Recombinant Lectin-Like Domain of Thrombomodulin Suppresses Vascular Inflammation by Reducing Leukocyte Recruitment via Interacting With Lewis Y on Endothelial Cells

Wei-Ling Lin, Chuan-Fa Chang, Chung-Sheng Shi, Guey-Yueh Shi, Hua-Lin Wu

Objective—The N-terminal lectin-like domain (domain 1 [D1]) of thrombomodulin (TM) is known to have an anti-inflammatory function. We previously showed that recombinant TM domain 1 (rTMD1) interacts with a carbohydrate molecule, Lewis Y (Leα), which is found to be expressed on adhesion molecules and involved in cell adhesion. Here, we tested the effect of rTMD1/Leα interaction on leukocyte recruitment in inflammation.

Approach and Results—The expression of Leα on the surface of human umbilical vein endothelial cells was increased by tumor necrosis factor-α stimulation. Direct binding of rTMD1 to Leα on the cell surface was observed. rTMD1 inhibited Leα-mediated leukocyte adhesion on the Leα-immobilized flow chamber and activated endothelium under a shear flow. The following leukocyte transmigration to endothelium was also reduced by rTMD1 through binding Leα. In vivo, treatment of rTMD1 reduced leukocyte recruitment to the inflammatory sites in carotid ligation injury and thioglycollate-induced peritonitis. rTMD1 administration in apolipoprotein E–deficient mice effectively suppressed atherosclerotic plaque formation and macrophage infiltration in atherosclerotic lesions. Increased Leα expression, as well as administered rTMD1, was observed in inflamed vessels.

Conclusions—rTMD1 suppresses vascular inflammation by inhibiting leukocyte recruitment to endothelium through attenuating Leα-mediated adhesion and further protects against atherosclerosis progression. The present study provides a mechanism showing that rTMD1 can inhibit inflammation by binding to its carbohydrate ligand Leα. (Arterioscler Thromb Vasc Biol. 2013;33:2366-2373.)

Key Words: atherosclerosis ■ inflammation ■ leukocytes ■ Lewis Y antigen ■ thrombomodulin

Vascular inflammation induced by proinflammatory mediators is an initial pathological condition that leads to chronic vascular diseases, such as atherosclerosis and restenosis.1 In the inflammatory conditions, vascular endothelium becomes activated and is characterized by increased expression of adhesion molecules, chemokines, and cytokines. Circulating leukocytes are subsequently attracted and stimulated by activated endothelium, triggering leukocyte recruitment into the vessel wall. The recruitment requires sequential steps, including rolling, firm adhesion, and transendothelial migration, that involve the interaction of various adhesion and cell–cell junction molecules on the surface of leukocytes and endothelial cells.2 Leukocyte infiltration and accumulation in injured vessel wall lead to various proinflammatory stimuli release and consequently augment the inflammatory responses. The sustained recruitment of these inflammatory cells may contribute to vascular diseases progression.3,4 Thus, reducing leukocyte recruitment by interfering with the interaction between leukocytes and endothelial cells is considered to be an effective strategy to suppress inflammation and disease progression.

Thrombomodulin (TM) is a type I transmembrane glycoprotein consisting of multiple domains. TM/thrombin complex on the endothelial cell surface is essential for anticoagulation, and activated protein C generated by this complex functions as a key factor in anticoagulation, anti-inflammation, and anti-apoptosis.5 In addition, several biological functions of TM in other cell types have been identified. The N-terminal C-type lectin-like domain (domain 1 [D1]) of TM has been known to have anti-inflammatory properties.6 Conway et al7 initially reported that TM domain 1 (TMD1) can inhibit inflammation using transgenic mice lacking TMD1 (TMLead/Lead). These transgenic mice have more severe inflammatory responses, including reduced survival after lipopolysaccharide challenge, increased leukocyte accumulation in lung injury, and larger infarcts after myocardial ischemia/reperfusion. Moreover,
recombinant TMD1 (rTMD1) has been shown to attenuate inflammation by inhibiting complement activation and sequestering high mobility group box 1 protein. However, except for high mobility group box B1, other interacting molecules of TMD1 that may participate in anti-inflammation are still unknown.

Previously, we demonstrated that Lewis Y (Le⁰; Fuc⁰1–2Galβ1–4(Fucβ1–3GlcNAβ1-R) is a specific carbohydrate ligand of rTMD1. Le⁰ is known to be highly expressed in epithelial cells, especially in cancer tissues, where Le⁰ mediates cell adhesion, proliferation, and apoptosis. Meanwhile, recent studies have demonstrated that Le⁰ is upregulated in synovial endothelium and joint fluid in rheumatoid arthritis. Le⁰ is also found to be expressed on intercellular adhesion molecule-2 (ICAM-2) on endothelial cells. ICAM-2 is a ligand of dendritic cell–specific C-type lectin, dendritic cell–specific ICAM-3–grabbing nonintegrin. The interaction of dendritic cell–specific ICAM-3–grabbing nonintegrin with ICAM-2 on vascular endothelium is Le⁰ dependent and is essential for dendritic cell–specific ICAM-3–grabbing nonintegrin recruitment into tissues to mediate immune responses. These findings suggest that interaction of Le⁰ with lectin-containing receptor might be involved in recruitment of leukocytes in inflammatory responses. Because Le⁰ is a specific ligand of rTMD1, we hypothesize that rTMD1/Le⁰ interaction on endothelial cells would influence leukocyte recruitment, thereby suppressing inflammation.

