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Pharmacological inhibition of MALT1 protease activity suppresses endothelial activation via enhancing MCPIP1 expression

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Abstract

Mucosa associated lymphoid tissue lymphoma translocation protein 1 (MALT1) is not only an intracellular signaling scaffold protein but also a paracaspase that plays a key role in the signal transduction and cellular activation of lymphocytes and macrophages. However, its role in endothelial cells remains unknown. Here we report that pharmacological inhibition of MALT1 protease activity strongly suppresses endothelial activation via enhancing MCPIP1 expression. Treatment with MALT1 protease inhibitors selectively inhibited TNF α -induced VCAM-1 expression in HUVECs and LPS-induced VCAM-1 expression in mice. In addition, Inhibition of MALT1 protease activity also significantly inhibited TNF α -induced adhesion of THP-1 monocytic cells to HUVECs. To explore the mechanisms, MALT1 inhibitors does not affect the activation of NF- κ B signaling pathway in HUVEC. However, they can stabilize MCPIP1 protein and significantly enhance MCPIP1 protein level in endothelial cells. These results suggest that MALT1 paracaspase also targets MCPIP1 and degrade MCPIP1 protein in endothelial cells similar as it does in immune cells. Taken together, the study suggest inhibition of MALT1 protease activity

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Disclosure of Conflicts of Interest

No conflicts of interests were disclosed by all authors.

Authors' contributions

YL, SH, XH, XL, AF and AS carried out the experiment; FG, SL, ZJ and HX provided consults; YL and MF designed the study and wrote the manuscript.

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may represent a new strategy for prevention/therapy of vascular inflammatory diseases such as atherosclerosis.

Keywords

endothelial activation; inhibitors of MALT1; VCAM-1; MCP1; vascular inflammation

1. Introduction

Vascular endothelial activation, featured by the expression of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), is an early and important event in atherosclerosis [1]. Although the use of preventive pharmacological approaches, such as lipid lowering and antihypertensive drugs, have been beneficial, there is no treatment available to reduce these early steps of atherogenesis. VCAM-1 is a member of the immunoglobulin gene superfamily that mediates leukocyte binding to endothelial cells through interaction with its integrin counterreceptor, very late activation antigen-4 (VLA-4). Because selective expression of VLA-4 on monocytes and lymphocytes, VCAM-1 plays an important role in mediating mononuclear leukocyte adhesion, and recruit them to the activated endothelium, leading to a chronic inflammatory state [2]. Thus, targeting the expression of VCAM-1 gene may provide novel therapeutic strategy for vascular inflammatory diseases.

MALT1 (mucosa associated lymphoid tissue lymphoma translocation protein 1) is a component of a signalosome CARMA1-BCL10-MALT1, which is critical for the signal transduction in immune cells [3–5]. However, the role of MALT1 in endothelial cells remains unknown. Recent studies demonstrated that MALT1 also acts as an arginine-specific protease, by which it contributes to the activation of immune cells via cleaving the negative regulators of inflammatory signaling such as CYLB and A20 [6, 7]. MCP1 is a newly identified RNase that acts as a master controller of macrophage inflammation, immune cell activation as well as endothelial activation [8–10]. Recent studies suggest that MCP1 is also a target of MALT1 protease in lymphocytes and macrophages [11, 12]. Pharmacological inhibition of MALT1 protease activity with MI-2 (a reported covalent inhibitor of MALT1) can selectively enhance the expression of MCP1 in macrophages and reverse the LPS-induced systemic inflammation and acute lung injury [12].

In this study, we have further examined the effect of pharmacological inhibition of MALT1 protease activity on endothelial activation. We found that inhibition of MALT1 protease activity significantly suppressed the expression of VCAM-1 both in vitro and in vivo. As a result, MALT1 inhibitors also significantly repressed monocyte adherence to the activated endothelial cells. The effect of MALT1 inhibitors on endothelial activation is dependent on the increased expression of MCP1 but not NF- κ B signal pathway. These results suggest that pharmacological inhibition of MALT1 protease activity may provide a new therapeutic strategy for vascular inflammatory diseases such as atherosclerosis.

2. Materials and methods

2.1 Cell culture

The primary human vascular endothelial cells including human aortic ECs (HAEC), human coronary artery ECs (HCAEC), human dermal microvascular ECs (HDMEC), human lung microvascular ECs (HLMEC) and human umbilical vein ECs (HUVEC) were purchased from Lonza Walkersville Inc. (Walkersville, MD) and cultured in EGM or EGM2 medium according to the manufacturer's instruction. These cells were used for no more 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).

