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Full Paper

Thrombomodulin induces anti-inflammatory effects by inhibiting the rolling adhesion of leukocytes *in vivo*

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ABSTRACT

Thrombomodulin (TM) is an integral membrane protein expressed on the surface of vascular endothelial cells that suppresses blood coagulation. Recent studies have shown that TM exhibits anti-inflammatory effects by inhibiting leukocyte recruitment. However, the actual modes of action of TM *in vivo* remain unclear. Here, we describe the pharmacological effects of recombinant human soluble TM (TM alfa) on leukocyte dynamics in living mice using intravital imaging techniques. Under control conditions, neutrophils exhibited three distinct types of adhesion behavior in vessels: 1) “non-adhesion”, in which cells flowed without vessel adhesion; 2) “rolling adhesion”, in which cells transiently interacted with the endothelium; and 3) “tight binding”, in which cells bound strongly to the endothelial cells. Compared to control conditions, local lipopolysaccharide stimulation resulted in an increased frequency of rolling adhesion that was not homogeneously distributed on vessel walls but occurred at specific endothelial sites. Under inflammatory conditions, TM alfa, particularly the D1 domain which is a lectin-like region of TM, significantly decreased the frequency of rolling adhesion, but did not influence the number of tight bindings. This was the first study to demonstrate that TM alfa exerts anti-inflammatory effects by inhibiting rolling adhesion of neutrophils to vascular endothelial cells in living mice.

Introduction

Inflammation is a defense mechanism that maintains homeostasis via the removal of exogenous materials. When infection occurs, leukocytes recognize pathogens and produce inflammatory cytokines, activated endothelial cells produce selectins, and integrin ligands activate the migration of leukocytes to the inflammatory site through blood vessels. During leukocyte migration, there are three steps necessary for cell adhesion. First, rolling adhesion involves the reversible binding of leukocytes to the vascular endothelium through selectin interaction. In the second step, tight binding depends on firm interactions between leukocyte function-associated antigen-1 (LFA-1), which is a leukocyte integrin, and adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1), on the endothelium so that cells can firmly attach to the endothelium. In the final step, leukocytes crawl and transmigrate through the endothelial cells.[1] In this way, the leukocyte migration system plays an important role in defense against infection.

Thrombomodulin (TM) is an integral membrane protein that is expressed on the surface of vascular endothelial cells and forms complexes with thrombin that cause protein C activation to suppress blood coagulation.[2] TM alfa is a recombinant human soluble TM that is used in Japan as a disseminated intravascular coagulation (DIC) medication.[3,4] TM alfa is composed of 557 amino acids and is a type I glycosylated transmembrane protein with three extracellular domains. Domain 1 (D1) is an NH_2-terminal lectin-like

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region, Domain 2 (D2) has six epidermal growth factor (EGF)-like structures, and Domain 3 (D3) is an O-glycosylation site-rich domain.[5] It was recently reported that TM exerts anti-inflammatory activities by activating protein C,[6] absorbing lipopolysaccharide (LPS) and high mobility group box 1 (HMGB-1),[7–10] and/or reducing leukocyte recruitment via the interaction of D1 with endothelial Lewis Y.[11,12] However, it remains unclear whether TM inhibits inflammation *in vivo*.

Although conventional methods such as histology and flow cytometry can be used to analyze the localization and function of cells at inflammatory sites, they cannot be used to evaluate cellular movement. Thus, rolling adhesion, which is the first step in leukocyte migration, cannot be analyzed using these techniques. However, the recent development of fluorescence imaging techniques allows for a more pronounced understanding of *in vivo* cellular dynamics in multiple organs and tissues.[13–15] Thus, in the present study, we investigated the pharmacological effects of TM alfa on leukocyte dynamics in the blood vessels of living mice using intravital imaging techniques that have recently been established by our group.

Materials and methods

Animals

The present study included 8-week-old male C57BL/6 mice that expressed enhanced green fluorescent protein (EGFP) under the lysozyme M promoter (LysM-EGFP).[16] All mice were housed at a maximum of three animals per cage and were randomly selected for the experiments. All mice were fed a normal diet (MF diet; Oriental Yeast Co., Ltd., Tokyo, Japan) and maintained at 23 ± 1.5 °C and 45 ± 15 % relative humidity under a 12-h/12-h light/dark cycle in the specific pathogen-free animal facilities at Osaka University. All animal experiments were approved by the Institutional Animal Experimental Committee of Osaka University.

