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THE C-TERMINAL DOMAIN OF THROMBOMODULIN REGULATES MONOCYTE MIGRATION WITH INTERLEUKIN-6 STIMULATION

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Thrombomodulin (TM) is expressed on the surface of monocyte, which is important in the regulation of cell migration, proliferation, and inflammatory responses. In a previous study, we demonstrated that TM on monocyte is negatively associated with cell migration. However, the mechanisms involved in this process are unclear, therefore, we explored the mechanisms in this study. Chemotactic assays and immunofluorescence showed that TM siRNA increased the chemotaxis of the IL-6-activated THP-1, and aggravated actin assembly relative to the IL-6-treated control. In contrast, cells overexpressing plasmids containing full-length or domain 5 of TM followed by IL-6 treatment displayed lower chemotaxis and less actin assembly. Western blot analysis showed that TM knockdown markedly increased cytoskeleton components cofilin and LIMK1 phosphorylation in IL-6-treated THP-1, whereas, transfected cells with HA-TM FL or HA-TM D5, but not HA-TM D1-3 plasmids, reversed the effects. Activation of ERK1/2 and JNK/SAPK, upstream regulators of cytoskeleton components, were also inhibited in overexpressed group. Immunoprecipitation assay demonstrated that actin interacts with TM and intersectin1 in THP-1. Decreased interaction between intersectin1 and actin in TM knockdowns suggested that the interaction is mediated by TM. Our findings indicate that TM domain 5 is a negative regulator and seems to have the ability to inhibit paxillin, cofilin, LIMK1, and actin activation. The mechanisms for the repression effect of domain 5 may be mediated by inhibition of the ERK1/2 and JNK/SAPK activation. Expression of domain 5 of TM may represent a promising approach for controlling monocyte migration, and TM may have potential applications in treatment of inflammatory diseases.

Thrombomodulin (TM) acts as an anticoagulant, through the conversion of thrombin activity towards the activation of protein C. TM is a single-chain transmembrane glycoprotein with five distinct domains, including a C-type lectin-like domain, an epidermal growth factor (EGF)-like peptide (six sequential EGF-like structures are included), a serine/threonine-rich domain, a transmembrane domain and a cytoplasmic tail (1). TM was first discovered on endothelial cells where it may increase

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thrombin/protein C activation. The functional EGF-like domain is the major site that functions in anti-coagulation. EGF 3 is the region responsible for a thrombin-thrombomodulin complex interaction with the thrombin-activatable fibrinolysis inhibitor (TAFI). The EGF 4-6 regulatory site includes binding sites for calcium and thrombin and is important for the activation of protein C (2). Some residues within the fourth domain of TM may primarily interact with protein C but others are functionally important for altering the way TM interacts with thrombin (3). TM is a high-affinity receptor for thrombin. The TM-thrombin complex modulates the activation of protein C that is associated with coagulation (4). In addition, TM is also expressed on keratinocytes, megakaryocytes, platelets, and monocytes (5), the anti-inflammatory properties of which depend on the relative vigor of plasma protein C and TAFI.

Monocytes have multiple biological functions that involve the inflammatory response and coagulation. High levels of TM expression on monocytes are involved in the regulation of inflammatory and coagulation pathways (6). Decreases in TM levels result in the diminished activation of protein C and coupling of inflammation, as well as coagulopathic complications. The expression of TM by monocytes is regulated by a wide variety of pathophysiological conditions in humans. Down-regulation of TM on monocytes is associated with TNF-α production and poor early outcomes in patients following serious surgery (7). Additionally, knockdown of TM aggravates the chemotactic ability of monocytes in response to IL-6 induction (7). Although the underlying mechanism is unknown, it has been demonstrated that TM is involved in the migratory and inflammatory abilities of monocytes.

The TM extracellular domain consists of an *N*-terminal globular domain that is similar to C-type lectins. This domain plays a key role in regulating inflammation by maintaining the integrity of cell-cell interactions and preventing the transmigration of leukocytes (8). Additionally, this domain might prevent the disturbance of endothelial cells by suppressing intercellular vascular adhesion molecule-1 (VCAM-1) production and downregulating the nuclear factor kappa B (NF-kB) (9) and mitogen-activated protein kinase (MAPK) (10) pathways. Although overexpression of TM can inhibit the invasiveness and migratory ability of A549 cells (non-small-cell lung cancer cells) (11), endogenous TM supports smooth muscle cell migration (12) and decreases hepatocellular carcinoma cell migration through a decrease in E-cadherin gene expression (13). Previously, the function of TM in monocyte migration remained unknown. Therefore, the goal of this investigation was to study the role of full-length TM, as well as the function of individual TM domains, in IL-6-dependent monocyte migration.

MATERIALS AND METHODS

Cell culture

THP-1 cells, a human promyelomonocytic cell line, were obtained from the American Type Culture Collection (ATCC, VA, USA) and grown in RPMI 1640 medium with 2 mM L-glutamate, 4.5 g/L glucose, 10 mmol/L HEPES, 1.0 mmol/L sodium pyruvate, 10% fetal bovine serum, and 1% antibiotic-antimycotic mixture. Cell density was maintained between 5×10^4 and 8×10^5 viable cells/mL, and the medium was refreshed every 2-3 days.

Monocyte chemotaxis assay

Monocyte chemotaxis was measured using 24-well Micro Chemotaxis Transwells (Corning Costar, Cambridge, MA, USA). The cells were suspended at 2×10^6/mL in RPMI medium and loaded into the upper chamber. The 20 nM of recombinant IL-6 (R&D Inc., Minneapolis, MN, USA) with 1 mg/mL of bovine serum albumin was added to the lower chamber. The lower and upper chambers were separated by a polycarbonate membrane (5-mm pores). The THP-1 cells were left to transmigrate for 24 h at 37°C under humidified 5% CO_2 in air. After incubation, the number of monocytes that had migrated to the lower compartment was determined by counting the cells using light microscopy. Chemotactic activity was defined as the chemotactic index (the migrating cell number/the total cell number).