2.2 Reagents

Human recombinant TNF α and LPS were purchased from Sigma (Saint Louis, MO). VCAM-1 (sc-13160), ICAM-1 (sc-1511-R) and β -actin (sc-1616) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). MCP1 rabbit polyclonal antibody was purchased from GeneTex (GTX110807). MALT1, BLC10, A20, CYLD, RelB, p-P65, P65, pIKK α / β , IKK α , IKK β , pIKB α , and IKB α antibodies were purchased from Cell Signaling Technology. Small interfering RNAs (siRNAs) targeting MALT1 and control siRNAs were purchased from Santa Cruz Biotechnology. QPCR primers were from IDT. MI-2 was purchased from R&D systems. MLT-827 was synthesized at Novartis (13). Mepazine was from Calbiochem.

2.3 Western blot

Cells or lung tissues from mice were solubilized in the extraction buffer containing 0.5% Brij® L23 solution (Sigma-Aldrich), 50 mM KCl, 2 mM CaCl₂, 20% glycerol, 50 mM Tris-HCl, and proteases inhibitors and phosphatases, pH 7.4. Cell lysates were cleaned by centrifugation at 12,000 rpm for 10 min and assayed for protein concentration using a standard BCA protein assay kit (Pierce, Rockford, IL). 20 μ g protein samples were separated using SDS-PAGE and then transferred onto a NC membrane (Corning). The membrane was blocked for 20 min at 4 °C with 5% skim milk dissolved in PBST (PBS, pH 7.4, containing 0.1% Tween 20). The membrane was incubated overnight with primary antibodies at 4 °C with gentle rocking. Anti-rabbit IgG or anti-mouse IgG conjugated with horseradish peroxidase (Thermo) was incubated for 1 hours. The membranes were detected with HRP chemiluminescence kit (Pierce, Rockford, IL) and exposed to X-ray film.

2.4 Real-time PCR analysis

Total RNA from HUVEC cells was harvested using TRIzol® Reagent (Life Technologies™) for Real-Time PCR analysis. Briefly, 2 μ g of RNA was reverse-transcribed (RT) to synthesize cDNA using High-capacity cDNA Reverse Transcription Kit (Life Technologies). 1/400th of RT reaction was used as a template for real-time quantification using SYBR Green master mix (ABI). Data was acquired using ABI StepOne Plus real-time PCR system instrument. Data was normalized to β -actin and compared to control sample. Data represents mean $\pm$ SEM from at least three independent biological experiments.

2.5 Monocyte adhesion assay

Monocyte adhesion assay was performed as previously described [13]. HUVECs were grown in 6-well plate and treated with 10 ng/mL TNF α at 37 °C for 6 h after pretreatment with MI-2 for 30 min, and then washed twice with PBS. THP-1 cells were labeled with fluorescein isothiocyanate using a PKH67 fluorescent staining kit (Zynaxis, Inc., Malvern, PA) according to the instruction of the manufacturer. Add 5×10^5 fluorescence dye-labeled THP-1 cells into each well and allowed to interact with HUVECs for 1 hour at 37°C. Non-adherent cells were removed by gently washing with cold PBS. The number of adherent THP-1 cells was determined by measuring the fluorescence intensity under Cytation 3 Cell Imaging Multi-mode Reader (Biotek Instruments).

2.6 MTT (3-[4,5-Dimethyl-Triazolyl-2]2,5-Diphenyl Tetrazolium Bromide) Assay

THP-1 monocyte cells were plated in a 96-well plate format and treated with different concentrations of MI-2 for 2 hours. The cells were incubated in serum-free RPMI 1640 containing MTT (0.5 mg/mL) for 4 hours. Then the medium was removed and the cells were incubated for 15 min with equal volume of DMSO to dissolve the formazan crystals. The absorbance of MTT formazan was determined at 570 nm in Cytation 3 Cell Imaging Multi-mode Reader (Biotek Instruments). Viability was expressed as a percentage of absorbance of treated cells to untreated cells.

