypothesis, we performed cross-coimmunoprecipitation using anti-IκBα and anti-SM22α antibodies. The results showed that IκBα was associated with SM22α in quiescent VSMCs, and was almost dissociated from SM22α following TNF-α stimulation (Fig. 4A). This interaction was also confirmed by coimmunoprecipitation experiments in HEK293 cells (Fig. 4B). Furthermore, VSMCs of SM22α$^{-/-}$ mice infected with Ad-GFP-SM22α to rescue expression of SM22α displayed a marked reduction of the interaction between SM22α and IκBα upon TNF-α stimulation (Fig. 4C). In addition, we also showed that SM22α bound to IκBα of IκBα–NF-κB complex at normal expression levels using cross-coimmunoprecipitation (Fig. S5). Collectively, these results indicate that SM22α forms a complex with IκBα under basal conditions, which may be disrupted by TNF-α stimulation.

3.5. CKII mediates TNF-α-induced SM22α phosphorylation

To elucidate the mechanism by which TNF-α induces dissociation of SM22α from IκBα, we focused on the effect of posttranslational modification of SM22α on interaction between the both proteins. Based on the two potential CKII consensus target motifs (S/T-X-X-D/E) in SM22α structure [23], we first measured CKII activity in VSMCs upon TNF-α stimulation, and found that CKII activity was enhanced in cells after treatment with TNF-α (Fig. S6). We then sought to determine whether TNF-α induces SM22α phosphorylation in VSMCs. The results showed that the phosphorylation of SM22α at Ser and Thr sites increased, while total SM22α levels did not change during short-time TNF-α stimulation (Figs. 5A, S7A). The pre-incubation with CX-4945, a CKII