F-actin and immunofluorescence staining

THP-1 cells (1×10^5) were fixed with 4% paraformaldehyde and quickly plated onto coverslips using a Shandon CytoSpin III Cytocentrifuge (GMI, MS, USA). Cell membranes were fenestrated with 0.4% Triton X-100-PBS, and nonspecific binding sites were blocked with 2% BSA-PBS-Tween 20 (0.1% v/v), followed by staining with TRITC-conjugated phalloidin, mouse anti-TM (Abcam, MA, USA), rabbit anti-b-actin (GeneTex, CA, USA), or chicken anti-intersectin1 (GeneTex, CA, USA) antibodies and incubation with secondary

antibodies that were Alexa-conjugated. Hoechst stain was used to identify the nuclei of THP-1 cells. All slides were examined by confocal microscopy (LSM510, ZEISS Inc., GERMANY) or fluorescent microscopy.

Knockdown of gene expression by RNA interference

The knockdown of TM gene expression was performed by siRNA transfection. The cells (3×10^6) were suspended in 2.5 mL of serum-free medium, and 10 or 25 mM of TM siRNA duplexes (Invitrogen Catalog THBD-HSS110719, 110721, 186320, NY, USA) were transfected according to the manufacturer's instructions. Silencer Validated siRNA (negative control siRNA, Santa Cruz Catalog sc-44230, CA, USA) was used to validate the knockdown. The cells were seeded into six-well plates immediately following transfection for further experiments 24 h after transfection. Following knockdown of gene expression by RNA interference TM expression was analyzed by flow cytometry and confocal microscopy.

Construction of HA-tagged TM-FL, TM D5, and TM D1-3 expression vectors

The pCDNA3.1/ V5-His-TM plasmid containing a segment of the TM open reading frame was a gift from Professor Yu-Jia Chang, Taipei Medical University, Taiwan. For the construction of the influenza hemagglutinin (HA) epitope-tagged full-length TM expression plasmid (HA-TM FL), the pCDNA3.1/ V5-His-TM plasmid was first digested with the SacI and XhoI enzymes, filled in with Klenow, and subsequently cloned in-frame into the SmaI sites of the pXJN-HA vector which contains the chick b-globin intron downstream (3') of the CMV promoter. The HA-TM D5 construct that contained a fragment of TM domain 5 and the HA-TM D1-3 construct that contained a fragment of TM domain 1-3 were obtained by using HA-TM FL as a template for the Reaction of the PCR-mediated deletion of plasmid DNA (14).

Western blot analysis

Total cell lysates were extracted from THP-1 cells. Proteins were separated by reducing SDS-PAGE and transferred to PVDF membrane. The membranes were probed with rabbit anti-phospho-paxillin (Abcam, MA, USA), rabbit anti-paxillin (Abcam, MA, USA), rabbit anti-phospho-cofilin (Abcam, MA, USA), mouse anti-cofilin (Abcam, MA, USA), rabbit anti-phospho-LIMK1 (Abcam, MA, USA), mouse anti-LIMK1 (Abcam, MA, USA), rabbit anti-p38, rabbit anti-phospho-p38, rabbit anti-JNK/SAPK, rabbit anti-phospho-JNK/SAPK, rabbit anti-p44/p42 MAPK, or mouse anti-phospho-p44/p42 MAPK antibody. All MAPK antibodies were purchased from Cell Signaling Technology in the USA. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Merk Millopore, Darmstadt, Germany) or HRP-conjugated goat anti-mouse IgG antibody (Merk Millopore, Darmstadt, Germany). The proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, NJ, USA). Mouse anti-b-actin (Labvision/NeoMarkers, CA, USA) antibody was used as loading control.

Immunoprecipitation

THP-1 cells with or without TM siRNA transfection were lysed in buffer containing 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 1% NP-40, and 1X protease inhibitor cocktail (Sigma, MO, USA). Lysed cells were put on ice for 30 min and centrifuged at 10,000 x g for 30 min to remove cell debris. Protein concentration was determined using a Bio-Rad Protein Assay Kit (BioRad Inc., CA, USA) with BSA as the standard. Approximately 1 mg of protein extract was pre-cleaned with 20 mL of a 50% protein A/G suspension mixture (protein A: BioRad, Inc., CA; protein G: Amersham, IL, USA). Pre-cleaned lysates were immunoreacted with anti-actin antibody (Millipore, MA, USA) at 4°C for 16 h, then immunoprecipitated by adding 50 mL of a 50% protein A/G sepharose mixture at 4°C for 1.5 h. The beads were washed three times with ice-cold lysis buffer, the bound proteins were eluted with 2X SDS–PAGE loading buffer and subjected to Western blot analysis. The blots were probed with mouse monoclonal anti-TM antibody (Abcam, MA, USA) or a chicken polyclonal antibody against intersectin1 (GeneTex, CA, USA).

Statistical analyses

The values were expressed as the means ± SD. Statistical evaluations were performed using a Student's *t*-test and one- or two-way ANOVA followed by Dunnett's test. A P value of < 0.05 was considered significant.

RESULTS

TM is associated with monocyte chemotaxis and actin organization

Previously, we demonstrated that IL-6 induces monocyte chemotaxis (7). In this study, we show that the chemotactic index of THP-1 cells was higher in the IL-6-chemotactic groups compared with the random migration group in a dose- and time-dependent manner (data not show). The efficiency of TM siRNA was identified as FACS and immunofluorescent staining. As shown in Fig. 1A, 10 or 25 nM TM-specific siRNA increased the chemotactic index of THP-1 cells relative

