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Human CD99L2 Regulates a Unique Step in Leukocyte Transmigration:

"Human CD99L2 in Transmigration"

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Abstract

CD99-like 2 (CD99L2; L2) is a highly glycosylated 52 kDa type-1 membrane protein that is important for leukocyte transendothelial migration (TEM) in mice. Inhibiting L2 using function blocking antibody significantly reduces the recruitment of leukocytes to sites of inflammation *in vivo*. Similarly, L2 knockout mice have an inherent defect in leukocyte transmigration into sites of inflammation. However, the role of L2 in inflammation has only been studied in mice. Furthermore, the mechanism by which it regulates TEM is not known.

In order to study the relevance to human inflammation, we studied the role of L2 on primary human cells *in vitro*. Our data show that like PECAM and CD99, human L2 is constitutively expressed at the borders of endothelial cells and on the surface of leukocytes. Inhibiting L2 using antibody blockade or genetic knockdown significantly reduces transmigration of human neutrophils and monocytes across endothelial cells. Furthermore, our data also show that L2 regulates a specific, sequential step of TEM between PECAM and CD99, rather than operating in parallel or redundantly with these molecules. Similar to PECAM and CD99, L2 promotes transmigration by recruiting the lateral border recycling compartment (LBRC) to sites of TEM, specifically downstream of PECAM initiation. Collectively, our data identify a novel functional role for human L2 in TEM and elucidate a mechanism that is distinct from PECAM and CD99.

INTRODUCTION

Recruitment of leukocytes to sites of inflammation is essential for maintaining host homeostasis. This recruitment is controlled by a series of adhesive interactions between leukocytes and endothelial cells lining the blood vessel wall. CD99-like 2 (CD99L2; henceforward L2) is a type-1 transmembrane protein that shares ~32% amino acid identity with CD99 (1), a known regulator of TEM (2, 3). Previous studies demonstrate an

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important role for L2 in leukocyte recruitment during inflammation (4–7). It is expressed on mouse leukocytes and endothelial cells (1, 4, 5). L2 has been shown to play a regulatory role in TEM of monocytes, neutrophils, and lymphocytes across endothelial cell borders *in vivo* (4, 5, 7). Inhibition of L2 using polyclonal antibodies reduced leukocyte recruitment in peritonitis models (4, 5). Furthermore, mice genetically deficient in L2 showed reduced transmigration of monocytes and neutrophils (7, 8) and T cells (6, 7) to sites of inflammation.

While the importance of L2 for leukocyte extravasation in mice is agreed upon, the step(s) in extravasation at which L2 plays its critical role has not been examined in living animals. In this paper, we show that in FVB/n mice, in which PECAM and CD99 antibodies block at different stages of TEM than in C57BL/6 mice (2, 9–12), endothelial cell L2 deficiency has no effect on neutrophil rolling or adhesion, but arrests leukocytes at the stage of TEM, apparently beyond the step blocked by anti-PECAM. Furthermore, we show that mouse neutrophils are arrested at the step of migrating across the junction in FVB/n mice, which is different from the stage controlled by L2 in C57BL/6 mice (10).

More important, all previous studies on L2 were performed in mice. The relevance of these studies to human health is unknown. In this paper, we show that L2 is constitutively expressed on human leukocytes and at human endothelial cell borders. TEM of human neutrophils and monocytes requires L2 expressed on both cells, presumably interacting with each other in a homophilic manner. Antibody blockade, knockdown by shRNA, or competitive inhibition by soluble L2 selectively prevents TEM of both human monocytes and neutrophils across human endothelial cell monolayers. Mechanistically, we also show that L2 is regulates a step in TEM that is functionally between the steps regulated by PECAM and CD99, and it is necessary for the continued recruitment of the lateral border recycling compartment to the site of TEM.

