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Article

TRIM65 E3 ligase targets VCAM-1 degradation to limit LPS-induced lung inflammation

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Although the adhesion molecules-mediated leukocyte adherence and infiltration into tissues is an important step of inflammation, the post-translational regulation of these proteins on the endothelial cells is poorly understood. Here, we report that TRIM65, an ubiquitin E3 ligase of tripartite protein family, selectively targets vascular cell adhesion molecule 1 (VCAM-1) and promotes its ubiquitination and degradation, by which it critically controls the duration and magnitude of sepsis-induced pulmonary inflammation. TRIM65 is constitutively expressed in human vascular endothelial cells. During TNFα-induced endothelial activation, the protein levels of TRIM65 and VCAM-1 are inversely correlated. Expression of wild-type TRIM65, but not expression of a TRIM65 mutant that lacks E3 ubiquitin ligase function in endothelial cells, promotes VCAM-1 ubiquitination and degradation, whereas small interference RNA-mediated knockdown of TRIM65 attenuates VCAM-1 protein degradation. Further experiments show that TRIM65 directly interacts with VCAM-1 protein and directs its polyubiquitination, by which TRIM65 controls monocyte adherence and infiltration into tissues during inflammation. Importantly, TRIM65-deficient mice are more sensitive to lipopolysaccharide-induced death, due to sustained and severe pulmonary inflammation. Taken together, our studies suggest that TRIM65-mediated degradation of VCAM-1 represents a potential mechanism that controls the duration and magnitude of inflammation.

Keywords: TRIM65, VCAM-1, endothelial activation, lung inflammation, ubiquitination

Introduction

Inflammation is required for the activation of innate and adaptive immunity, which is essential for host defense against invading pathogens such as viruses and bacteria. The inflammatory response must be resolved after the pathogens are cleared, because unchecked inflammation can cause tissue damage and organ failure in the host. However, the mechanism that controls resolution of the inflammatory response is incompletely understood (Cao, 2016).

The vascular endothelium forms the interface between blood and tissues and plays a major role in promoting inflammatory response, including the recruitment of circulating leukocytes to the vessel wall and surrounding tissues at sites of inflammation (Aird, 2003). One of the initial and key events in the endothelium's response to inflammatory stimuli is the expression of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecular 1 (ICAM-1) and E-selectin. These proteins mediate early leukocyte attachment and rolling on the endothelial surface (Pober and Sessa, 2007). Subsequent events such as firm adhesion and transmigration across the endothelial lining then set the stage for a developing inflammatory response within tissues. Given the importance of these adhesion molecules in inflammation, the molecular mechanisms regulating their expression have been the subject of considerable investigation. However, though the transcriptional mechanisms regulating their expression are well documented, the post-translational regulation of these proteins is not clear (Osborn 1990; Montgomery et al., 1991; Hou et al., 1994; Zhou et al., 2007).

TRIM65 is a member of the tripartite motif (TRIM) protein family. The TRIM protein family comprises ~75 members

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and is involved in various cellular processes, including cell proliferation, differentiation, cell death, and immunity (Ozato et al., 2008). Human TRIM65 is a 517-amino acid protein containing an N-terminal RING domain, a B-box, a coiled-coil domain, and a SPRY domain. Several reports suggest that TRIM65 acts as an ubiquitin E3 ligase, targeting GW182, p53, ANXA2, Axin1, and MDA5 to regulate carcinogenesis, innate immunity and miRNA pathway (Li et al., 2014, 2016; Lang et al., 2017; Yang et al., 2017; Wei et al., 2018). However, the physiological role of TRIM65 remains unknown. Human genome-wide association studies suggest that TRIM65 gene mutation is associated with human white mater hyperintensities (WMH), which is commonly caused by cerebral small vascular disease (Fornage et al., 2011; Freudenberger et al., 2012). However, the function of TRIM65 in vasculature is never reported.

In our study, TRIM65-deficient (TRIM65$^{-/-}$) mice were growing normally and had normal life span but were sensitive to lipopolysaccharide (LPS)-induced death due to sustained and severe lung inflammation. The lung inflammation of TRIM65$^{-/-}$ mice is mainly caused by over-infiltration of monocytes/-macrophages. Further experiments defined that TRIM65 E3 ligase selectively targeted VCAM-1 to promote its ubiquitination and degradation, by which it controls monocyte adherence to endothelium and infiltration into tissues. Certainly, the other mechanisms may also contribute to the phenotypes. These results suggest that TRIM65-mediated VACM-1 ubiquitination and degradation represents a possible mechanism that controls vascular inflammation and contributes to human inflammatory diseases.

Results

Genetic deletion of TRIM65 augments LPS-induced death and lung inflammation of mice

To define the physiological role of TRIM65 *in vivo*, we have obtained the mice with TRIM65-targeted allele from the UC DAVIS KOMP Repository (Stock# ET12501), with a 'knockout-first allele' promoter-driven selection cassette, allowing both the generation of TRIM65$^{-/-}$ mice in F2, and also conditional deletion following exposure to site-specific recombinases Cre and Flp (Figure 1A). Genotyping and western blotting confirmed insertion of the cassette (Figure 1B) and consequent deletion of TRIM65 protein in lungs (Figure 1C), aorta, and spleen (data not shown). TRIM65$^{-/-}$ mice were born in Mendelian ratios and had normal survival rates (data not shown). To test if the expression of TRIM65 is critical in the protection of LPS-induced death, TRIM65$^{+/+}$ and TRIM65$^{-/-}$ mice at 2-month-old (12 mice for each genotypes) were intraperitoneally (i.p.) injected with 45 mg/kg body weight of LPS. All mice were closely monitored, and a 'moribund status' was equated to death to minimize the discomfort of the mice. The survival rate was recorded. As shown in Figure 1D, TRIM65$^{-/-}$ mice were more sensitive to LPS-induced death compared to TRIM65$^{+/+}$ mice ($P = 0.0256$). To test whether TRIM65 expression is essential in the protection of LPS-induced inflammation and lung injury, TRIM65$^{-/-}$ and TRIM65$^{+/+}$ mice were injected intraperitoneally (i.p.) with sublethal dose of LPS (25 mg/kg body weight) for 12 h. Lung inflammation and injury were analyzed by lung edema, histology, and leukocyte infiltration in bronchoalveolar lavage fluid (BAL). As shown in Figure 1E, pulmonary edema, measured as a significant increase in the wet-to-dry lung weight ratio, was significantly increased in LPS-treated TRIM65$^{-/-}$ mice compared to that in LPS-treated TRIM65$^{+/+}$ mice. Hematoxylin-and-eosin (H&E) staining showed that without LPS challenge, there were barely inflammation in the lungs from both TRIM65$^{+/+}$ and TRIM65$^{-/-}$ mice; with LPS challenge, both TRIM65$^{+/+}$ and TRIM65$^{-/-}$ mice developed severe lung inflammation and injury, but the inflammation and injury was much more severe in the lungs from TRIM65$^{-/-}$ mice than TRIM65$^{+/+}$ mice (Figure 1F). To determine the cell types that infiltrated into the lungs, we analyzed the BAL collected from these mice. LPS challenge significantly increased the total cell numbers and protein amount in the BAL from both TRIM65$^{+/+}$ mice and TRIM65$^{-/-}$ mice, but the cell numbers and protein amount were much more in the BAL from TRIM65$^{-/-}$ mice than TRIM65$^{+/+}$ mice. Further analysis showed that the infiltrated cells in BAL were mainly monocytes/macrophages but not neutrophils (Figure 1G).