To test our hypothesis, we examined the expression and function of Le⁰ in endothelial cells under inflammatory conditions and studied the effect of rTMD1/Le⁰ binding on anti-inflammation in vitro and in vivo. We discovered that leukocyte recruitment was interfered by rTMD1/Le⁰ interaction. Animal inflammation models of thioglycollate-induced peritonitis, carotid ligation injury, and atherosclerosis were used to study the effect of Le⁰ in endothelial cells under inflammatory conditions and studied the effect of rTMD1/Le⁰ binding on anti-inflammatory responses.12,21,22 These studies suggest that interaction of Le⁰ with lectin-containing receptor might be involved in recruitment of leukocytes in inflammatory responses. Because Le⁰ is a specific ligand of rTMD1, we hypothesize that rTMD1/Le⁰ interaction on endothelial cells would influence leukocyte recruitment, thereby suppressing inflammation.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Le⁰ Mediates Leukocyte Adhesion on Inflammation
To investigate the role of Le⁰ in inflammation, we initially tested whether Le⁰ expression in endothelial cells would be induced by stimulation of the proinflammatory cytokine, tumor necrosis factor-α (TNF-α). As shown in Figure 1A, mRNA levels of fucosyltransferase 1 (FUT1), FUT2, and FUT4 for Le⁰ synthesis in endothelial cells were measured. The expression of FUT4 and FUT1 was significantly increased after TNF-α stimulation for 12 and 24 hours, respectively; meanwhile, no significant difference was found in the expression of FUT2. In addition, higher Le⁰ expression on the surface of endothelial cells was observed using flow cytometry and confocal microscope analysis (Figure 1B and 1C). These results indicate that Le⁰ expression in endothelial cells is increased in response to TNF-α stimulation.

Figure 1. Lewis Y (Le⁰) expression is increased in tumor necrosis factor-α (TNF-α)-activated endothelial cells and mediates leukocyte adhesion. A, Real-time polymerase chain reaction analysis of fucosyltransferase 1 (FUT1), FUT2, and FUT4 mRNA expression in human umbilical vein endothelial cells (HUVECs). The relative fold changes were obtained by normalization of resting condition. Data are mean±SEM (n=3). **P<0.01 vs resting condition. B, Representative histogram of cell surface Le⁰ expression in resting and 24 hours of TNF-α–activated HUVECs detected by flow cytometry. Bar graph, Mean fluorescence intensity (MFI) of cell surface Le⁰ expression in HUVECs. Data are mean±SEM (n=3–4 experiments). **P<0.05 and ***P<0.001. C, Resting and 24 hours of TNF-α–activated HUVECs were stained by Le⁰ mAb (5 µg/mL) or isotype control Ab (5 µg/mL), and the signal was detected using the confocal microscope. Representative photos are 1 of 3 experiments. D, Adhesion of human leukocytes to immobilized biotinylated Le⁰–polycrylamide (PAA) or biotinylated Galβ1–3(Fuc⁰1–2)Galβ1–4PAA (control sugar) at a shear rate of 0.5 dynes/cm² was studied in the absence or presence of recombinant thrombomodulin domain 1 (rTMD1). Adherent cells were counted after 3 minutes of perfusion. Adhesion is shown as the number of adherent cells per field (magnification, ×100). Data are mean±SEM (n=3 experiments). ***P<0.001. E, Adhesion of human leukemia mononuclear cells (PBMCs) to Le⁰–immobilized slides under the shear flow condition. Human neutrophils and PBMCs both were arrested by Le⁰ under a shear rate of 0.5 dynes/cm².
rTMD1 Binding to Endothelial Le<sup>e</sup> Interferes With Leukocyte Adhesion and Transmigration

Our previous studies have shown that rTMD1 binds to soluble Le<sup>e</sup> specifically,<sup>10,11</sup> To test whether rTMD1 could bind Le<sup>e</sup> on endothelial cells, we first analyzed the binding of fluorescein isothiocyanate–labeled rTMD1 on the cell surface using flow cytometry. The results showed that rTMD1 dose dependently bound on endothelial cells (Figure 2A and 2E). The binding was enhanced while cells were activated by TNF-α (Figure 2B and 2F) and reduced in the presence of soluble Le<sup>e</sup> specifically (Figure 2C and 2G), showing that soluble Le<sup>e</sup> can sequester rTMD1 from binding to endothelial cells. Next, we tested whether Le<sup>e</sup> would be the ligand of rTMD1 on endothelial cells. Le<sup>e</sup> antibody specifically recognized Le<sup>e</sup> on the cell surface; however, preincubation of unlabeled rTMD1 with cells decreased the capacity of the antibody recognizing Le<sup>e</sup> on cells (Figure 2D and 2H), indicating that rTMD1 bound to cell surface Le<sup>e</sup>.

Because rTMD1 inhibited leukocyte adhesion to immobilized Le<sup>e</sup> (Figure 1D and 1E), we proceeded to examine whether rTMD1/endothelial Le<sup>e</sup> interaction may have an inhibitory effect on leukocyte/endothelium adhesion. As shown in Figure 3A, rTMD1 effectively reduced monocyte adhesion to TNF-α–activated HUVECs when compared with control at a shear rate of 0.5 dynes/cm<sup>2</sup>. Preincubation of soluble Le<sup>e</sup> with rTMD1 specifically reversed the inhibitory effect of rTMD1 on monocyte adhesion. Leukocyte adhesion followed by transendothelial migration occurs in leukocyte trafficking in inflammation. Therefore, we next used transwell assay to test whether transendothelial migration of monocyte would also be inhibited by rTMD1. The results showed that the number of transmigrated cells across endothelium to the lower chamber of transwell was significantly decreased by rTMD1. Similarly, the inhibitory effect of rTMD1 was reversed merely by soluble Le<sup>e</sup> preincubation (Figure 3B). Then, we assessed the effect of rTMD1 on inflammatory cell recruitment using acute thioglycollate-induced peritonitis model.<sup>14</sup> Twenty-four hours after thioglycollate stimulation, elicited inflammatory cells were detected in the peritoneal cavity. Preadministration of rTMD1 30 minutes before thioglycollate significantly reduced total white blood cell, neutrophils, and macrophage infiltration in peritoneal cavity (Figure 3C).