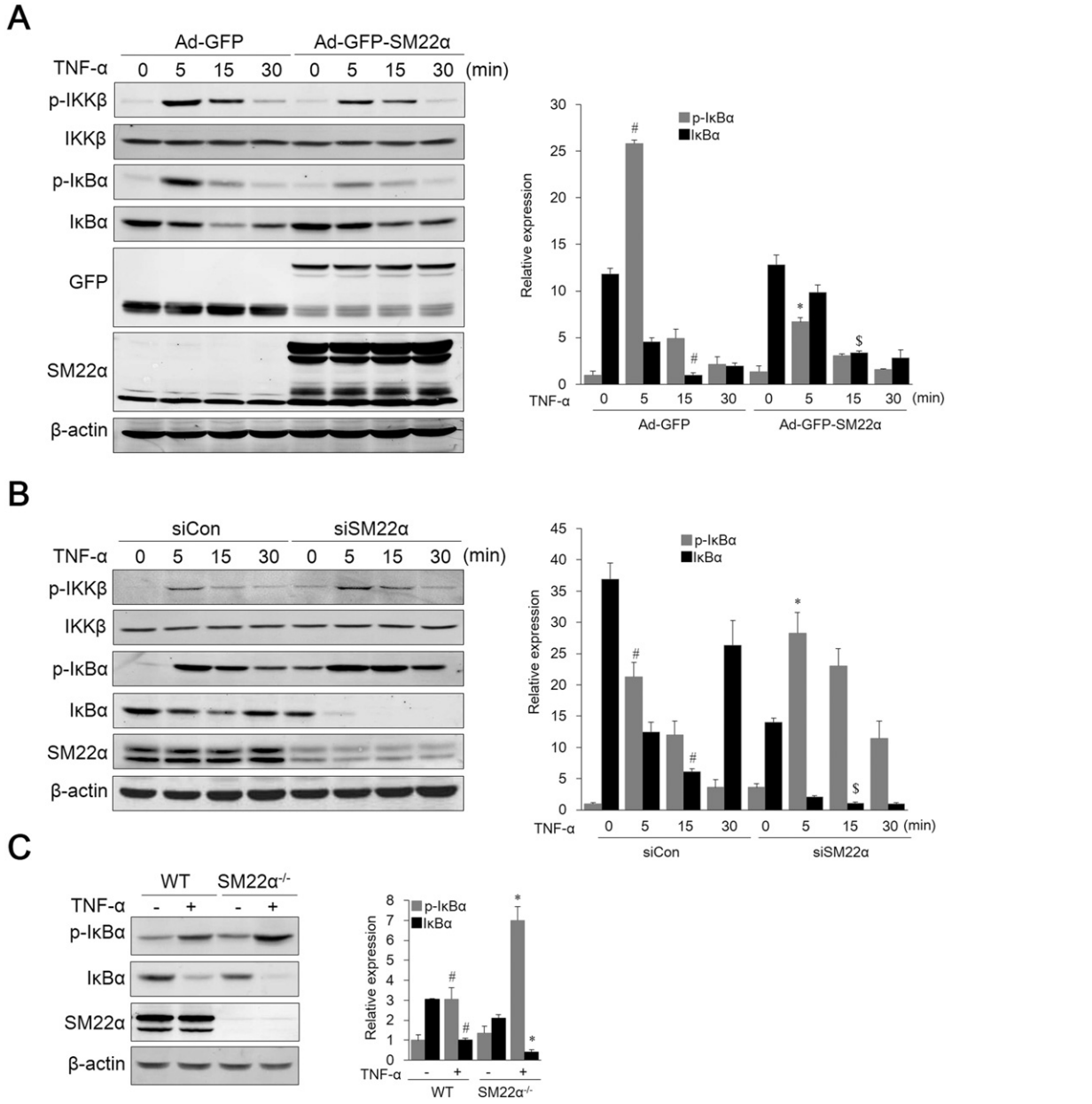

Fig. 3. SM22α inhibits TNF-α-induced IκBα phosphorylation and degradation. A, Western blot for NF-κB signaling-related proteins. VSMCs were infected with Ad-GFP or Ad-GFP-SM22α, and then treated with TNF-α for indicated times. Endogenous SM22α is 22 kDa (lower band), and GFP-tagged SM22α is 49 kDa (upper bands). Data are presented as the mean ± SEM, $^{\#}P < 0.01$ versus Ad-GFP (0 min), $^{*}P < 0.01$ versus Ad-GFP (5 min) and $^{\$}P < 0.01$ versus Ad-GFP (15 min). B, VSMCs were transfected by siCon or siSM22α, and then treated with TNF-α for indicated times. Data are presented as the mean ± SEM, $^{\#}P < 0.01$ versus siCon (0 min), $^{*}P < 0.01$ versus siCon (5 min) and $^{\$}P < 0.01$ versus siCon (15 min). C, Western blot for p-IκBα and IκBα. Data are presented as the mean ± SEM, $^{\#}P < 0.01$ versus WT (−) and $^{*}P < 0.01$ versus WT (+).

inhibitor, significantly attenuated TNF-α-induced phosphorylation of SM22α at Thr but not Ser site (Figs. 5B, S7B). To verify this argument, the specific CKII siRNA or a dominant-negative form of CKII (CKIIDN) was transfected into VSMCs to disrupt CKII. We showed that disruption of CKII abolished TNF-α-induced Thr phosphorylation of SM22α (Figs. 5C–D, S7C–D). In addition, in a rescue experiment, re-expression of CKII could restore TNF-α-induced SM22α phosphorylation in CKII-knocked down VSMCs (Figs. 5E, S7E). Furthermore, SM22α WT and the mutants containing CKII site were transducted into the VSMCs of SM22α$^{-/-}$ mice. We observed the phosphorylation of SM22α WT, but not the mutants of T139D (to mimic threonine phosphorylation) or T139A (to inhibit SM22α phosphorylation) (Figs. 5F, S7F), suggesting that CKII may selectively phosphorylate SM22α Thr139 in response to TNF-α stimulation. These data indicate that CKII mediates TNF-α-induced phosphorylation of SM22α in VSMCs.