Intravital imaging

For the imaging procedures, LysM-EGFP mice were anesthetized with isoflurane (Wako Pure Chemical Industries, Ltd., Tokyo, Japan; 2.0% vaporized in 100% oxygen) and their epigastric veins were exposed. For each mouse, the abdominal area was shaved, the skin flap was fixed onto the stage, and blood vessels were visualized by intravenously injecting Texas Red-conjugated dextran (10 mg/mL; Molecular Probes, Inc., Eugene, OR, US). The vascular cavity was observed via spinning disk confocal microscopy (CSU-X1; Yokogawa Electric Corporation, Tokyo, Japan) at 30 ms per frame. During the experiments, a heater was used to keep mice warm.

LPS-induced inflammation model and drug administration

During the imaging experiments, LPS (1 mg/ml, 200 μl; Sigma–Aldrich, St. Louis, MO, USA) was locally applied into the visual fields of the mice to induce local inflammation. At 30 min after LPS administration, the mice received an intravenous injection of either TM alfa (3 mg/kg; Asahi Kasei Pharma Corporation, Tokyo, Japan), D1 (1.09 mg/kg; Asahi Kasei Pharma Corporation), or D2 (1.58 mg/kg; Asahi Kasei Pharma Corporation).

Imaging data analysis

All imaging data were edited and analyzed using the MetaMorph (Molecular Devices, Sunnyvale, CA, USA) and Imaris (Bitplane, Zurich, Switzerland) software programs. The automatic 3D object tracking feature of Imaris Spots was used with manual correction to retrieve the cell spatial coordinates over time. The frequency of rolling adhesion events per cell and the number of cells showing tight binding per visual field were counted. A density plot analysis was performed using the EBImage package of R/Bioconductor.[17]

Statistics

All data were analyzed using GraphPad Prism software (GraphPad, San Diego, CA, US) and are presented as the mean ± standard error of the mean (SEM) unless otherwise specified. Two-tailed t-tests were used to calculate p values. A P-value < 0.05 was considered to reflect statistical significance.

Results

Visualization of neutrophil dynamics in blood vessels using intravital imaging

The vascular cavities of epigastric veins of LysM-EGFP mice, in which EGFP was mainly expressed in neutrophils, were observed (Fig. 1A and Supplementary video 1). The neutrophils exhibited three distinct adhesion behaviors in the vascular cavity: 1) “non-adhesion”, in which cells flowed without vessel adhesion; 2) “rolling adhesion”, in which cells transiently interacted with the endothelium (Fig. 1B); and 3) “tight binding”, in which cells firmly bound to the endothelial cells (Fig. 1C).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.jphs.2020.01.001.

Next, the duration of adhesion between the neutrophils and endothelial cells was quantitatively analyzed. First, the trajectory of the cells was semi-automatically analyzed with Imaris software and then the frequency of rolling adhesions per cell as they flowed from **a** to **b** was determined (Fig. 1A). More than 95% of cells had a duration within the range of 0.06–0.30 s (Fig. 1D). Thus, in the present study, rolling adhesion was defined as a cell staying at the same point for 0.09–0.30 s and tight binding was defined as a cell staying at the same point throughout the 60-s observation period.