MATERIALS AND METHODS

Antibodies and Reagents

Sheep anti-human L2 polyclonal antibody was purchased from R&D systems (AF5185). The antibody was designed using a peptide (Val20-Ala188) from the predicted sequence of human CD99L2. Recombinant human CD99L2 was derived from Chinese hamster ovary cell line CHO. L2 antibody was purified using standard antigen affinity purification methods. Monoclonal mouse antibodies against human PECAM (clone hec7, IgG_{2a}) (13), human VE-cadherin (clone hec1, IgG_{2a}) (14), human CD99 (clone hec2, IgG_1) (3), and human CD18 (clone IB4, IgG_{2a}) (15) were produced in the laboratory using standard hybridoma methodologies. The Armenian hamster anti-mouse PECAM (clone 2H8) (16) was purified from hybridoma cultures. The nonblocking mouse anti-human PECAM antibody (clone P1.1) (17) was purified from mouse ascities fluid, a gift from Peter Newman (Blood Center of Wisconsin, Milwaukee, WI) and digested to F(ab) fragments using standard protocols. The rabbit anti–human β-actin was purchased from Sigma Aldrich (A2228, clone AC-74). PE mouse anti-human CD3 IgG_{2a} (555340), BV510 mouse anti-human CD19 IgG_1 (562947), and BV711 mouse anti-human CD31 IgG_1 (740777) were purchased from BD Bioscience. Alexa Fluor 700 mouse anti-human Siglec-8 IgG_1

(FAB7975N) was purchased from Novusbio. The rabbit anti–mouse collagen IV (ab19808) and rat anti–mouse MRP-14 (ab105472) were purchased from abcam. The nonblocking rat anti-mouse PECAM (clone 390, CBL1337) was purchased from EMD Millipore (Billerica, MA). Croton oil was purchased from Sigma Aldrich (C6719). Recombinant mouse IL-1b was purchased from R&D Systems (401-ML). Recombinant human TNF-α was purchased from R&D Systems. Perfusion buffer used in intravital microscopy experiments was Tyrode's Salts solution purchased from Sigma-Aldrich supplemented with sodium bicarbonate (1 g/L, Thermo Fisher Scientific). Ketamine was purchased from Henry Shein. Xylazine was purchased from Akron. Dylight 488 anti-rat, DyLight 550 anti-Armenian hamster, and DyLight 649 anti-rabbit collagen IV were all purchased from Jackson ImmunoResearch and conjugated using kits purchased from Thermo Fisher Scientific. Donkey anti-sheep (unconjugated and FITC) IgG, goat anti-mouse IgG, goat anti-rabbit IgG, goat anti-Armenian hamster IgG, anti-human Fc, human gamma globulin, and horseradish peroxidase conjugated anti-human Fc were purchased from Jackson ImmunoResearch Laboratories. Hanks Buffered Saline Solution (HBSS) was purchased from Mediatech Inc. Ficoll-Plaque gradient medium was purchased from GE Healthcare. Human Serum Albumin (HSA) was purchased from Grifols Biological. M199 medium was purchased from Invitrogen. Polymorph prep was purchased from Axis-Sheild.

Animals

All procedures were approved by the Institutional Animal Use and Care Committee of Northwestern University. All in vivo studies used 8 to 12-week-old mice. $L2^{-/-}$ mice backcrossed into the FVB/n background were described previously (8) and housed under barrier conditions at the Feinberg School of Medicine animal facility.

Bone Marrow Chimeras

Bone Marrow Chimeras were generated for intravital microscopy studies as previously described (18). Recipient FVB/n Wild-type and L2 knockout mice were irradiated using a Gammacell 40 Exactor ^{137}Cs irradiator (1,000 cGy dose). Isolated Bone marrow from donor FVB/n LysM-GFP mice were administered to recipient mice through retroorbital injection. Both donors and recipients were age/sex matched. Mice were allowed to recover for 4 weeks prior to intravital microscopy studies. Successful bone marrow transfer was confirmed using blood smears for the presence of GFP positive leukocytes.

