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TC1(C8orf4) Is a Novel Endothelial Inflammatory Regulator Enhancing NF-κB Activity

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Endothelial inflammation is regulated by a complex molecular mechanism. TC1(C8orf4) is a novel regulator implicated in cancer and inflammation. It is a small protein conserved well among vertebrates. In zebrafish embryos, it is mostly expressed in angiogenic and hematopoietic system and the overexpression induces edema. In human aortic endothelial cells and umbilical vein endothelial cells, TC1 transfection up-regulates key inflammatory cytokines, enzymes, and adhesion proteins including IL-6, IL-1α, COX-2, CXCL1, CCL5, CCL2, IL-8, ICAM1, VCAM1, and E-selectin, while TC1 knockdown down-regulates them. TC1 also enhances inflammatory parameters such as monocyte-endothelial adhesion and endothelial monolayer permeability. TC1 is up-regulated by IL-1β, TNF-α, LPS, and phorbol ester, and the up-regulation is inhibited by I-κB-kinase inhibitors. TC1, in turn, enhances the nuclear translocation of RelA and the DNA binding activity, suggesting a biological role of amplifying NF-κB signaling via a positive feedback. Our findings suggest that TC1 is a novel endothelial inflammatory regulator that might be implicated in inflammatory vascular diseases. The Journal of Immunology, 2009, 183: 3996–4002.

Blood vessels are exposed to various infectious agents and hazardous stimuli causing inflammation. The endothelial regulation is of critical importance in vascular inflammation and immunity (1–4). Under inflammatory conditions, NF-κB is activated to turn on downstream cytokines, chemokines, and intercellular adhesion molecules in endothelial cells. However, the complex molecular mechanism of inflammatory regulation in endothelial cells is not completely understood. It is probable that the inflammatory regulation in higher organisms would require multiple regulators functioning in a complex way for the fine tuning of inflammatory reaction to diverse stimuli and biological situations.

TC1(C8orf4) is a novel gene up-regulated in certain cancers (5–9). It is associated with metastasis and poor survival (9). It is a small protein of 106 amino acids without known functional domain but a nuclear localization signal and a leucin zipper-like sequence (10). It is rapidly degraded through ubiquitin-proteasome pathway (10), and is suggested to be a natively disordered protein (6, 11).

The biological function of TC1 begins to be elucidated. We have shown that TC1 enhances the Wnt/β-catenin signaling pathway by relieving the antagonistic function of Cby on β-catenin-mediated transcription (10). TC1 up-regulates the expression of downstream genes to enhance proliferation and invasiveness of cancer cells (9, 10). Interestingly, TC1 is up-regulated by IL-1β and TNF-α, and enhances the proliferation of follicular dendritic cells (12), which are essential for B cell development (13). TC1 is also up-regulated by heat shock and cellular stresses in various cells (14). Together, TC1 appears to be implicated in diverse biological regulations with a potential role in the inflammatory and immune regulation in response to various stimuli.

In this study, we show that TC1 is a novel inflammatory regulator of endothelial cells. It is expressed mostly in blood vessels and hematopoietic cells of developing zebrafish, and the over-expression induces edema in the embryos. TC1 enhances key inflammatory mediators and inflammatory response in human endothelial cells. TC1 is regulated by NF-κB and, in turn, enhances the NF-κB transcriptional regulatory activity, suggesting a potential amplification of inflammatory signals.

Materials and Methods

Cells and reagents

Human aortic endothelial cells (HAEC)† and human umbilical vein endothelial cells (HUVEC) (Lonza) were cultured in EGM-2 basal medium (Lonza) at 37°C in humidified atmosphere with 5% CO2. Experiments were done using cultures of passages 6–9. THP-1 cells were cultured in RPMI 1640 (Life Technologies), supplemented with 10% FBS and 100 μg/ml penicillin/streptomycin. HEK293 cells were grown in DMEM supplemented with 5% FBS and antibiotics. Human IL-1β, TNF-α, and CCL2 were purchased (Sigma-Aldrich). BMS-345541, LPS, 12-O-tetradecanoylphorbol-13-acetate (TPA), resveratrol, and (-)-epigallocatechin-3-O-gallate (EGCG) were also purchased from Sigma-Aldrich.