3.6. SM22α Thr 139 phosphorylation results in dissociation of SM22α from IκBα

To determine that CKII-mediated Thr 139 phosphorylation results in dissociation of SM22α from IκBα, we detected the interaction of IκBα with SM22α WT, as well as T139D and T139A mutants using coimmunoprecipitation. SM22α T139D mutant revealed a reduced interaction with IκBα, while SM22α T139A had an increase in binding to IκBα compared with SM22α WT (Fig. 6A), suggesting that phosphorylation of SM22α is required for IκBα dissociation. To assess whether this dissociation contributes to IκBα phosphorylation and degradation, SM22α WT and these two phosphorylation mutants were transducted into SM22α$^{-/-}$ mice VSMCs. SM22α T139A mutant markedly decreased TNF-α-induced IκBα phosphorylation and degradation compared to SM22α WT or T139D mutant, accompanied by decreased

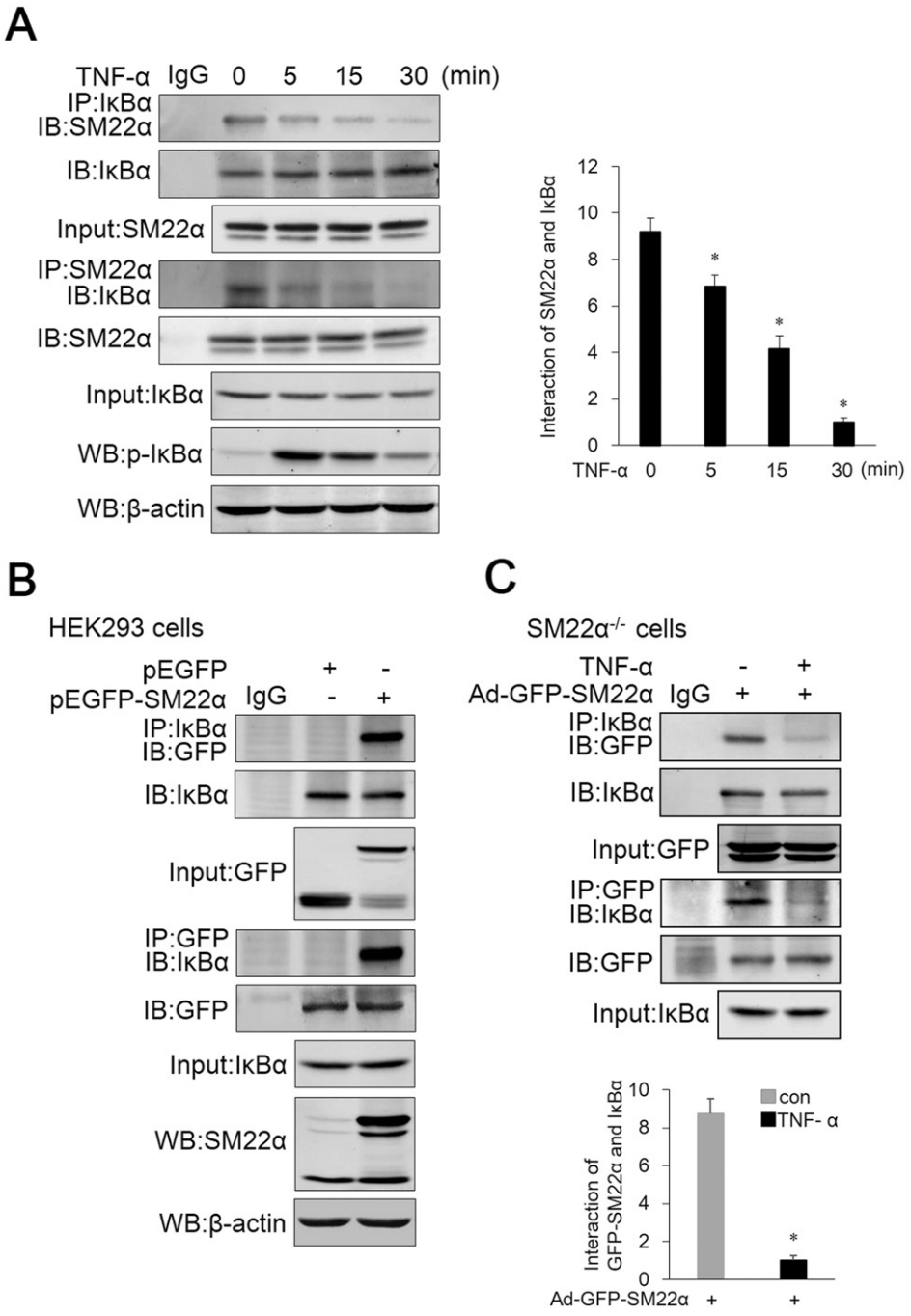

Fig. 4. SM22α interacts with and stabilizes IκBα. A, VSMCs were pretreated with the proteasome inhibitor MG132 (10 μmol/L) or vehicle control (0.1% DMSO), and then treated with TNF-α for 15 min. The graph showed the quantification of SM22α–IκBα interaction, data are presented as the mean ± SEM, $^{*}P < 0.05$ versus 0 min. B, HEK293 cells were transfected with GFP or GFP-tagged-SM22α. C, SM22α$^{-/-}$ mice VSMCs were infected with Ad-GFP-SM22α and pretreated with MG132, and then treated with TNF-α for 15 min. The anti-SM22α, anti-IκBα and anti-GFP antibodies were used for immunoprecipitation (IP) or Western blot (IB). Input