2.7 LPS challenge in mice

Adult C57BL/6 mice from Jackson Laboratory were challenged by the injection (i.p.) of 25 mg/kg LPS (O127:B8; Sigma-Aldrich) in 200 μ l sterile saline. They were divided into three groups. The first group of mice was treated with MI-2 (25 mg/body weight, i.p.) for 2 hours, then the mice were treated with LPS (25 mg/kg body weight, i.p.); the second group of mice were treated with PBS for 2 hours, then the mice were treated with LPS; the third group of mice were treated with PBS only. After LPS injection, the mice were closely monitored for general condition and survival. All mice were sacrificed after 8 hours of LPS injection and the lungs were collected for immunoblotting analysis. All experiments were approved by the Institutional Animal Care and Use Committee of University of Missouri Kansas City.

2.8 Statistical analysis

Data were expressed as the mean $\pm$ SD from three independent experiments. Statistical analysis was performed using SPSS18.0 for Windows. Comparisons between two groups were performed by paired t-test. Significance was considered to be $p < 0.05$. Experiments were repeated at least three times.

3. Results

3.1 MALT1 inhibitors suppresses endothelial activation

Endothelial activation is characterized by the expression of multiple chemokines and adhesion molecules such as VCAM-1 and ICAM-1. To explore the role of MALT1 in endothelial activation, first we detected the expression of MALT1 in human vascular endothelial cells. As shown in Figure 1A, MALT1 protein is easy to detect in human

endothelial cells from different tissues including aorta, coronary artery, dermal, lung and umbrell vein. Next, we examine the effect of MI-2 treatment (a reported inhibitor of MALT1) on the expression of VCAM-1 and ICAM-1 in activated endothelial cells. As shown in Figure 1B, TNF α , LPS and IL-1 β greatly induced both expression of VCAM-1 and ICAM-1. Pretreatment with MI-2 significantly suppressed VCAM-1 expression, but not ICAM-1 expression in activated endothelial cells. Furthermore, MI-2 treatment significantly inhibited TNF α -induced VCAM-1 mRNA expression, but not ICAM-1 mRNA expression. The mRNA levels of TNF α , IL-1 β , MCP-1 and CXCL1 were also reduced by MI-2 in activated endothelial cells (Figure 1C). To further confirm the role of MALT1 in the endothelial activation, siRNAs targeting MALT1 were transfected into HUVECs to knockdown the expression of MALT1. The transfected cells were then activated by TNF α . As shown in Figure 1D, MALT1 expression was efficiently decreased by more than 90% with si-MALT1. The expression of VCAM-1 was also significantly decreased by si-MALT1 treatment. MI-2 is an active site irreversible inhibitor of MALT1 protease activity but it lack specificity (14). Mepazine is the first allosteric MALT1 inhibitor reported (15). MLT-827 is a recent reported potent and selective MALT1 inhibitor (13). To test if MLT-827 and Mepazine also have similar effect on endothelial activation, we performed similar experiments using MLT-827 and mepazine. As shown in Figure 1E and 1F, Pretreatment with MLT-827 or mepazine significantly suppressed TNF α -induced VCAM-1 expression, but not ICAM-1 expression in human endothelial cells. Taken together, these results suggest that MALT1 protease activity is essential for the expression of VCAM-1 in activated endothelial cells. Pharmacological inhibition of MALT1 protease activity represents a new strategy to suppress endothelial activation.

3.2 Administration of MI-2 inhibits the expression of VCAM-1 in vivo

To examine whether MI-2 treatment could inhibit VCAM-1 expression in vivo, mice were treated with or without MI-2 (25 mg/body weight, i.p.). After 2 hours of injection, the mice were challenged with LPS (25 mg/kg body weight, i.p.) for 8 hours. Lungs were harvested for Western blotting analysis. As shown in Figure 2, LPS dramatically induced VCAM-1 expression in the lungs. Pretreatment with MI-2 significantly inhibited LPS-induced VCAM-1 expression, but not affected ICAM-1 expression. These results further confirmed the role of MALT1 in the regulation of endothelial activation in vivo.