Plasmids and transfection

HA-TC1 construct in pcDNA3 vector (Invitrogen) was described previously (9, 10). shTC1 constructs using pRNA H1.1 vector (Genscript) were described previously (10, 14). For the control, sh-Control vector containing unrelated sequence 5′-GGCGATTCACCCACGTCG-3′ and/or vector were transfected similarly. All plasmids were sequenced.

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HAEC and/or HUVEC transfection was done using Effectene transfection reagent (Qiagen), following the manufacturer’s instructions. For the transfection, 400 ng DNA was applied to 1.2 × 10⁵ cells in a six-well chamber, unless otherwise specified. Controls were mock and/or vector-transfected. The transfection efficiency was monitored using transfected β-galactosidase expression.

Real-time and semiquantitative RT-PCR

Real-time PCR was done as described previously (12, 14). Total RNA was extracted using TRizol reagent (Invitrogen), and cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen). Quantitative PCR was performed using a continuous fluorescence detecting thermal cycler ABI PRISM 7000 Sequence Detection System (ABI), and a SYBR Green real-time PCR master mix (Toyobo). Measurements were done in triplicate using β-actin as endogenous control. Semiquantitative RT-PCR was described previously (10, 15). The sequences of PCR primers are summarized in supplementary Tables I and II.

ELISA-based DNA binding analysis

The DNA binding capacity of p65-containing NF-κB dimers was assessed using ELISA plates containing fixed NF-κB binding site consensus sequences following manufacturer’s instructions (Panomics). In brief, HAEC and HUVEC were transfected using TC1 or vector only. Ten micrograms of nuclear extract was diluted into binding buffer and incubated for 1 h at room temperature. Following three washes, primary Ab specific for p65 was added to each well, incubated again at room temperature for 1 h, followed by HRP-conjugated secondary Ab incubation and chromogen reaction. Experiments were done in triplicate, and optical densities were measured using a SpectraMax microplate spectrophotometer (Molecular Devices).

Western blotting and cytokine array

After TC1 transfection, HAEC were washed and harvested. Total cellular, nuclear, and cytoplasmic fractions were obtained using Nuclear/ cytosol fractionation kit (BioVision Research Products). For immunoprecipitation, harvested cells were lysed in the lysis buffer (20 mM LiCl, 1.5 mM LiCl2, 1 mM EGTA, 1 mM PMSF, 0.5% NP40, 10% glycerol, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) at 4°C. After the centrifugation at 10,000 × g for 30 min, expressed proteins were pulled down from the supernatant using anti-hemagglutinin immunomorx (Roche Applied Sciences), as described previously (10). Samples were solubilized in the sample buffer and loaded, 20 μg per lane, on 12% SDS-PAGE. Proteins were blotted onto nitrocellulose membrane and probed using rabbit anti-VCAM-1, anti-COX-2, anti-RelA, anti-NF-kB1, and/or anti-NF-kB2 antisera (Santa Cruz Biotechnology). The rabbit anti-TC1 antiserum was described previously (10). β-actin was used as loading controls. After donkey anti-rabbit second Ab (Amersham Biosciences) was applied, blots were visualized using ECL method (Amersham Biosciences). Alternatively, 50 μg proteins were applied to a human cytokine array (Raybiotech), according to manufacturer’s instructions.

Zebrafish in situ hybridization and RNA microinjection

Zebrafish embryos were obtained from natural spawning and incubated at 28.5°C in 1/3 Ringer’s solution (39 mM NaCl, 0.97 mM KCl, 1.8 mM CaCl2, and 1.7 mM HEPES (pH 7.2)), as described previously (16, 17). The developmental stages of the embryos were determined by the hours post fertilization. Monocyte adhesion assay

HAEC, 4 × 10⁵ per well, were seeded in 24-well plates, and TC1-transfection was done as described. THP-1 cells suspended in phenol red-free RPMI 1640 medium containing 0.1% BSA were stimulated with 10 ng/ml CCL2 for 30 min to activate integrins. THP-1 cells, 1 × 10⁵/well, were added to the HAEC monolayers, and incubated for 30 min at 37°C. Non-adherent monocytes were removed by gentle washing for three times with PBS and the bound monocytes were counted under the microscope. Experiments were repeated in triplicate.

