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LFA-1 and Mac-1 integrins bind to the serine/threonine-rich domain of thrombomodulin

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ABSTRACT

LFA-1 (αLβ2) and Mac-1 (αMβ2) integrins regulate leukocyte trafficking in health and disease by binding primarily to IgSF ligand ICAM-1 and ICAM-2 on endothelial cells. Here we have shown that the anti-coagulant molecule thrombomodulin (TM), found on the surface of endothelial cells, functions as a potentially new ligand for leukocyte integrins. We generated a recombinant extracellular domain of human TM and Fc fusion protein (TM-domains 123-Fc), and showed that pheripheral blood mononuclear cells (PBMCs) bind to TM-domains 123-Fc dependent upon integrin activation. We then demonstrated that αL integrin-blocking mAb, αM integrin-blocking mAb, and β2 integrin-blocking mAb inhibited the binding of PBMCs to TM-domains 123-Fc. Furthermore, we show that the serine/threonine-rich domain (domain 3) of TM is required for the interaction with the LFA-1 (αLβ2) and Mac-1 (αMβ2) integrins to occur on PBMCs. These results demonstrate that the LFA-1 and Mac-1 integrins on leukocytes bind to TM, thereby establishing the molecular and structural basis underlying LFA-1 and Mac-1 integrin interaction with TM on endothelial cells. In fact, integrin-TM interactions might be involved in the dynamic regulation of leukocyte adhesion with endothelial cells.

1. Introduction

Integrins represent an important family of immune receptors that mediate the cell adhesion of leukocytes to endothelial cells. The adhesive interactions of leukocytes and integrins with their cognitive ligands on endothelial cells allow immune cells to patrol the surface of the vasculature. During this intravascular surveillance, some immune cells encounter inflammatory signals displayed on endothelial cells, inducing further activation and facilitating stable, firm adhesions and subsequent crawling, which leads finally to transendothelial migration and extravasation. Intercellular adhesion molecules (ICAMs) and junctional adhesion molecules (JAMs) are the major known immunoglobulin superfamily (IgSF) endothelial ligands for leukocyte integrins that mediate extravasation to sites of inflammation. Of note, a few anti-coagulant molecules present on the surface of endothelial cells {e.g., activated protein C (APC), endothelial protein C-receptor (EPCR)} have recently been shown to interact with leukocyte integrins [1,2], thereby suggesting a potential link between the immune and coagulation systems.

Thrombomodulin (TM) is an anti-coagulant molecule expressed on endothelial cells that has demonstrated anti-inflammatory properties. TM has a large extracellular portion (domains 1–3), a single transmembrane domain (domain 4), and a short cytoplasmic part (domain 5). The extracellular portion contains three distinct domains including the N-terminal C type lectin domain (domain 1), the epidermal growth factor (EGF)-like domain (domain 2), and the membrane proximal serine/threonine-rich domain (domain 3). The anti-coagulant activities are primarily included in the TM-domains 2 and 3, through which TM forms a complex with thrombin to

Abbreviations: TM, thrombomodulin; rhsTM, recombinant human soluble thrombomodulin; rhAPC, recombinant human activated protein C; CBB, Coomassie Brilliant Blue; PBMCs, pheripheral blood mononuclear cells.

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generate APC that degrades factor Va and VIIIa, thereby regulating balance among the coagulation pathways. By contrast, anti-inflammatory activities occur, at least partly, in the TM-domain 1, which has been shown to interact not only with the High Mobility Group Box 1 (HMGB1) released from necrotic cells [3] and activated immune macrophages [4], but also with Lews Y antigen released either from gram-negative bacteria or expressed on endothelial cells [5]. More recently, the TM-domains 2 and 3 have been shown to interact with CD14, and to inhibit the CD14-mediated inflammatory response [6]. The ability of TM to support both anti-coagulation and anti-inflammatory activities is believed to contribute to the therapeutic effects of a recombinant soluble TM (Recomodulin™ or ART-123) administered for the treatment of septic shock in animals [7].

Exogenously administered soluble TM is, therefore, thought to interact with receptors on immune cells and endothelial cells, thereby alleviating inflammation. However, whether endogenous endothelial TM would interact with leukocytes, and if so by what molecular and structural mechanisms, remain to be elucidated. Here we have shown that the leukocyte integrins LFA-1 (αLβ2) and Mac-1 (αMβ2) specifically bind to TM on the substrate and have identified the TM-domain 3 as the integrin binding site.