lanes correspond to the original cell extracts used for the immunoprecipitation assays. IgG was used as negative control. The graph showed the quantification of SM22α-IκBα interaction, data are presented as the mean ± SEM, $^{*}P < 0.01$ versus control.

expression of nuclear RelA/p65 (Fig. 6B). Taken together, these data indicate that the phosphorylation-mediated dissociation of SM22α from IκBα facilitates IκBα phosphorylation and degradation, and subsequent NF-κB nuclear translocation upon TNF-α stimulation (Fig. 6C). We identify a novel regulatory mechanism by which the degree of inflammation is controlled by SM22α expression.

4. Discussion

Our recent studies have demonstrated that SM22α was an anti-proliferation factor and molecular switch for oxidative stress in VSMCs [14,15]. The previous study demonstrated that vascular inflammation was aggravated in SM22α$^{-/-}$ mice compared with wild-type mice [20]. The present *in vitro* and *in vivo* study showed that SM22α was a novel suppressor of vascular inflammation, demonstrating that overexpression of SM22α was sufficient to cause the repression of NF-κB activity and pro-inflammatory molecular expression. We found that endogenous SM22α inhibited RelA/p65 nuclear translocation via binding to and stabilizing IκBα in the cytoplasm. The interaction of SM22α with IκBα was regulated by SM22α phosphorylation status upon TNF-α stimulation. Thus, SM22α is identified as a phosphorylation-regulated suppressor of NF-κB, which may play a functional role in