3.3 MI-2 treatment attenuates monocyte adherence to activated endothelial cells

Next, we further tested the functional significance of pharmacological inhibition of MALT1 protease activity on endothelial activation by performing monocyte adherence assay. HUVECs were pretreated with or without MI-2 and then activated with TNF α . Fluorescent-labeled THP-1 cells were incubated with activated endothelial cells and the washed twice with cold PBS. The adherent THP-1 cells were imaged and counted by Cytation 3 Cell Imaging Multi-mode Reader. As shown in Figure 3A, TNF α dramatically increased the number of THP-1 cells attached to activated endothelial cells. MI-2 treatment significantly inhibited the adhesion of THP-1 cells to TNF α -stimulated HUVEC cells. Cell viability was measured to demonstrate that the inhibition of THP-1 attachment by MI-2 was not due to its cytotoxicity, as the doses of MI-2 used in the study did not show any cytotoxic effect on THP-1 cells (Figure 3B). To further confirm that VCAM-1 mediates the MI-2-induced

monocyte adherence, we transfected VCAM-1 or empty vector into HUVECs and the transfected cells were treated as described above. As shown in Figure 3C, MI-2 treatment significantly inhibited TNF α -induced monocyte adherence, whereas overexpression of VCAM-1 can rescued this inhibition. The expression of MCP1, VCAM-1 and ICAM-1 was verified by Western blot (Figure 3D).

3.4 MI-2 treatment does not affect NF- κ B signaling in TNF α -induced endothelial cells

Since MALT1 acts as a scaffold protein that plays a key role in NF- κ B signaling in response to antigen receptor stimulation in lymphocytes [3] and the expression of VCAM-1 is controlled by NF- κ B signaling [17], we tested if MI-2 treatment affect TNF α -induced NF- κ B signaling in endothelial cells. As expected, the phosphorylation of IKK, I κ B α and NF- κ B subunit P65 were increased in TNF- α -induced HUVECs. However, these protein phosphorylation were not affected by MI-2 treatment (Figure 4). These results indicate that MI-2 treatment suppresses endothelial cell activation not through a NF- κ B-dependent mechanism.

3.5 MALT1 inhibitors enhances MCP1 expression in human endothelial cells

It is well-known that MALT1 can cleave CYLD, A20, BCL10 and RelB through its protease activity, by which it fine-tune lymphocyte activation signaling [6, 7] We recently reported that pharmacological inhibition of MALT1 protease activity enhanced MCP1 protein levels in macrophages [12]. To investigate if MALT1 protease also targets MCP1 in endothelial cells, HUVECs were incubated with MI-2 and MG132. As shown in Figure 5A, MI-2 treatment can prevent MCP1 protein cleavage and enhance its protein levels in a dose-dependent manner. Moreover, MI-2 treatment significantly induced MCP1 expression in time-dependent manners in HUVECs (Figure 5B). MI-2 treatment did not affect MCP1 mRNA levels (Figure 5C). Interestingly, MI-2 treatment did not affect the protein levels of CYLD, A20, BCL10 and RelB in HUVECs (Figure 5D). Similarly, MLT-827 and mepazine also selective increased MCP1 protein levels in human endothelial cells (Figure 5E, 5F and 5G). Taken together, these results suggest that MALT1 protease selectively targets MCP1 to degradation in human endothelial cells.

3.6 MCP1 mediates MI-2-induced inhibition of VCAM-1 expression in human endothelial cells

We previously reported that MCP1 can protect ECs against TNF α -induced endothelial activation by attenuating the expression of VCAM-1 other than ICAM-1 [10]. Overexpression of MCP1 significantly attenuated TNF α -induced VCAM-1, but not ICAM-1 (Figure 6A), suggesting that MCP1 may mediated MI-2-induced inhibition of VCAM-1 expression in human endothelial cells. To test this idea, HUVECs were transfected with si-MCP1 or si-Control. The transfected cells were then treated with MI-2 and/or TNF α . As shown in Figure 6B, knocking down of MCP1 at least partially reversed MI-2-mediated inhibition on VCAM-1 expression. To further verify that MCP1 mediated inhibition of VCAM-1 expression is dependent on its RNase activity, we transfected HUVECs with HA-MCP1 and HA-MCP1-mutant (which lost its RNase activity). The cells were treated as described above. As shown in Figure 6C, overexpression of wild-type MCP1, but not its mutant decreased VCAM-1 expression.