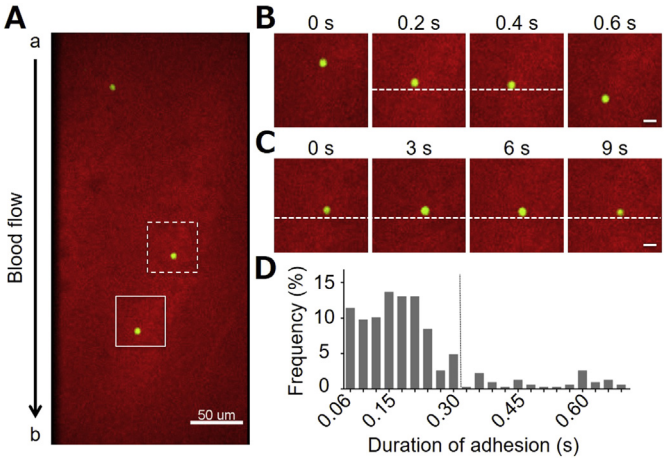

Fig. 1. Visualization of neutrophil migration in blood vessels using intravital imaging. (**A**) Representative intravital image of epigastric veins in the vascular cavity in LysM-EGFP mice under the control condition; cells flowed from **a** to **b**. Green: LysM-EGFP-positive cells; red: blood vessels visualized by an intravenous injection of Texas Red-conjugated 70 kDa dextran. Scale bar: 50 μm. (**B**) Representative images of rolling adhesion in which cells transiently interacted with the endothelium (the cell outlined in **A**). Scale bar: 10 μm. (**C**) Representative images of tight binding in which cells firmly bound to the endothelial cells (the cell delineated by dotted line in **A**). Scale bar: 10 μm. (**D**) Histogram of the duration of cell adhesion.

Quantitative analysis of neutrophil dynamics under the acute inflammation

In the control condition, most neutrophils flowed through the vascular cavity without adhering to the endothelial cells (Fig. 2A). However, some neutrophils exhibited rolling adhesion behavior and a few cells showed tight binding. Next, the changes in neutrophil dynamics were investigated when acute inflammation was induced by LPS administration. The frequency of rolling adhesion was obviously increased at 10 and 30 min after LPS stimulation (Fig. 2A–C and Supplementary video 2). Subsequently, a density plot analysis was performed to determine how many times a rolling adhesion event occurred in each area during the 60-s observation period, revealing that rolling adhesion events were not distributed homogeneously on the blood vessel walls but occurred on specific endothelial sites at a higher rate (Fig. 2B). Additionally, the number of cells showing tight binding was increased and the mean tracking velocity of the neutrophils was