Genetic deletion of TRIM65 leads to increased expression of VCAM-1 in vascular endothelial cells

To understand why TRIM65$^{-/-}$ mice are more sensitive to LPS-induced lung inflammation, injury and death, we analyzed the mRNA expression of inflammatory cytokines in the lungs. As shown in Supplementary Figure S1, the mRNA levels of CCL2, CXCL1, IL-6, IL-17, IL-9, GCSF, CXCL13, and IFNβ were significantly increased in LPS-treated TRIM65$^{-/-}$ mice compared to that in LPS-treated TRIM65$^{+/+}$ mice. The mRNA levels of CXCL2, IL-1β, CXCL10, TLR2, TLR4, and IFNγ were significantly induced by LPS but not changed between TRIM65$^{-/-}$ and TRIM65$^{+/+}$ mice. As the transcription of most of these inflammatory cytokines are controlled by MAPK and NF-κB signaling pathways (Read et al., 1997; Milstone et al., 2015), we next observed the changes of MAPK and NF-κB signaling pathways in the lungs from both TRIM65$^{-/-}$ and TRIM65$^{+/+}$ mice. As shown in Supplementary Figure S2, the levels of phospho-c-Jun, JNK, ERK1/2, p38, p65, and IκBα were not changed in the lungs between TRIM65$^{-/-}$ and TRIM65$^{+/+}$ mice, suggesting that the increased inflammation in TRIM65$^{-/-}$ lungs was not due to over activation of MAPK and NF-κB signaling pathways. The effect of TRIM65 expression on MAPK and NF-κB signaling pathways was further tested on cultured human umbilical vein endothelial cells (HUVECs) (Supplementary Figure S3). Next, we detected the protein levels of vascular adhesion molecules including VCAM-1, ICAM-1, E-selectin, and VE-cadherin in the lungs by western blotting. As shown in Figure 2A and B, TRIM65 expression was completely abolished in the lungs from TRIM65$^{-/-}$ mice, further confirming the global deletion of TRIM65 in the mice. Among the examined vascular adhesion molecules, only VCAM-1 protein levels were significantly increased in LPS-treated TRIM65$^{-/-}$ mice compared to TRIM65$^{+/+}$ mice. Similar results were also observed in aorta

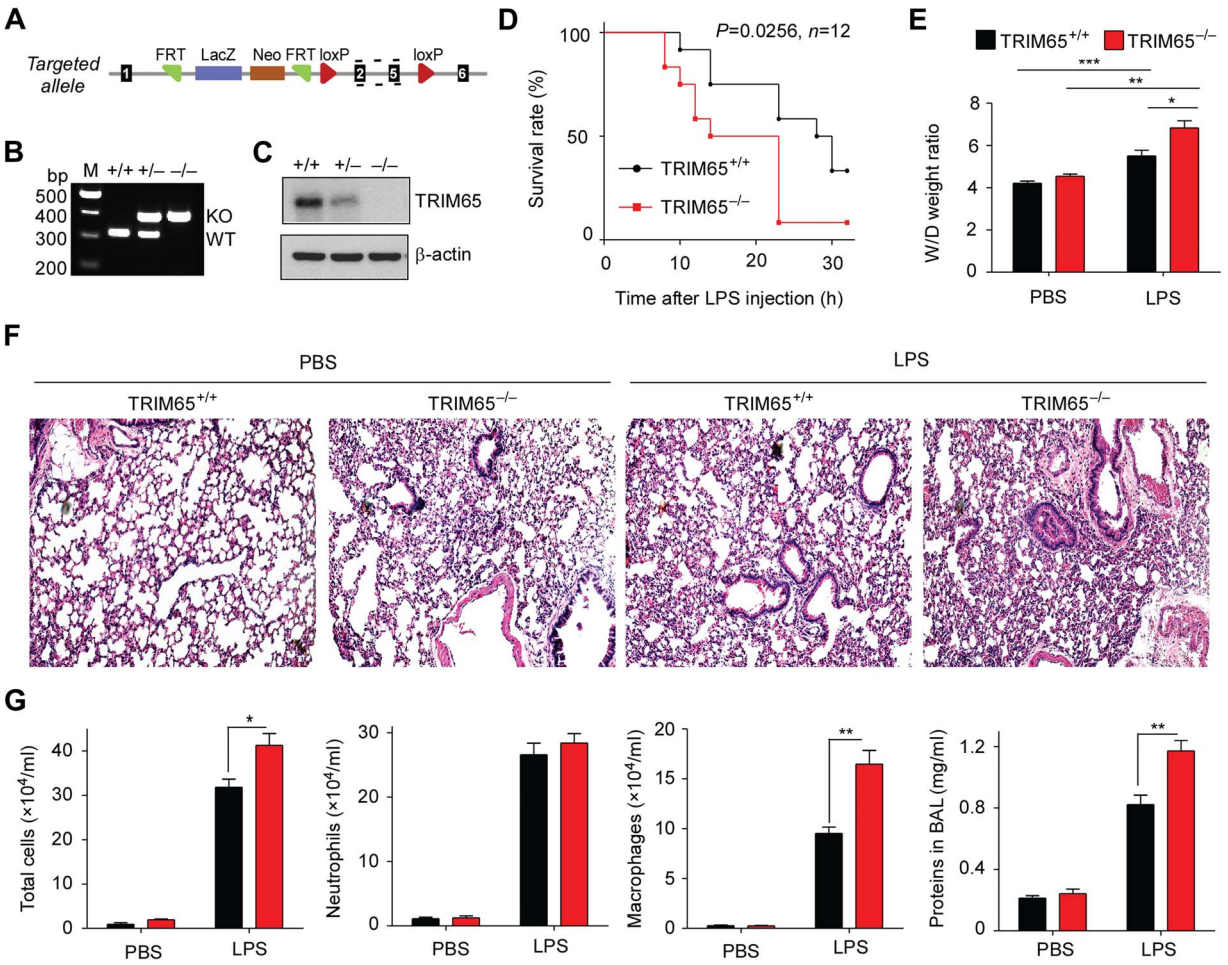

Figure 1 Genetic deletion of TRIM65 augments LPS-induced lung inflammation. (**A**) Schematic strategy of generation of TRIM65-targeted allele. (**B**) Genotyping of TRIM65$^{+/+}$, TRIM65$^{+/-}$, and TRIM65$^{-/-}$ mice. (**C**) Lungs were collected from TRIM65$^{+/+}$, TRIM65$^{+/-}$, and TRIM65$^{-/-}$ mice and subjected to western blot analysis for TRIM65. (**D**) Survival rate of mice challenged with 45 mg/kg body weight of LPS injection (i.p.). (**E**–**G**) TRIM65$^{+/+}$ and TRIM65TRIM65$^{-/-}$ mice were injected with PBS or 25 mg/kg body weight of LPS and examined for lung inflammation after 12 h. (**E**) The lung wet-to-dry ratio. (**F**) H&E staining of lung sections. Original magnification, 100×. (**G**) Cell numbers of BAL collected. Data are representative of three independent experiments and presented as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by Student's *t*-test.

from these mice (Supplementary Figure S4). To further verify that TRIM65 deficiency results in increased expression of VCAM-1 in vascular endothelial cells, we isolated microvascular endothelial cells from the lungs of TRIM65$^{-/-}$ and TRIM65$^{+/+}$ mice. The cultured primary vascular endothelial cells were stimulated with LPS (1 μg/ml) for 0, 4, 8, and 24 h. The cell lysates were then extracted for western blot analysis. As expected, LPS-induced VCAM-1 expression in TRIM65$^{+/+}$ endothelial cells in a time-dependent manner. The expression of VCAM-1 but not ICAM-1, E-selectin, and VE-cadherin was markedly increased in TRIM65$^{-/-}$ cells compared to TRIM65$^{+/+}$ cells (Figure 2C and D). We also observed the mRNA levels of these adhesion molecules by quantitative polymerase chain reaction (QPCR). The mRNA levels of VCAM-1, ICAM-1 and E-selectin were significantly induced by LPS but not changed between TRIM65$^{-/-}$ and TRIM65$^{+/+}$ cells (Supplementary Figure S5). Taken together, these results suggest that TRIM65 may control VCAM-1 protein turn over in vascular endothelial cells, by which it protects mice from LPS-induced lung inflammation and injury.