The binding affinity of mammalian-rTMD1 (m-rTMD1) to Le<sup>e</sup> was similar to Pichia-expressed rTMD1 (Figure I in the online-only Data Supplement); therefore, m-rTMD1 was also used to examine whether the protein would interfere with leukocyte recruitment. The results showed that m-rTMD1 had similar inhibitory effects as Pichia-rTMD1 (Figure II in the online-only Data Supplement). These data indicate that rTMD1 inhibits leukocyte adhesion and transmigration to
endothelium under inflammatory conditions by blocking Le\textsuperscript{\textalpha} on endothelial cells.

rTMD1 Inhibits Leukocyte Adhesion Induced by Carotid Ligation

Arterial ligation triggers leukocyte recruitment to the injured vessel that contributes to inflammation and neo-intima formation.\textsuperscript{25} The upregulation of vascular adhesion molecules and leukocyte adhesion can be observed near the ligation site at the early time points after ligation.\textsuperscript{26} Consistently, CD45-positive leukocytes were recruited to the ligated vessel at day 3 after ligation (Figure 4A). We examined whether rTMD1 would inhibit leukocyte adhesion by carotid artery ligation in mice. rTMD1 was administered intraperitoneally to mice once a day after ligation. At day 3, increased adherent polymorphonuclear leukocytes, identified as neutrophils,\textsuperscript{27} were observed at the luminal surface in ligated vessels of saline-treated mice, whereas there was fewer leukocyte adhesion in unligated vessels (Figure 4B). Furthermore, the number of leukocyte recruited to the ligation site was dose dependently reduced by rTMD1 treatment (Figure 4B and 4C). The data indicate that rTMD1 can reduce leukocyte adhesion on vascular endothelium after ligation-induced injury.

rTMD1 Reduces Macrophage Accumulation to Suppress Atherosclerosis

In chronic vascular inflammation, such as atherosclerosis, recruitment of leukocytes (especially monocytes/macrophages) promotes atherosclerotic plaque formation and consequently vessel occlusion.\textsuperscript{1,28} Therefore, the inhibitory effect of rTMD1 on leukocyte adhesion and transmigration in atherosclerosis progression was tested. Atherosclerosis was induced in apolipoprotein E-deficient mice feeding a high-fat chow diet for 20 weeks. During this period, mice received rTMD1 by intraperitoneal injection every other day. At the end of the treatment, the atherosclerotic plaque formation in the descending aorta and the atherosclerotic lesion composition in aortic root were measured. The descending aortas derived from rTMD1-treated mice exhibited less plaque formation than that of saline-treated mice (Figure 5A). In addition, histology analysis of lesion components in the aortic root showed less lipid-rich lesion (Figure 5B and 5C) and fewer macrophage accumulation (Figure 5D and 5E) in mice that received rTMD1 compared with saline control. In contrast, no significant difference in smooth muscle cell content between groups was observed (Figure 5F and 5G). Thus, rTMD1 can reduce macrophage accumulation in atherosclerotic lesions to attenuate atherosclerotic plaque formation.
Le⁺ Expression Is Increased in Inflamed Vessels
We analyzed Le⁺ expression in the vessels after carotid ligation–induced and atherogenesis–induced inflammation. The unligated–contralateral and left ligated carotid arteries were harvested after 3 days of ligation, and then tissue extracts of the vessels were subjected to tissue Western blotting analysis for Le⁺. Several bands were highly expressed in ligated arteries in comparison with the unligated control (Figure 6A, left). ICAM-1, as an inflammatory marker of endothelium, was only expressed in ligated vessels (Figure 6A, right upper). Immunofluorescence staining for Le⁺ in the carotid arteries showed that Le⁺ expression was obvious in the ligated vessel surface, whereas it was barely detectable in unligated vessel (Figure 6B, left). We used CD31 as the endothelial marker. The lining of CD31–positive endothelial cells was observed at the surface of both unligated and ligated vessels (Figure 6B, right), suggesting that Le⁺ expression was increased in inflammatory endothelial cells.

Le⁺ expression was further examined in the aortic arch sections from apolipoprotein E–deficient mice fed with high-fat chow diet. Oil red O staining for lipids showed the atherosclerotic plaque in the blood vessel (Figure 6C, left). Both of normal and atherosclerotic vessels were used for immunofluorescence staining. Le⁺ expression had a sparse and weak pattern at the surface of normal vascular wall (Figure 6C, middle upper, arrowheads). In atherosclerotic vessel, a stronger signal of Le⁺ was detected at the plaque surface (Figure 6C, middle lower, arrows), whereas Le⁺ expression pattern at the surface without plaque was as similar as normal vessel (Figure 6C, middle lower, arrowheads). Using CD31 as the endothelial cell marker (Figure 6C, right), we measured Le⁺ expression area specifically at the endothelial layer and found higher Le⁺ expression at the surface of plaque in atherosclerotic vessel when compared with its expression in normal vessel (Figure 6D).

Administrated rTMD1 Is Observed in Inflamed Vessels
To verify the treatment of rTMD1, we directly detected the presence of administered rTMD1 on the inflamed vessel...
surface. In the carotid ligation model, we found that rTMD1 was present on the ligated vessel surface, whereas it was undetectable on the unligated vessel surface (Figure IIIA in the online-only Data Supplement, left). In addition, the expression pattern of rTMD1-positive signals in ligated vessels was similar to that of CD31 (Figure IIIA, in the online-only Data Supplement, right), indicating that rTMD1 would bind on the damaged endothelial cells after ligation-induced injury. Besides, rTMD1 was not found on leukocytes (Figure IIIA in the online-only Data Supplement, asterisks) or the surface without endothelial cell attachment (Figure IIIA, in the online-only Data Supplement, arrows). In atherosclerotic tissues, we found that rTMD1 mostly was located on vessel surface without plaque (Figure IIIB in the online-only Data Supplement, left, open arrowheads). However, it was hardly detected on the plaque area (Figure IIIB in the online-only Data Supplement, left, dashed line). Endothelial denudation was found on the surface of an atherosclerotic plaque according to the CD31 staining result (Figure IIIB in the online-only Data Supplement, right, dashed line); hence, rTMD1 may have been lost or was difficult to be detected around the area. Taken together, these findings suggest that Le^e expression is increased in inflamed vessels where it can facilitate leukocyte recruitment by promoting leukocyte/endothelium interaction, and rTMD1 treatment inhibits this effect by directly blocking Le^e.