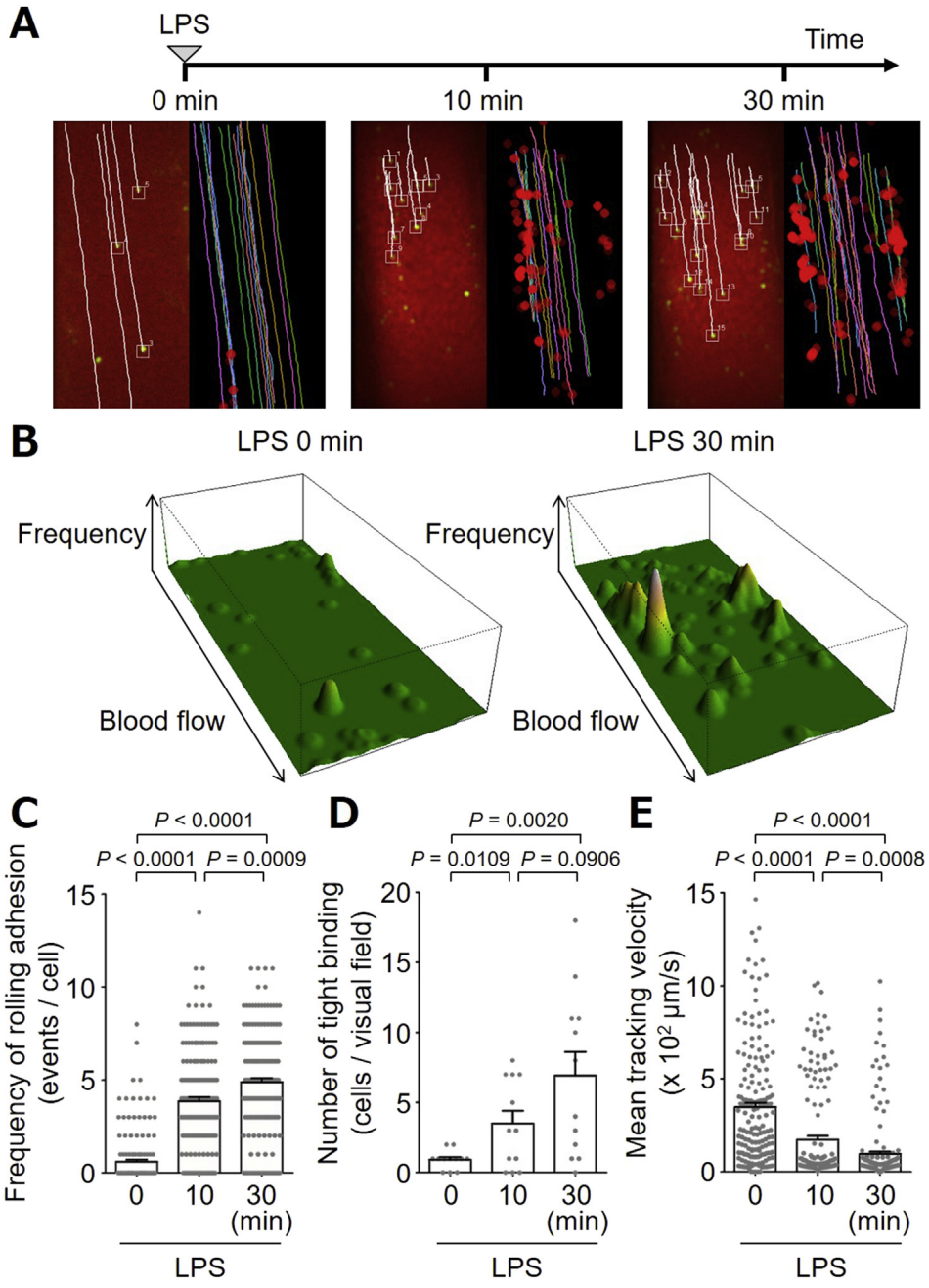

Fig. 2. Quantitative analysis of neutrophil dynamics under the acute inflammation. (**A**) Representative intravital images of epigastric veins in the vascular cavity in LysM-EGFP mice under the acute inflammatory condition. During the imaging experiments, LPS was locally applied into the visual fields of mice to induce local inflammation. Images of the same visual field are shown. Green: LysM-EGFP-positive cells; red: blood vessels visualized by an intravenous injection of Texas Red-conjugated 70 kDa dextran (*left*). Colored lines show the associated trajectories of cells. Red: rolling adhesion spot (*right*). Some of the cells were selected and plotted. (**B**) Density plot of the frequency of rolling adhesion events; both the color and height indicate how many times the events occurred in each area during the 60-s observation period. Green: low frequency of rolling adhesion events. Yellow/Orange/White: high frequency of rolling adhesion events. (**C**–**E**) The frequency of rolling adhesion events per cell (**C**), number of cells showing tight binding per visual field (**D**), and mean tracking velocity of the neutrophils (**E**). Images were obtained from twelve independent experiments per group. All data are presented as the mean ± SEM.

decreased at 10 and 30 min after LPS administration (Fig. 2D and E). These changes in neutrophil dynamics after LPS administration are important for neutrophils to migrate to the inflamed region.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.jphs.2020.01.001.

Pharmacological effects of TM alfa on neutrophil dynamics

Next, the effects of the intravenous injection of TM alfa were evaluated at 30 min after LPS administration (Fig. 3A and B and Supplementary video 3, 4). TM alfa significantly decreased the frequency of rolling adhesion at 30 min after its administration (Fig. 3C). Although the number of cells showing tight binding did not change after TM alfa treatment (Fig. 3D), the mean tracking velocity of neutrophils was significantly increased at 30 min after its administration (Fig. 3E). Taken together, these results indicate that TM alfa inhibits the rolling adhesion of neutrophils.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.jphs.2020.01.001.

