nature cell biology

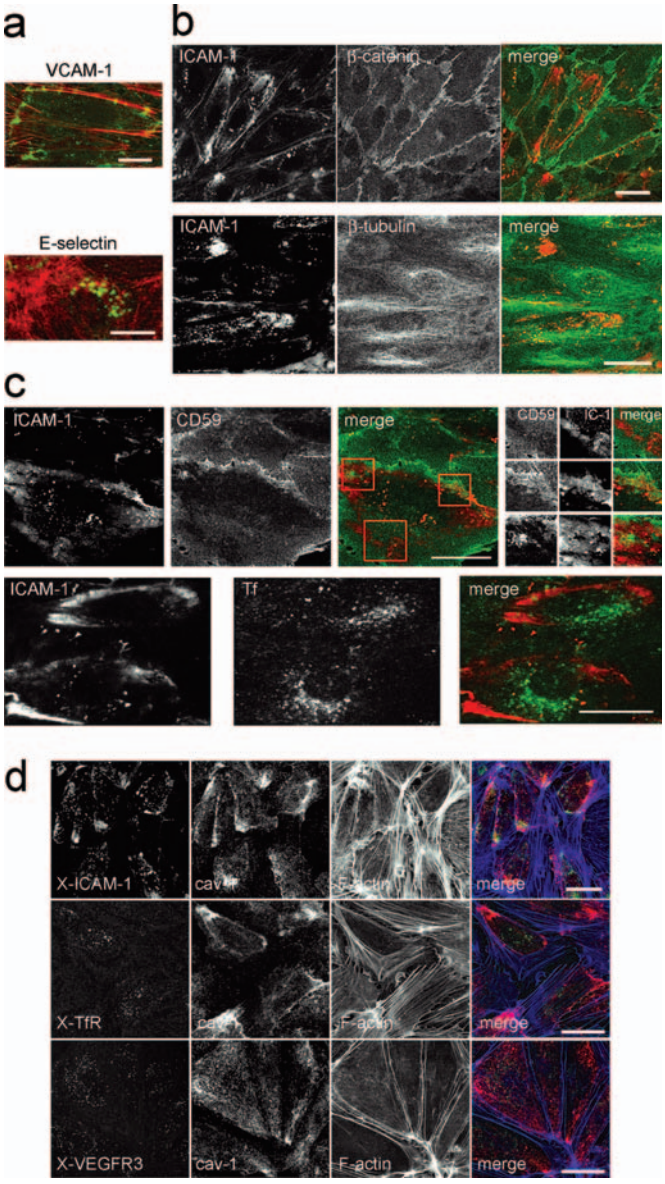

Figure S1 Clustering of surface receptors on HUVECs and HDMVECs. (a-c) Localization of antibody-crosslinked ICAM-1, VCAM-1 and E-selectin in HUVECs. (a) VCAM-1 and E-selectin (green) were clustered on confluent TNF-α-stimulated HUVECs by incubation at 37°C with specific antibodies followed by FITC-labelled secondary antibodies. Cells were stained with TRITC-phalloidin to visualize F-actin. (b) ICAM-1 (red) was clustered on confluent TNF-α-stimulated HUVECs by incubation at 37°C with anti-ICAM-1 antibodies followed by TRITC-labelled secondary antibodies as in Fig. 1. Cells were stained with anti-β-catenin antibody to visualize adherens junctions (green) or FITC-conjugated anti-β-tubulin to detect microtubules. (c) CD59 and transferrin (Tf) do not colocalise with clustered ICAM-1. HUVECs were treated and ICAM-1 was clustered crosslinked as in (b) and stained using anti-CD59 specific antibodies (green). For Tf localization TNF-α-stimulated HUVECs were preloaded with 100 μg/ml of human holo-transferrin during antibody-mediated ICAM-1 crosslinking. Bars, 10 μm. (d) ICAM-1, transferrin receptor (TfR) or VEGFR3 were crosslinked (X-) on confluent TNF-α-stimulated HDMVECs by incubation at 4°C for 45 min with specific mouse monoclonal antibodies followed by FITC-labelled secondary antibodies for 60 min at 37°C (merged in green). Cells were stained with TRITC-phalloidin to visualize F-actin (merged in blue) and with specific antibodies for caveolin-1 (merged in red). Each panel shows a single projection of a z-stack of between 6 and 8 confocal sections. Bars, 20 μm