**Discussion**

In this study, we investigated the effect of rTMD1 and Le^e interaction on leukocyte recruitment under inflammatory conditions. Le^e expression was elevated on endothelium on inflammatory stimulation in vitro and in vivo, suggesting the involvement of Le^e in inflammation. Following studies demonstrated that rTMD1/endothelial surface Le^e interaction interfered with Le^e-mediated leukocyte adhesion to reduce leukocyte recruitment and further ameliorated vascular inflammation. These studies provide a novel mechanism demonstrating and emphasizing the significant role of the carbohydrate-binding ability of the lectin-like domain of TM in anti-inflammation.

About the role of carbohydrate and carbohydrate-binding protein interaction in leukocyte recruitment, selectins and selectin ligands are well-studied adhesion molecules that critically facilitate the initial steps of capture and rolling in leukocyte trafficking. Selectin bind to the carbohydrate structure, sialyl-Lewis X (sialyl-Le^e), on selectin ligands with low affinity. This interaction plays an important role in slowing down leukocytes rolling velocity that helps the following attachment of leukocytes to endothelium. The expression of sialyl-Le^e is mostly on leukocytes; however, it can also be found on high endothelial venues in lymph node to mediate lymphocytes homing. In inflammatory conditions, the selectin ligands and glycosyltransferases for sialyl-Le^e synthesis are induced to participate in immune responses. In the pathological circumstance, selectin inhibitors are thought to be able to ameliorate inflammation. Direct interference of selectin and sialyl-Le^e interaction is mostly the target to develop selectin inhibitors. Thus, small molecules of carbohydrate or protein are generated to be applied for further trials.

In comparison with our present studies, Le^e is also a carbohydrate determinant that mediates leukocyte adhesion and was upregulated during inflammation. Different from sialy–Le^e, Le^e is preferentially presented on endothelium. We here speculate that the finding of Le^e involving in the attachment of leukocyte to endothelium may be another carbohydrate molecule contributing in leukocyte recruitment beyond selectin and sialyl-Le^e. The function of Le^e probably has the synergistic effect on leukocyte/endothelium interaction. Notably, the binding affinity between m-rTMD1 and soluble Le^e as reported by our previous studies showing the dissociation constant was 3.97×10^-6 mol/L, which is higher than that in binding between sialyl-Le^e and selectins. Moreover, the binding affinity of rTMD1 and Le^e was much higher than that of other carbohydrates, including other Lewis antigen members. It might be proposed that leukocyte TM and endothelial Le^e interaction could play a prominent role in mediating leukocyte/endothelium adhesion in inflammation. rTMD1 might be served as the soluble inhibitor that could functionally block membrane TM/Le^e interaction and reduce inflammation. Further studies should be required to investigate this hypothesis.

The adhesive ability between neutrophils and PBMCs to Le^e-coated surface was different as observed in our experiments (Figure 1D). Neutrophils had the higher and more rapid adhesion to Le^e than PBMCs. According to the previous reports, different rolling characteristics of polymorphonuclear leukocytes and mononuclear leukocytes may be related to their distinct binding interaction to endothelial selectins. Thus, the difference in the number between neutrophils and PBMC adhesion on Le^e-coated slide probably suggests that there may be different types or distribution of receptors that have dissimilar binding properties for Le^e among the leukocyte subpopulations.

The mRNA levels of FUT1, FUT2, and FUT4 were used to determine Le^e expression in HUVECs before and after TNF-α activation (Figure 1A). Within the structure of Le^e (Fucα1,2Galβ1,4(Fucα1,3GlcNAcβ1-R), FUT1 and FUT2 are responsible for Fucα(1,2)-linkage, and FUT4 is responsible for Fucα(1,3)-linkage). FUT4 and FUT1 were found to be significantly elevated after 12 and 24 hours of stimulation, but FUT2 mRNA level was not affected by TNF-α. Because the expression of FUT genes is tissue- and cell-type dependent and FUT2 expression is much lower than FUT1 in HUVECs, it is expectable that there was no significant change of FUT2 mRNA levels in TNF-α–activated HUVECs.

Previous studies have shown that FUT4 and FUT7 controlled susceptibility in atherosclerosis in apolipoprotein E–deficient mice, indicating that the elevation of these 2 FUTs contributes to atherosclerosis. FUT4 is one of the glycosyltransferases for Le^e synthesis, and presently, we found higher expression of Le^e in the vessel wall with atherosclerotic plaque than without plaque (Figure 6C and 6D). The observation implies the positive correlation of Le^e and atherosclerosis progression. Although CD31 staining showed the intact endothelial lining in the vessel wall without plaque, the signal of Le^e was weak and sparse in that area, indicating the lower expression of Le^e in normal endothelium and an inflammatory environment results in Le^e upregulation. In another in
vivo investigation, increased Le\textsuperscript{o} expression has been found on vascular endothelium from patients with rheumatoid arthritis where Le\textsuperscript{o} was cytokine inducible.\textsuperscript{20} These results certainly identify the significance of Le\textsuperscript{o} in vascular inflammation. It is more interesting to notice that the result of tissue Western blot showed several increasing bands in vessels after ligation (Figure 6A), indicating that there are some specific molecules carrying Le\textsuperscript{o} that are induced in the injured vasculature and probably involve in leukocyte adhesion. Further studies are needed to identify these molecules.

By comparing the efficacy of rTMD1 proteins expressed by *Pichia* and mammalian expression systems, there were no significant differences in their functions between both of the proteins. Our previous studies\textsuperscript{10} have illustrated that the efficiency of *Pichia*-rTMD1 in the inhibition of lipopolysaccharide-induced inflammation is comparable with that of m-rTMD1 by several in vitro assays. These findings suggest that although the glycosylation of these 2 rTMD1 proteins are very different, their functions are similar, meaning that the glycosylation would not affect the Le\textsuperscript{o}-binding ability of TMD1.