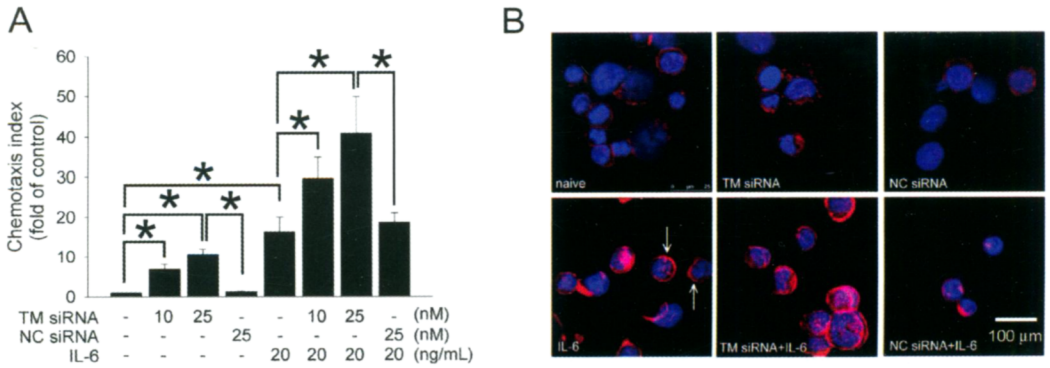


Fig. 1. *TM is associated with monocyte chemotaxis and actin organization.* ***A)*** *THP-1 cells were transfected with 10 or 25 nM TM siRNA for 24 h followed by 20 ng/mL IL-6 stimulation for 24 h. Cell chemotaxis was then analyzed. Silencer-validated siRNA (NC siRNA) was used for knockdown validation. Data represent the results of 5 independent experiments (mean ± SD; *P < 0.05).* ***B)*** *Actin organization of THP-1 cells was monitored with TRITC-labeled phalloidin and observed by confocal microscopy. Hoechst stain was used to label the nucleus. The scale bar indicated 100 mm.*

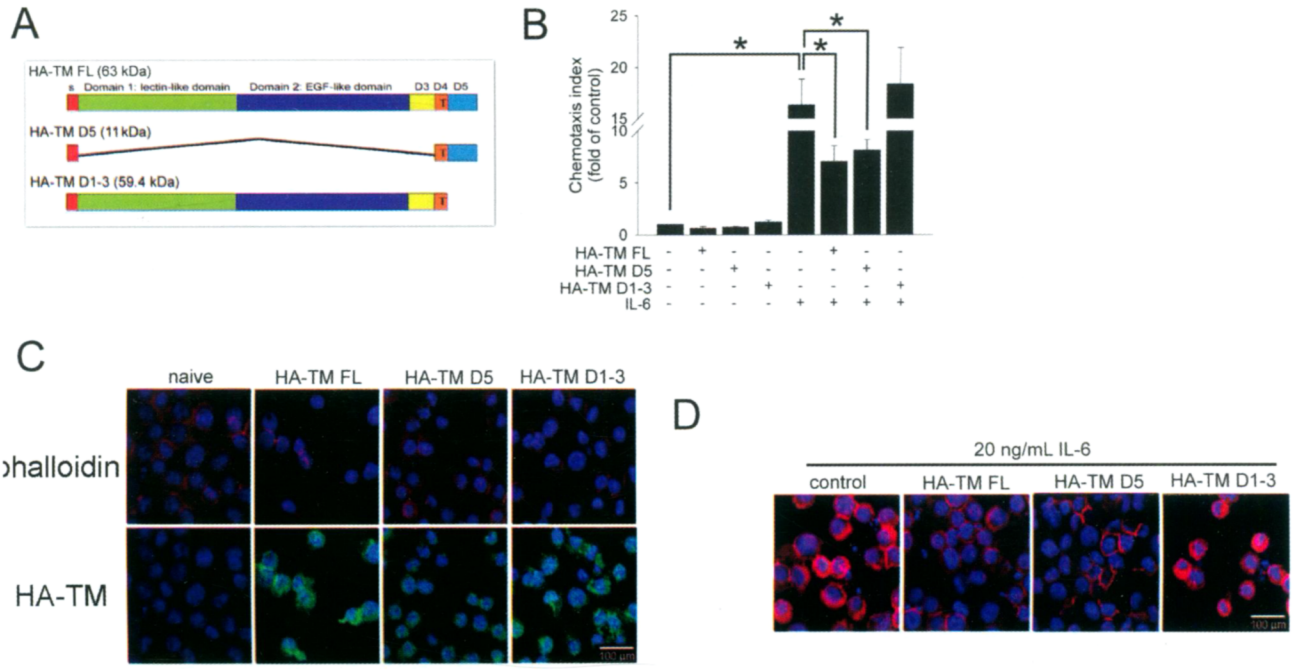


Fig. 2. *Domain 5 of TM is required for IL-6-induced THP-1 chemotaxis and actin organization.* ***A)*** *Schematic representation of the various plasmids. containing full length TM, domain 5 or domain 1-3 of TM. Labels are: S, signal peptide; D1, lectin-like domain; D2, EGF-like domain; D3, serine/threonine-rich domains; D4, transmembrane domain; D5, cytoplasmic tail domain.* ***B)*** *THP-1 cells were transfected with various plasmids (HA-TM FL, HA-TM D5 or TM D1-3) for 24 h followed by 20 ng/mL of IL-6 stimulation for 24 h. Cell chemotaxis levels were analyzed. Data represent the results of 5 independent experiments (mean ± SD; *P < 0.05).* ***C)*** *THP-1 cells were transfected with HA-TM FL, HA-TM D5 or TM D1-3 plasmid and 24 h later actin organization was monitored with TRITC-labeled phalloidin. TM was identified using an anti-HA Alexa488-conjugated antibody and immunofluorescence.* ***D)*** *THP-1 cells transfected with the HA-TM FL plasmid, the HA-TM D5 plasmid or the TM D1-3 plasmid were treated with 20 ng/mL IL-6 for 24 h. Actin organization was monitored with TRITC-labeled phalloidin. Hoechst stain was used to label the nucleus. The scale bar indicated 100 mm.*