TRIM65 promotes VCAM-1 degradation

Although several reports suggest that TRIM65 gene mutation is associated with human WMH (Fornage et al., 2011; Freudenberger et al., 2012), which is commonly caused by cerebral small vascular disease, the expression and function of TRIM65 in vascular endothelial cells is never reported. Here, we observed that TRIM65 is highly enriched in human endothelial cells from various locations such as human aortic endothelial cells (HAECs), human coronary artery endothelial cells (HCAECs), human dermal microvascular endothelial cells (HDMECs), human lung microvascular endothelial cells (HLMECs), and HUVECs (Supplementary Figure S6). It is well known that the

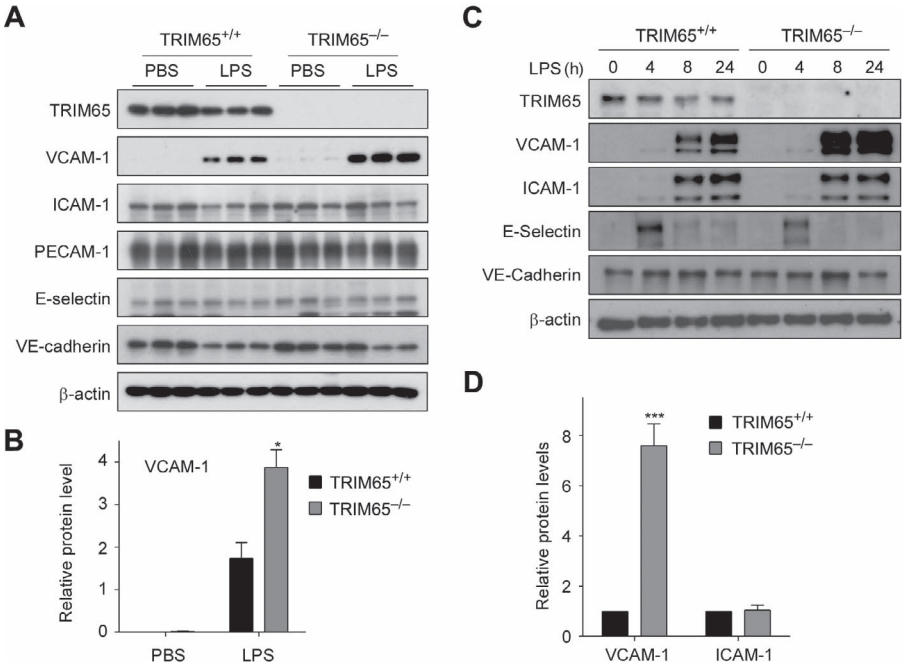

Figure 2 Genetic deletion of TRIM65 leads to increased expression of VCAM-1 in vascular endothelial cells. (**A** and **B**) The lung extracts were collected from mice after 12 h of PBS or LPS injection. The tissue lysates were subjected to western blot analysis. (**C** and **D**) Pulmonary microvascular endothelial cells were isolated and treated with LPS (1 μg/ml) for different times as indicated. The cell lysates were subjected to western blot analysis. Fold change of the protein levels were determined by densitometry and normalized to β-actin. Quantitative data are presented as mean ± SD ($n = 3$); $^{*}P < 0.05$, $^{***}P < 0.001$ vs. TRIM65$^{+/+}$ group by Student's *t*-test.

expression of VCAM-1 on endothelial cells is precisely controlled at transcriptional level (Neish et al., 1992; Marui et al., 1993; Manning et al., 1995). However, if VCAM-1 protein is also regulated at post-translational level remains unknown. We have examined the protein half-life of VCAM-1 in activated endothelial cells. As shown in Figure 3A, VCAM-1, but not ICAM-1 was quickly degraded after inhibition of translation with cycloheximide (CHX). The half-life of VCAM-1 protein is about 4 h. Interestingly, MG132, a proteasome inhibitor, significantly inhibited VCAM-1 protein degradation, suggesting that VCAM-1 may be subjected to the regulation of ubiquitin–proteasome system. Moreover, during activation of endothelial cells, VCAM-1 expression was increased at 4 h of TNFα stimulation, peaked at 8 h, then gradually declined, and disappeared at 40 h. In contrast, TRIM65 expression was decreased after TNFα stimulation, reached the lowest point at 8 h, and then gradually came back to basal line at 40 h (Figure 3B). The dynamic changes of TRIM65 and VCAM-1 protein levels were inversely co-related during endothelial activation.

To examine whether TRIM65 affects VCAM-1 protein expression, HUVECs were transiently transfected with Flag-TRIM65 or empty vector (EV). Twenty-four hours later, the transfected cells were stimulated with TNFα for 8 h. The cells were harvested for western blot analysis. As expected, VCAM-1 was not expressed in resting endothelial cells, but dramatically induced by TNFα. Interestingly, overexpression of TRIM65 significantly decreased VCAM-1 protein level, but did not affect ICAM-1 or VE-cadherin levels (Figure 3C). QPCR showed that overexpression of TRIM65 did not affect the mRNA expression of VCAM-1 in HUVECs (Supplementary Figure S7A). These results suggest that TRIM65 may regulate VCAM-1 expression at post-translational level, but not at transcriptional level. To further determine if TRIM65-mediated VCAM-1 degradation is dependent on TRIM65 E3 ligase activity, HEK293 cells, a human embryonic kidney cell line (VCAM-1 is normally not expressed in this cell line), were co-transfected with VCAM-1 and Flag-TRIM65 or Flag-TRIM65 RING mutant. The cell lysates were examined by western blotting with VCAM-1 and Flag antibodies. As shown in Figure 3D, overexpression of TRIM65 significantly decreased the protein levels of VCAM-1, whereas TRIM65 RING mutant, which lost its E3 ligase activity, has barely effect. These results suggest that TRIM65 E3 ligase activity is necessary for VCAM-1 degradation.

To address whether endogenous TRIM65 regulates VCAM-1 degradation, HUVECs were transfected with control siRNA or TRIM65 siRNAs. After 24 h, the cells were stimulated with TNFα for 8 h. The cell lysates were examined by western blotting with TRIM65 and VCAM-1 antibodies. As expected, siRNA-induced knockdown of TRIM65 resulted in a marked increase in VCAM-1 protein levels (Figure 3E). TRIM65 did not affect VCAM-1 mRNA expression (Supplementary Figure S7B). Furthermore, the half-life of VCAM-1 protein in HUVEC cells was detected. siRNA-mediated knockdown of TRIM65 significantly increased VCAM-1 protein half-life from ~4.5 h to >16 h