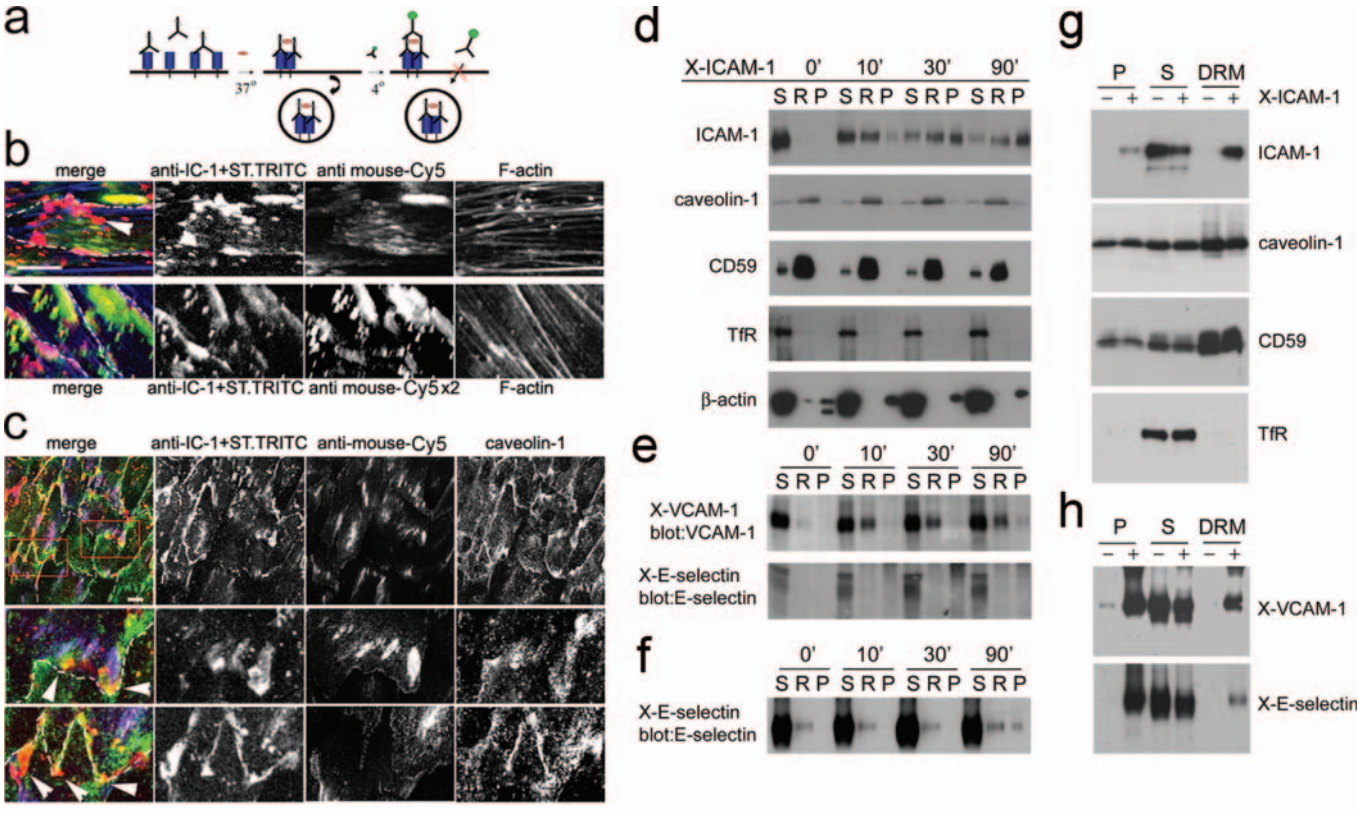

Figure S2 ICAM-1 is internalized at caveolin-enriched domains near cell edges. (a) Schematic representation of the protocol used to discriminate surface ICAM-1 from the total ICAM-1 pool. TNF-α-stimulated HUVECs were incubated for 45 min at 37°C with biotinylated mouse anti-ICAM-1 antibody followed by TRITC-streptavidin for 90 min at 37°C. Cells were then incubated at 4 °C with FITC- or Cy5-coupled anti-mouse secondary antibody for 15 min, fixed and permeabilized. (b) In the upper panels, internalized ICAM-1 is labelled only with TRITC-streptavidin (arrowhead), whereas surface ICAM-1 is labelled with both TRITC-streptavidin (red) and Cy5 anti-mouse antibody (green). Further staining of permeabilized cells with the same secondary antibody (anti-mouse-Cy5x2) was performed (lower panels) to demonstrate that biotinylation and/or streptavidin incubation does not impair secondary antibody binding to the anti-ICAM-1 antibody. F-actin was detected with FITC-phalloidin (shown in blue in merged images). (c) Further staining of permeabilized cells with anti-caveolin-1 antibody (green). Regions outlined with red boxes (upper panel in merged image, c) are shown at 3-fold higher magnification in the lower two sets of panels. Discontinuous lines indicate cell perimeters, arrowheads in (c) show caveolin-1 enriched domains colocalizing with internal ICAM-1. (d-h) Antibody-clustered ICAM-1 and VCAM-1 are recruited to caveolin-1-enriched detergent-resistant lipid rafts. HUVEC monolayers were treated with TNF-α for either 15 h (d,e,g,h) or 4 h (f). ICAM-1 (d), VCAM-1 (e) and E-selectin (e and