2. Materials and methods

2.1. Construction of recombinant human thrombomodulin domains 1, 2, and 3− immunoglobulin Fc portion fusion protein (TM-domains 123-Fc) expression vectors

A DNA fragment containing TM-domains 123 was PCRed out from a pSV2TMJ2 vector that contained the entire sequence for human TM [8] using the KOD FX Neo reagents (TOYOBO, Osaka, Japan). A forward primer with an engineered HindIII restriction enzyme site and a reverse primer with an engineered BamHI site were used. The TM-domains 123 fragment was digested with Hind III and BamHI, and subcloned into the Hind III/BamHI site in frame to the human IgG1 Fc fragment of the pcDNA3.1 (+) vector (Supplementary Table S1a.).

A panel of domain-deleted mutants of soluble TM-Fc fusion protein (i.e., TM-domains 23-Fc that lacks domain 1, TM-domains 13-Fc that lacks domain 2, TM-domains 12-Fc that lacks domain 3, and TM-domains 3-Fc that lacks domains 1 and 2) were generated by inverse PCR using a KOD Plus Mutagenesis Kit (TOYOBO) on the pcDNA3.1 (+) containing TM-domains 123-Fc plasmid as a template. In addition to the universal outer primers, muTM-1 and muTM-2, the following inner primers were used: muTM-3 and muTM-4 for deleting TM domain 2; muTM-6 and muTM-5 for deleting TM domain 3; muTM-3 and muTM-2 for deleting TM domains 1 and 2 (Supplementary Table S1b.).

2.2. Expression and purification of TM domain-Fc fusion proteins

TM-domains 123-Fc or mutant TM-Fc plasmids (TM-domains 12-Fc, TM-domains 23-Fc, TM-domains13-Fc and TM-domain 3-Fc) were transiently transfected into human embryonic kidney cell-line 293T (HEK293T) cells (ATCC number CRL-1573) using Lipofectamine 2000 reagents (Invitrogen, Tokyo, Japan). HEK293T cells were cultured for 7 days in Opti-MEM (Gibco, Invitrogen, Tokyo, Japan) without fetal bovine serum, and supernatants were collected. TM-domains 123-Fc and other mutant proteins secreted in the supernatants were Protein A-affinity purified by the Amicon Pro purification system (Millipore-Japan, Tokyo, Japan). The purity of TM-domains 123-Fc and mutant proteins was confirmed by SDS-PAGE. Two micrograms of each protein were mixed with 2-mercaptoethanol and heated at 90 °C for 3 min. After electrophoresis, gels were stained with the CBB stain one dye (Nacalai Tesque, Kyoto, Japan). To detect the TM-domains 123-Fc protein bands, a Western blotting analysis was carried out using polyclonal rabbit anti-THBD (Sigma−Aldrich, Tokyo, Japan) antibody and goat anti-rabbit IgG (H + L)-HRP conjugate (Bio-Rad, Tokyo, Japan). The Fc portion was detected using a goat anti-human IgG (Fc specific)-antibody conjugated with peroxidase (Sigma−Aldrich). Each blot was developed using an enhanced chemiluminescence kit (Nacalai Tesque, Kyoto, Japan) and analyzed by ImageQuant LAS 4000mini (GE healthcare, Tokyo, Japan).

2.3. Preparation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from sodium citrate-treated blood samples of healthy volunteers as previously described [9]. Blood donors were all given informed consent for the procedure. Blood samples were subjected to Percoll Plus (GE Healthcare) density gradient centrifugation at 1000 *g* for 15 min. PBMCs were suspended in HEPES-buffered saline (HBS).

2.4. Cell adhesion assay

A Cell adhesion assay using 96-well V-bottom plates was performed as previously described [10]. Briefly, a 50-μl aliquot of phosphate-buffered saline (PBS) containing 0.25 μM of wild-type or mutant TM-Fc fusion protein, or control human IgG1 Fc recombinant protein (Millipore-Japan), was dispensed in each well. ICAM-1-Fc chimera protein (0.05 μM ICAM-1-Fc, R&D systems, Minneapolis, MN, USA) and 0.1 μM fibronectin (FN, Sigma−Aldrich, Tokyo, Japan) were included as references. Plates were incubated at 4 °C overnight and blocked by PBS containing 2% BSA for 2 h at 37 °C. One hundred microliter aliquot of HBS containing 1×10^4 calcein-labeled PBMCs either in the presence of 1 mM $MgCl_2$ and 1 mM $CaCl_2$; 0.1 mM $MnCl_2$, 1 mM $MgCl_2$, and 1 mM $CaCl_2$; or 2 mM EDTA was dispensed in each well. In some experiments, 10 μg/ml of blocking monoclonal antibodies to integrins or control IgG were added and incubated at room temperature for 5 min. The plates were then centrifuged at 1000 rpm for 5 min using a swinging bucket rotor (EX-125, TOMY SEIKO Co, Ltd.). Accumulated non-adhered cells at the nadir of V-bottom wells were detected by a 2030 ARVO X-2 Multilabel Reader (PerkinElmer Japan, Kanagawa, Japan).