4. Discussion

Endothelial activation is a critical process involved in many vascular diseases including atherosclerosis. Upon inflammatory stimulation, such as TNF α , IL-1 β and LPS, the endothelial cells express adhesion molecules such as VCAM-1 and ICAM-1, which mediate monocytes and neutrophils recruitment into vascular wall or tissues [18]. Targeting the expression of VCAM-1 and/or ICAM-1 is a potential therapeutic strategy for vascular inflammatory diseases [19]. In this paper, we have reported that pharmacological inhibition of MALT1 protease activity with MI-2 potently suppresses VCAM-1 expression both in vitro and in vivo, which is dependent on MALT1 protease-mediated MCP1 protein degradation in vascular endothelial cells. Findings were corroborated with alternative MALT1 inhibitors, in particular MLT-827, which is a recently described potent and selective inhibitor (13). In addition, as long term-treatment with MI-2 is not toxic in mice (20), our study suggest that MI-2 may provide an opportunity for drug development targeting to vascular inflammatory diseases.

It is reported that GPCR ligands such as angiotensin II can trigger NF- κ B activation via CARMA3-Bcl10-MALT1 signalosome in endothelial cells, which further results in the expression of VCAM-1 and ICAM-1 and recruitment of immune cells to vascular wall or tissues [21–23]. Since the transcription of VCAM-1 gene is controlled by NF- κ B activation, we have examined the effect of MI-2 treatment on NF- κ B signaling activation. Our results suggest that MI-2 treatment does not affect NF- κ B signal activation in endothelial cells.

As several groups have demonstrated that MALT1 targeted MCP1 for degradation in lymphocytes and macrophages [11, 12], we hypothesize that pharmacological inhibition of MALT1 may increase MCP1 protein expression via stabilizing its protein. We observed that MI-2 treatment, or treatment with MLT-827 or mepazine, selectively enhanced MCP1 expression in endothelial cells. Furthermore, siRNA-mediated knocking down of MCP1 at least partially reversed the effect of MI-2 on VCAM-1 expression. The other mechanisms by which that MALT1 inhibitors suppresses endothelial activation may also exist and need to be further explored.

Recently, it is reported that MALT1 could cleave CYLD into two fragments. The fragments can alter endothelial barrier integrity which is unrelated to NF- κ B signaling [24]. In this study, we observed that MI-2 treatment did not affect CYLD protein level in human endothelial cells. The discrimination may be caused by different cells and cultured conditions.

In conclusion, we here demonstrate that pharmacological inhibition of MALT1 protease activity selectively enhances MCP1 expression and by which it suppresses VCAM-1 expression and endothelial inflammation. These results suggest MALT1 as a promising therapeutic target for vascular inflammatory diseases such as atherosclerosis.