We verified the treatment of rTMD1 by directly detecting the presence of administered rTMD1 on the inflamed vessels of carotid ligation and atherosclerosis models. The signals of rTMD1 were partially detected at the endothelial layer. Because the denudation occurred in aortic arches, we observed that the intensity of rTMD1 staining was weak in atherosclerotic samples. However, rTMD1-positive staining pattern was similar to CD31 and Le\textsuperscript{o}, indicating that rTMD1 was supposed to deposit on inflamed endothelium by binding Le\textsuperscript{o}. Notably, the half-life of rTMD1 in circulation reported by our group is 3 to 4 hours, and the injected rTMD1 exists in circulation last for 12 hours.\textsuperscript{10} In ligated arteries, rTMD1 was still detectable in samples harvested at 16 hours after injection, suggesting that the binding of administered rTMD1 to inflamed endothelium may prevent the rapid rTMD1 clearance in circulation; and further, the duration of the efficacy of injected rTMD1 might be extended.

Various in vivo inflammatory models of the anti-inflammatory ability of rTMD1 have been well exhibited.\textsuperscript{7–10} Here, we initially administrated rTMD1 into apolipoprotein E-deficient mice to verify whether the protein would control atherosclerosis progression. The results showed that atherosclerotic plaque formation and macrophage accumulation were significantly reduced by rTMD1 treatment. TM is known to be the membrane protein representing as a protector on vascular endothelium. When endothelial cells are damaged, TM expression level is reduced. Loss of TM on endothelium is also found in atherosclerotic coronary arteries,\textsuperscript{35} which dampens the anti-coagulation and anti-inflammation mediated by TM/thrombin complex. Further thrombosis and inflammation occur in the atherosclerotic region. In addition, soluble TM can be detected in plasma of the patients with coronary heart disease, and some clinical data showed that the levels of soluble plasma TM may be inversely associated with coronary heart disease.\textsuperscript{36,37} According to our studies, it suggests that rTMD1 has the potential effect on suppressing atherosclerosis.

In summary, the present works provide evidences that Le\textsuperscript{o} is an inflammation-induced carbohydrate on endothelial cells and mediates leukocyte adhesion. However, rTMD1 functions as an inhibitor in Le\textsuperscript{o}/leukocyte binding; therefore, leukocyte recruitment is consequently suppressed in vitro and in vivo. This study yields an important mechanism underlying the therapeutic function of rTMD1 in vascular diseases.

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**Disclosures**

None.

**References**

Recombinant thrombomodulin domain 1 (rTMD1) decreases leukocyte infiltration in various inflammatory models as has been observed; however, the direct molecular mechanism remains unknown. We report that rTMD1 can attenuate leukocyte/endothelial cell interaction via a direct targeting on the carbohydrate molecule, Lewis Y, on endothelium. Upregulation of Lewis Y is observed in inflamed vascular endothelium in carotid ligation and atherosclerosis. Administration of rTMD1 effectively reduces leukocyte recruitment in response to vascular injury via binding to Lewis Y. Notably, we first demonstrate that rTMD1 treatment can suppress atherosclerosis progression that highlights a new therapeutic effect of rTMD1 on the chronic vascular disease.
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In the article by Lin et al, which appeared in the October 2013 issue of the journal (Arterioscler Thromb Vasc Biol. 2013;33:2366-2373. DOI: 10.1161/ATVBAHA.113.301221), figure 6 was incorrect. The correct figure is below:

The online version of the article has been corrected and is available at http://atvb.ahajournals.org/content/33/10/2366.full.
Supplement materials

The Recombinant Lectin-like Domain of Thrombomodulin Suppresses Vascular Inflammation by Reducing Leukocyte Recruitment via Interacting with Lewis Y on Endothelial Cells

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**Supplemental Figure I.** rTMD1 proteins from mammalian and *Pichia* expression system have similar binding ability to Le\(^x\). The interactions of rTMD1 proteins with Le\(^x\) and Le\(^y\) were analyzed by using AlphaScreen assay. m-rTMD1 represents as mammalian-expressed rTMD1. p-rTMD1 represents as *Pichia*-expressed rTMD1. Relative intensity was obtained by normalization of the binding to control carbohydrate (Con, Galα1-3(α1-2Fuc)Galβ). Data are Mean ± SEM (n=4 experiments). n.s.: not significant; **P < 0.01; ***P < 0.001.
Supplemental Figure II. rTMD1 proteins from mammalian and Pichia expression system both interfere with leukocyte recruitment. 

A, Adhesion of THP-1 monocytes to endothelium was assessed in the indicated conditions. m-rTMD1 represents as mammalian-expressed rTMD1. p-rTMD1 represents as Pichia-expressed rTMD1. Adherent cells represent as the number of cells per millimeter squares at a shear rate of 0.5 dynes/cm². Data are Mean ± SEM (n=3 experiments). ***P < 0.001 versus TNF-α only; ###P < 0.001; n.s.: not significant. 

B, Transmigration of THP-1 monocytes across endothelium in the indicated conditions is shown. Transmigration represents as the number of cells in the lower wells of the transwell plate. Data are Mean ± SEM (n=3 experiments). *P < 0.05 and ***P < 0.001 versus TNF-α only; ###P < 0.001; n.s.: not significant. 