2.5. Antibodies against integrins

Anti-integrin αL monoclonal antibody (mAb) clone TS1/22 (Thermo scientific, Waltham, MA, USA), anti-integrin αM mAb clone M1/70 (BD Biosciences, Tokyo, Japan), anti-integrin β1 mAb clone P5D2 (R&D systems, Minneapolis, MN, USA) and mouse IgG1 isotype control antibody clone MOPC-21 (Sigma−Aldrich) were purchased from suppliers as noted. Anti-integrin αL mAb clone MHM24 was obtained from the Developmental Studies Hybridoma Bank (The University of Iowa, Department of Biology, Iowa City, IA 52242). Anti-human integrin β2 mAbs were used as previously described [11].

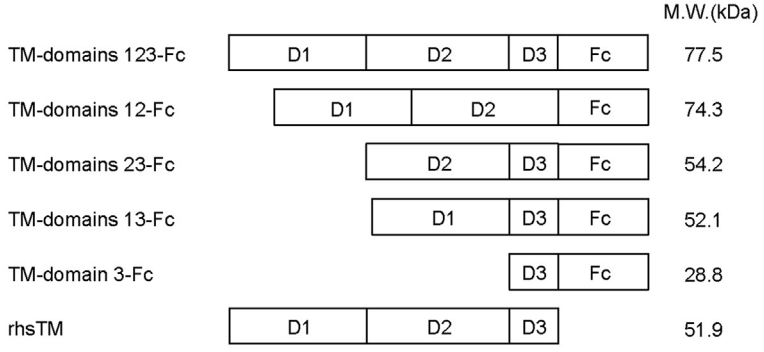
2.6. Statistical analyses

Statistical analyses were performed using SPSS software v.18.0 (IBM Corp, Armonk, NY, USA). Results are presented as mean ± standard deviation. Wilcoxon and Mann−Whitney tests were used for within-group comparison. P-value < 0.05 was considered statistically significant.

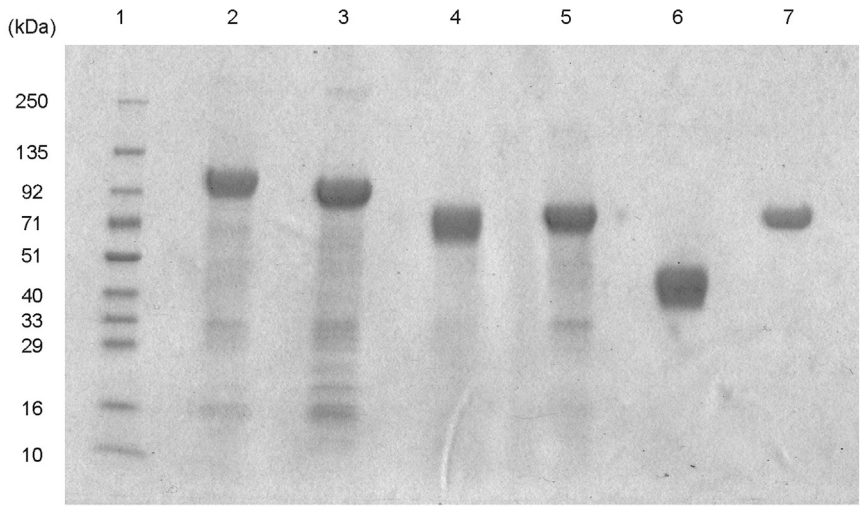
(a)

TM domain	Amino acid sequence
D1 (1-226)	PAEPQPGGSQCVEHDCFALYPGPATFLNASQICDGLRGHLMTVRSSVAA DVISLLLNGDGGVGRRRLWIGLQLPPGCGDPKRLGPLRGFQWVTGDNNT SYSRWARLDLNGAPLCGPLCVAVSAAEATVPSEPIWEEQQCEVKADGFL CEFHFPATCRPLAVEPGAAAAAVSITYGTPFAARGADFQALPVGSSAAV APLGLQLMCTAPPGAVQGHWAREAPGAWD
D2 (227-462)	CSVENGGCEHACNAIPGAPRCQCPAGAALQADGRSCTASATQSCNDLCE HFCVPNPDQPGSYSCMCETGYRLAADQHRCEDVDDCILEPSPCPQRCVN TQGGFECHCYPNYDLVDGECVEPVDPCFRANCEYQCQPLNQTSYLCVCA EGFAPIPHEPHRCQMFCNQTACPADCDPNTQASCECPEGYILDDGFICT DIDECENGGFCSGVCHNLPGTFECICGPDSALARHIGTDC
D3 (463-497)	DSGKVDGGDSGSGEPPPSPTPGSTLTPPAVGLVHS
D4 (498-521)	GLLIGISIASLCLVVALLALLCHL
D5 (522-557)	RKKQGAARAKMEYKCAAPSKEVVLQHVRTERTPQRL