14 C-sucrose permeability test

HUVEC, 4 × 10⁴ cells/well, were seeded on a Transwell filter (Corning Costar), and incubated until a complete monolayer was formed changing the medium every 2 days. TC1 transfection was done using 50 ng DNA per mL.

FIGURE 1. TC1 sequence conservation among vertebrates and the expression in developing zebrafish. A, Multiple alignment of TC1 amino acid sequence among vertebrates. Identical amino acid residue is written in red, high similarity in green, and low similarity in blue letters. Amino acid sequences of Homo sapiens NP_064515.1, Mus musculus NP_081207.1, Rattus norvegicus NP_001108515.1, Bos taurus NP_001030567.1, Gallus gallus XP_001232955.1, Danio rerio tc1a XP_001393155.1, and Danio rerio tc1b XM_682605.1. B, Whole mount in situ hybridization of tc1a in zebrafish embryo 36 h postfertilization. In two-color in situ hybridization, tc1a expression (blue) is detected mostly in the blood vessel overlapping with fil1 (brown), an endothelial marker (inset). Mild expression of tc1a is also present in the brain region. C, The expression of tc1b (blue) mostly correlates with gata1 (brown), a hematopoietic precursor cell marker. D, Microinjection of tc1a and tc1b showing trunk edema (arrow) and mildly decreased head-size (red-line) in comparison with controls at 36 h postfertilization. Human TC1 microinjection also shows identical phenotypic changes.

mA mRNA was dissolved in distilled water and ~100pg RNA was microinjected into one blastomere of two-cell stage embryos as described previously (17). At least 50 embryos were injected for each experiment.

Monocyte adhesion assay

HAEC, 4 × 10⁵ per well, were seeded in 24-well plates, and TC1-transfection was done as described. THP-1 cells suspended in phenol red-free RPMI 1640 medium containing 0.1% BSA were stimulated with 10 ng/ml CCL2 for 30 min to activate integrins. THP-1 cells, 1 × 10⁵/well, were added to the HAEC monolayers, and incubated for 30 min at 37°C. Non-adherent monocytes were removed by gentle washing for three times with PBS and the bound monocytes were counted under the microscope. Experiments were repeated in triplicate.

14 C-sucrose permeability test

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well as described. Controls were transfected using the same amount of empty vector. At 24 h posttransfection, 50 μCi/ml of 14C-sucrose (Amersham Pharmacia) was added to the upper compartment. After incubation for 30 min, the amount of radioactivity that diffused into the lower compartment was measured using a liquid scintillation counter, Tri-Carb 3100TR (Packard Bioscience). For comparison, HUVEC monolayer was treated using 10 ng/ml IL-1 for 6 h. Experiments were repeated in triplicate.

Statistical methods
All measurements are presented as the mean ± SD. Significance was determined using ANOVA test.

Results
TC1 gene is conserved among vertebrates
TC1 is present in vertebrates, conserved well from human to zebrafish (Fig. 1A). From human to chicken, TC1 proteins consist of 106 amino acids: chicken TC1 has amino acid sequence of 76.4% identity and 91.5% similarity to the human orthologue. Unlike other vertebrates, two homologues, tc1a and tc1b, are present in zebrafish consisting of 100 amino acids each. tc1a has the amino acid sequence of 48.1% identity and 81.1% similarity to human TC1. Such conservation suggests an essential biological role of TC1 among vertebrates.

Zebrafish tc1 is expressed in angio-hematopoietic system
To investigate the potential biological function, we analyzed the tissue-specific expression of tc1 in developing zebrafish embryos. Both tc1a and tc1b mRNAs were detected from blastula and gastrula stage (data now shown). At 24–36 h postfertilization, tc1a expression was detected in association with developing blood vessels. In two-color in situ hybridization, tc1a mostly overlapped with fli1, an endothelial marker (Fig. 1B). tc1b expression was distinct from tc1a: it mostly overlapped with gata1, a marker of hematopoietic precursor cells (Fig. 1C). Both tc1a and tc1b appeared to be expressed in the brain at low level.

We then investigated the effect of tc1 over-expression in zebrafish by microinjecting tc1 RNAs. Both tc1a and tc1b over-expression models showed identical phenotypic changes: edema and mildly reduced head size compared with control (Fig. 1D). Human TC1 over-expression also showed identical phenotypic changes, suggesting a conserved biological role among vertebrates. TC1-induced edema suggested a potential role in the inflammatory regulation.