Croton Oil-induced Dermatitis

Position of leukocyte arrest was scored relative to the endothelium using a standard croton oil dermatitis model (19). 20 μl of 1% Croton oil, an inflammatory stimulus, diluted in an olive oil/acetone mixture (4:1 mixture; carrier) was distributed dropwise to the inner and outer skin of the ear of wild-type or L2 deficient mice. An equal volume of carrier control (olive oil: acetone) was applied to the contralateral ear. Mice were sacrificed and ears were harvested after 5 hours. Ears were fixed overnight in freshly made 4% Paraformaldehyde solution at 4°C. After fixing, the tissue was incubated in a permeabilization/blocking solution (0.3% TritonX-100, 2.5 % BSA, 2.5% antibody host-species serum) overnight at 4°C in preparation for staining. The ear leaflets were stained using 1 μg/mL anti-MRP14, 10 μg/mL anti-PECAM (clone 2H8), and 1 μg/mL anti-collagen IV diluted in blocking

buffer overnight at 4°C. Ears were then washed three times and incubated with Dylight550 conjugated anti-Armenian hamster, Alexa Fluor 488 anti-rat, and Alexa Fluor 647 anti-rabbit in BS solution for 4 hours at RT. Ear leaflets were mounted and sealed on coverslips with vasculature facing upward. At least 8 fields per sample were imaged. Contralateral ears that received carrier served as internal negative controls.

Isolation of Mouse Cremaster and Intravital Confocal MicroscopyAnalysis

Mice were injected intrascrotally with 100 mg of fluorescently conjugated nonblocking PECAM antibody (390) to label vessels and 5 ng recombinant mouse IL-1 β to induce inflammation or 1x PBS for control. 4 hours post injection, mice were anesthetized by intraperiotoneal injection of ketamine/xylazine (100 mg/kg and 10 mg/kg body weight, respectively). Supplemental doses of ketamine/xylazine (~25 μL) were administered upon reaction to foot pad pinch or whisker movement (every 30–45 minutes). Fur on the scrotum was removed using Nair™.

The mouse was then secured facing upward on a Plexiglass platform containing an internal heating pad set to ~37°C. The lower legs were positioned so that the scrotum could be extended using a threading needle over a quartz pedestal embedded in silicone. Under a dissecting microscope, a small ~1 cm incision was made along the left side of the scrotum and the cremaster muscle was carefully exteriorized across the quartz pedestal and secured using suture needles as previously described (9). The tissue was continuously hydrated using warmed perfusion buffer consisting of Tyrode's Salt and 1 g/L sodium bicarbonate at pH 7.4. Connective tissue was carefully removed with forceps and the muscle was gently opened using microsurgery scissors. The microvasculature was then imaged. Ideal fields contained postcapillary venules with normal undisturbed flow 30–50 μm in size. Three separate sets of recordings were collected for each field. The first image is a 3D stack in the red (PECAM) channel to determine vessel dimensions. Next, we captured the green channel (GFP expressing PMN) for 60s in a single plane recording (1 frame/s) in the middle of the vessel to determine rolling velocity and flux. Last, we captured a 30 to 60-minute recording (4 frames/s) of both channels in 3D to quantify TEM events.

Vessel measurements were collected using Volocity® software. Vessel length and width were used to calculate vessel surface area (vessel surface area=πdl). Neutrophil adhesion, rolling flux and velocity were determined using the single-plane 60 sec rolling video. Cells were considered adherent if the observed neutrophil had not moved for more than one cell diameter for ≥30 s during the recording. Rolling flux was determined by quantifying the number of neutrophils that rolled passed an arbitrary point in the vessel and divided by the total number of neutrophils present in the vessel, using the 60 sec rolling recording. TEM was quantified using frame-by-frame analysis of the 30–60 min 3D recordings. Neutrophils were considered to have transmigrated if they were originally inside the vessel and then migrated outside the vessel with no vessel contact during the recording (9).

Isolation of Primary Endothelial Cells and Leukocytes

Primary human umbilical vein endothelial cells (HUVEC), PBMCs and PMNs were isolated as previously described (13), (20), (21). At the second passage, HUVECs were cultured on 3D type-I collagen in 96-well plates.