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References

1. Rajendran P, Rengarajan T, Thangavel J, Nishigaki Y, Sakthisekaran D, Sethi G & Nishigaki I The vascular endothelium and human diseases. *International journal of biological sciences* 9, 1057–1069 (2013).24250251
2. Sprague AH & Khalil RA Inflammatory cytokines in vascular dysfunction and vascular disease. *Biochemical pharmacology* 78, 539–552 (2009).19413999
3. Thome M CARMA1, BCL-10 and MALT1 in lymphocyte development and activation. *Nature reviews. Immunology* 4, 348–359 (2004).
4. Shen W, Du R, Li J, Luo X, Zhao S, Chang A, Zhou W, Gao R, Luo D, Wang J, Hao N, Liu Y, Chen Y, Luo Y, Sun P, Yang S, Luo N, Xiang R. TIFA suppresses hepatocellular carcinoma progression via MALT1-dependent and -independent signaling pathways. *Signal Transduct Target Ther.* 2016 7 22;1:16013.29263897
5. Rebeaud F, Hailfinger S, Posevitz-Fejfar A, Tapernoux M, Moser R, Rueda D, Gaide O, Guzzardi M, Iancu EM, Rufer N, Fasel N & Thome M The proteolytic activity of the paracaspase MALT1 is key in T cell activation. *Nature immunology* 9, 272–281 (2008).18264101
6. Staal J, Driege Y, Bekaert T, Demeyer A, Muyliaert D, Van Damme P, Gevaert K & Beyaert R T-cell receptor-induced JNK activation requires proteolytic inactivation of CYLD by MALT1. *The EMBO journal* 30, 1742–1752 (2011).21448133
7. Coornaert B, Baens M, Heyninck K, Bekaert T, Haegman M, Staal J, Sun L, Chen ZJ, Marynen P & Beyaert R T cell antigen receptor stimulation induces MALT1 paracaspase-mediated cleavage of the NF-kappaB inhibitor A20. *Nature immunology* 9, 263–271 (2008).18223652
8. Fu M and Blackshear PJ. RNA-binding proteins in immune regulation: a focus on CCCH zinc finger proteins. *Nature Review Immunology.* 2017, 17:130–143.
9. Miao R, Huang S, Zhou Z, Quinn T, Van Treek B, Nayyar T, Dim D, Jiang ZS, Papasian CJ, Wang Y, Liu G, Chen YE and Fu M#. Targeted disruption of MCPIP1/Zc3h12a results in fatal inflammatory disease. *Immunol Cell Biol*, 2013, 91(5):368–76.23567898
10. Qi Y, Liang J, She ZG, Cai Y, Wang J, Lei T, Stallcup WB & Fu M MCP-induced protein 1 suppresses TNFalpha-induced VCAM-1 expression in human endothelial cells. *FEBS letters* 584, 3065–3072 (2010).20561987
11. Uehata T, Iwasaki H, Vandenbon A, Matsushita K, Hernandez-Cuellar E, Kuniyoshi K, Satoh T, Mino T, Suzuki Y, Standley DM, Tsujimura T, Rakugi H, Isaka Y, Takeuchi O & Akira S Malt1-induced cleavage of regnase-1 in CD4(+) helper T cells regulates immune activation. *Cell* 153, 1036–1049 (2013).23706741
12. Li Y, Huang X, Huang S, He H, Lei T, Saaoud F, Yu XQ, Melnick A, Kumar A, Papasian CJ, Fan D & Fu M Central role of myeloid MCPIP1 in protecting against LPS-induced inflammation and lung injury. *Signal transduction and targeted therapy* 2, 17066 (2017).29263935
13. Bardet M, Unterreiner A, Malinverni C, et al. The T-cell fingerprint of MALT1 paracaspase revealed by selective inhibition. *Immunol Cell Biol.* 96, 81–99 (2018).29359407
14. Unterreiner A, Stoehr N, Huppertz C, Calzascia T, Farady CJ, Bornancin F. Selective MALT1 paracaspase inhibition does not block TNF- α production downstream of TLR4 in myeloid cells. *Immunol Lett.* 2017 12;192:48–51.29079202
15. He H, Guo F, Li Y, Saaoud F, Kimmis BD, Sandhu J, Fan M, Maulik D, Lessner S, Papasian CJ, Fan D, Jiang Z & Fu M Adiporedoxin suppresses endothelial activation via inhibiting MAPK and NF-kappaB signaling. *Scientific reports* 6, 38975 (2016).27941911
16. Schlauderer F, Lammens K, Nagel D, et al. Structural analysis of phenothiazine derivatives as allosteric inhibitors of the MALT1 paracaspase. *Angew Chemie-Int Ed.* 52, 10384–10387 (2013).
17. Min JK, Kim YM, Kim SW, Kwon MC, Kong YY, Hwang IK, Won MH, Rho J & Kwon YG TNF-related activation-induced cytokine enhances leukocyte adhesiveness: induction of ICAM-1 and VCAM-1 via TNF receptor-associated factor and protein kinase C-dependent NF-kappaB activation in endothelial cells. *J Immunol* 175, 531–540 (2005).15972689
18. Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, Davis V, Gutierrez-Ramos JC, Connelly PW & Milstone DS A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *The Journal of clinical investigation* 107, 1255–1262 (2001).11375415

19. Galkina E & Ley K Vascular adhesion molecules in atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology* 27, 2292–2301 (2007).
20. Fontan L , Yang C , Kabaleeswaran V , Volpon L , Osborne MJ , Beltran E , Garcia M , Cerchietti L , Shakhovich R , Yang SN , Fang F , Gascoyne RD , Martinez-Climent JA , Glickman JF , Borden K , Wu H & Melnick A MALT1 small molecule inhibitors specifically suppress ABC-DLBCL in vitro and in vivo. *Cancer cell* 22, 812–824 (2012).23238016
21. Delekta PC , Apel IJ , Gu S , Siu K , Hattori Y , McAllister-Lucas LM & Lucas PC Thrombin-dependent NF- κ B activation and monocyte/endothelial adhesion are mediated by the CARMA3-Bcl10-MALT1 signalosome. *The Journal of biological chemistry* 285, 41432–41442 (2010).21041303
22. McAllister-Lucas LM , Jin X , Gu S , Siu K , McDonnell S , Ruland J , Delekta PC , Van Beek M & Lucas PC The CARMA3-Bcl10-MALT1 signalosome promotes angiotensin II dependent vascular inflammation and atherogenesis. *The Journal of biological chemistry* 285, 25880–25884 (2010).20605784
23. Martin D , Galisteo R & Gutkind JS CXCL8/IL8 stimulates vascular endothelial growth factor (VEGF) expression and the autocrine activation of VEGFR2 in endothelial cells by activating NF κ B through the CBM (Carma3/Bcl10/Malt1) complex. *The Journal of biological chemistry* 284, 6038–6042 (2009).19112107
24. Klei LR , Hu D , Panek R , Alfano DN , Bridwell RE , Bailey KM , Oravec-Wilson KI , Concel VJ , Hess EM , Van Beek M , Delekta PC , Gu S , Watkins SC , Ting AT , Gough PJ , Foley KP , Bertin J , McAllister-Lucas LM & Lucas PC MALT1 Protease Activation Triggers Acute Disruption of Endothelial Barrier Integrity via CYLD Cleavage. *Cell reports* 17, 221–232 (2016). 27681433