TC1 up-regulates inflammation genes in endothelial cells
We then analyzed the expression of TC1 in human endothelial cells. TC1 was expressed in HAEC and HUVEC whereas the expression in a monocyte line THP-1 and HEK293 cells was negligible (supplementary Fig. 1). The expression of endogenous TC1 was relatively low. Upon real-time PCR analysis, the mRNA expression level in endothelial cells was <1/100 of β-actin mRNA in HAEC under normal culture condition (data not shown).
To investigate the potential function in endothelial cells, HAEC and HUVEC were transfected transiently using TC1 in a mammalian expression vector (9, 10). The transfection efficiency monitored by β-gal expression was between 20 and 30% of cells consistently (data not shown). No cellular damage was noticed under our experimental procedures. Control cells were transfected using empty vector and/or mock transfection. Upon TC1-transfection, TC1 protein of ~15KD was detected in Western blotting of whole cell preparations and immunoprecipitated samples using HA-immunomatrix (supplementary Fig. 2). Endogenous TC1 was not detected well in control HAEC, indicating that the expression of endogenous TC1 protein was kept at a low level as we described in other cell types (10). The expression of inflammatory cytokines was measured using real-time PCR. In HAEC, TC1 up-regulated the expression of IL-6, IL-1α, and ICAM1 × 4.8, 7.8, and 2.1 times of control values, respectively (Fig. 2A). Other key inflammatory cytokines and adhesion proteins were also up-regulated, including COX-2, CCL5, CXCL1, IL-8, VCAM1 and E-selectin (supplementary Fig. 3A). In HUVEC, TC1 transfection also enhanced the expression of IL-6, IL-1α, ICAM1 (supplementary Fig. 4A), and other inflammatory genes (supplementary Fig. 4B).

We then knocked down TC1 in HAEC to investigate the loss-of-function. As TC1 was knocked down using shTC1 transfection, the expression of IL-6, IL-1α, and ICAM1 was significantly down-regulated in comparison with control, showing the normal requirement of TC1 in endothelial inflammatory gene regulation (Fig. 2B and supplementary Fig. 3B).

TC1-dependent downstream regulation was also analyzed at the protein level. Upon Western blotting, VCAM1 and COX-2 were up-regulated significantly in TC1-transfected HAEC compared with mock- and/or vector-transfected controls (Fig. 2C). A cytokine array also showed significant up-regulation of IL-6, IL-8, and CXCL1 in TC1-transfected HAEC compared with empty vector-transfected controls (Fig. 2D). Together, our data indicated a biological role of TC1 as a major inflammatory regulator of endothelial cells.

**TC1 enhances monocyte-endothelial adhesion and endothelial permeability**

To investigate the potential biological function of TC1 in endothelial regulation in vivo, we analyzed the effect of TC1 on the two critical steps of inflammatory regulation, i.e., monocyte-endothelial adhesion and endothelial monolayer permeability. Activated THP-1 monocytes showed enhanced adhesion to TC1-transfected HAEC compared with the vector-transfected control (p < 0.001) (Fig. 3A). TC1 knockdown in HAEC inhibited the THP-1 adhesion significantly compared with the control (Fig. 3B). To investigate the role of TC1 in the endothelial activation mediated by inflammatory mediators, we then measured the THP-1 adhesion to TC1-knocked down HAEC after 10 ng/ml IL-1β treatment for 6 h. IL-1β enhanced monocyte adhesion significantly in control-transfected HAEC (Fig. 3C). However, IL-1β did not enhance monocyte adhesion in TC1-knocked down HAEC compared with control cells. Our data suggested that IL-1β-mediated endothelial activation was dependent on TC1.

TC1 effect on the permeability of endothelial monolayer was analyzed using the 14C-sucrose permeability test. HUVEC monolayer was transfected using low-dose DNA, 50 ng/ml. No cellular damage was noted in either TC1- or vector-transfected cells. Upon 14C-sucrose application for 30 min, the permeability of TC1-transfected HUVEC monolayer was enhanced mildly, 26.6% in average, compared with the control (p < 0.02) (Fig. 3D). The TC1-induced permeability was about two-thirds of positive control treated using 10 ng/ml IL-1β.