(b)



(c)



	2	3	4	5	6	7
D1	+	+		+		+
D2	+	+	+			+
D3	+		+	+	+	+
Fc	+	+	+	+	+	

Fig. 1. The amino acid sequence and domain organization of thrombomodulin and illustrations depicting the primary structures of the recombinant extracellular portion of wild-type (intact) and domain-deleted mutant TM-Fc fusion proteins. (a) The amino acid sequences of D1-5 are shown. (b) Schematic representations of the wild-type intact and domain-deleted mutant TM-Fc fusion proteins are shown. For comparison Recomodulin, a soluble form of TM (rhsTM), is shown. (c) A representative SDS-PAGE image of intact and mutant thrombomodulin-Fc fusion proteins (Lane 2–6). Lane 1, molecular weight markers (masses shown at left in kDa); Lane 7, rhsTM.

3. RESULTS

3.1. Preparation of intact and domain-deleted mutant TM-Fc fusion proteins

To study the adhesive interactions of TM with integrins on leukocytes, we expressed in HEK293T cells, and purified, the recombinant soluble form of the extracellular portion of TM fused to the Fc portion of human immunoglobulin (TM-domains 123-Fc) that contained the TM domains 1 to 3. The presence of the Fc portion was expected not only to facilitate expression and purification of the recombinant proteins, but also to maintain the integrity of the TM moiety upon immobilization to substrates. In addition, as endothelial TM is thought to form a homophilic multimer via inter-molecular interactions of the transmembrane domain [12], a dimer formation of TM-Fc could partly mimic physiologic conditions. In addition to wild-type TM-domains 123-Fc, a panel of domain-deleted TM-Fc mutants was created (Fig. 1b). SDS-PAGE analyses of the intact and domain-deleted TM-Fc proteins showed good purity, as well as molecular weights consistent with those predicted (Fig. 1c). A subsequent western blotting examination using antibodies to TM and Fc confirmed the integrity of the proteins; i.e., the proteins were not inappropriately cleaved.

3.2. Integrins support cell adhesion of PBMCs to the TM extracellular portion

To study whether leukocytes would interact with TM on substrates, we performed cell adhesion assays. In control experiments, human PBMCs showed effective binding both to ICAM-1-Fc, a ligand for β2 integrins such as αLβ2 (LFA-1) and αMβ2 (Mac-1) and to fibronectin, a ligand for β1 integrins such as α4β1 (VLA-4) and α5β1 (VLA-5). In contrast, PMBCs exhibited only minimal binding to BSA and isotype-matched IgG1 Fc (a negative reference for TM-Fc proteins) (Fig. 2). Binding of PBMC to ICAM-1 and fibronectin was enhanced by stimulation with the Mn^{2+} ion, an experimental stimuli that mimics inside-out integrin activation, while binding was abolished by EDTA-treatment, which depleted divalent cations including Mg^{2+} (Fig. 2). Mg^{2+}-dependent and Mn^{2+}-enhanced ligand binding are both hallmarks of integrin-mediated adhesive interactions, and thus indicative that the cell adhesion assay used in this study was optimized for examining the potential interaction of integrins expressed on leukocytes with TM. Remarkably, human PBMCs exhibited good, specific binding to TM-Fc in a divalent cation-dependent manner, compared with their binding to controls such as BSA and IgG1 Fc. In addition, cell adhesion of PBMCs to TM-Fc was significantly upregulated upon integrin stimulation with Mn^{2+} (Fig. 2). These results indicated that PBMCs exhibited integrin-mediated cell adhesion to the extracellular portion of TM (Fig. 2).

3.3. β2 integrin is essential for the binding of PBMCs to TM

β1 and β2 integrins are the major integrins expressed on human PBMCs that include lymphocytes and monocytes [13]. To determine which integrin(s) would support the interactions of leukocyte with TM, we examined the effects β1 integrin blocking had on mAb P5D2 and β2 integrin blocking on mAb TS1/18 (Fig. 3a). Treatment with the β2 integrin blocking mAb TS1/18 completely inhibited the specific binding of PBMCs to TM, thus decreasing binding to levels as low as those observed in the non-specific binding of PBMC to Fc proteins. By contrast, treatment with the β1 integrin blocking mAb displayed little inhibitory effects on the binding of PBMCs to TM. These results demonstrated that β2, but not β1, integrin is responsible for the binding of PBMC to TM.