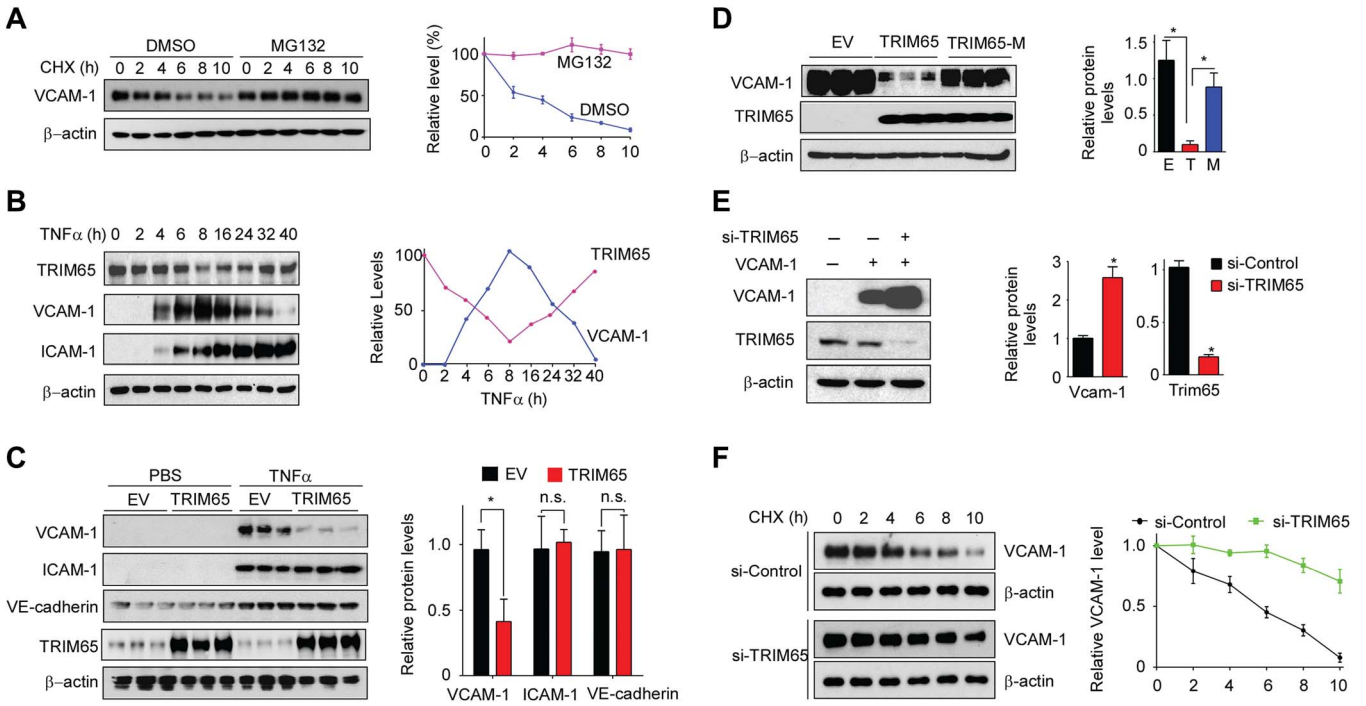

Figure 3 TRIM65 expression promotes VCAM-1 degradation. (**A**) HUVECs were stimulated with TNFα for 8 h and then treated with CHX plus DMSO or MG132 to repress protein translation and degradation through proteasome. (**B**) HUVECs were stimulated with TNFα for different times as indicated. (**C**) HUVECs were transiently transfected with Flag-TRIM65 or EV for 24 h and then treated with 10 ng/ml of TNFα for 8 h. (**D**) HEK293 cells were co-transfected with VCAM-1 plasmid and Flag-TRIM65 (T), Flag-TRIM65 mutant (M), and EV (E) for 24 h. (**E**) HUVECs were co-transfected with VCAM-1 plasmid and siRNA for TRIM65 for 24 h. (**F**) HUVECs were stimulated with TNFα for 8 h and then treated with CHX for different times as indicated. Cell lysates were extracted after above-mentioned treatments and analyzed by western blotting for detection of the indicated protein levels. β-actin served as a loading control. In **A**, **B**, and **F**, western blot bands were quantified using Gel-Pro Analyzer software and presented as fold changes on the right of the images. In **C**–**E**, relative fold changes of proteins were determined by densitometry and normalized to β-actin. Data are presented as mean ± SD ($n = 3$). *$P < 0.05$ by Student's t-test. n.s. indicates not significant.

(Figure 3F). Over-expression of TRIM65 but not vector and mutant can markedly decrease the half-life of VCAM-1 (Supplementary Figure S8). Taken together, these results suggest that TRIM65 may control VCAM-1 protein degradation via its direct ubiquitin E3 ligase activity.

TRIM65 mediates ubiquitination of VCAM-1

As previous study demonstrated that TRIM65 acted as ubiquitin E3 ligase for GW182 and other molecules (Li et al., 2014), we then investigated whether TRIM65 mediated VCAM-1 ubiquitination to promote its degradation. First, we found that overexpression of TRIM65 promoted VCAM-1 ubiquitination in HEK293T cells but failed to induce ICAM-1 ubiquitination, suggesting the specific role of TRIM65 in VCAM-1 ubiquitination (Figure 4A and B). In addition, the RING finger domain was critical for TRIM65-mediated VCAM-1 ubiquitination (Figure 4C). We also investigated which type of ubiquitin linkage was occurring on TRIM65 and found that the ubiquitin mutant that contains only one lysine at position 48 (K48) was sufficient for TRIM65-mediated VCAM-1 ubiquitination, but K63 mutant was not (Figure 4D). In addition, TRIM65 could not catalyze the linkage of K48R ubiquitin mutant, which contains a single lysine to arginine mutation at position 48, to VCAM-1 (Figure 4E), suggesting that TRIM65 mediates K48-linked VCAM-1 ubiquitination. To confirm VCAM-1 ubiquitination during endothelial activation, HUVECs were treated with or without TNFα for 8 h, the cell lysates were subjected to immunoprecipitation (IP) with VCAM-1 antibody. The immunoprecipitates were analyzed by western blotting with anti-Ub-K48 or anti-Ub-K63 as well as anti-VCAM-1. As shown in Figure 4F, TNFα treatment significantly induced K48-type ubiquitination of VCAM-1. To further confirm the role of TRIM65 in VCAM-1 ubiquitination, HUVECs were co-transfected with VCAM-1 and Flag-TRIM65 or Flag-TRIM65 RING mutant. The cell lysates were subjected to IP with VCAM-1 antibody. The immunoprecipitates were examined by western blotting with anti-Ub-K48 or anti-Ub-K63 or VCAM-1 antibodies. As shown in Figure 4G, overexpression of TRIM65 significantly increased the ubiquitinated protein levels of VCAM-1, whereas TRIM65 RING mutant, which lost its E3 ligase activity, has barely effect. Taken together, these results suggest that TRIM65 may function as an ubiquitin E3 ligase and specially regulate VCAM-1 degradation through ubiquitin–proteasome system.