f) were clustered with antibodies for the indicated times as described in Fig. 1. Cells were lysed and fractionated using selective solubilisation with β-octyl-glucoside. Equivalent volumes of each fraction were separated by SDS-PAGE and the indicated proteins detected by western blotting. (S) TX100-soluble fraction; (R) TX100-resistant /βOG-sensitive lipid rafts; (P) pellet. (g, h)) Cell treated with (+) or without (-) crosslinking antibodies to ICAM-1, VCAM-1 or E-selectin for 30 min were lysed, fractionated by sucrose density gradient centrifugation and analysed by western blotting. (P), pellet; (S) TX100-soluble fraction; (DRM), detergent-resistant membranes. ICAM-1 and VCAM-1 are recruited to insoluble lipid rafts in both methods, a smaller fraction of E-selectin was recruited using sucrose density gradient centrifugation (h) as previously described [45] but remained mostly soluble using the β-octyl-glucoside method (e,f).

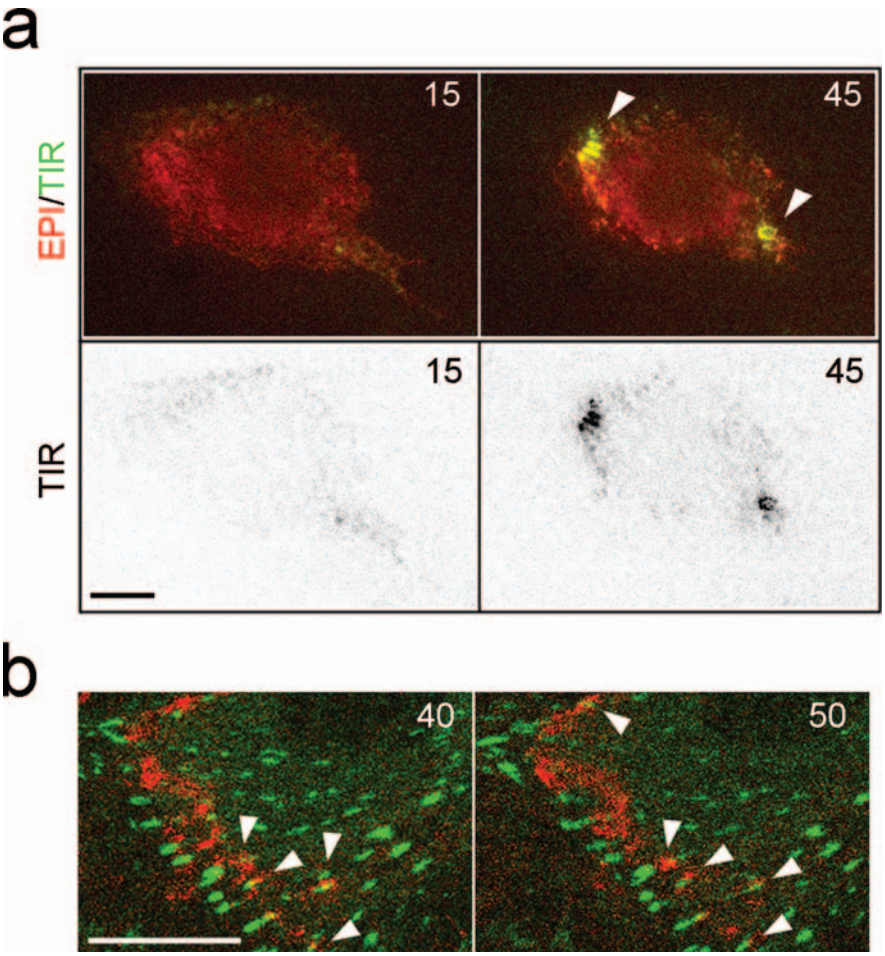

Figure S3 ICAM-1 translocation does not require intercellular junctions and occurs close to focal adhesions. (a) TIRFM/EPI time-lapse microscopy of antibody-clustered ICAM-1 in subconfluent cells. Cells were clustered and analyzed as in Fig. 2. Note the shape of ICAM-1 clusters in contact with the basal membrane after 45 min of croslinking. At the top left corner they form three converging straight lines whereas at the bottom right clusters are organized into circular structures that are internalised (b) Time-lapse confocal microscopy of a basal section showing partial co-localization of clustered ICAM-1 (red) with paxillin-GFP (green). Bar, 20 μm.