PBMCs were isolated from whole blood diluted 1:1 with warm HBSS. The diluted sample was then gently layered over a ficoll-plaque density gradient and centrifuged at 2200 rpm for 20 minutes with the brake off to avoid disturbing layers of separation. The thin layer of PBMCs was collected, diluted to a final volume of 50 mL with HBSS and centrifuged at 1500 rpm for 10 minutes at 4°C. PBMCs were resuspended in EDTA-treated platelet poor plasma to eliminate platelets and centrifuged at 1200 rpm for 5 min at 4°C. The PBMCs were washed 3 times in HBSS+0.1% human serum albumin. After the final wash, PBMCs were resuspended in M199 containing 0.1% HSA to the concentration needed for experiment purposes.

For primary neutrophils, whole blood was drawn from healthy donors. The undiluted blood was carefully layered over Polymorphprep (Cosmo Bio USA INC., Carlsbad, CA) and centrifuged at 1800 rpm for 30 minutes at 20 ^{o}C with the brake off. All steps were performed at room temperature. The middle layer was collected and diluted in HBSS+0.1% HSA, washed three times and centrifuged at 750 rpm for 5 minutes. Neutrophils were then resuspended in M199+0.1% HSA to the final concentration needed for experiments. Plastic transfer pipettes coated with 0.1% human serum albumin were used during resuspensions to avoid agitation and unintentional cell activation.

Cloning of L2 constructs

The human L2 short hairpin RNA (L2 shRNA) (5’-CCGGCGATGTCAAGAACGAGCATCAAATCGATTTGATGCTCGTTCTTGACATCGTTTTT-3’) was generated against the 3’ untranslated region using Gene Link and RNAi Designer from Life Technologies. The shRNA construct was cloned into a pENTR4 plasmid containing a hU6 promoter. The newly generated pENTR4 plasmid containing the shRNA construct was cloned into the adenoviral vector pAd/PL-DEST (Invitrogen) via recombinase reaction using LR Clonase according to manufactures protocol (Invitrogen). 5 μl of the recombinase reaction was transformed into 45 μl of freshly thawed competent E. coli cells and transformed on an agarose plate. After purification, the plasmid was transfected into 293A cells using Lipofectamine 2000 according to standard protocols. After cell lysis, cells and media were collected and freeze/thawed three times. Cell debris was pelleted via centrifugation at 3000 rpm for 30 minutes at 4°C. Supernatant containing virus was passed through a micron filter, aliquoted and stored at −80°C.

The full-length CD99-GFP in the pENTR4 vector (2) was used as a template to generate the full-length L2-GFP construct. Full-length CD99 was removed from the plasmid using the restriction endonucleases HindIII and BamHI. The resulting plasmid was recombined with the pAd/CMV/V5-DEST vector (Invitrogen) using LR clonase (Invitrogen). The resulting DEST vector was subjected to PacI digestion and transfected into 293A cells for adenoviral production using Lipofectamine 2000.

DNAM-Fc in the pBR1 vector (22) was used as a template to generate L2-Fc and double digested with restriction enzymes BamHI and SalI. The Fc portion of the vector was previously mutated to avoid possible Fc binding to Fcγ receptors (23). The extracellular domain of human L2 was amplified using the following primers: (5'-GAT*GGATCC*GTGCCAGGCTCTGCCACCATG -3') and (5'-ACGC*GTCGAC*CCACCATGGTGGCCTGGCGCTC −3') from full-length L2-GFP. The amplified extracellular domain of L2 was inserted into the Fc containing pBR1 vector via ligation. The resulting vector was transformed using standard protocols and plasmid was purified using Qiagen Miniprep Kit. NS-1 cells were then transfected using the Neon Transfecting System (Invitrogen). 3 μg of plasmid and 10 μl of 10^7 NS-1 cells were mixed in "R" buffer. 10 μl of the mixture was drawn up into the Invitrogen Neon 10 μl tips and electroporated at 1400V, 3 pulses for 10 ms. Subsequently the mixture was ejected into 500 μl of RPMI complete medium without antibiotics. After 2 days, medium was replaced with geneticin containing medium. Antibiotic resistance was used to confirm transfection and cells were then serial diluted using standard methods to select clones. Fc producing clones were confirmed using a sandwich ELISA and expanded to a cell-line flask. Production of L2-Fc was performed using standard hybridoma methodologies.