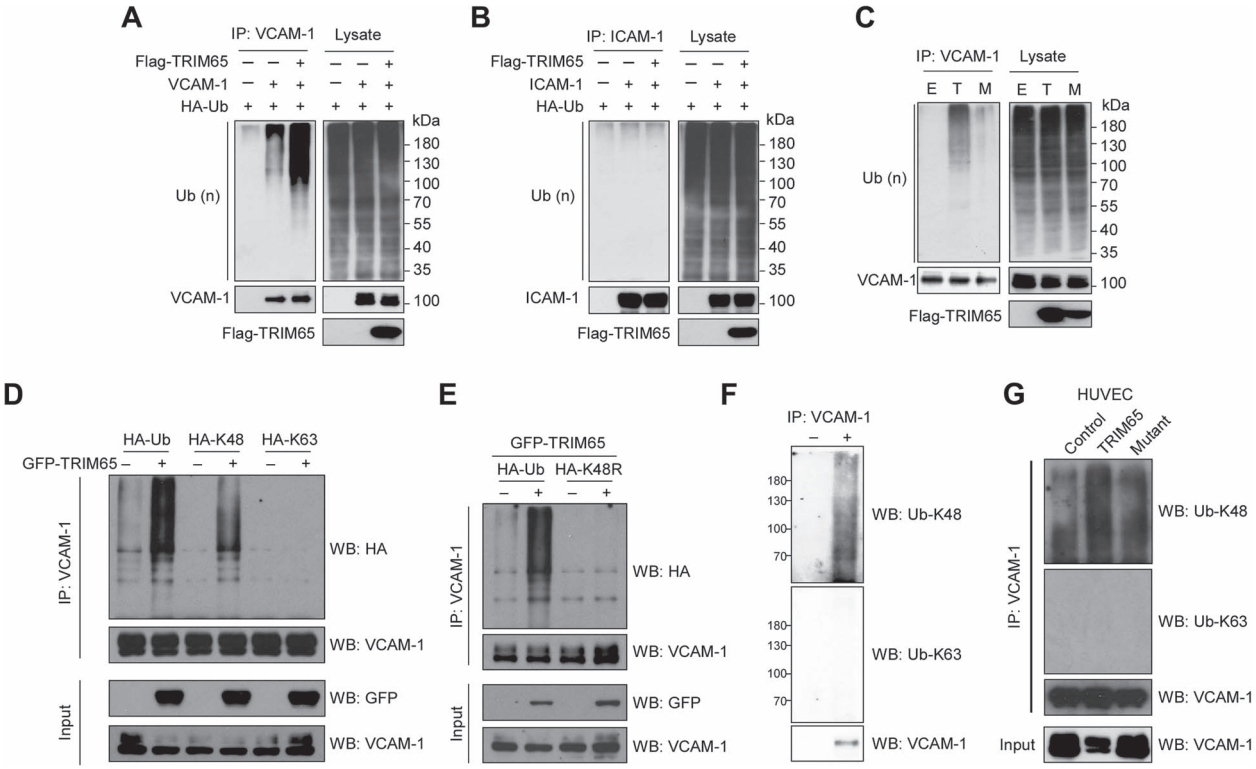

Figure 4 TRIM65 mediates ubiquitination of VCAM-1. (**A** and **B**) HEK293 cells were co-transfected with VCAM-1 (**A**) or ICAM-1 (**B**) plus HA-ubiquitin and/or Flag-TRIM65. After 16 h, the cells were treated with 10 μM of MG132 for 4 h. The cell lysates were immunoprecipitated with anti-VCAM-1 (**A**) or anti-ICAM-1(**B**). (**C**) HEK293 cells were co-transfected with VCAM-1 plus EV (E), Flag-TRIM65 (T), or Flag-TRIM65 mutant (M). After 16 h, the cells were treated with 10 μM of MG132 for 4 h. The cell lysates were immunoprecipitated with anti-VCAM-1. (**D**) HEK293 cells were co-transfected with VCAM-1 and GFP-TRIM65 plus HA-UB,HA-K48, or HA-K63. After 16 h, the cells were treated with 10 μM of MG132 for 4 h. The cell lysates were immunoprecipitated with anti-VCAM-1. (**E**) HEK293 cells were co-transfected with VCAM-1 and GFP-TRIM65 plus HA-UB or HA-K48R. After 16 h, the cells were treated with 10 μM of MG132 for 4 h. The cell lysates were immunoprecipitated with anti-VCAM-1. (**F**) HUVECs were stimulated with TNFα for 8 h. The cell lysates were immunoprecipitated with anti-VCAM-1. (**G**) HUVECs were transfected with control vector, Flag-TRIM65, or Flag-TRIM65 mutant. After 16 h, the cells were treated with TNFα for 8 h. The cell lysates were immunoprecipitated with anti-VCAM-1. All collected immunoprecipitates and cell lysates were examined by western blotting with indicated antibodies.

TRIM65 interacts with VCAM-1

To determine whether TRIM65 physically interacts with VCAM-1, we first co-transfected VCAM-1 plasmid with Flag-TRIM65 into HEK293 cells. To prevent TRIM65-mediated VCAM-1 degradation, the transfected cells were treated with MG132, an inhibitor of proteasome. We immunoprecipiated TRIM65 or VCAM-1 from the cell lysates using Flag or VCAM-1 antibodies. The immunoprecipitates and cell lysates were analysed by western blotting with VCAM-1 or Flag antibodies. As shown in Figure 5A, either TRIM65 or VCAM-1 can co-IP each other in the cell lysates, suggesting that TRIM65 is associated with VCAM-1 *in vivo*. To further confirm that TRIM65 physically interacts with VCAM-1, GST or GST-TRIM65 protein was synthesized and purified from *Escherichia coli* and incubated with the cell lysates of VCAM-1 transfected cells. After pulldown, the beads were washed and the bound proteins were eluted and processed to western blotting with VCAM-1 or TRIM65 antibodies. As shown in Figure 5B, VCAM-1 can be pulled down by GST-TRIM65 but not GST. Furthermore, we confirmed that TRIM65 can directly interact with VCAM-1 but not ICAM-1 (Figure 5C and D). To further identify a common docking site in TRIM65 protein that bind VCAM-1, we cloned sequences encoding full-length TRIM65 and several deletion mutants into pEGFP vector (Figure 5E). The plasmids were co-transfected with VCAM-1 expression plasmid into HEK293 cells. We immunoprecipitated VCAM-1 from the cell lysates using VCAM-1 antibody. The immunoprecipitates and cell lysates were analysed by western blotting with GFP or VCAM-1 antibodies. As shown in Figure 5F, only TRIM65 full-length or TRIM65 SPRY domain can be co-immunoprecipitated with VCAM-1, suggesting that VCAM-1 binds to SPRY domain of TRIM65. To further determine the region of VCAM-1 that TRIM65 binds, we cloned sequences encoding full-length of VCAM-1 and the deletion mutants into pcDNA3.1 vector. The plasmids were transfected into HEK293 cells. The cell lysates were harvested and subjected to GST-pulldown *in vitro*. As shown in Figure 5G and H only full-length of VCAM-1, but not the two

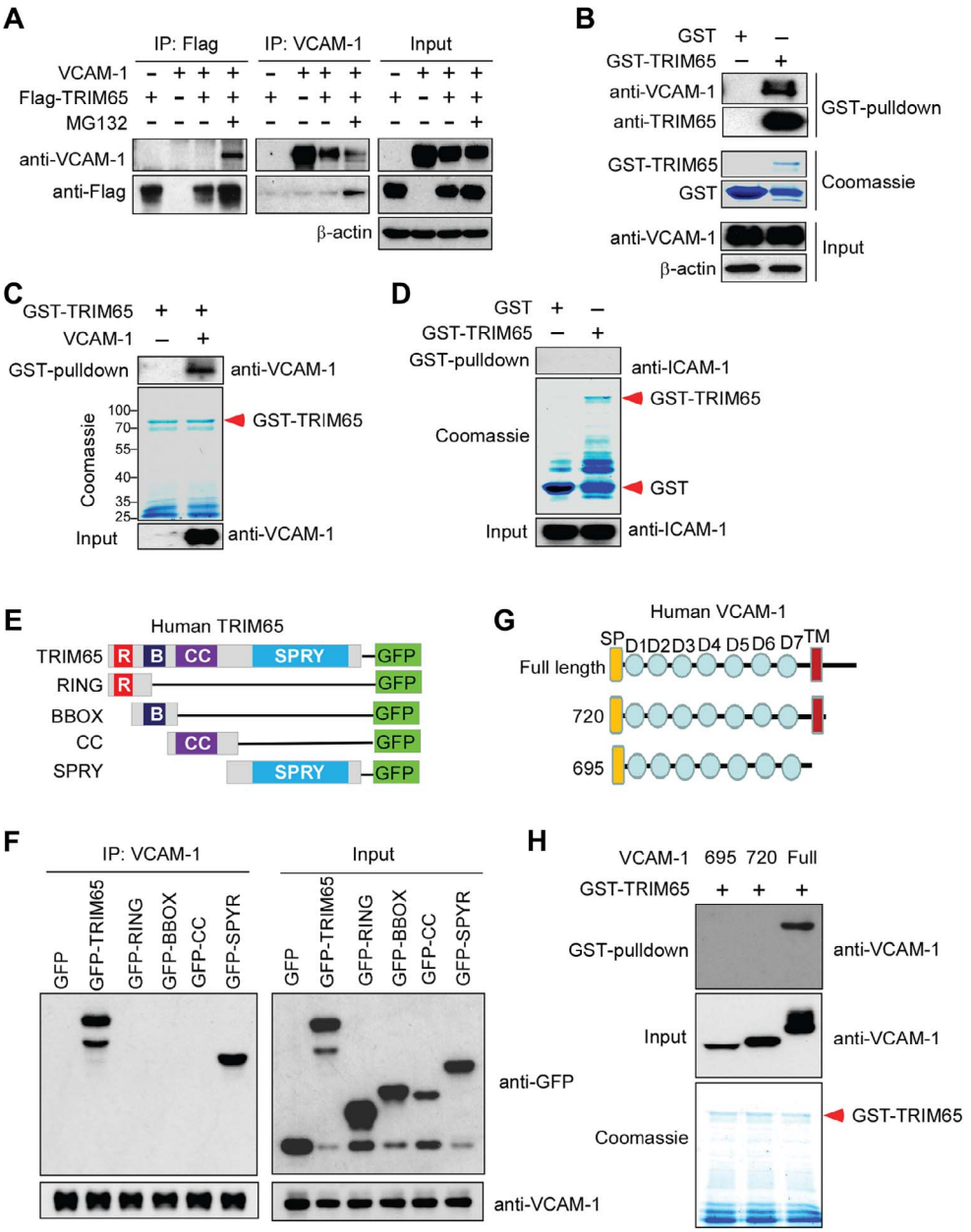

Figure 5 TRIM65 interacts with VCAM-1. (**A**) IP and western blot analysis of the interaction of Flag-TRIM65 with VCAM-1 in the lysates of HEK293 cells transfected with VCAM-1 and Flag-TRIM65 and treated with or without MG132. (**B**–**D**) Purified GST or GST-TRIM65 protein was incubated with the cell lysates of HEK293 cells transfected with or without VCAM-1 (**B** and **C**) or ICAM-1 (**D**). After GST-pulldown experiment, the elute protein and cell lysates were analyzed by western blotting with indicated antibodies. The purity of GST-TRIM65 protein was determined by Coomassie blue staining. (**E**) Schematic of generation of serial deletion mutant plasmid for TRIM65 fused with GFP. (**F**) IP and western blot analysis of the interaction of Flag-TRIM65 and its deletion mutants with VCAM-1 in the lysates of HEK293 cells transfected with VCAM-1 and Flag-TRIM65 deletion mutants and treated with or without MG132. (**G**) Schematic of generation of serial deletion mutant plasmid for VCAM-1. SP, signal peptide; D1–D7, Ig-like domains; TM, transmembrane domain. (**H**) Purified GST-TRIM65 protein was incubated with the cell lysates of HEK293 cells transfected with VCAM-1 deletion mutants. After GST-pulldown experiment, the elute protein and cell lysates were analyzed by western blotting with indicated antibodies. The coomassie blue staining image of GST-TRIM65 protein serves as a loading control.