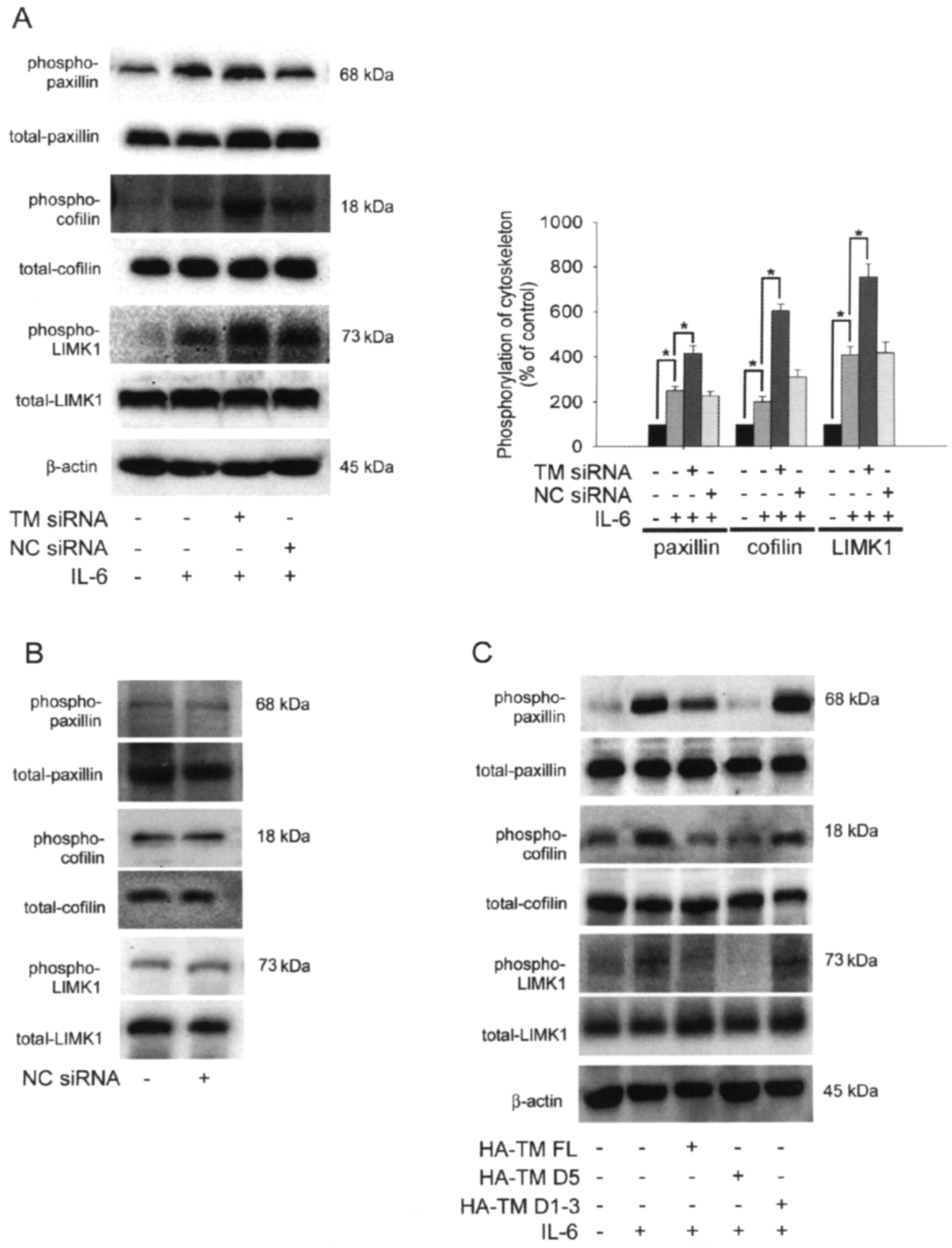


Fig. 3. *Domain 5 of TM regulates the activation of paxillin, cofilin, and LIMK1. **A** and **B**) Naïve or TM siRNA-transfected THP-1 cells were treated for 12 h with 20 ng/mL IL-6 (**A**) or without IL-6 treatment (**B**). Paxillin, cofilin, and LIMK1 activation (phosphorylation) was analyzed by western blot analysis. NC siRNA was used for knockdown validation. The density of band was quantified using densitometry and showed as bar graph. Data are expressed as % of control, presented as the mean ± SD and represent the results of three independent experiments (n=3, *P < 0.05 was considered significant). **C**) THP-1 cells were transfected with HA-TM FL, HA-TM D5 or TM D1-3 plasmid followed by 20 ng/mL IL-6 treatment for 12 h. Paxillin, cofilin, and LIMK1 phosphorylation was analyzed by Western blot analysis. Total paxillin, cofilin, LIMK1 and b-actin were used as loading controls.*