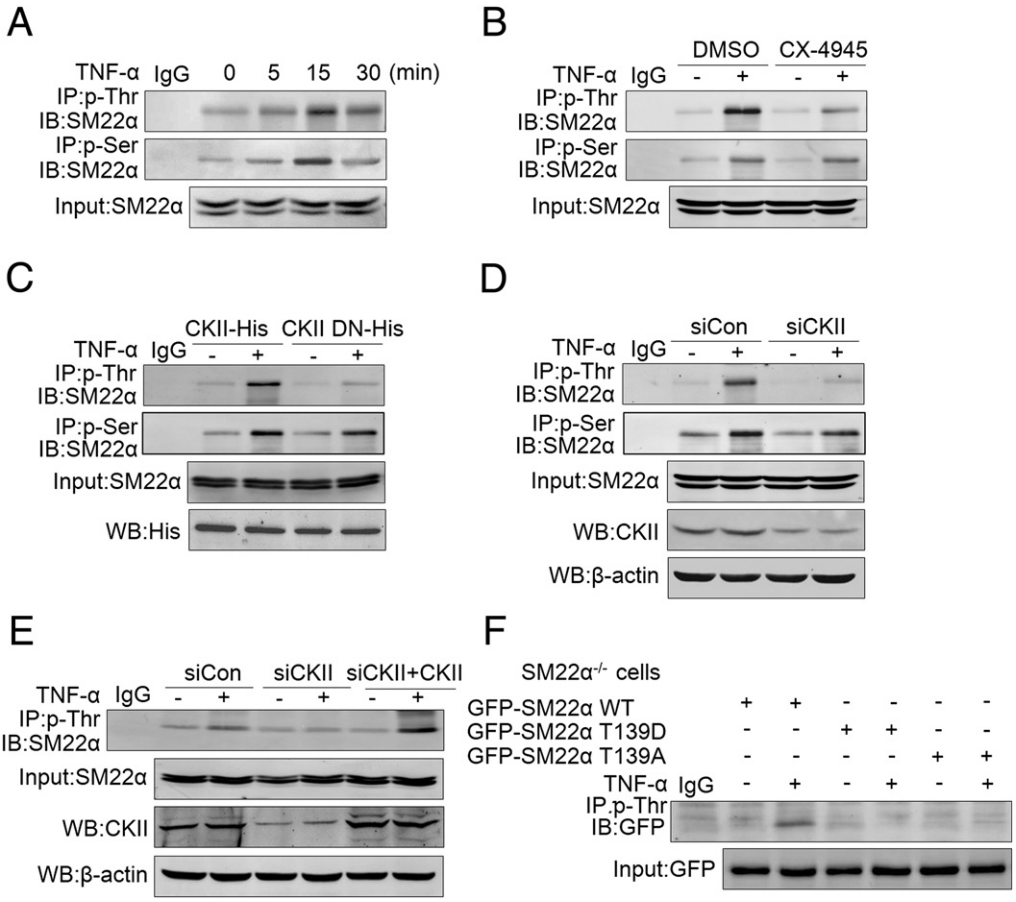

Fig. 5. CKII mediates TNF-α-induced SM22α phosphorylation. A–F, Immunoprecipitation (IP) and Western blot (WB) for serine/threonine phosphorylation (p-Ser/p-Thr) of SM22α. A, VSMCs were treated with TNF-α for indicated times. B, VSMCs were pretreated with CKII inhibitor CX-4945 (10 μmol/L) or vehicle control (0.1% DMSO), and then treated with or without TNF-α for 15 min. C, VSMCs were transfected first with CKII siRNA for 24 h, then transfected with dominant-negative mutant form of CKII (pcDNA3.1-His-CKII DN) or pcDNA3.1-His-CKII, and cell were treated with TNF-α. D, VSMCs were transfected by siCon or siCKII, and then treated with TNF-α. E, VSMCs were transfected by siCon or siCKII, and then rescued with CKII. F, VSMCs of SM22α$^{-/-}$ mice were transfected with SM22α WT or its mutants, and then treated with TNF-α. The anti-p-Ser and anti-p-Thr antibodies were used for immunoprecipitation (IP), anti-SM22α and anti-GFP antibodies were used for Western blot (IB). Input lanes correspond to the original cell extracts used for the immunoprecipitation assays. IgG was used as negative control.

maintaining a low inflammatory state in VSMCs. A working model depicting the role of SM22α in cellular responses to TNF-α is proposed in Fig. 6C, which displays an intriguing link between CKII, SM22α, and IκBα, related with modulation of NF-κB activity. Our study suggested that SM22α bonds to signal molecules and regulated their functions by itself phosphorylation.

We note that more studies support a role for SM22α in smooth muscle biology. Specifically, deletion of SM22α delayed the downregulation of SMC differentiation markers and enhanced neointimal formation [14]. However, the role of SM22α in a chronic vascular inflammation like experimental atherogenesis remains an important unanswered question. Lili *et al.* [20] found SM22α$^{-/-}$ mice developed higher inflammatory responses after artery injury, and proposed that disruption of SM22α expression in stressed VSMCs results in increased ROS production, thereby leading to NF-κB activation. Our data confirm that SM22α is a direct regulator of NF-κB. SM22α$^{-/-}$ mice may be a sensitive model of early vascular inflammation [16]. Importantly, these findings may also shed more light on the role of downregulation of SM22α in cancers, abdominal aortic aneurysm, atherosclerosis and other inflammatory vascular diseases. Therefore, homeostasis balance of SM22α expression may serve as a therapeutic strategy to repress the dysregulated inflammatory responses in arterial diseases as well as in other diseases. The precise mechanism underlying down-regulation of SM22α in inflammatory responses is worthy of further investigation. Regardless, the current work, coupled with these previous observations, indicates that SM22α is a novel modulator of vessel homeostasis in health and diseases.