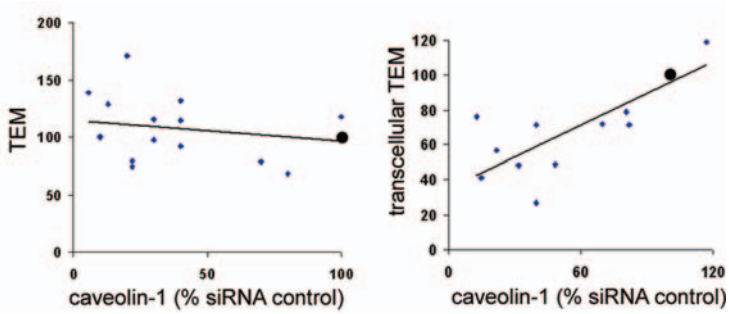

Figure S4 Decrease of transcellular TEM correlates with low levels of caveolin-1. Relative total TEM (TEM) and transcellular TEM were plotted against relative levels of caveolin-1, determined by western blotting for each siRNA transfection from the 4 different experiments in Fig. 6. Black rounded dots in the graphs represent values from siRNA control (x4) taken as 100%.

SUPPLEMENTARY METHODS

Detergent extraction procedures. *Selective solubilisation with β-octyl-glucoside.* TNF-α-stimulated HUVECs were lysed for 20 min in TST (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Triton X-100, 10 μg ml^{-1} each leupeptin and aprotinin, 1 mM PMSF) at 4ºC. Lysates were centrifuged at 14,000 g for 3 min at 4ºC and the supernatant (S) and pellet separated. Soluble proteins, solubilised membrane proteins and lipid raft domains not big enough to pellet by conventional centrifugation were separated in the S fraction. Pellets were then solubilised in 1 ml of TST plus 60 mM β-octyl-glucoside (OG) for 20 min at 37ºC. Homogenised pellet was then centrifuged at $3x10^4$ g for 3 min at room temperature and supernatant (R) (OG-sensitive lipid rafts) and nuclei and cytoskeleton pellet (P) were separated. Equivalent volumes of P, S and R fractions were separated by SDS-PAGE, transferred to PVDF membranes and proteins detected by western blotting. *Sucrose density gradient centrifugation.* TNF-α-stimulated HUVECs were lysed for 20 min in 1 ml TST at 4ºC. Low-density detergent resistant membranes (DRMs) were isolated by standard procedures [41]. The pellet (P), soluble fractions (S) (1-2) and insoluble fractions (DRMs) (30-5% interphase) were pooled separately. Equivalent volumes of each fraction were separated by SDS-PAGE and the distribution of proteins detected by western blotting.

Movie 1 Dynamics of ICAM-1 movement to the cell pole. HUVECs were transfected with pEYFP-actin, stimulated with TNF-α and ICAM-1 was clustered with antibodies as described in Fig. 2 and Materials and Methods. Images of clustered ICAM-1 (red) and actin-YFP (green) were recorded at 10-sec intervals with a timelapse EPI microscope. The video is displayed at 30 frames/sec.

Movie 2 ICAM-1 segregation to the cell periphery is accompanied by translocation to the basal membrane. HUVECs stimulated with TNF-α and ICAM-1 was clustered as described in Fig. 2 and materials and Methods. ICAM-1 movement was followed by simultaneous TIRFM/EPI timelapse microscopy at 37ºC. TIR (green) and EPI (red) images were recorded at 10-sec intervals. The video is displayed at 8 frames/sec.

Movie 3 ICAM-1 segregation to the cell periphery does nor require intercellular junctions ICAM-1 was clustered on subconfluent HUVECs as described in Fig. 2 and Suppl. Fig. 3a. ICAM-1 movement was followed by simultaneous TIRFM/EPI timelapse microscopy at 37ºC. TIR (green) and EPI (red) images were recorded at 10-sec intervals. The video is displayed at 8 frames/sec.

Movie 4 Dynamics of ICAM-1 and caveolin-1 movement following ICAM-1 clustering. TNF-α-stimulated HUVECs expressing caveolin-1-YFP were analysed by EPI time-lapse microscopy to follow the movement of caveolin-1-YFP (green) and antibody-clustered ICAM-1 (red), as described in Fig. 2. Images were recorded at 10-sec intervals and the video is displayed at 5 frames/sec.