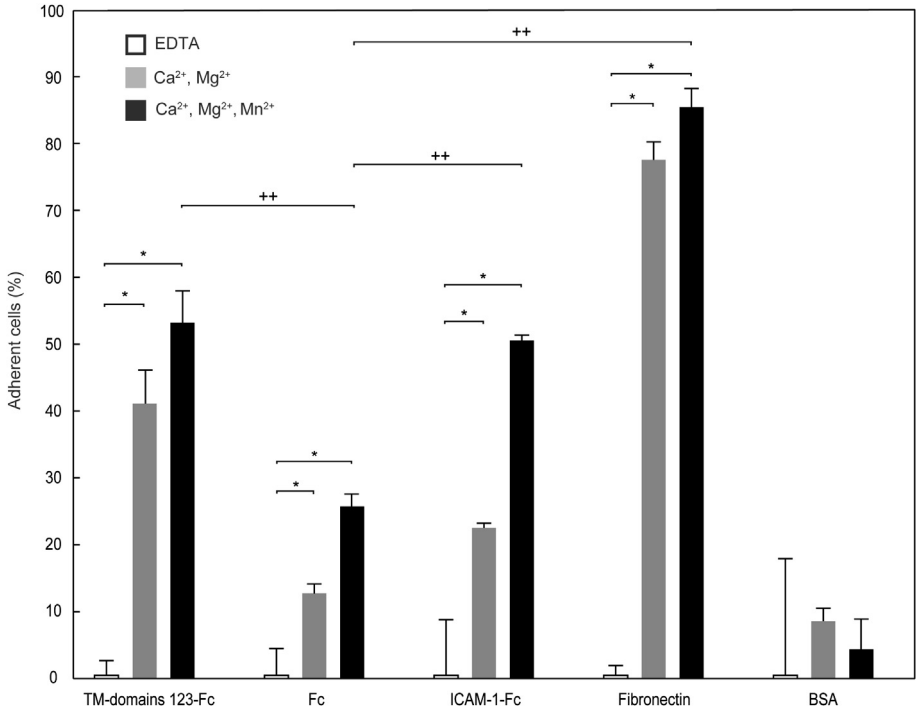


Fig. 2. Mg^{2+}-dependent and Mn^{2+}-enhanced adhesion of human PBMCs to thrombomodulin substrates. Binding of PBMCs to TM-domains 123-Fc, Fc, ICAM-1-Fc, Fibronectin and BSA was studied in the presence or absence of $MgCl_2$ and $CaCl_2$ (gray bar) and $MgCl_2$, $CaCl_2$ and $MnCl_2$ (black bar), or none (i.e., EDTA-treatment; white bar). Data are expressed as the mean ± SD. *P < 0.05 compared with EDTA at each group. ++P < 0.05 compared with Fc.