Analysis of functional sites of TM alfa on neutrophil dynamics

Finally, the functional sites of TM alfa (i.e., the two extracellular domains referred to as D1 and D2) were assessed (Fig. 4A). To determine whether either domain can inhibit the rolling adhesion of neutrophils, each domain was intravenously administered 30 min after LPS administration (Fig. 4B and C and Supplementary video 5, 6). Compared to D2 domain, D1 domain significantly decreased the frequency of rolling adhesion and increased the mean tracking velocity of neutrophils at 30 min, whereas the number of cells showing tight binding did not change after treatment with either D1 or D2 domain (Fig. 4D–F). Taken together, these results suggest that D1 domain is the main functional site associated with the inhibition of the rolling adhesion of neutrophils after LPS administration *in vivo*.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.jphs.2020.01.001.

Discussion

The present study was the first to employ intravital imaging techniques to visualize the effects of TM alfa on neutrophil dynamics in living mice. We demonstrated that TM alfa suppressed the rolling adhesion of neutrophils to vascular endothelial cells, which subsequently increased neutrophil velocity. In addition, we evaluated the functional sites of TM alfa (i.e., two primary extracellular domains: D1 and D2) involved in neutrophil dynamics, and found that D1, but not D2, inhibited rolling adhesion to the same extent as TM alfa. These results indicate that D1 on TM alfa plays an important role in the anti-inflammatory effects of TM *in vivo*.

During the initial stages of inflammation, vascular endothelial cells are activated by inflammatory cytokines, such as tumor necrosis factor (TNF), and then P- and E-selectins are synthesized within a few minutes and a few hours, respectively. These selectins reversibly adhere to Sialyl-Lewis X of P-selectin glycoprotein ligand-1 (PSGL-1), which is expressed on neutrophils, and then rolling adhesion occurs. Within the next 6 h, neutrophil migration