C, Differential cell count in peritoneal fluid 24 hours after intraperitoneal injection of thioglycollate in mice is shown. Mice were intravenously injected with saline and rTMD1 proteins 30 minutes before thioglycollate administration. Data are Mean ± SEM (n=9 to 12 each group). **P < 0.01 and ***P < 0.001 versus saline; n.s.: not significant.
Supplemental Figure III. Administered rTMD1 is present on the endothelial cells of inflamed vessels. Representative sections of carotid arteries in ligation model (A) and aortic arches in atherosclerosis model (B) stained for rTMD1 (anti-His tag) and endothelial cells (CD31). Filled arrowheads indicate the positive staining of rTMD1 and CD31. Filled arrows indicate the negative staining of rTMD1 and CD31. Asterisk indicates the leukocyte. Open arrowheads indicate the positive staining of rTMD1 and CD31 on endothelium without plaque formation. Dash lines indicate the plaque area. Scale bar: 50 μm. n=3 experiments.
### Supplemental Table. Primers used in quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
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| GAPDH  | Fwd: aggfcatccctgagctgaacgg  
        | Rev: cgctgcttcaccaccccttg |
| FUT1   | Fwd: gcaggccatggactggtt    
        | Rev: cctggaggtgtcagttt    |
| FUT2   | Fwd: ctcgctacagctccctcatctt  
        | Rev: cgtggagggtcaatgttct  |
| FUT4   | Fwd: gagctagctgtccacatcacc  
        | Rev: cagctggccaaagttcgtatg |
Materials and Methods

Mice
Apolipoprotein E-deficient (ApoE\(^{-/-}\)) mice, backcrossed 10 times on the C57BL/6 background, were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). C57BL/6 mice were purchased from BioLASCO Taiwan Co., LTD. All animal experiments were approved by the Institutional Animal Care and Use Committee, National Cheng Kung University.

Cells
Human leukemia monocytic THP-1 cells were purchased from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) and cultured in RPMI-1640 medium (GIBCO BRL, Grand Island, USA). Human umbilical vein endothelial cells (HUVECs) were purchased from Invitrogen (NY, USA) and cultured in Medium 199 (GIBCO BRL, Grand Island, USA). Cells were cultured by the recommended condition. HUVECs from passage 2 to 4 were used in the experiments.

Isolation of Human Neutrophils and Peripheral Blood Mononuclear Cells (PBMCs)
As previously described,\(^1\) whole blood was collected from healthy volunteers into tubes containing heparin. After RBC sedimentation by 3% dextran T-100 (Sigma-Aldrich, USA), leukocyte-rich plasma was centrifuged at 1000 rpm for 10 minutes to pellet cells. Cells were then resuspended by saline, and Ficoll-Hypaque (Pharmacia, USA) solution was layered beneath the cell suspension. After centrifuging at 1400 rpm for 40 minutes, the cloudy band of mononuclear cells in Ficoll-Hypaque/saline interface was aspirated, followed by removing the top layer and Ficoll-Hypaque and leaving pellet consisting of neutrophils and residual RBC. Mononuclear cells was then washed and resuspended by serum-free RPMI medium. After removing RBC by hypotonic lysis, neutrophils were resuspended in ice-cold PBS/glucose. Cells were used within 2 hours.

rTMD1 expression and purification
The DNA sequence coding for TMD1 was clones into the pPICZ\(\alpha\)A and pCR3 vectors (Invitrogen, USA). pPICZ\(\alpha\)A vector was used for expression and secretion of human rTMD1 protein containing 6 \(\times\) His tag and c-Myc epitope by Pichia pastoris expression system; while, pCR3 was used in human embryonic
After a period of culture in appropriate media, rTMD1 protein containing conditional medium was collected, dialyzed, and applied to nickel-chelating Sepharose column (Amersham Pharmacia Biotech., Piscataway, NJ). An imidazole gradient was used for elution. The purified products were verified by silver staining and Western blotting. The fractions containing pure rTMD1 proteins were then collected, concentrated and quantified. The purity of rTMD1 was identified with mass spectrometry analysis (Applied Biosystems, USA). Amino acid sequence determinations were performed automatically by Edman degradation with a model 477A sequencer (Applied Biosystems).

**Flow Cytometry Analysis**

For determining Le⁺ expression, 5 μg/ml of Le⁺ monoclonal antibody (mAb) (F3, Abcam: ab3359, USA) or isotype control antibody (mouse IgM, Invitrogen: MGM00, USA) was incubated with cells, followed by Alexa Fluor 546 conjugated goat anti-mouse IgM secondary antibody (Invitrogen, USA). After wash, fluorescence was analyzed in the FACS-Calibur (Becton-Dickinson, USA). In the blocking condition, rTMD1 was preincubated with cells at RT for 30 minutes before incubating with antibodies. For determining rTMD1 and HUVECs interaction, FITC labeled-rTMD1 protein was prepared as described and incubated with cells. After wash, the binding of rTMD1 was detected by fluorescence and analyzed by FACS-Calibur. In the blocking condition, soluble polyacrylamide (PAA, Glycotech Corp., USA) or carbohydrate-PAA polymers were preincubated with rTMD1 at RT for 30 minutes; then, cells were incubated with the mixture of rTMD1 and PAA or carbohydrate-PAA polymers. Fluorescence intensity was analyzed by using FlowJo software (Treestar, Ashland, OR).

**Quantitative Real-time PCR**

Total cell RNA was extracted using Total RNA mini extraction kit (RBC Bioscience, Taiwan) and cDNA was synthesized for 60 minutes at 42°C in the reverse transcription reaction containing a mixture of 5x M-MLV reverse transcriptase reaction buffer (Promega, USA), 2 μg total RNA, 1 mM dNTPs (Viogene, Taiwan), 200 ng oligo(dT)₁₅ primer (Promega, USA), 20 units RNase inhibitor (Promega, USA), and 200 units M-MLV reverse transcriptase (Promega, USA). The reaction mixture for real-time PCR was prepared by a total 15 μl volume of 200 nM forward and reverse primers, 25 ng cDNA and two times dilution of SYBR Green Master Mix (Promega, USA). Then, the
reactions were performed by ABI 7500 sequence detection system (Applied Biosystem, USA) and the detailed method was described elsewhere.\textsuperscript{3} The relative mRNA expression level was calculated by using $2^{-\Delta C_T}$ method, where $\Delta C_T$ was equal to $(C_T \text{target gene} - C_T \text{reference gene})$.\textsuperscript{4} Glyceraldehyde 3-phosphate dehydrogenase was used as the reference gene. The relative fold change was obtained by the equation of $2^{-\Delta \Delta C_T}$, where $\Delta \Delta C_T$ was equal to $(\Delta C_T \text{TNF-}\alpha_{\text{activated}} - \Delta C_T \text{Resting})$. Thus, the relative mRNA expression level of resting condition represented as 100% for normalization of stimulation condition. Primers (Supplemental Table) used in the real-time PCR have been designed as previously described\textsuperscript{5-7} and synthesized by MDBio. (MDBio, Inc. Taiwan).