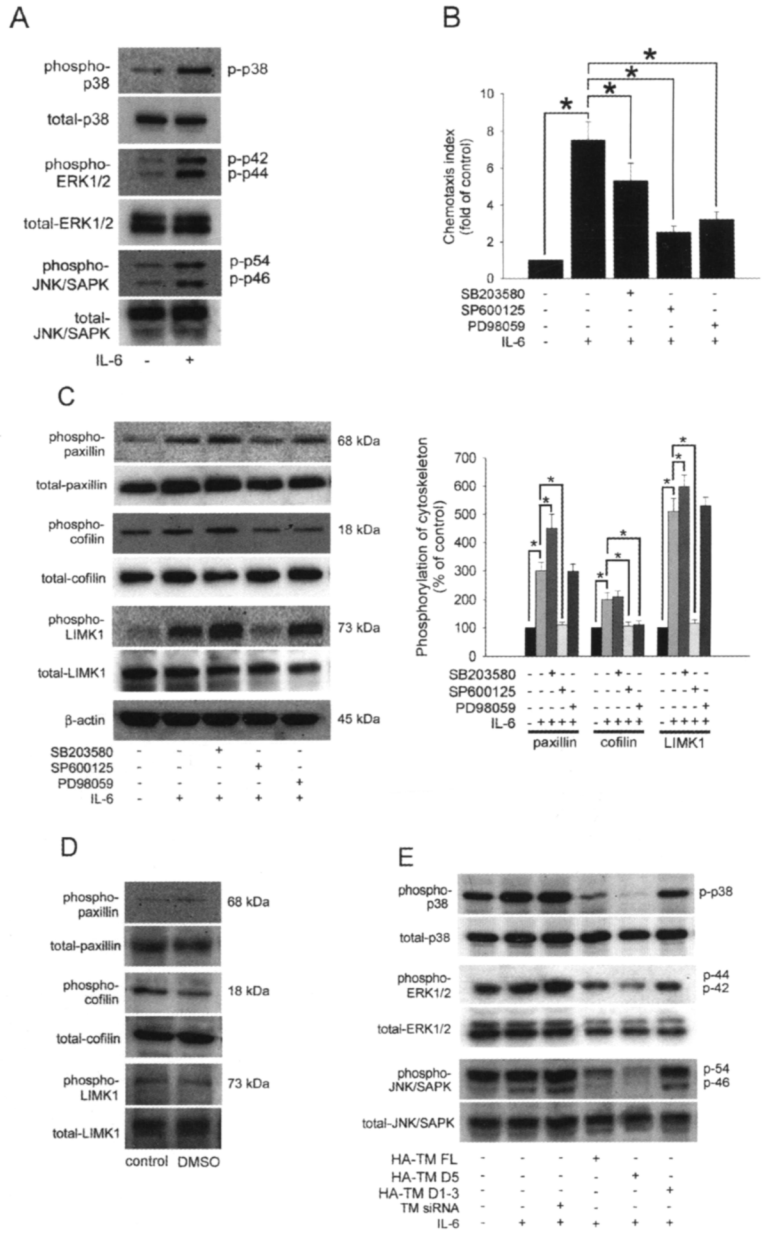


Fig. 4. *Domain 5 of TM may regulate MAPK activation in a process that requires cytoskeleton activation.* ***A****) THP-1 cells were treated for 6 h with 20 ng/mL IL-6. The phosphorylation states of p38 MAPK, ERK1/2, and JNK/SAPK were analyzed by western blot analysis. Total p38 MAPK, ERK1/2, and JNK/SAPK were used as loading controls.* ***B****) THP-1 cells were pretreated with 10 μM SB203580 (SB), SP600125 (SP), or PD98059 (PD) for 1 h prior to stimulation with IL-6 for 24 h. THP-1 cell chemotaxis was then analyzed. Data represent the results of 5 independent experiments (mean ± SD; *P < 0.05).* ***C****) THP-1 cells were pretreated with SB, SP, or PD for 1 h prior to stimulation with IL-6 for 12 h. Paxillin, cofilin, and LIMK1 phosphorylation was analyzed by Western blot analysis. Total paxillin, cofilin, LIMK1 and b-actin were used as loading controls. The density of band was quantified using densitometry and showed as bar graph. Data are expressed as % of control, presented as the mean ± SD and represent the results of three independent experiments (n=3, *P < 0.05 was considered significant). (D) THP-1 cells were pretreated with SB, SP, or PD for 12 h. Paxillin, cofilin, and LIMK1 phosphorylation was analyzed by Western blot analysis. b-actin was used as a loading control. (E) THP-1 cells were transfected with HA-TM FL, HA-TM D5 or TM D1-3 plasmid followed by 20 ng/mL IL-6 treatment for 6 h. Phosphorylation of p38 MAPK, ERK1/2, and JNK/SAPK was monitored by western blot analysis. Total p38 MAPK, ERK1/2, and JNK/SAPK were used as loading controls.*

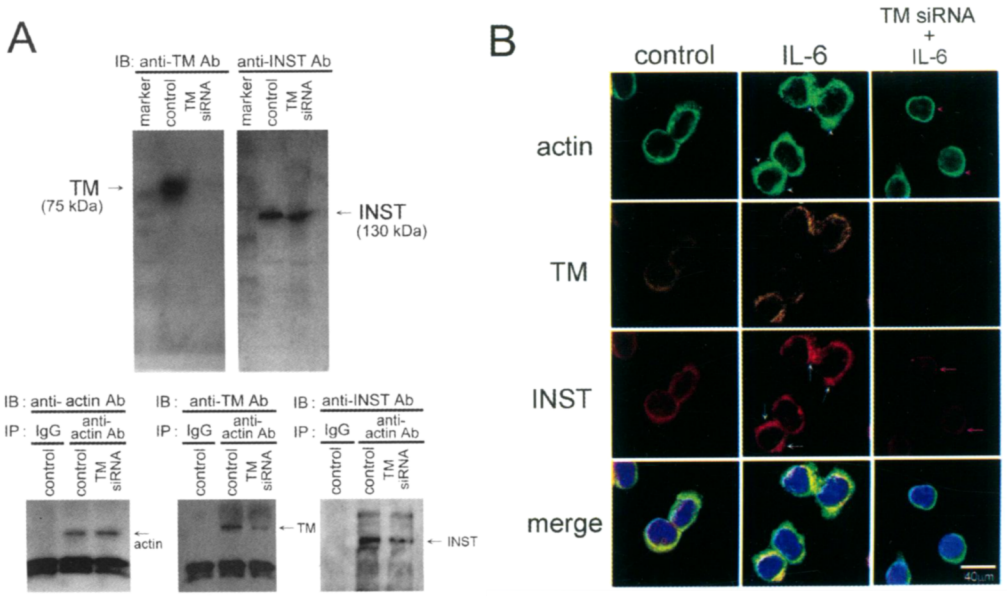


Fig. 5. *TM and intersectin1 interact with the actin cytoskeleton. **A**, upper panels) Total lysate from control THP-1 cells or TM siRNA-transfected cells was immunoblotted with anti-TM antibody or anti-intersectin1 antibodies, respectively. **A**, lower panels) Lysates of THP-1 cells with or without TM siRNA transfection were immunoprecipitated by using an anti-b-actin or a control IgG antibody followed by immunoblotting with antibodies against b-actin, TM or intersectin1 (INST). **B**) THP-1 cells with or without TM siRNA transfection were stimulated with 20 ng/mL IL-6 for 12 h. Intracellular b-actin, INST and TM were identified with specific antibodies by immunofluorescence and observed by confocal microscopy. Hoechst stain was used to label the nucleus. The scale bar indicated 40 mm.*

to that of the control group (6.92±1.23 fold and 10.62±1.40-fold relative to the control, respectively). Furthermore, the TM siRNA, but not the negative control (NC) siRNA, exacerbated the chemotactic index in IL-6-induced THP-1 cells. As shown in Fig. 1B by immunofluorescence, naïve THP-1 cells showed prominent phalloidin staining. F-actin staining with phalloidin was enhanced following stimulation with 20 ng/mL IL-6 for 24 h. Compared with the naïve control group, IL-6 appeared to induce actin assembly and organization (white arrow). Interestingly, TM siRNA knockdown, but not the NC siRNA treatment, slightly increased actin assembly in THP-1 cells. Furthermore, TM siRNA knockdown enhanced IL-6-stimulated actin assembly. These results strongly suggest that TM level on cell surface is associated with F-actin assembly in response to IL-6-stimulated cell migration in monocytes.