ELISA

To confirm the production of L2-Fc we used a standard sandwich ELISA to measure the presence of Fc. 96-well polystyrene plates were coated with anti-human Fc at RT for 2 hours. The standard curve for this assay was human gamma globulin that was serial diluted (curve range was 0 ng/ml to 100 ng/ml). The supernatant from the non-secreting mouse myeloma cells (NS-1) that were transfected with L2-Fc chimera (described above) and the standards served as the antigen in this assay. The antigen was incubated on the coated wells for 1 hour at RT. Wells were washed and incubated with anti-human Fc. The signal was amplified using a horseradish peroxidase secondary incubation of bovine anti-goat secondary antibody. The ELISA was developed using AZTBS substrate. Plates were then read on a spectrophotometer at a wavelength of 405 nm in 10-minute intervals for 40 minutes. DNAM-Fc served as positive control for ELISA. Secondary screening was confirmed using western blot analysis probing Fc positive samples with anti-L2. Concentration of recombinant L2-Fc was determined using standard curve and further confirmed with absorbance.

Transendothelial Migration (TEM) assays

TEM assays were performed as previously described (3) For all experiments, HUVEC monolayers were activated with 20 ng/μL TNF-α for 4 hours. PBMCs and activated HUVEC were co-cultured at 37°C for 1 hour. For antibody blockades, antibodies were diluted to 20 μg/mL in 100 μl of PBMCs resuspended to a concentration of 2.0×10^6 cells/ml. For genetic inhibition studies, HUVEC were transfected with adenovirus containing shRNA against L2 72 hours prior to activation. For rescue experiments, leukocytes were transfected with adenovirus expressing full-length L2-eGFP 24 hours after knockdown and 48 hours before activation. For crosslinking experiments, crosslinking antibodies were added to PBMC: HUVEC co-cultures 10 min prior to the end of the incubation. After 1 hour co-culture, wells were washed 1x with 1 mM EDTA to remove loosely bound lymphocytes.

Immediately following EDTA wash, wells were washed 3x in cold PBS with Ca^{2+} and Mg^{2+} and fixed in 4% PFA for immunofluorescence imaging or 2% glutaraldehyde in 0.1 M cacodylate solution for Modified Wright/Giemsa staining. TEM was quantified using either Wright-Giemsa staining or from orthogonal projections of confocal images of the monolayers (24). For assays in which PBMC were used, monocytes were distinguished from lymphocytes. In this assay, lymphocytes do not transmigrate, are much smaller and morphologically distinguishable from monocytes (20). Monocytes were scored as either above or below the monolayer. Percent TEM was calculated as monocytes below the monolayer divided by total monocytes (above + below). In assays of neutrophil TEM, the starting preparations were ~ 99% neutrophils; the percent TEM for neutrophils was calculated the same manner as for monocytes.

Binding Assay

The wells of a 96-well polystyrene ELISA plate were coated with soluble L2 for 1 hour at RT. Wells were washed with 1x PBS and blocked with 0.1% BSA. Primary leukocytes were preincubated with non-specific IgG, BSA, or L2-Fc for 1 hour on ice. Cells were then added to the pre-coated wells for 15 minutes at RT. Wells were extensively washed with 0.1% EDTA to remove any unbound cells. Bound cells were quantified using phase microscopy.

Sequential TEM block assay

Sequential block studies were modified from the TEM assay as previously described (3, 22). Leukocytes were allowed to transmigrate for 1 hour in the continuous presence of blocking antibody at 37°C. Because antibody inhibition of TEM is reversible once antibody bound to the endothelial cell or leukocyte is degraded, we can remove the initial blockade through extensive washing at 4°C (to remove any unbound antibody) followed by an extended period at 37°C during which time the antibody is internalized and degraded below the critical concentration required to block TEM. A new antibody, targeting a different molecule, is added during this time. If the molecule targeted by the antibody added during the second incubation functions downstream of the molecule targeted during the first incubation, then TEM will remain blocked (3, 24). If not, TEM will proceed.