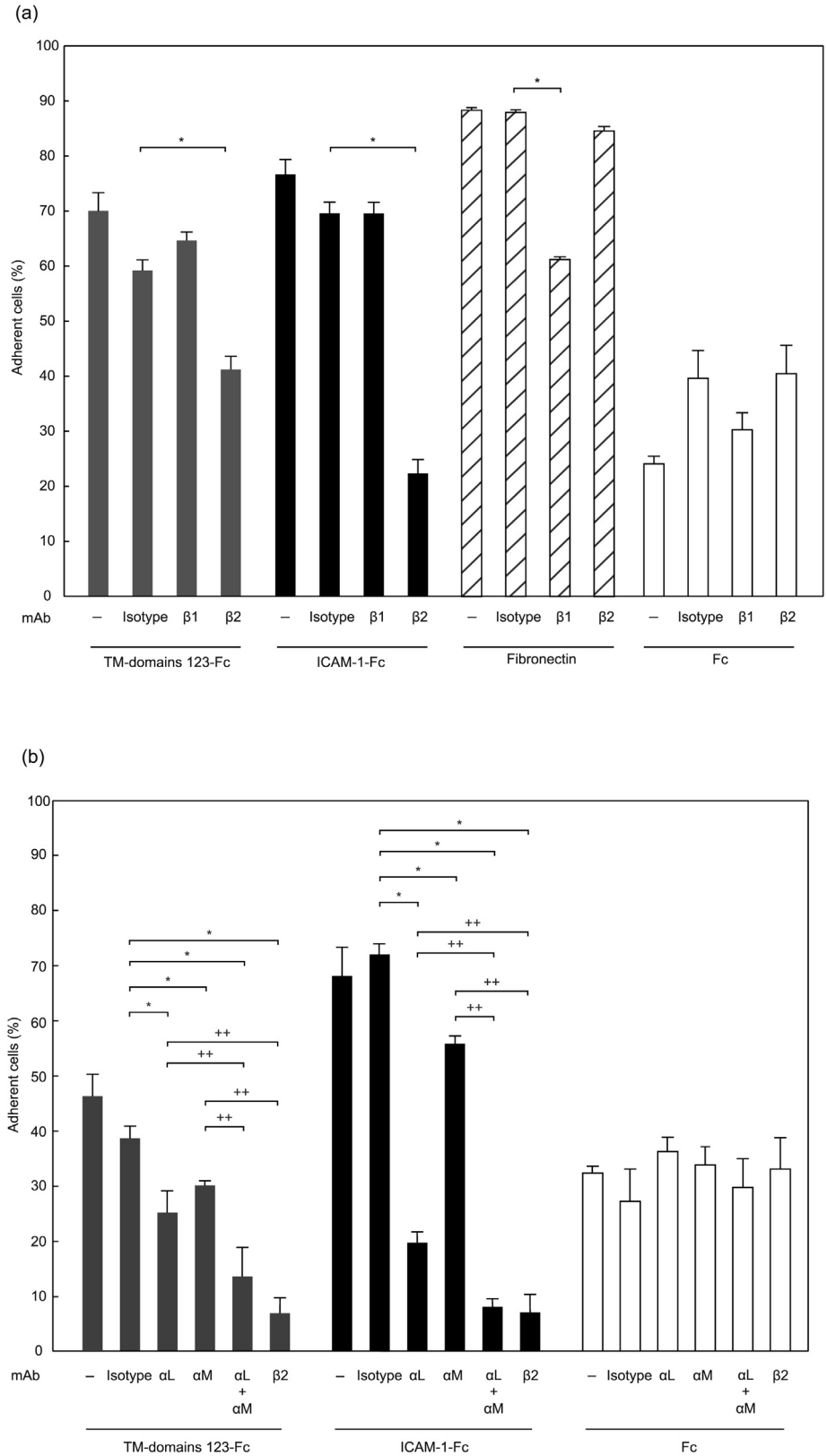


Fig. 3. (a) β2 integrin is required for the binding of PBMCs to thrombomodulin substrates. Binding of PBMC to TM-domains 123-Fc, ICAM-1-Fc, Fibronectin and Fc was examined in the presence of β1 integrin blocking mAb (P5D2) or β2 integrin blocking mAb (TS1/18) or control IgG. (b) Interaction of αL and αM integrins with thrombomodulin substrates. Binding of PBMCs to TM-domains 123-Fc was studied in the presence of anti-CD11a (αL) mAb (MHM24), anti-CD11b (αM) mAb (M1/70), and anti-β2 integrin mAb (TS1/18), or isotype control IgG. Data are expressed as the mean ± SD. *P < 0.05 compared with isotype control. ++P < 0.05 compared with αL or αM integrin mAb.