**Immunofluorescence for Confocal Microscopy Analysis**

Cells were grown on glass coverslips in 24-well plate. After washing by PBS, cells were fixed in 4% paraformaldehyde in PBS, then permeabilized in 0.2% Triton X-100. Nonspecific binding was blocked by 3% FBS, followed by incubation with 5 μg/ml of Le$^\gamma$ mAb (F3, Abcam: ab3359, USA) or isotype control antibody (mouse IgM, Invitrogen: MGM00, USA) at 4°C overnight. Then, secondary antibody conjugated with Alexa-546 (Invitrogen, USA) was used to detect Le$^\gamma$ antibody. Images were analyzed by using FV-1000 confocal microscope (Olympus, Japan).

**Cell Adhesion Assay**

For adhesion of leukocytes to immobilized carbohydrates under flow, 1 μM of the biotinylated carbohydrate-PAA (Glycotech Corp., USA) was immobilized on a laminar flow chamber slide (μ-slide I 0.8$^{\text{luer}}$, ibidi, Martinsried, Germany) coated with 0.1 μg/ml streptavidin (Sigma Aldrich, USA). After 2-3 hours of immobilization at RT, the chamber was rinsed with PBS three times and preincubated with or without rTMD1 (0.5 μM) for 30 minutes at RT. Fresh isolated human neutrophils or PBMCs (5x10$^4$/ml) were perfused at a constant flow rate of 0.5 dynes/cm$^2$. The number of adherent cells (stationary over 5 seconds) was determined after 3 minutes of perfusion. For adhesion of THP-1 cells on endothelium under flow, HUVECs were grown to confluence on the flow chamber slide and activated by tumor necrosis factor (TNF)-α (10 ng/ml) for 24 hours. Then, HUVECs were preincubated with or without inhibitor for 30 minutes at 37°C. To reverse the effect of rTMD1, soluble PAA or carbohydrate-PAA polymers were preincubated with rTMD1 at RT for 30 minutes before incubation with HUVECs. After washing HUVECs by serum-free medium, THP-1 cells (5x10$^5$/ml) were perfused in the flow chamber
at a constant flow rate of 0.5 dynes/cm². The number of adherent THP-1 cells was determined after 30 minutes of perfusion. In these experiments, the shear flow was generated by using the perfusion loop system and an air pressure pump (ibidi, Martinsried, Germany), and the image was recorded every 10 seconds by time lapse. The number of adherent cells was calculated in 10-15 random fields in a single experiment and the statistic was calculated by 3-5 experiments.

**Transmigration Assay**

Transmigration assay was performed as previously described. Briefly, the assay was carried out by using a 24-well transwell plate (Costar, Corning Inc. USA) with a 5 μm pore size. HUVECs (1 x 10⁵/well) were seeded and grown for 48 hours to reach confluence. Then, cells were activated with or without TNF-α (10 ng/ml) for 24 hours. Followed by activation, HUVECs were preincubated with or without rTMD1 for 30 minutes at 37°C. To reverse the effect of rTMD1, soluble PAA or carbohydrate-PAA polymers were preincubated with rTMD1 at RT for 30 minutes before incubation with HUVECs. After washing HUVECs by serum-free medium, 3x10⁵/well THP-1 monocytes were added into the upper chamber and medium with 10% serum was added into the lower chamber. After 4 hours of incubation, monocytes in the lower chamber were collected and cell number was counted by using a counting chamber.

**Peritonitis Model**

Peritonitis was induced by intraperitoneal injection of thioglycollate (4% wt/vol in 1 ml sterile saline; Sigma Aldrich, USA). Saline or rTMD1 (0.4 and 1 mg/kg) treatment was performed 30 minutes before thioglycollate by intravenous administration. 24 hours after thioglycollate injection, mice were killed by CO₂ overexposure, and 10 ml of sterile saline was injected into the peritoneal cavity. Cells were obtained by aspirating peritoneal lavage. Differential cell count was determined by using a hemocytometer (XT1800iV; Sysmex, USA)

**Carotid Artery Ligation Model**

Complete ligation was operated in the left common carotid artery in C57BL/6 (8 weeks old) mice. On day 0, the left carotid artery in mice was completely ligated with 5-0 silk, just proximal to the carotid bifurcation to disrupt blood flow. rTMD1 (0, 0.4 and 1 mg/kg/day) was given intraperitoneally on day 0, 1, and 2. On day 3 after the surgery, the animals were sacrificed, and the segments of
the right and the left common carotid arteries just proximal to the ligation were excised, fixed in 4% paraformaldehyde, and embedded in OCT compound. A total of 5 transverse sections per animal were cut at 100 μm intervals and stained with hematoxylin-eosin (Sigma Aldrich, USA). Leukocytes were characterized by polymorphic nuclear morphology. The average number of adherent leukocyte on the luminal surface of the five sections in each artery was counted.

**Tissue Western Blot**
Unligated right carotid artery and ligated left carotid artery were harvested and washed by PBS. The vessels were immersed in liquid nitrogen and immediately grounded into powder, then lysed in cell lysis buffer (Cell Signaling Technology, USA) by sonication. Total protein concentration of the vessel lysate was quantified by bicinchoninic acid protein assay. Samples were equally (50 μg/lane) loaded and subjected to electrophoresis. After electrophoresis, the SDS-PAGE gel was blotted on to a nitrocellulose membrane (Millipore, The Netherlands) and incubated with primary antibodies which were detected by peroxidase conjugated-secondary antibodies. Signals were visualized by enhanced chemiluminescence.