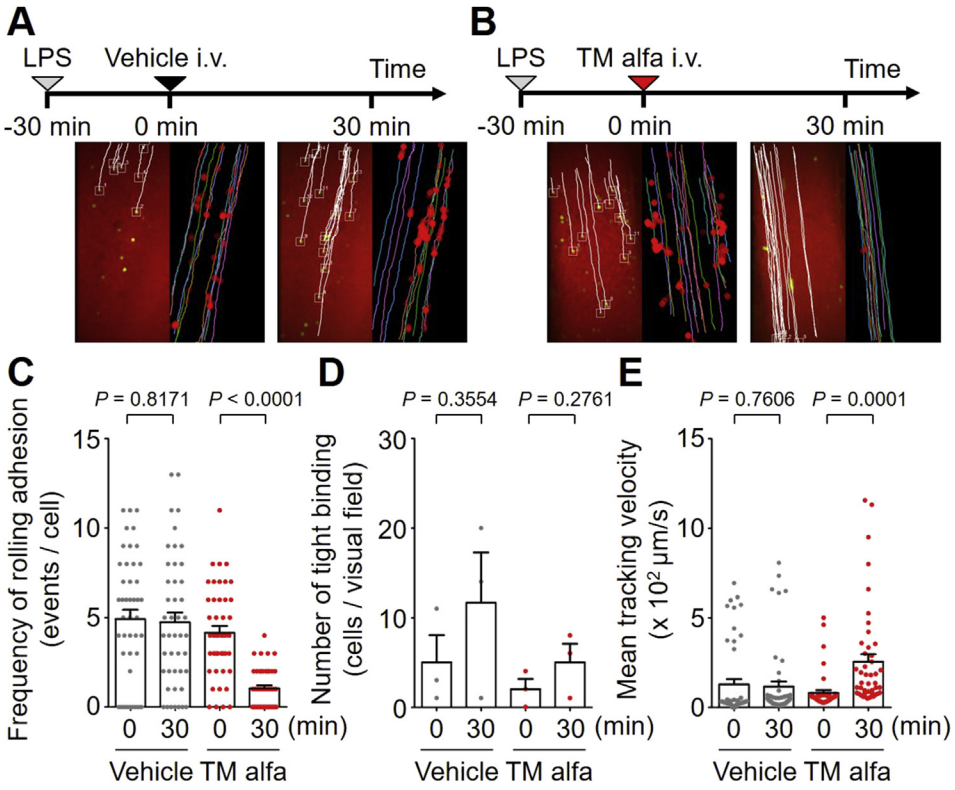

Fig. 3. Pharmacological effects of TM alfa on neutrophil dynamics following LPS-induced inflammation. (**A-B**) Representative intravital images of epigastric veins in the vascular cavity of LysM-EGFP mice treated with vehicle (**A**) or TM alfa (**B**) under the LPS-induced inflammatory condition. During the imaging experiments, LPS was locally applied into the visual fields of mice to induce local inflammation. At 30 min after LPS administration, vehicle or TM alfa was intravenously injected. Images of the same visual field are shown. Green: LysM-EGFP-positive cells; red: blood vessels visualized by intravenous injection of Texas Red-conjugated 70 kDa dextran (*left*). Colored lines show the associated trajectories of cells. Red: rolling adhesion spot (*right*). (**C**–**E**) The frequency of rolling adhesion events per cell (**C**), number of cells showing tight binding per visual field (**D**), and mean tracking velocity of the neutrophils (**E**). Images were obtained from three independent experiments per group. All data are presented as the mean ± SEM.

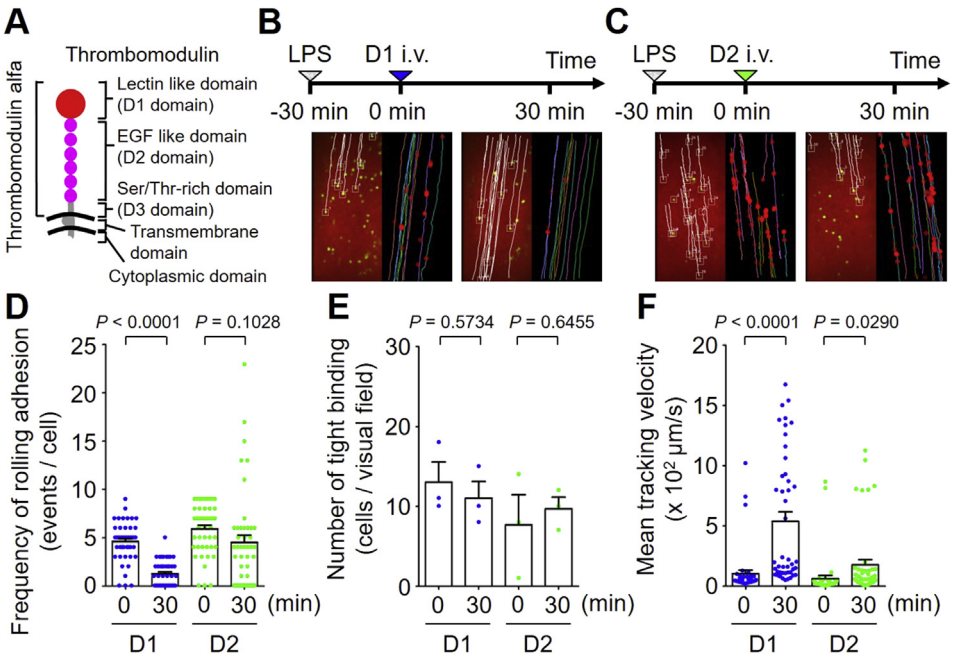

Fig. 4. Analysis of functional sites of TM alfa on neutrophil dynamics. (**A**) The structures of TM and TM alfa. TM alfa consists of three extracellular domains: Domain 1 (D1), Domain 2 (D2), and Domain 3 (D3). (**B**–**C**) Representative intravital images of epigastric veins in the vascular cavity of LysM-EGFP mice treated with D1 (**B**) or D2 (**C**) under the LPS-induced inflammatory condition. During the imaging experiments, LPS was locally applied into the visual fields of mice to induce local inflammation. At 30 min after LPS administration, D1 or D2 was intravenously injected. Images of the same visual field are shown. Green: LysM-EGFP-positive cells; red: blood vessels visualized by intravenous injection of Texas Red-conjugated 70 kDa dextran (*left*). Colored lines show the associated trajectories of cells. Red: rolling adhesion spot (*right*). (**D**–**F**) The frequency of rolling adhesion events per cell (**D**), number of cells showing tight binding per visual field (**E**), and mean tracking velocity of the neutrophils (**F**). Images were obtained from three independent experiments per group. All data are presented as the mean ± SEM.