**TC1 expression is NF-κB-dependent**

Our data together indicated a role of positive inflammatory regulator of TC1 in endothelial cells. Previously, we have shown that IL-1β and TNF-α, the prototype activator of NF-κB, up-regulated TC1 expression in follicular dendritic cells (12). In HAEC, IL-1β and TNF-α also enhanced the expression of TC1 in dose-dependent manners (Fig. 4, A and B). Significant TC1 up-regulation was shown in 6 h after IL-1β and/or TNF-α treatment and lasted for longer than 24 h (Fig. 4, A and B). IL-1β enhanced TC1 expression up to 25 times of the baseline in 6 h, suggesting that IL-1β might be a more potent enhancer of TC1 expression than TNF-α in endothelial cells. A similar result was obtained in our previous study using follicular dendritic cells (12). TC1 was also up-regulated by LPS and TPA in 6 h in dose-dependent manners (Fig. 4C).

The up-regulation of TC1 by IL-1β/TNF-α suggested a NF-κB-dependent regulation. Because NF-κB activation requires IκB-kinase (IKK)-mediated IκB degradation (18), we investigated the effects of IKK inhibitors on TC1 expression. BMS-345541, a specific IKK inhibitor, suppressed TC1 expression considerably, reducing the mRNA level to one-fifth of untreated controls by 10

**FIGURE 3.** TC1 enhances monocyte adhesion and endothelial monolayer permeability. A, HAEC-THP-1 adhesion assay: HAEC seeded in 24-well plates, 4 × 10⁴ cells/well, and transfected using TC1. THP-1 cells stimulated with 10 ng/ml CCL2 for 30 min, are added to the HAEC monolayers, 1 × 10⁵/well, and incubated for 30 min at 37°C. After removal of nonadherent monocytes by gentle washing for three times with PBS, bound monocytes are counted under the microscope. Statistically significant differences are asterisked on the bars. B, THP-1 adhesion assay in TC1 knock-down HAEC using shTC1 in comparison with sh-Con. C, THP-1 adhesion assay after 10 ng/ml IL-1β treatment for 6 h in TC1 knocked-down and control HAEC. D, 14C-sucrose permeability test. Four × 10⁴ cells/well HUVEC are seeded on a Transwell filter (Corning), and incubated until a complete monolayer is formed. After a 30-min incubation, the amount of radioactive measurement is measured in the lower compartment using a liquid scintillation counter. For comparison, the same HUVEC monolayers are treated using 10 ng/ml IL-1β for 6 h. Experiments are repeated in triplicate.
TC1 regulates endothelial inflammation.

**FIGURE 4.** TC1 regulation by inflammatory mediators in HAEC. A, TC1 regulation by IL-1β. Dose-dependent expression at 6 h as indicated (left) and time-dependent expression at 1 ng/ml IL-1β (right). Real-time PCR measurements are done in triplicate using β-actin endogenous controls. One and two asterisks indicate statistical difference with \( p < 0.01 \) and \( p < 0.001 \), respectively, in comparison with the previous dose. B, TC1 regulation by TNF-α. Dose-dependent expression at 6 h as indicated (left) and time-dependent expression at 10 ng/ml TNF-α (right). C, Dose-dependent up-regulation of TC1 expression after LPS and/or TPA treatments for 6 h as indicated.

μM BMS-345541 in 6 h (Fig. 5A). Sulindac also suppressed TC1 expression mildly (Fig. 5A). We then analyzed the effect of BMS-345541 on TC1 up-regulation by inflammatory mediators. IL-1β-mediated TC1 up-regulation was inhibited completely by 10 μM BMS-345541 (Fig. 5B), supporting the NF-κB-dependent TC1 regulation in endothelial cells. Phorbol esters are also known to activate NF-κB activity (19). TPA-mediated TC1 up-regulation was also inhibited by BMS-345541 completely (Fig. 5B).

To further investigate the potential clinical implication of TC1 in inflammatory vascular diseases such as atherosclerosis, we analyzed the effects of cardiovascular protective agents on TC1 expression in HAEC. Resveratrol and EGCG inhibited TC1 expression in 24 h in dose-dependent manners from relatively low concentrations (supplementary Fig. 5).