deletion mutants can be pulled down by GST-TRIM65, suggesting that TRIM65 interacts with the cytosol region of VCAM-1 (amino acids 720–749). A previous report showed that TRIM65 protein is localized in cytosol, but not nuclear (Li et al., 2014). To determine if TRIM65 can be translocated onto cell membrane after TNF stimulation, we have conducted an experiment and the data have been presented in Supplementary Figure S9. The results showed that although VCAM-1 protein was induced by

TNF and appeared in both cytosol and membrane, TRIM65 is only localized in cytosol and decreased by TNF stimulation consistent with previous data.

TRIM65 expression inhibits human monocyte adherence to activated HUVECs

The expression of VCAM-1 on the surface of endothelial cells contributes to the monocyte adherence on endothelial cells (Chuluyan et al., 1995; Supplementary Figure S10). To examine if TRIM65 also suppresses the monocyte adherence on activated endothelial cells, HUVECs were transfected with Flag-TRIM65 or EV overnight, the cells were stimulated with TNFα for 8 h and then the PKH67 fluorescent-labelled human THP1 cells were co-cultured with the activated endothelial cells for 1 h. After washed, the adherent cells were visualized by a fluorescent microscopy and counted in a double-blind way (He et al. 2016). As shown in Figure 6A, TNFα significantly increased monocyte adherence on endothelial cells compared with that in control group. As expected, overexpression of wild-type TRIM65, but not TRIM65 mutant, significantly suppressed TNFα-induced monocyte adherence on endothelial cells. Conversely, siRNA-induced knockdown of TRIM65 significantly increased THP1 cell adherence to the transfected endothelial cells (Figure 6B). Similarly, it was observed that more THP-1 cells adherence to activated TRIM65$^{-/-}$ microvascular endothelial cells compared to TRIM65$^{+/+}$ cells (Figure 6C). Finally, forced expression of VCAM-1 rescued the inhibitory effect of TRIM65 on monocyte adherence (Figure 6D and E). The proper expression of transfected plasmids was determined by western blotting (Figure 6F). Taken together, these results suggest that TRIM65 E3 ligase selectively targets VCAM-1 degradation to limit the inflammatory cell adherence to endothelium and infiltration into tissues, by which it may control the magnitude and duration of LPS-induced lung inflammation and injury.

Discussion

In the present study, we provide evidence showing that TRIM65 is a physiological E3 ubiquitin ligase of VCAM-1. Although many studies have shown that VCAM-1 mRNA expression is regulated by many signals, including LPS and cytokines, in endothelial cells, the mechanisms that regulate the VCAM-1 abundance at the posttranslational level have never been reported. In the current study, we offer the first evidence to show that VCAM-1 protein is degraded through the ubiquitin–proteasome pathway. TRIM65 E3 ligase negatively regulates VCAM-1 protein levels through ubiquitination and degradation, by which it suppresses monocyte adherence to endothelium and infiltration into tissues. TRIM65$^{-/-}$ mice are sensitive to LPS-induced death due to unchecked inflammatory cell infiltration and lung injury. Our findings provide a novel mechanism for the regulation of VCAM-1 abundance and function in endothelial cells and the TRIM65-VCAM-1 axis may be a possible mechanism controlling the magnitude and duration of LPS-induced lung inflammation and injury.

The vascular endothelium represents a critical interface between blood and all tissues. Endothelial dysfunction contributes to the development of both acute inflammatory disease states, such as sepsis, and chronic inflammatory disease states, such as atherosclerosis, diabetes, rheumatoid arthritis, and inflammatory bowel disease (Guerci et al., 2001;Galkina and Ley, 2007; Ley et al., 2007; Roifman et al., 2009; Khan et al., 2010). In response to inflammatory stimuli, the vascular endothelium expresses a number of adhesion molecules including VCAM-1, ICAM-1, and E-selectin that play key roles in the recruitment of leukocytes to the sites of inflammation (Cook-Mills, 2002). Although the transcription of VCAM-1, ICAM-1, and E-selectin is regulated by NF-κB signaling pathway, only VCAM-1 protein is subjected to be regulated by ubiquitin–proteasome pathway. We here identified that TRIM65 E3 ligase selectively targets VCAM-1 degradation, but not affect ICAM-1 abundance. As VCAM-1 is a receptor for the ligand VLA4 that is specially expressed on the surface of monocytes and lymphocytes, thus expression of VCAM-1 contributes to the recruitment of monocytes and lymphocytes into tissues (Vestweber, 2015). ICAM-1 mediates the recruitment of neutrophils to the tissues (Yusuf-Makagiansar et al., 2002). In animal studies, we observed that monocytes/macrophages were higher in the BAL from TRIM65$^{-/-}$ mice, whereas neutrophils were not changed in the BAL of TRIM65$^{-/-}$ mice compared to that of TRIM65$^{+/+}$ mice. These results further support that TRIM65 selectively target VCAM-1 degradation but not ICAM-1. Monocytes/macrophages and lymphocytes are the major sources for cytokine production. The key role of neutrophils is killing bacteria during acute infection with limited cytokine release. As the overproduction of inflammatory cytokines is the key factor for the development of sepsis, we postulate that TRIM65 is a key factor to control the inflammatory cytokine production but not affect neutrophil-mediated pathogen killing, suggesting that TRIM65 may be a better target for the development of therapeutic treatment for sepsis and other inflammatory diseases.

In the normal condition, TRIM65 protein is enriched in endothelial cells. When endothelial cells are activated by LPS or cytokines, TRIM65 protein levels are declined at the early time points. Conversely, VCAM-1 expression is rapidly increased at this time period. This expression changes actually let the host fully response to the infection at the early time point to generate an inflammatory environment to fight with the pathogens. After the early time points, TRIM65 expression was gradually come back to normal levels, whereas VCAM-1 protein levels were gradually declined to basal levels, which is believed to be important for resolving the inflammation. The mechanisms that precisely regulate TRIM65 protein levels in activated endothelial cells remain unknown. As TRIM65 is an ubiquitin E3 ligase, we postulate that TRIM65 protein may be subjected to phosphorylation by inflammatory signaling that may further trigger its auto-ubiquitination and degradation. Further studies are necessary to understand the regulatory mechanisms that control TRIM65 protein turn over.