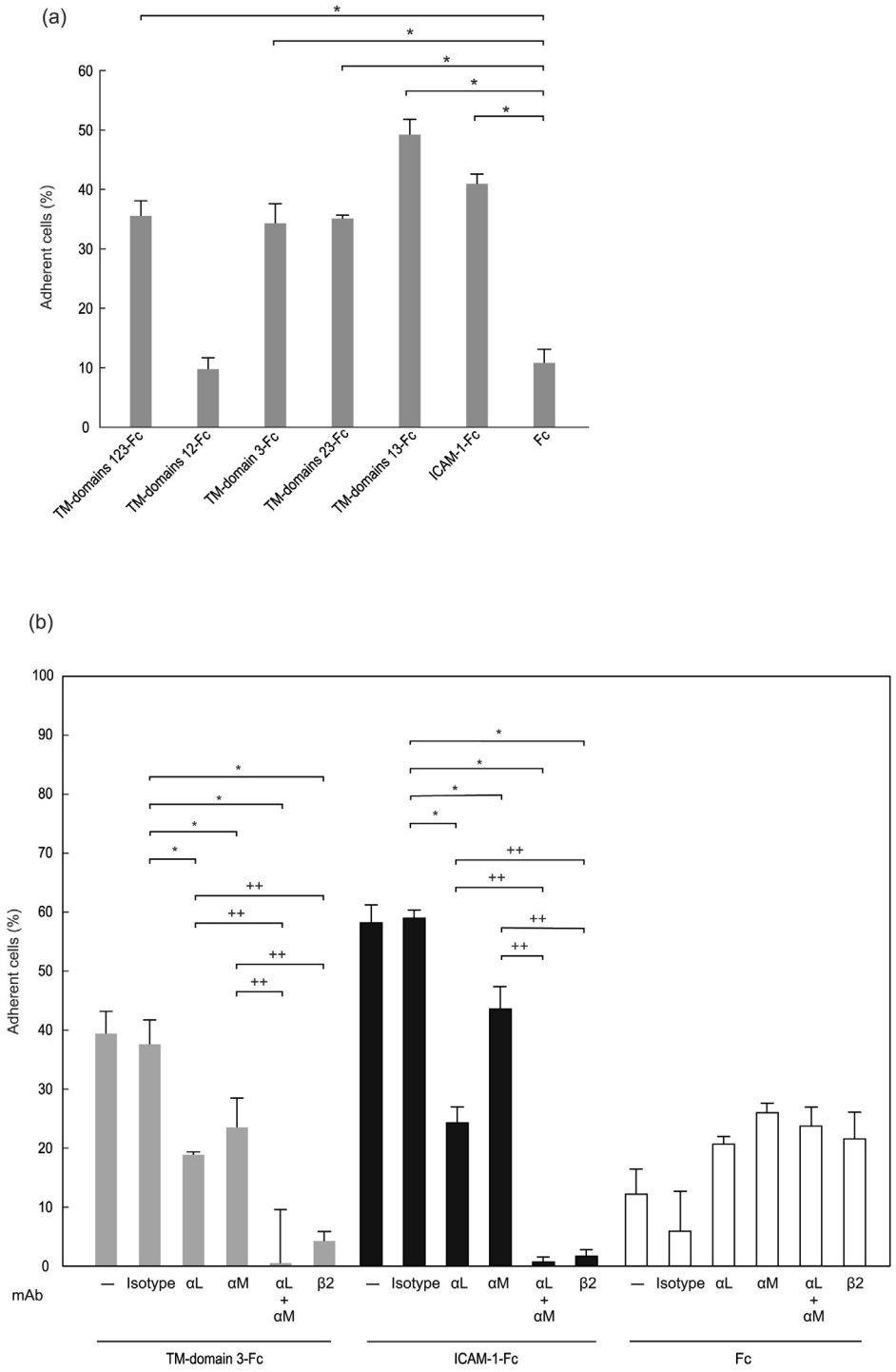


Fig. 4. Thrombomodulin domain 3 contains the integrin binding site. (a) Thrombomodulin domain 3 is indispensable for PBMC binding. Binding of PBMC to a panel of thrombomodulin domain-deleted mutants was studied. (b) Effects of anti-αL, anti-αM and anti-β2 integrin antibodies on PBMCs binding. Data are expressed as the mean ± SD. *P < 0.05 compared with control IgG Fc protein.

3.4. αLβ2 (LFA-1) and αMβ2 (Mac-1) integrins both support PBMC binding to TM

αLβ2 (LFA-1) and αMβ2 (Mac-1) are the two predominant members of β2 integrins on human leukocytes. Thus, we sought to determine which specific β2 integrin heterodimer, αLβ2 or αMβ2, is responsible for binding PBMCs to TM. Treatment with an inhibitory mAb MHM24 specific to αLβ2 suppressed the binding of PBMCs to TM. In addition, treatment with an inhibitory mAb M1/70 specific to αMβ2 also reduced the binding of PBMCs. Treatment with both mAb MHM24 and mAb M1/70 resulted in inhibitory effects as promising as those achieved with the β2 integrin inhibitory mAb TS1/18, which blocks both αLβ2 and αMβ2. These results convincingly show that both αLβ2 and αMβ2 integrins support the binding of PBMCs to the TM-domains 123-Fc (Fig 3b).

3.5. TM domain 3 supports αLβ2 and αMβ2 integrin binding

The results thus far have shown that αLβ2 and αMβ2 integrins on human PBMCs play a critical role in the binding to TM (Fig. 3b). To determine to which TM domains β2 integrins bind, we engineered a panel of domain-deleted TM mutants-Fc fusion protein (Fig. 1), and then performed cell adhesion assays with the TM mutants. We found that specific binding to TM was abolished in the TM-domains 12-Fc lacking domain 3 (Fig. 4a). Conversely, the TM mutant that contains only domain 3 showed binding as effective as the intact TM containing domains 1, 2, and 3. To substantiate the specific interaction of integrin αLβ2 and αMβ2 with domain 3 of TM, we studied the effects of anti-αL, αM and β2 mAbs on PBMC binding to the TM domain 3 (Fig. 4b). Anti-αL mAb MHM24, anti-αM mAb M1/70 and anti-β2 mAb TS1/18 reduced PBMCs adhesion to TM domain 3, compared to the isotype control. These results demonstrate that domain 3 of TM contains the binding site(s) for the αLβ2 and αMβ2 integrins.

4. Discussion

Here we have demonstrated that TM on the substrate support αLβ2 and αMβ2 integrin-mediated leukocyte adhesion. The results provided in this study strongly suggest that endothelial TM functions as a novel integrin ligand that facilitates leukocyte adhesion to the endothelial surface in cooperation with known integrin IgSF ligands such as ICAM-1 and ICAM-2. Expression of multiple integrin ligands enables leukocytes to make complex and elaborate adhesive interactions with endothelial cells both in health and disease. ICAM-1 and ICAM-2 have been shown to play a role in supporting leukocyte crawling and transendothelial migration [14]. It is possible that endothelial TM contributes to a fine-tuning of ICAM-1- and ICAM-2-mediated leukocyte adhesive interactions with endothelial cells. While ICAM-1 expression is induced by endothelial inflammation, ICAM-2 is constitutively expressed [15]. Notably, expression of endothelial TM has been shown to decrease in inflammation due to activation-induced proteolytic cleavage of the extracellular portion at the membrane proximal site [16]. Thus, relative expression levels of TM/ICAM-1/ICAM-2 are likely to vary significantly in health and inflammation, thereby allowing for the dynamic regulation of integrin-mediated leukocyte interactions with inflamed as well as uninflamed endothelial cells.