Domain 5 (intracellular domain) of TM is a negative regulator for IL-6-induced THP-1 chemotaxis

To investigate which domain of TM regulates monocyte migration, expression plasmids containing variable TM domains were transfected into THP-1 cells. A schematic representation of the various plasmids containing the TM domains is depicted in Fig. 2A. Expression of plasmids encoding different TM domains in HeLa and THP-1 cells was confirmed by using confocal microscopy, Western blot analysis or flow cytometry, respectively (data not show). Cells transfected with the HA-TM FL, HA-TM D5 or TM D1-3 plasmid had no effect in chemotactic index relative to the control THP-1 cells. Interestingly, cells transfected with HA-TM FL or HA-TM D5 plasmid followed by IL-6 treatment displayed a lower chemotactic index compared with the IL-6-treated group. In contrast, the TM D1-3 plasmid did not affect IL-6-induced cell chemotaxis (Fig. 2B). HA-tagged TM expression and actin organization were analyzed by immunofluorescence. Transfection with plasmid resulted in expression of HA-TM in the cells and did not change the distribution of actin in THP-1 cells (Fig. 2C). Similar to the data presented in Fig. 2B, cells that were transfected with HA-TM

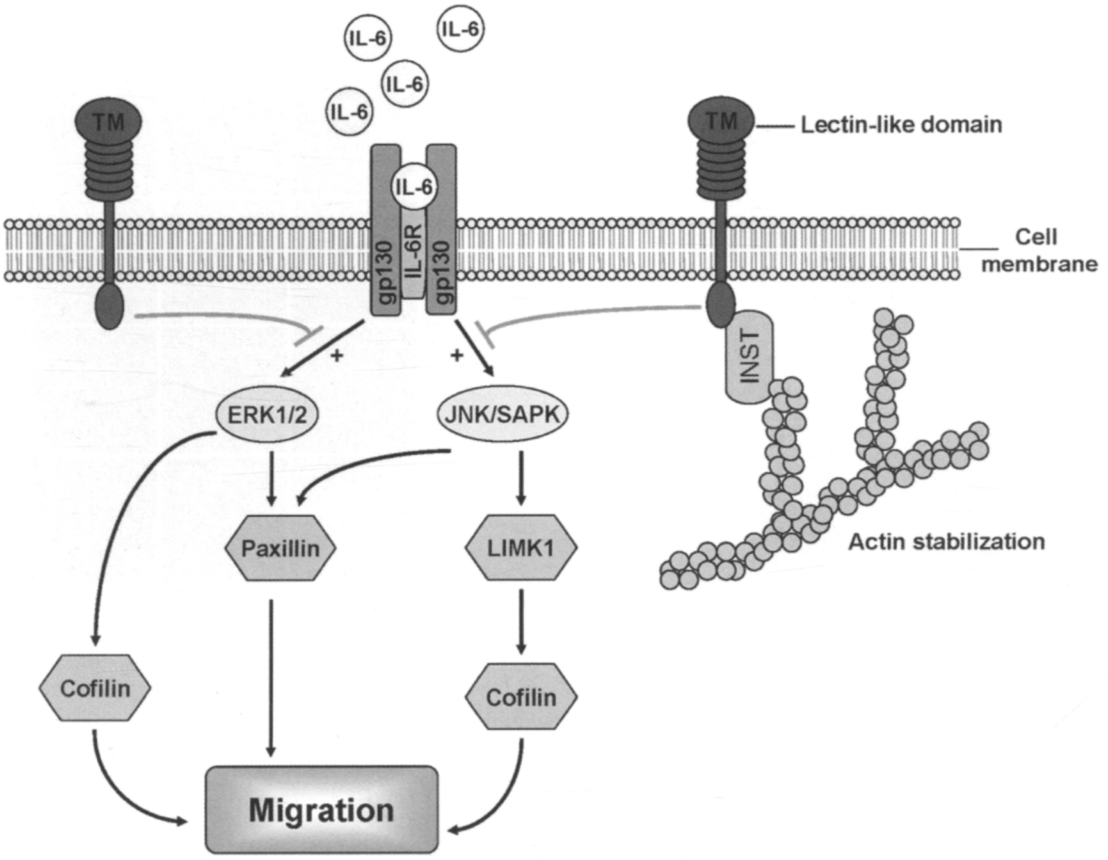


Fig. 6. *The mechanism by which TM contributes to IL-6-mediated migration in monocytic cells. TM may regulate cell migration via its intracellular domain (the fifth domain), which acts as an extinguisher for p38 MAPK ERK1/2 and JNK/SAPK activation, as well as further downstream paxillin, cofilin, and LIMK1 phosphorylation. Interaction between TM, actin cytoskeleton, and intersectin, an actin filament assembly inducer, further supported a role for TM in cell migration.*

FL or HA-TM D5 and treated with IL-6 displayed less actin assembly relative to the IL-6-treated group. In contrast, the TM D1-3 plasmid did not decrease IL-6-induced actin assembly (Fig. 2D). These results demonstrate that domain 5 (the intracellular domain) of TM is responsible for regulation of THP-1 migration, and act as a negative regulator for IL-6-induced THP-1 chemotaxis.