Highlights

- We studied the role of MALT1 protease in endothelial activation. > Pharmacological inhibition of MALT1 protease activity significantly suppresses VCAM-1 expression and endothelial activation. > Pharmacological inhibition of MALT1 protease activity with MI-2 may be a novel therapeutic strategy for vascular inflammatory diseases such as atherosclerosis.

Figure 1. MALT1 inhibitors suppresses endothelial activation.

(A) MALT1 protein levels in HUVEC, HAEC, HCAEC, HDMEC and HLMEC were measured by Western blot. (B) HUVECs were pretreated with DMSO or MI-2 (1 μ M) for 30 minutes and then treated with or without inflammatory stimuli (10 ng/mL TNF α , 1 μ g/mL LPS or 10 ng/mL IL1 β) for 8 hours. The protein levels of VCAM-1, ICAM-1 were detected by Western blot. The bands were quantified by Gel-Pro Analyzer software and presented as fold changes; n=3, *p<0.05 or **p<0.001 vs DMSO treatment. (C) HUVECs were pretreated with MI-2 (1 μ M) for 30 minutes and then incubated with or without TNF α .

(10 ng/mL) for 4 hours. The relative mRNA levels of VCAM-1, ICAM-1, IL1 β , TNF α , MCP1 and CXCL1 were detected by QPCR using $2^{-\Delta\Delta CT}$ method. Data represent mean $\pm$ SD, n=4. (D) HUVECs were transiently transfected with short interfering RNA targeting on MALT1 (si-MALT1) or nonspecific short interfering RNA (si-Control) by electroporation (Amaxa). Transfected cells were quiescent for 48 hours and then treated with or without TNF α (10 ng/mL) for 8 hours. Expression of MALT1 and VCAM-1 were detected using Western blot. Band intensity of VCAM-1 was quantified by Gel-Pro Analyzer software; n=3, **p<0.001 vs treated si-Control cells. **(E&F)** HUVECs were pretreated with DMSO or 1 μ M of MLT-827 (**E**) or mepazine (**F**) for 30 minutes and then treated with or without inflammatory stimuli (10 ng/mL TNF) for 8 hours. The protein levels of VCAM-1, ICAM-1 were detected by Western blot. The bands were quantified by Gel-Pro Analyzer software and presented as fold changes; n=3, *p<0.05 or **p<0.001 vs DMSO treatment.

Figure 2. Administration of MI-2 inhibits the expression of VCAM-1 in vivo.

Adult C57BL/6 mice were intraperitoneally injected (i. p.) with PBS or LPS (25 mg/kg body weight) for 8 hours. One group of mice was pretreated with MI-2 (25 mg/kg body weight) for 2 hours and then injected with LPS for 8 hours. Mice were euthanized 8 hours after LPS injection. The lungs were harvested for Western Blot with indicated antibodies, β-actin serves as a loading control. Fold-changes of protein levels were determined by densitometry and normalized to β-actin. Data quantifications from Western blot were expressed as the mean ± SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$ by Student's *t*-test.

Figure 3. MI-2 treatment attenuates monocyte adherence to activated endothelial cells.