to the inflammatory site occurs due to the activation of integrins (LFA-1) and/or chemokines (C-X-C motif chemokine ligand 8 [CXCL8]), which is associated with strong and irreversible adhesions. Endothelial cells are covered with an endothelial surface layer (ESL) composed of glycosaminoglycans $\geq$ 500 nm thick, while P- and E-selectins extend $\leq$ 38 nm above the endothelial plasma membrane. This surface layer is expected to shield adhesion molecules completely.[18] However, in the present *in vivo* experiments, the events of rolling adhesion after LPS administration occurred on specific endothelial sites at higher rates. Previous reports indicated that the ESL thickness can be reduced by inflammatory stimuli, leading to increased availability of endothelial surface adhesion molecules to circulating neutrophils. Schmidt et al reported that LPS induced degradation of endothelial glycocalyx via TNF-dependent mechanisms at 30 min after administration, leading to neutrophil recruitment and inflammation.[19] These results suggest that ESL shedding may accelerate the rolling adhesion of neutrophils in hotspots we detected, although further studies are needed to elucidate the molecular mechanism involved with the formation of hotspots.

In this study, TM alfa inhibited rolling adhesion at 30 min after LPS administration but did not affect tight binding. There are several hypotheses regarding the mechanisms underlying the inhibitory effects of TM alfa on rolling adhesion of neutrophils. Previously, Shi et al examined the ligand specificity of recombinant lectin-like domain of TM (rTMD1) and showed that Lewis Y antigen was a specific ligand for rTMD1, whereas Lewis X antigen was not.[7] In addition, Lin et al demonstrated that rTMD1 could bind to Lewis Y on the endothelium and suppress vascular inflammation by reducing leukocyte recruitment.[11] Furthermore, it was also reported that rTMD1 could bind to LPS by interacting with Lewis Y antigen, and that it could inhibit the production of LPS-induced inflammatory mediators, such as TNF.[7] Notably, TNF is known to induce robust neutrophil adhesion via induction of P- and E-selectins on the endothelium.[20] These results indicate another possible mechanism, in which TM alfa could interact with the Lewis Y antigen of LPS, suppress LPS-induced TNF production, and inhibit the induction of endothelial adhesion molecules; this could result in the inhibition of neutrophil rolling adhesion to the endothelium. Taken together, these observations indicated that TM alfa has the capability to reduce the frequency of cell adhesion between neutrophils and vascular endothelial cells by binding to either Lewis Y antigen on endothelial cells or LPS. The development of an intravital imaging system for visualizing biological phenomena at the single molecule levels would allow determination of the additional mechanism of action of TM alfa *in vivo*.

In the treatment of patients with DIC, TM alfa can confer protection against sepsis caused by bacterial infections. First, TM alfa could suppress excess neutrophil migration to inflammatory sites, thus protecting against neutrophil-mediated tissue damage. Previously, Shi et al showed that rTMD1 could cause the agglutination of bacteria and enhance their phagocytosis by macrophages, thereby resulting in bacterial clearance during sepsis. In addition, rTMD1 could bind to Lewis Y antigen on LPS and inhibit LPS-induced production of inflammatory mediators.[7] These results suggest that TM alfa has a protective function against bacterial infections and would be useful in the treatment of severe inflammation in sepsis.

In conclusion, this was the first study to demonstrate that TM alfa exerts anti-inflammatory effects *in vivo* by inhibiting the rolling adhesion of neutrophils in living mice. Because TM alfa possesses both anti-coagulatory and anti-inflammatory effects *in vivo*, it can be utilized as a pharmacological treatment for a broad range of inflammatory diseases as well as DIC.

Author Contributions

J.K. and M.I. conceived and designed the study. S.N. performed the imaging experiments and data analysis with the assistance of J.K., H. Mizuno, T.S. and T.A.. S.S. and H. Matsuda contributed to the imaging data analysis. M.K. and R.T. discussed the experiments and results. J.K., S.N. and M.I. wrote the manuscript.

Declaration of Competing Interest

M.K. and R.T. are full-time employees of Asahi Kasei Pharma Corporation. All other authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphs.2020.01.001.