Leukocyte integrins have a unique property in that they bind strongly only to ligands present on the cell surface, but not to ligands in solution. ICAM-1 is released from the cell surface by endothelial inflammation and damage, resulting in the circulation of soluble ligand [17]. It has been shown that soluble ICAM-1 only minimally binds to leukocyte integrins stimulated with physiologic activators [18]. Thus, while the presence of soluble ICAM-1 in serum has been shown to serve as a maker for endothelial inflammation [19], soluble ICAM-1 in serum is unlikely to interfere in the interaction of leukocyte integrins with endothelial ICAM-1. Recent investigation into the interaction of αLβ2 with ICAM-1 has revealed the mechanism that explains why leukocyte integrins robustly bind only to immobilized, but not soluble, ligand. Burkhardt and colleagues have shown that a mechanical tensile force applied to the integrin cytoplasmic tail via actin fiber is critical for the induction of ligand-competent high-affinity conformation, and that such a tensile force is effectively applied when integrin ligand is immobilized [20]. Indeed, our flow-cytometric experiments indicated that Recomodulin™, a soluble form of recombinant TM, failed to bind to leukocyte integrins on PBMCs (not shown). Thus, the leukocyte integrin-TM interaction shown in this study would make a minimal contribution to the reported ability of Recomodulin to inhibit leukocyte cell adhesion [21]. Rather, leukocyte integrins would primarily interact with endogenous endothelial TM, but not with soluble TM, in serum.

Leukocyte recruitment is regulated by cell adhesion molecules and ligands, and the dysregulation of leukocyte adhesion can lead to inflammatory diseases [22]. Leukocytes increase integrin expression and activity in response to pro-inflammatory stimulations in order to attach to endothelial cells [23]. Integrins are involved not only in cell adhesion, but also in the augmentation of inflammatory responses via leukocyte activation. Therefore, integrin-targeted therapy is particularly attractive for certain inflammatory diseases. Recent clinical trials suggest that the blockade of integrins is a promising strategy for treating several diseases. Natalizumab, an anti-α4 integrin monoclonal antibody, is a humanized monoclonal antibody that blocks leukocyte migration to the inflamed brain in multiple sclerosis [24,25]. Abciximab, a glycoprotein IIb/IIIa receptor antagonist, is a platelet aggregation inhibitor. It is principally used to treat patients with coronary heart disease in order to prevent thrombus formation following coronary artery intervention [26]. These studies suggest that adhesion molecule-targeting therapies are one of the more effective therapies against inflammatory diseases. We have shown that TM domain 3 binds to LFA-1 and Mac-1 integrins on human PBMCs, which suggests that TM-domains 123 regulates integrin-mediated leukocyte adhesion on endothelium. A next important goal towards determining the potential of integrin-TM interactions as a therapeutic target would be to elucidate how integrin-TM interactions regulate leukocyte migration to inflamed tissues.

TM is now a new addition to the list of anti-coagulant molecules capable of interacting with integrins on immune cells such as APC and EPCR. APC inhibits coagulation by degrading coagulation factors Va and VIIIa. Recombinant human APC exerts anti-inflammatory [27] and cytoprotective effects [28] through the activation of protease activated receptor 1. Moreover, it has been reported that rhAPC directly binds to β1 and β3 integrins, resulting in the blockade of neutrophil adhesion [1], as the rhAPC contains an Arg-Gly-Asp (RGD) sequence that is a critical to β1 and β3 integrin binding site. In this manner, rhAPC inhibits neutrophil adhesion on endothelial cells and migration into inflamed tissues. In addition, EPCR, which has been linked to protein C activation via the thrombin-TM complex on endothelial cells, also interacts with leukocyte integrins [29]. Thus, the coagulation system is intimately connected to the immune system. We herein have shown that integrins bind to TM (Fig. 4) via TM domain 3, but not via TM domain 1 or TM domain 2, which are essential to achieving anti-inflammatory and anti-coagulant effects. Although integrin binding to TM at domain 3 is not expected to interfere directly with the anti-coagulant activity of TM, indirect and allosteric effects might be mediated by integrin binding to TM. Therefore, further investigations are needed to address how integrins modify TM functionality in the coagulation system.

Conflict of interests

The authors have no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.04.007.

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