**Atherosclerosis Model**
ApoE−/− mice (10 weeks old) were fed with a high-fat diet containing 0.15% cholesterol and 20.2% fat (TestDiet 57BD) for 20 weeks. During this period, these mice were intraperitoneally injected with saline or rTMD1 (0.4 mg/kg) every other day. At the end of the treatment, hearts, aortic arches and descending aortas were dissected from mice when sacrificed. The tissues were embedded in OCT compound and frozen at -80°C.

**Histology Analysis**
Frozen sections of aortic root were dissected as previously described. A total of 8 sections per animal were cut at 100 μm intervals and stained for lipid-rich plaque (Oil Red O), macrophages, (MOMA-2, Abcam: ab33451, USA), and smooth muscle cells (αSMA, clone 1A4, Sigma Aldrich, USA). The percentage of lesion area was obtained by Oil Red O-positive area relative to total aortic root area, and the percentage of macrophages and SMA was obtained by positive staining area relative to lesion area. All these data were quantified in an average of 4 cross sections containing aortic valve. In mice descending aortas, the atherosclerotic plaque was en face stained with Oil Red O and the
percentage of plaque was quantified by plaque area relative to whole vessel area. The lesion area in aortic roots and descending aortas were all measured by using image analysis software (Image J 1.45s). In mice aortic arches, the whole arches were cut at 100 μm intervals and the sections were stained for lipid-rich plaque (Oil Red O), nuclei (DAPI), endothelial cells (CD31, Abcam: ab28364, USA) and Le\(\gamma\) (A70-C/C8, Abcam: ab23911, USA). Le\(\gamma\) positive staining area in endothelial layer was quantified as follows. Parallel tissue sections of 5 μm intervals were used for Le\(\gamma\) and CD31 staining, respectively. CD31 was used as the endothelial cell marker to identify the endothelial layer. The area of Le\(\gamma\)-positive signal for quantification was restricted to the CD31-positive endothelial layer only, and the area with positive signal was measured by using image analysis software (Image J 1.45s) under 200x magnification. Positive area was calculated as percentage of total vessel wall area or plaque area.

**Immunohistochemistry**

5 μm cryo-sections of hearts and vessels were used for staining. Briefly, sections were fixed in ice-cold acetone for 5 minutes. The endogenous peroxidase activity was blocked by incubating with 0.3% H\(\text{2}O\text{2}\) for 10 minutes, followed by nonspecific blocking with 1 hour incubation of blocking buffer (DAKO). After PBS rinse, the sections were incubated with primary antibodies overnight at 4°C. Then, appropriate secondary antibody-conjugated with horseradish peroxidase was applied to the sections for 2 hours at RT. After washing by PBS, the signal of horseradish peroxidase was detected by 3-amino-9-ethylcarbazole (AEC) substrate chromogen (DAKO, Denmark). Nuclei were counterstained with hematoxylin.

**Immunofluorescence**

5 μm cryo-sections of aortic arch were used for staining. Briefly, sections were fixed in ice-cold acetone for 5 minutes, followed by nonspecific blocking of 1 hour incubation of blocking buffer (DAKO). After PBS rinse, the sections were incubated with primary antibodies overnight at 4°C. Then, appropriate Alexa 546 conjugated secondary antibodies (Invitrogen, USA) were applied to the sections for 2 hours at RT. After washing by PBS, nuclei were counterstained with DAPI. The fluorescent signal was examined by using a fluorescence microscope (Leica, Germany).
**Immunohistochemistry of administered rTMD1**

Ligated carotid arteries and unligated-contralateral arteries were harvested on the third day after ligation and at 16 hours after the final injection of rTMD1. In the atherosclerosis model, the aortic arches were extracted from samples harvested after 20 weeks of rTMD1 treatment. 5 μm cryo-sections of carotid arteries and aortic arches were used for staining. Sections were fixed in ice-cold acetone for 5 minutes. The endogenous peroxidase activity was blocked by incubating with 0.3% H₂O₂ for 10 minutes, followed by nonspecific blocking with 1 hour incubation of blocking buffer (DAKO). After PBS rinse, the sections were incubated with anti-His tag polyclonal antibody (H15, Santa Cruz, USA) overnight at 4°C. Then, appropriate secondary antibody-conjugated with horseradish peroxidase was applied to the sections for 2 hours at RT. After washing by PBS, the signal of horseradish peroxidase was detected by 3-amino-9-ethylcarbazole (AEC) substrate chromogen (DAKO, Denmark). Nuclei were counterstained by using hematoxylin.

**AlphaScreen™ assay**

The binding between rTMD1 and carbohydrate polymers was analyzed as described previously. Briefly, biotinylated carbohydrate-PAA (Glycotech Corp., USA), rTMD1, anti-His tag polyclonal antibody (H15, Santa Cruz, USA), donor and acceptor beads (PerkinElmer Life Sciences, Inc., USA) were diluted with the assay buffer to an appropriate concentration. The anti-His tag antibody and acceptor beads were incubated in the assay buffer for 1 hour before use. Biotinylated carbohydrate-PAA, rTMD1 proteins and donor beads (5 μl for each) were added separately to the wells of the ProxiPlate-384 assay plates (PerkinElmer Life Sciences, Inc., USA) and incubated for 1 hour. An aliquot of the acceptor mixture was added into the wells and incubated at for another 2 hours. The results were obtained on the PerkinElmer Envision instrument using the AlphaScreen™ program. Relative intensities were obtained by normalization of the binding intensity of control.

**Statistics**

Data are represented as mean ± SEM. Statistical significance between two groups was analyzed by unpaired Student t-test. 3 or more groups of comparison were done by one-way ANOVA with post test of Tukey correction. A P-value < 0.05 was considered statistically significant.
References


10. Chang CF, Pan JF, Lin CN, Wu IL, Wong CH, Lin CH. Rapid characterization of sugar-binding specificity by in-solution proximity