**FIGURE 5.** IKK inhibitors down-regulate TC1 in HAEC. A, BMS-345541 and Sulindac inhibit TC1 expression in dose-dependent manners. Real-time PCR measurements are done in triplicate using β-actin endogenous controls. Statistically significant differences are asterisked on the bars. B, BMS-345541 inhibits IL-1β- and TPA-mediated TC1 up-regulation. HAEC are pre-treated with BMS-345541 for 1 h as indicated before 10 ng/ml IL-1β and/or 100 nM TPA treatment for 6 h. Measurements are done in triplicate using β-actin controls.

TC1 enhances NF-κB nuclear translocation and DNA binding activity

TC1-regulated inflammation genes were also known to be NF-κB-downstream genes. We then questioned whether TC1 would be involved in NF-κB activation. The NF-κB activity was analyzed in TC1-transfected or knocked-down endothelial cells using ELISA-based DNA binding analysis for RelA. Nuclear proteins from TC1-transfected or knocked-down endothelial cells using ELISA-based DNA binding consensus sequence oligonucleotides. TC1 enhanced the RelA DNA-binding activity 2.4 times of the empty vector transfected control (Fig. 6A). TC1-transfected HUVEC also showed significant enhancement of NF-κB activity compared with the control (Fig. 6B). TC1 knockdown inhibited the NF-κB activity significantly in HAEC and HUVEC, suggesting a normal requirement of TC1 for the NF-κB activity (Fig. 6, A and B).

We then analyzed the intracellular distribution of RelA in TC1-transfected HAEC using β-actin as loading control. β-actin is present in cellular nuclei as well as cytoplasm (20). In TC1-transfected HAEC, nuclear RelA was enhanced prominently in comparison with vector-transfected control (Fig. 6C). In contrast, RelA appeared to be up-regulated minimally in whole cell and cytoplasmic fractions (Fig. 6C). Together, our data suggested that TC1 enhanced nuclear translocation of NF-κB. Nuclear p52 and p50 were not detected well using the same samples (data not shown).

**Discussion**

We have shown that TC1 up-regulates key inflammation genes in endothelial cells. It also enhances monocyte-endothelial adhesion and permeability of endothelial monolayer. TC1 knockdown inhibits downstream inflammation gene expression and IL-1β-mediated monocyte-endothelial adhesion, indicating a requirement of TC1 in the endothelial inflammatory regulation.

TC1 is well conserved among vertebrates, suggesting an essential biological role. It does not share any identifiable motif with other genes, suggesting that it is a relatively new gene developed in the evolution. Intriguingly, two homologues are present in zebrafish, showing distinct distributions in developing embryos. tc1a mostly distributes in blood vessels while tc1b is in hematopoietic precursor cells. The overexpression of zebrafish and TC1 shows edema and reduced head size in zebrafish embryos. Edema may be caused by aberrant inflammatory regulation. It is not clear how the overexpression of tc1a and tc1b shows identical phenotypic changes despite distinct distributions. Our data suggest that TC1 might regulate hematopoietic as well as endothelial cells. The
implicated in atherosclerosis (2–4). It is of note that TC1 is downregulated with the vascular regulation. TC1 downstream genes have been characterized. The interaction with Cby (10). Recently, novel coregulators of TC1 (C8orf4) have been reported (23–25), suggesting the presence of a fine-tuning system for the regulation of innate immunity needs to be investigated. TC1 appears to have a potential clinical relevance in association with cardiovascular protective agents such as resveratrol and EGCG. Resveratrol, a polyphenol compound of red wine, and EGCG, a major catechin in green tea, have been shown to have significant anti-inflammatory activities (26–29). Our data together suggest that TC1 might be a therapeutic target for the vascular inflammatory diseases. TC1-induced enhancement of endothelial permeability might also be implicated in cancer metastasis. We have reported that TC1 expression is associated with cancer metastasis and lymphatic permeation (9).

TC1 expression is enhanced by various stimuli including TPA and LPS, suggesting that it may be implicated in flexible inflammatory response to diverse stimuli and biological situations. LPS-induced activation of innate immunity via TLR4 has been implicated in atherosclerosis (30, 31). A potential role of TC1 in the regulation of innate immunity needs to be investigated. TC1 function in vivo is investigated using genetically manipulated mice.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