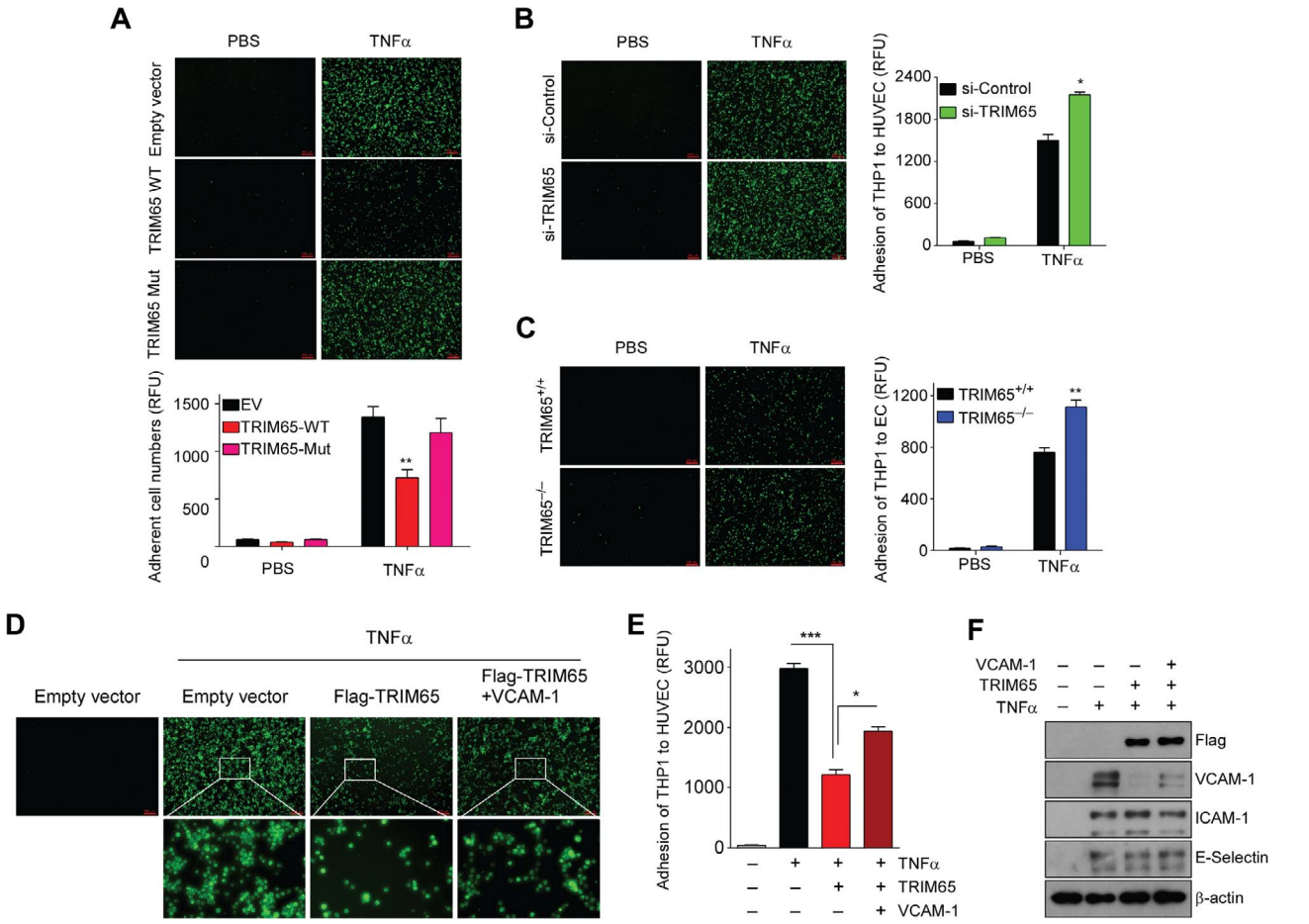

Figure 6 TRIM65 expression suppresses human endothelial cells recruiting monocytes. (**A**) HUVECs were transfected with Flag-TRIM65, Flag-TRIM65 mutant, or EV. (**B**) HUVECs were transfected with TRIM65 or control siRNAs. (**C**) Pulmonary microvascular endothelial cells were isolated from TRIM65$^{+/+}$ or TRIM65$^{-/-}$ mice. (**D**–**F**) HUVECs were co-transfected with Flag-TRIM65 with or without VCAM-1. (**A**–**E**) These cells were incubated with TNFα or PBS for 8 h and then co-cultured with fluorescence-labeled THP1 cells for 1 h. After carefully washing, adhesive cells were visualized and attached cells were counted from five random pictures in three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (**F**) The cell lysates harvested in **D** were analyzed by western blotting with indicated antibodies.

Subcortical vascular dementia results from cerebral small vessel disease leading to ischemic and hemorrhagic brain tissue damage that can be observed on magnetic resonance imaging. Such brain lesions are WMH (Schmidt et al., 2012). In a genome wide gene association study to search gene mutations for WMH burden in 9361 stroke-free individuals of European descent, an SNP (A/C) on the second intron of human TRIM65 gene locus was most significant in association with WMH (Fornage et al., 2011). However, the functional relationship of TRIM65 with WMH is still unknown. Our TRIM65$^{-/-}$ mice would be a useful tool to examine the physiological involvement of TRIM65 in WMH. In addition, whether the SNP mutation (A/C) of TRIM65 gene actually affects its mRNA transcription is under investigation. It is well known that in inflammatory conditions and in cardiac allografts undergoing rejection, VCAM-1 is upregulated in endothelium of postcapillary venules (Cook-Mills et al., 2011). Arterial expression of VCAM-1 is also found in experimental models of atherosclerosis in the human and mouse (Getz and Reardon, 2012; Mu et al., 2015). Overexpression of VCAM-1 definitely contributes to many inflammatory diseases such as atherosclerosis (Ley and Huo, 2001). The role of TRIM65–VCAM-1 axis in the pathogenesis of human inflammatory diseases would be an interesting subject for further research.

In summary, the present study is at the first to identify the central role of TRIM65 E3 ligase in the protection from LPS-induced inflammation and lung injury via controlling VCAM-1 protein ubiquitination and degradation in endothelial cells. TRIM65-mediated VCAM-1 degradation represents a potential mechanism that controls the duration and magnitude of inflammation. Targeting TRIM65/VCAM-1 axis may represent a useful strategy to treat human inflammatory diseases such as sepsis and atherosclerosis-associated diseases.

Materials and methods

Generation of TRIM65$^{-/-}$ mice

To define the physiological role of TRIM65 *in vivo*, we have obtained the mice with heterozygous TRIM65-targeted allele from the UC DAVIS KOMP Repository (Stock# ET12501) and generated the mice with homozygous TRIM65-targeted alleles by interbreeding. The mice were created in C57BL/6 background. As shown in Figure 1A, two loxp sites flanked the two sides of exons 2–5 of TRIM65 gene. A LacZ-Neo cassette was inserted in the region of the first intron. Two flippase recognition target sites flanked the two sides of the LacZ-Neo cassette. Genotyping was done with two sets of primers: one for wild-type allele (forward: 5′-tgtctcaaagcaacaatgagaaggg-3′ and reverse: 5′-tccaaacctcatccctaactcaggc-3′) and the other for targeted allele (forward: 5′-gggatctcatgctggagttcttcg-3′ and reverse: 5′-tccaaacctcatccctaactcaggc-3′) (Figure 1B). The lung tissue extracts were examined by western blot analysis and the results showed that TRIM65 protein was abolished in the homozygous targeted allele, due to the insertion of the LacZ-Neo cassette (Figure 1C), which generated the global knockout mouse (designated as TRIM65$^{-/-}$). The mice were housed in a pathogen-free animal facility at 25°C and were illuminated by 12-h:12-h light–dark cycles. The mice were provided with standard rodent chow and water *ad libitum*. All of the animal breeding and other procedures were approved by the Institutional Animal Care and Use Committee of University of Missouri Kansas City and followed the Institutional and US National Institutes of Health guidelines.

LPS challenge in mice

TRIM65$^{+/+}$ and TRIM65$^{-/-}$ mice at 2 months of age were used. Mice were challenged by the i.p. injection of LPS at a dose of 45 mg/kg (O127:B8; Sigma-Aldrich) or PBS in 200 µl sterile saline. After injection, the mice were closely monitored for general condition and survival for 32 h. The other groups of mice were challenged by the i.p. injection of LPS at a dose of 25 mg/kg or PBS in 200 µl sterile saline, 12 h after the LPS challenge, the mice were euthanized, and the lungs were collected for dry/wet ratio, histological examinations and QPCR analysis. All experiments were approved by the Institutional Animal Care and Use Committee of University of Missouri Kansas City.