TM domain 5 (intracellular domain) has the ability to inhibit the activation of paxillin, cofilin, and LIMK1 mediated by IL-6 stimulation in THP-1 cells

The cytoskeleton is a key component that allows cells to continue to proliferate and maintain migration (15,16). To study for cytoskeleton molecule activation, THP-1 cells under different treatment were collected for Western analysis by using paxillin, cofilin, and LIMK1 specific antibodies. Fig. 3A shows that naïve THP-1 exhibited less paxillin, cofilin, and LIMK1 phosphorylation. However, treatment with 20 ng/mL of IL-6 resulted in the increased phosphorylation of paxillin, cofilin, and LIMK1. Additionally, TM knockdown resulted in a marked aggravation of cofilin and LIMK1 phosphorylation in IL-6-treated THP-1 cells; however, this treatment had only minor effect on paxillin (Fig. 3A). In contrast, transfection with NC siRNA did not affect the phosphorylation of paxillin, cofilin, and LIMK1 in either naïve or IL-6-stimulated THP-1 cells (Fig. 3A and B). To explore the TM domain that is involved in regulation

of cytoskeletal molecules phosphorylation, we engineered THP-1 cells to express various TM domains. As shown in Fig. 3C, HA-TM FL or HA-TM D5 plasmid but not HA-TM D1-3 transfections inhibited the phosphorylation of paxillin, cofilin, and LIMK1 in IL-6-treated cells. These results indicate that intracellular domain (domain 5) of TM plays a role in inhibiting the activation of paxillin, cofilin, and LIMK1 mediated by IL-6-stimulation in THP-1 cells.

TM domain 5 (intracellular domain) acts as an extinguisher for MAPK activation that is involved in downstream paxillin, cofilin, and LIMK1 activation

IL-6 activates MAPK signaling pathways, leading to the inflammatory response. In the present study, IL-6 markedly induced MAPK phosphorylation, including p38 MAPK, ERK1/2, and JNK/SAPK (Fig. 4A) following exposure to 20 ng/mL IL-6 for 6 h. To determine the specific role of the MAPKs in IL-6-induced chemotaxis and cytoskeleton activation in THP-1 cells and to clarify whether TM is involved in the activation of MAPK signaling pathways, we treated THP-1 cells with SB203580 (a p38 MAPK inhibitor), SP600125 (a JNK/SAPK inhibitor), or PD98059 (an ERK1/2 inhibitor) prior to IL-6 treatment. SB203580, SP600125, and PD98059 significantly decreased chemotaxis in the IL-6-stimulated THP-1 cells (IL-6 group, 7.5±0.9-fold; SB203580 group, 5.3±0.9-fold; SP600125 group, 2.5±0.3-fold and PD98059 group, 3.2±0.4-fold, all relative to the control) (Fig. 4B). Additionally, preincubation of the cells with 10 mM SP600125 or PD98059 for 1 h decreased cofilin phosphorylation, respectively, compared with 20 ng/mL IL-6 treatment alone. LIMK1 and paxillin phosphorylation in IL-6-treated cells were decreased by pretreatment with SP600125. In contrast, pretreatment with SB203580 did not decreased paxillin, cofilin and LIMK1 activation (Fig. 4C). Treatment of 1% dimethyl sulfoxide (DMSO, a solvent of SP600125, PD98059 and SB203580) were used to comparisons (Fig. 4D). Furthermore, Western blot analysis demonstrated that TM siRNA knockdown slightly increased intracellular MAPKs activation in IL-6 stimulated THP-1 cells. Interestingly, transfection with the HA-TM FL or HA-TM D5 plasmids inhibited p38 MAPK, ERK1/2, and JNK/SAPK activation in IL-6-stimulated THP-1 cells. The HA-TM D1-3 plasmid only slightly decreased the activation of ERK1/2 in IL-6-stimulated THP-1 cells (Fig. 4E). These results suggest that intracellular domain (domain 5) of TM seems to be a repressor for IL-6-activated p38 MAPK, ERK1/2 and JNK/SAPK phosphorylation that further regulates the downstream activation of the paxillin, cofilin, and LIMK1 signaling pathways. In short, the cytoplasmic tail domain of TM has the ability to effectively regulate the migration of THP-1 cells via controlling the MAPK activity and cytoskeletal activation.

Interaction of TM and intersectin1 with the actin cytoskeleton

Because TM plays a role in regulation of THP-1 migration, we wanted to study whether TM interacts with the actin cytoskeleton in THP-1 cells. We performed immunoprecipitation assays to verify that actin could coimmunoprecipitate TM and intersectin1 from THP-1 cells. As shown in Fig. 5A, Western blot analysis showed that TM was present in untreated THP-1 cell lysates and was absent in cells transfected with TM siRNA. Immunoprecipitation of b-actin from THP-1 cells with an anti-actin monoclonal antibody was performed, and interactions were analyzed for the presence of TM by Western blotting. The immunoprecipitation-Western data showed that TM interacts with b-actin. The interaction between TM and b-actin was specific because a decreased TM signal was observed in cells transfected with TM siRNA (Fig. 5A, bottom left). Intersectin1 is an actin filament assembly inducer. As we wanted to establish that an interaction occurs between intersectin1 and actin in THP-1 cells, immunocomplexes obtained by anti-actin antibody were also analyzed for the presence of intersectin1. Indeed, Intersectin1 interacts with actin in THP-1 cells. A decreased signal in TM knockdown THP-1 cells indicated that the interaction between actin and intersectin1 is mediated by TM level in THP-1 cells (Fig. 5A, bottom right). As shown by the immunofluorescence assays in Fig. 5B, control THP-1 cells showed dispersed actin, intersectin1 and TM staining. Compared with the control group, IL-6 induced actin assembly (white arrowhead), intersectin 1 aggregation (white arrow), and actin colocalization with TM and intersectin1 (merge

yellow staining). The siRNA knockdown of TM efficiently abolished all detectable TM. Interestingly, TM siRNA knockdown followed by IL-6 treatment significantly enhanced the actin assembly (red arrowhead) and decreased the intersectin 1 aggregation (red arrow) as well as the colocalization of intersectin1/actin compared with the behavior in naïve cells treated with IL-6. According to these results, we speculated that TM, intersectin1 and the actin cytoskeleton probably directly form immunocomplexes and suggest a role for TM in migration. In addition, interaction of intersectin1 with actin is involved in the TM-mediated regulation of migration in THP-1 cells.

DISCUSSION

The schematic diagram in Fig. 6 summarizes our current results. We demonstrated for the first time that TM might decrease cell migration in IL-6-stimulated monocytic cells. In fact, TM may regulate cell migration by its intracellular domain (the fifth domain), which acts as an extinguisher for p38 MAPK ERK1/2 and JNK/SAPK activation, as well as further downstream paxillin, cofilin, and LIMK1 phosphorylation. Furthermore, our *in vitro* findings suggest that TM interacts with the actin cytoskeleton and simultaneously interacts with intersectin. Interaction between TM, actin cytoskeleton, and intersectin, an actin filament assembly inducer, further supported a role for TM in cell migration.