References

1. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. 2007;7:678–689.
2. Dahlbäck B, Villoutreix BO. The anticoagulant protein C pathway. *FEBS Lett*. 2005;579:3310–3316.
3. Mohri M, Sugimoto E, Sata M, Asano T. The inhibitory effect of recombinant human soluble thrombomodulin on initiation and extension of coagulation–a comparison with other anticoagulants. *Thromb Haemost*. 1999;82:1687–1693.
4. Maruyama I. Recombinant thrombomodulin and activated protein C in the treatment of disseminated intravascular coagulation. *Thromb Haemost*. 1999;82:718–721.
5. Li YH, Kuo CH, Shi GY, Wu HL. The role of thrombomodulin lectin-like domain in inflammation. *J Biomed Sci*. 2012;27:19–34.
6. Abraham E, Laterre PF, Garg R, et al. Drotrecogin alfa (activated) for adults with severe sepsis and a low risk of death. *N Engl J Med*. 2005;353:1332–1341.
7. Shi CS, Shi GY, Hsiao HM, et al. Lectin-like domain of thrombomodulin binds to its specific ligand Lewis Y antigen and neutralizes lipopolysaccharide-induced inflammatory response. *Blood*. 2008;112:3661–3670.
8. Dettke M, Pálfi G, Loibner H. Activation-dependent expression of the blood group-related Lewis Y antigen on peripheral blood granulocytes. *J Leukoc Biol*. 2000;68:511–514.
9. Abeyama K, Stern DM, Ito Y, et al. The N-terminal domain of thrombomodulin sequesters high-mobility group-B1 protein, a novel anti-inflammatory mechanism. *J Clin Investig*. 2005;115:1267–1274.
10. Ito T, Kawahara K, Okamoto K, et al. Proteolytic cleavage of high mobility group box 1 protein by thrombin–thrombomodulin complexes. *Arterioscler Thromb Vasc Biol*. 2008;28:1825–1830.
11. Lin WL, Chang CF, Shi CS, Shi GY, Wu HL. Recombinant lectin-like domain of thrombomodulin suppresses vascular inflammation by reducing leukocyte recruitment via interacting with Lewis Y on endothelial cells. *Arterioscler Thromb Vasc Biol*. 2013;33:2366–2373.
12. Conway EM, Van de Wouwer M, Pollefeyt S, et al. The lectin-like domain of thrombomodulin confers protection from neutrophil-mediated tissue damage by suppressing adhesion molecule expression via nuclear factor kB and mitogen-activated protein kinase pathways. *J Exp Med*. 2002;196:565–577.
13. Ishii M, Egen JG, Klauschen F, et al. Sphingosine-1-phosphate mobilizes osteoclast precursors and regulates bone homeostasis. *Nature*. 2009;458:524–528.
14. Frommhold D, Tschada J, Braach N, et al. Protein C concentrate controls leukocyte recruitment during inflammation and improves survival during endotoxemia after efficient in vivo activation. *Am J Pathol*. 2011;179:2637–2650.
15. Braach N, Frommhold D, Buschmann K, et al. RAGE controls activation and anti-inflammatory signalling of protein C. *PLoS One*. 2014;9, e89422.
16. Faust N, Varas F, Kelly LM, Heck S, Graf T. Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages. *Blood*. 2000;96:719–726.
17. Pau G, Fuchs F, Sklyar O, Boutros M, Huber W. EBImage—an R package for image processing with applications to cellular phenotypes. *Bioinformatics*. 2010;26:979–981.
18. Marki A, Esko JD, Pries AR, Ley K. Role of the endothelial surface layer in neutrophil recruitment. *J Leukoc Biol*. 2015;98:503–515.
19. Schmidt EP, Yang Y, Janssen WJ, et al. The pulmonary endothelial glycocalyx regulates neutrophil adhesion and lung injury during experimental sepsis. *Nat Med*. 2012;18:1217–1223.
20. Weller A, Isenmann S, Vestweber D. Cloning of the mouse endothelial selectins. Expression of both E- and P-selectin is inducible by tumor necrosis factor alpha. *J Biol Chem*. 1992;267:15176–15183.