Preparation of recombinant TMD (rTMD) proteins using both *Pichia* and mammalian protein expression systems

The pPICZαA and pCR3-EK vectors were used for expression and secretion of human rTMD proteins containing 6×His tag and c-Myc epitope for purification and for protein detection in the *Pichia pastoris* and human embryonic kidney 293 (HEK293) mammalian protein expression systems. Briefly, DNA fragments coding for rTMD1 (residues Ala^{1}–Ala^{155}), rTMD2/EGF123 (residues Ala^{224}–Glu^{346}), and rTMD23 (residues Ala^{224}–Ser^{497}) were obtained by a PCR of human umbilical vein endothelial cell cDNA using specific primers. An enterokinase cutting site between the rTMD sequence and His/c-Myc tag allowed subsequent removal of the tag sequence. Yeast fermentation medium or HEK293 cell conditioned medium containing expressed rTMD proteins was applied to a nickel-chelating Sepharose column (Amersham Pharmacia Biotech., Piscataway, NJ), and rTMD-containing fractions were eluted in an imidazole gradient. The purified rTMD proteins were examined by SDS-PAGE and Western blotting, and were verified by protein identification with mass spectrometry analysis. The N-terminal amino acid sequence of rTMD proteins was also determined by Edman degradation with a model 477A sequencer (Applied Biosystems, Foster City, CA). The molecular masses of rTMD

proteins containing fusion peptides were predicted using the ExPAsy website for calculating protein concentration (rTMD1: 22,902 Da, rTMD2/EGF123: 16,473 Da, and rTMD23: 32,823 Da). A non-tagged rTMD1 was prepared by incubation of 1mg rTMD1 with 5 ng enterokinase (New England Biolab, Beverly, MA) at 4^{o}C for 16 hours. After incubation, the sample was applied to a nickel-chelating Sepharose column and the non-bound fraction containing the non-tagged rTMD1 was collected. The sample was further treated with immobilized soy bean trypsin inhibitor-Sepharose gel to adsorb the residual enterokinase. Each sample was analyzed before and after enterokinase treatment by SDS-PAGE and Western blot, and non-tagged rTMD1 having a molecular mass 2000 Da less than the original tagged rTMD1 was obtained.

rTMD1 ligand analysis

Biotin-polyacrylamide (Biotin-PAA)-sugars (GlycoTech, Rockville, MA), rTMD1, rabbit anti-mouse IgG antibody (Zymed), mouse anti-6× His tag antibody (Abcam, Cambridge, UK), and streptavidin-coated donor and protein A conjugated acceptor beads (PerkinElmer, Boston, MA) were diluted with assay buffer containing 25 mM Tris (pH 7.0), 25 mM $CaCl_2$, and 1 mg/mL BSA to appropriate concentrations. The anti-6× His tag antibody, rabbit anti-mouse IgG antibody, and acceptor beads were

incubated with assay buffer for 1 hour at 25^{o}C before use (as the acceptor mixture). Biotin-PAA-sugars, rTMD1, and donor beads were separately added to wells of ProxiPlate-384 assay plates (PerkinElmer) and incubated at 25^{o}C for 1 hour. An aliquot of the acceptor mixture was then added to the wells and incubated at 25^{o}C for another 2 hours. The results were read on a PerkinElmer Envision instrument using the AlphaScreen program. All the procedures and incubations were carried out in the dark.

Analysis of Lewis antigens in *E. coli* LPS O111:B4

E. coli O111:B4 LPS (5 μg/well) was coated onto wells of a high-binding microtiter plate. After the nonspecific binding blocked with binding buffer containing 50 mg/mL BSA, 5 μg/mL of various kinds of antibodies (Abcam) against Lewis a (Le^{a}), Lewis b (Le^{b}), Lewis X (Le^{x}), and Le^{y} were added to wells with binding buffer containing 1 mg/mL BSA and incubated at 37^{o}C for 2 hours prior to incubation with goat anti-mouse horseradish peroxidase-conjugated IgG or IgM at 37^{o}C for 2 hours. The peroxidase reaction was performed using 3,3′,5,5′-tetramethylbenzidine as a substrate and was stopped by 2N H_2SO_4. The products were detected by measuring absorbance at 450 nm.