Previous studies also focused on the ability of TM to mediate anti-inflammatory effects via its lectin-like domain. The TM lectin-like domain might regulate inflammation by maintaining the integrity of cell-cell interactions and preventing the transmigration of leukocytes (8). This domain might also prevent the disturbance of endothelial cell and neutrophil-mediated tissue damage by suppressingVCAM-1 production through a calcium-dependent interaction of lectin-like domain (17) and downregulation of the NF-kB pathway (10). The recombinant serine/threonine-rich domain or the EGF-like domain of TM enhanced the angiogenic response (18), suppressed inflammation in the vasculature (19), and protected the lung against tissue-damaging pro-inflammatory responses following ischemia-reperfusion (20), suggesting that TM fragments may play a role in the formation of new vessels and protection of reperfusion injury. Lysophosphatidic acid stimulates TM lectin-like domain shedding in human endothelial cells and may bind to its specific ligand Lewis Y antigen and neutralize the lipopolysaccharide-induced inflammatory response (21). Previous researchers have mostly focused on exploring the function and roles of the EGF-like domain of TM; however, little is known about the function and importance of the TM cytoplasmic tail. During chronic inflammation, the migration of monocytes is an important process. Expression of TM on monocytes is associated with early outcomes in patients with coronary artery bypass graft surgery (7) and adjustment of cancer cell growth (22). We are the first group to demonstrate that domain 5 of TM is important for monocyte migration.

TM is expressed on keratinocytes, megakaryocytes, platelets, and monocytes (5) and is also expressed and regulated in many kinds of tumor cell growth and metastasis, including pulmonary adenocarcinoma (23) and hepatocellular carcinoma (24). These studies demonstrated that while higher TM expression increases survival rate, malignant and metastatic cancer cells display lower TM expression compared with benign tumor cells. Knockdown of TM may enhance the ability of a lung adenocarcinoma cell line (A549 cells) to undergo invasion and migration (11). In contrast, overexpression of TM may regulate the activation of the PI3K/Akt signaling pathway that results in the inhibition of A549 cell migration (11). The migration of vascular smooth muscle cells (VSMCs) from media and the infiltration of monocytes (25) to subendothelial spaces play key roles during atherogenesis and in the process of restenosis after percutaneous transluminal coronary angioplasty. Interestingly, TM on VSMCs performs the opposite function in migration compared with its function in tumor cells. A high level of TM expression may induce VSMC proliferation and migration, which deteriorate atherosclerotic lesion formation (26). Previous research lead scientists to predict that platelet-derived growth factor (PDGF)-BB enhances PI3K/Akt activation that results in the binding of AP-1 components to the TM promoter which regulating cells migration (12). Although our recent studies predicted that monocytes that express TM

may be associated with inflammation (7) and that TM expression will also be influenced by drugs, we still do not understand the mechanisms that underlie these observations. In this study, we fortuitously discovered a potential role for TM in monocyte migration. We discovered a new, unprecedented role for TM domain 5 (the cytoplasmic tail), but not TM domain 1-3 (the EGF-like domain), in IL-6-stimulated monocytes. Additionally, the domain 5 of TM alone may recapitulate the effect of the full length of TM in IL-6-stimulated monocytes.

It is well known that IL-6 may induce actin reorganization that results in monocyte migration (27). When LIMK (a key regulator of cytoskeletal organization that is involved in cell migration) receives a chemotactic signal it activates actin depolymerizing factor/cofilin and induces actin reorganization (28). In addition to the dynamic changes in actin filaments, the formation and disassembly of cell adhesion sites are also involved in cell migration (29). Paxillin is a cytoskeletal adaptor protein that may associate with focal adhesion complexes and crucial regulate cell migration (30). The angiotensin II type 1 receptor activates Src, ERK1/2, and p38 MAPK signaling, which leads to the phosphorylation of PYK2 (protein tyrosine kinase 2) and paxillin, enhances cytoskeletal focal adhesion formation in monocytes, and increases cell migration (31). Our results predict that knockdown of TM might accelerate the activation (phosphorylation) of LIMK1 and cofilin as well as actin reorganization. Activated paxillin might transduce extracellular signals and transmit them to the intracellular cytoskeleton. Furthermore, we predict that overexpression of TM inhibits the activation of the cytoskeleton, p38 MAPK, ERK1/2 and JNK/SAPK, which is an affect that is also observed with TM domain 5 alone. We conclude that the intracellular domain of TM regulates the activation of MAPKs in a process that involves reorganization of the cytoskeleton. Additionally, consistent with a role for intersectin1 in the endocytic machinery and the induction of actin filament assembly (32) as well as the discovery by Professor Huey-Chun Huang that TM interacts with actin and intersectin-1 (as shown by LC-MS/MS analysis in HaCaT cells, a human keratinocyte; unpublished data at http://ndltd.ncl.edu.tw/cgi-bin/gs32/gsweb.cgi?o=dnclcdr&s=id=%22098CMCH5108016%22.&searchmode=basic), we have demonstrated that TM directly interacts with actin and intersectin-1, indicating that TM regulates cytoskeletal activation by the continual association of intersectin that inhibits actin reorganization and cell migration. According to a recent study, ERK1/2 and JNK/SAPK may play key roles in the regulation of endogenous TM expression in naïve THP-1 cells. However, domain 5 of TM is involved in IL-6-dependent ERK1/2 and JNK/SAPK phosphorylation that regulates paxillin, cofilin, and LIMK1 signaling pathway activation. Domain 5 of TM might be sufficient to regulate the migration of THP-1 cells, which mediates MAPK activity and cytoskeleton reorganization. Future research will focus on the relationship between TM and MAPKs to better understand this regulatory pathway.

In conclusion, our investigation explores that the c-terminal domain of thrombomodulin regulates the migration of interleukin-6-stimulated monocytes. These results could represent a promising approach to prevent inflammation and preserve intact immunity in patients via the regulation of TM expression.

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