(A) HUVECs were treated with or without 1μM/L MI-2 for 30 minutes, and then incubated with or without 10 ng/mL TNFα for 8 hours and co-cultured with PHK67-labeled THP-1 cells for another 1 hour. Following three washing with PBS, attached cells were visualized by Cytation 3 Cell Imaging Multi-mode Reader (Biotek Instruments). Adhesive cells were counted and analyzed. (B) Cell viability percentage of THP-1 cells treated with different concentration of MI-2 (0, 0.5μM, 1 μM, 2 μM) for 2 hours was estimated by MTT. Data is shown as mean±SEM of three separate experiments. Treatments significantly different from the untreated control at $p < 0.05$ are presented as *. (C) HUVECS were transfected with VCAM-1 or empty vector for 24 hours, then the transfected cells were treated and analyzed as described in (A). The cell lysates were also harvested for Western blot analysis (D).

Figure 4. MI-2 treatment does not affect NF-κB signaling in TNFα-induced endothelial cells. HUVECs were pretreated with 1 μM MI-2 for 30 minutes then exposed with 10 ng/mL of TNFα for 0, 5, 15 and 30 minutes as indicated. Cell lysates were extracted and Western blot were performed to detect phosphorylation of IκBα, P65 and IKKα/β with specific antibodies as indicated. Total protein levels of IκBα, P65, IKKα and IKKβ were also detected. Experiments were repeated three times and showed consistent results.

Figure 5. MALT1 inhibitors enhance MCPIP1 expression in human endothelial cells.

(A) Western blots for MCPIP1 after 30 minutes pretreatment with increasing concentration of MI-2 (0.25 μ M, 0.5 μ M, 1 μ M and 2 μ M) or DMSO, followed by proteasome inhibitor MG-132 (5 μ M) treatment for 2 hours in HUVEC. The full-length and cleavage products of MCPIP1 were marked. (B) HUVECs were treated with MI-2 for different durations as indicated. Cell lysates were collected to analysis by Western blots with the MCPIP1 antibody. β -actin served as a loading control. Fold changes of the MCPIP1 protein levels were determined by densitometry and normalized to β -actin. Data are representative of three

independent experiments. **(C)** The mRNAs of MCPIP1 in (A) cells were quantified by QPCR. Actin levels were used to normalize. Data represent mean $\pm$ SD. **(D)** HUVECs were pretreated with or without MI-2 (1 μ M) for 30 minutes and then stimulated with or without TNF α (10 ng/mL) for 8 hours. The protein levels of MALT1 and its targets (MCPIP1, A20, CYLD, BCL10 and RelB) were determined by Western blots with individual antibodies as indicated. β -actin served as a loading control. **(E)** HUVECs were treated with MLT-827 or mepazine for different durations or doses as indicated. Cell lysates were collected to analysis by Western blots with the MCPIP1 antibody. β -actin served as a loading control. Data are representative of three independent experiments. **(F&G)** HUVECs were pretreated with or without MLT-827 (F) or mepazine (G) for 30 minutes and then stimulated with or without TNF α (10 ng/mL) for 8 hours. The protein levels of MALT1 and its targets (MCPIP1, A20, CYLD, BCL10 and RelB) were determined by Western blots with individual antibodies as indicated. β -actin served as a loading control.

Figure 6. MCPIP1 mediates MI-2-induced inhibition of VCAM-1 expression in human endothelial cells.

(A) HUVECs were transiently transfected with Flag-MCPIP1 or empty vector for 48 hours. The cell lysates were harvested, and the protein levels of MCPIP1, VCAM-1 and ICAM-1 were measured by Western blots. The bands of VCAM-1 were quantified by Gel-Pro Analyzer software and presented as fold changes, $n=3$, $*p<0.05$ vs DMSO treatment. (B) HUVECs were transfected with si-Control or si-MCPIP1 for 48 hours. The transfected cells were pretreated with or without MI-2 (1 μ M) for 30 minutes and then stimulated with or without TNF α (10 ng/mL) for 8 hours. The protein levels of MCPIP1, VCAM-1 and ICAM-1 were determined by Western blots. β -actin served as a loading control. Fold changes of the MCPIP1 and VCAM-1 protein levels were determined by densitometry and normalized to β -actin. Data are representative of three independent experiments as the mean $\pm$ SD, $**p<0.01$ by Student's t -test. (C) HUVECs were transiently transfected with HA-MCPIP1 or HA-MCPIP1M (mutant) for 48 hours. The cell lysates were harvested, and the protein levels of MCPIP1, VCAM-1 and ICAM-1 were measured by Western blots.