It was reported that Wiskott–Aldrich syndrome (WAS) protein, a regulator of actin cytoskeleton, regulates nuclear translocation of NFAT2 and NF-κB p65 [24]. Gabriel *et al.* [25] have shown that overexpression of thymosin β4, an actin-binding protein, suppresses TNF-α-induced NF-κB activation in HCECs. In addition, up-regulation of NF-κB was found in human intestinal epithelial cells and monocytic cells following cytoskeleton depolymerization by cytochalasin D [26,27], implying that actin dynamics affects NF-κB activity. Our current study provides evidence that SM22α directly modulates inflammatory signaling molecule activity in inflammatory responses of VSMCs. SM22α interacted with IκBα in quiescent cells, and effectively prevented IκBα from phosphorylation and degradation, which allowed IκBα to trap RelA/p65 in the cytoplasm. Moreover, this interaction seems to increase the stability and ability of IκBα to associate with p65. Our findings elucidated a novel mechanism by which SM22α inhibits NF-κB activation via targeting IκBα as a partner, and explained its beneficial actions in the diverse pathologies.

The activity and function of proteins are regulated by posttranslation modification. CKII is a ubiquitously expressed and constitutively active serine–threonine kinase that exists in various cells [28]. CKII-mediated phosphorylation of IκBα has been implicated in the basal and signal-