Reagents

Human recombinant TNFα, LPS, and IL-1β were purchased from Sigma. VCAM-1 (sc-13160), ICAM-1 (sc-1511-R), E-Selectin (sc-14011), β-actin (sc-1616), Histone H2A (sc-10807) antibodies were from Santa Cruz Biotechnology. TRIM65 antibody (ARP34737) was purchased from Aviva Systems Biology. Phospho-p65 (3033), p65 (8242), IκBα (4812), phospho-IκBα (2859), phospho-IKKα/β (2078), IKKα (11930), IKKβ (8943), phospho-JNK (4668), JNK (9252), phospho-ERK1/2 (4370), ERK1/2 (4695), phospho-p38 (4511), p38 (8690), phospho-c-Jun (2361), c-Jun (9165), Flag (8146), α-tubulin (2125), and Hsp70 (4876) antibody were purchased from Cell Signaling Technology. Ubiquitin (ab7780), ubiquitin (K48, ab140601), and ubiquitin (K63, ab179434) antibodies were purchased from Abcam. SiRNAs targeting TRIM65 were purchased from Santa Cruz Biotechnology. QPCR primers were from IDT.

Cell culture and transfection

All human primary vascular endothelial cells (HAEC, HCAEC, HDMEC, HLMEC, and HUVEC) were purchased from Lonza Walkersville Inc., cultured in EGM or EGM2 medium according to the manufacturer instruction, and used for experiment in less than five passages. The human acute monocytic leukemia cell line THP-1 was obtained from American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium (Corning) containing 10% FBS (Sigma-Aldrich). HeLa, A549, RAW264.7, and U937 were purchased from ATCC and cultured in DMEM supplemented with 10% FBS. Transient transfection of TRIM65 vector and siRNA into HUVECs was performed by electroporation using Nucleofactor device (Lonza) and Nucleofector kits for HUVEC (Lonza) following the manufacturer's instruction. After electroporation, cells were plated into 35 mm dishes and incubated for 24 h at 37°C, 5% CO_2. Then, cells were treated with 10 ng/ml TNFα for indicated times and proteins from those cells were extracted and detected by western blotting.

Pulmonary microvascular endothelial cell isolation

The mice were euthanized under deep isoflurane anesthesia and lungs were rapidly removed on ice. The endothelial cells were isolated by MACS magnetic cell sorting (Milteryi Biotec) with positive selection by the CD31 MicroBeads (130-097-418, Milteryi Biotec) according to the manufacturer's instructions. Briefly, lung tissue was gently homogenized in glass homogenizer. These were digested by collagenase and DNAase for 45 min at 37°C. The supernatant passed through a 70-µm pore size cell strainer (Falcon) to enrich for single cells, and washed twice with PBS with 10% FBS/2 mM EDTA (Sigma-Aldrich) buffer. The cell suspension was spun down and washed 3 times in PBS with 2 mM EDTA and 0.5% BSA. Enriched primary endothelial cells were suspended in 90 µl PBS with 2 mM EDTA and 0.5% BSA and added 10 µl mouse CD31 Microbeads, followed by incubation at 4°C for 15 min. After washing with 500 µl PBS buffer with 10% FBS, the microbead-incubated cells were centrifuged at 300 *g* for 10 min and resuspended with 200 µl PBS with 10% FBS for immunomagnetic separation.CD31 positive cells were collected on a MACS LS column and washed three times with PBS with 10% FBS. The cells were resuspended and cultured in endothelial cell basal medium-2 (EBM-2, Lonza).

Protein isolation and western blotting

Protein isolation and western blotting were essentially performed as described previously (Liang et al., 2008). Tissue extracts and whole-cell lysates were prepared in radioimmunoprecipitation assay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, and 5% glycerol) or hypertonic lysis buffer (100 mM Tris-HCl, pH 8.0, 0.5 M NaCl,

5 mM EDTA, 1.25% Nonidet P-40, and 5% glycerol) containing protease inhibitors (Complete Mini), respectively, cleared by centrifugation, and quantified using the Bradford assay (Bio-Rad). Denatured lysates were separated on 8%–12% Criterion Tris-HCl gels and transferred onto nitrocellulose membranes. Blots were incubated at 4°C overnight with antibodies as indicated, followed by incubation with HRP-conjugated goat anti-rabbit $(Fab')_2$ (Pierce). Immune complexes were detected using SuperSignal West Pico chemiluminescent substrate (Pierce).

RNA isolation and QPCR

Total tissue or cellular RNA was isolated using TRIzol reagents, according to the manufacturer's instructions (Invitrogene). Residual genomic DNA was removed by on-column digestion with RNase-free DNase I. First-strand cDNAs were synthesized using oligo $(dT)_{12–18}$ primers and SuperScript III Reverse Transcriptase (Invitrogen). Real-time PCR was performed using SYBR Green and the ABI Step-One Plus Detection System (Applied Biosystems). Primers were designed and compared with the current mouse genome reference sequence using BLAST to ensure that no cross-reactivity with other genes would occur. Results were normalized against the β-actin transcript as an internal control, and were then used to calculate expression levels according to the ΔΔ cycle threshold method. All data were expressed in terms of fold change relative to the unstimulated sample, which was set as 1, unless otherwise specified. The primers were validated for their amplification efficiency and specificity prior to being used in the study.

Co-IP

For endogenous IP, the transfected HEK293 cells were lysed in CelLytic M Cell lysis buffer with protease inhibitors, phosphatase inhibitors, NEM, and ubiquitin aldehyde. Lysates were incubated with specific antibodies. The immune complexes were collected by incubation (2 h, 4°C) with protein G-agarose (Sigma). Co-IP assays were performed by using Anti-Flag matrix (Sigma) followed the manufacturer's instruction. After extensive washing, immunoprecipitated proteins were resolved by 6%–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by immunoblotting with FLAG, HA, or VCAM-1 antibodies. Membranes were developed with enhanced chemiluminescence (Amersham Biosciences).

Monocyte adhesion assay

The adhesion assay was performed as previously described (Li et al., 2018). After 24 h of transfection, HUVEC cells grown on 6-well plate were treated with 10 ng/ml TNFα for 8 h and then washed twice with PBS. THP-1 cells were labeled with fluorescein isothiocyanate using a PKH67 fluorescent staining kit (Zynaxis) according to the manufacturer's instructions. Then, 5×10^5 fluorescence dye-labeled THP-1 cells were added into each well and allowed to interact with HUVECs for 1 h at 37°C. Non-adherent cells were removed by gently washing with cold PBS. The images of adherent THP-1 cells and the number were determined under Cytation 3 Cell Imaging Multi-mode Reader (Biotek Instruments).

Histological analysis

Tissues were fixed in 4% phosphate-buffered paraformaldehyde and processed using standard procedures. The 5-μm paraffin tissue sections were cut and stained with H&E as previously described (Liang et al., 2010).

Ubiquitination assay in vivo

HEK293T cells were cotransfected with expression plasmids encoding HA-tagged full-length wild-type or mutant ubiquitin together with Flag-tagged TRIM65 or its mutant (C12A/C15A), using the standard calcium phosphate technique. The protease inhibitor MG132 (10 mM; Sigma-Aldrich) was added 4 h before harvest. At 24 h posttransfection, whole-cell lysate was prepared, and 500 μg of the lysate was used for IP using the antibodies against HA and VCAM-1, followed by the detection of respective proteins by immunoblotting. For the detection of VCAM-1 ubiquitination, 10 mM NEM (Sigma, E3876) was added to inhibit protein deubiquitination.

In vitro pulldown assay

Recombinant GST-TRIM65 protein was purified in *E. coli* (BL21) as reported (Li et al., 2014). Whole-cell lysates from HEK293T cells transfected with Flag-VCAM-1 and its mutant were incubated with GST or GST-TRIM65 protein in a buffer contacting 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% Triton buffer overnight at 4°C. Protein complexes were precipitated with cobalt-beads (Thermo) and subjected to immunoblotting with indicated antibodies.

Statistics

Data were expressed as mean ± SD. For comparison between two groups, the unpaired Student's *t*-test was used. For multiple comparisons, analysis of variance followed by unpaired Student's *t*-test was used. A value of $P < 0.05$ was considered significant.

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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