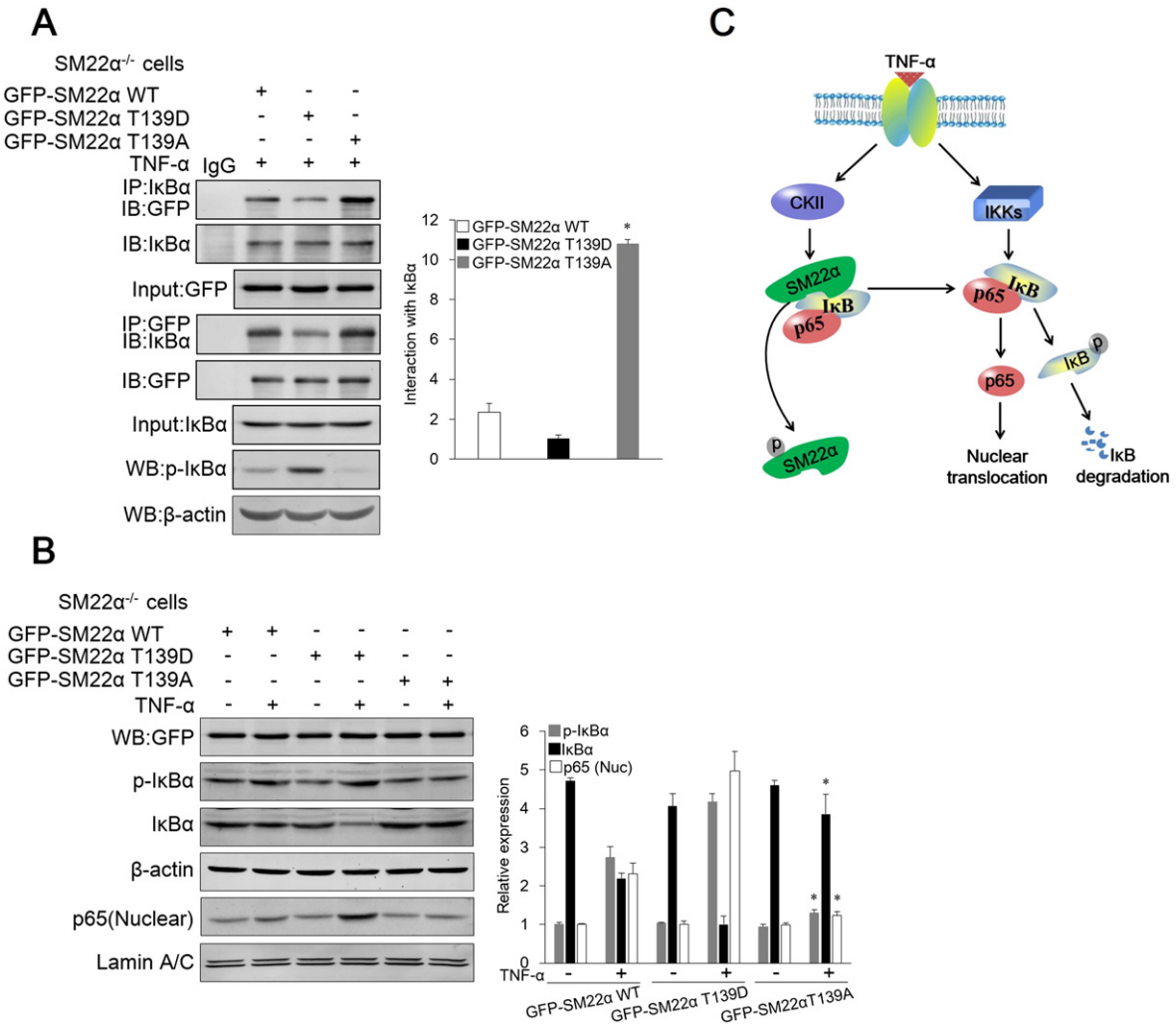

Fig. 6. SM22α Thr 139 phosphorylation results in dissociation of SM22α from IκBα. A, Coimmunoprecipitation for the interaction of IκBα with the WT and mutants of SM22α. VSMCs of SM22α$^{-/-}$ mice were transfected with SM22α WT or its mutants, pretreated with MG132, and then treated with TNF-α (+) for 15 min. Data are presented as the mean ± SEM, $^{*}P < 0.05$ versus GFP-SM22α T139D (+). The anti-IκBα and anti-GFP antibodies were used for immunoprecipitation (IP) or Western blot (IB). Input lanes correspond to the original cell extracts used for the immunoprecipitation assays. IgG was used as negative control. B, Western blot for p-IκBα and IκBα, and p65 nuclear translocation. Lamin A/C was used as a loading control of nuclear fraction. Data are presented as the mean ± SEM, $^{*}P < 0.05$ versus GFP-SM22α T139D (+). C, Diagram depicting the role of SM22α in response to TNF-α in VSMCs. Upon TNF-α stimulation, SM22α is phosphorylated by CKII, and subsequently dissociated from IκBα, which promotes IκBα phosphorylation and degradation, and then NF-κB nuclear translocation to activate the transcriptions of inflammatory genes.

dependent turnover of free and NF-κB-bound IκBα [29]. Previous work has revealed that CKII may be involved in various steps of the NF-κB activation process [30]. In the present study, we showed that CKII decreased IκBα stability via direct modification of SM22α, and knockdown of CKII reduced TNF-α-induced IκBα phosphorylation and degradation in VSMCs (Fig. S8). Phosphorylation of SM22α at Thr139 by CKII resulted in its dissociation from IκBα, and subsequent IκBα phosphorylation and degradation in VSMCs. The possible explanation for this is that IKK target site in IκBα may be masked by SM22α. These findings suggest that SM22α is required for stabilization of IκBα–NF-κB inhibitory complex in VSMCs. Although the contribution of SOD2 production to NF-κB activation has been suggested in the injured carotids of SM22α$^{-/-}$ mice [20], in the present study, we found that CKII and SM22α T139A mutant did not affect the TNFα-induced SOD2 expression (Fig. S9). One possible explanation is the phosphorylation of SM22α is not involved in regulation of SOD2 expression.

In summary, we identified that SM22α is a new operating point for NF-κB activation and vascular inflammation. The phosphorylation of SM22α switches IKK–IκBα–NF-κB signaling cascades. SM22α is essential for maintaining a low inflammatory state in VSMCs *in vitro* or *in vivo*. Our findings demonstrate that VSMCs have the potential to control inflammatory damage. The exploitation for the anti-inflammation function of SM22α may lead to the development of new therapeutics for human vascular disease and other inflammatory conditions.

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Disclosures

None declared.

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