Targeted Recycling Assay

To study the initial recruitment of the LBRC (targeted recycling), the assay was performed as previously described (25). HUVEC were labeled with nonblocking unconjugated P1.1 Fab (IgG2a) to label all PECAM at 37°C for 1 hour. Sheep anti-human L2 was used during initial LBRC labeling to block L2 function. Cells were washed in M199 and HEPES in 4°C ice water bath. We then used an unconjugated anti-mouse $F(ab')_2$ secondary to bind surface P1.1 at 4°C for 1 hour. Primary leukocytes were then diluted in Alexa Fluor 647 labeled anti-mouse $F(ab')_2$ and allowed to settle for 10 min on ice. Next, cells were rapidly warmed to 37°C for 8–10 minutes, washed and fixed in 4% PFA. To study the subsequent targeted recycling, the assay was modified to allow the initial recruitment of the LBRC to take place without being labeled (2). In this assay, after the initial incubation of the EC in P1.1 mAb, leukocytes are diluted in unlabeled anti-mouse $F(ab')_2$ and allowed to settle for 10 minutes on ice. Cells were then placed at 37°C for 10 minutes to allow the initial recruitment of the LBRC. After chilling and extensive washing on ice, cells were incubated with Alexa Fluor

647 labeled anti-mouse F(ab')$_2$ for 10 minutes at 37°C to allow targeted recycling and TEM to resume. Samples were then chilled, washed and fixed in 4% PFA for imaging. Leukocytes in both assays were labeled with IB4 post fixation.

Immunofluorescence

Confluent HUVECs were washed gently with 1x PBS with divalent cations three times. Monolayers were then fixed in 4% PFA for 10 minutes at room temperature. Cells were washed 3 times with 1x PBS and permeabilized with 0.1% Triton X-100 for 10 minutes at room temperature. Cells were then blocked with freshly made blocking buffer (1x PBS, 2.5 % BSA, 2.5% normal Goat, mouse, or donkey serum (species used is antibody host-dependent) for 1 hour at room temperature.

Western Blot

Confluent HUVEC monolayers grown on 24-well plates or 60-cm dishes were washed 2 times with cold 1x PBS with ions. Cells were then lysed in 50 μl or 150 μl lysis buffer (1% NP-40, protease inhibitors (Complete Mini), 1 μM sodium orthovanadate, 1 mM PMSF), respectively, for 5 minutes on ice and scraped. After scraping, lysates were gently pipetted up and down, collected and transferred to an Eppendorf tube. Lysates were centrifuged for 15 minutes at 4°C and 14,000 g. After centrifugation, supernatants were mixed with 5x Lamelli buffer and boiled at 95°C for 5 minutes. Samples were then loaded onto a 10% acrylamide gel at equal amounts, electrophoresed at 100V for 15 minutes to stack, and then at 150V for the remainder of the run. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane at 110V for 2 hours in 1x transfer buffer. The membrane was blocked in 5% milk containing PBS with 0.2% Tween-20 for 1 hour at room temperature. After blocking, the membrane was incubated with anti-L2 (1:1000) diluted in blocking buffer overnight at 4°C. Membrane was washed three times, 10 minutes each, in PBST at room temperature with gentle rocking. Signal was amplified using horseradish peroxidase conjugated anti-sheep secondary and developed using chemiluminesence.

Flow Cytometry

Peripheral blood was collected from 6 healthy donors. Using forward scatter vs side scatter, leukocyte populations were identified and further gated using the appropriate leukocyte surface markers. T-cells were identified as cells that were CD3+. B-cells were identified as cells that were CD19+. Monocytes and neutrophils were identified as CD14+ and CD15+, respectively. L2 expressing cell subsets were identified based on leukocyte marker and L2 double positivity. PECAM expression across leukocyte subtypes was used as positive control.

Statistical Analysis

In vitro experiments were performed independently three times. TEM and TR assays contained averages of at least six replicates per experiment. ELISAs were performed in triplicate. Flow cytometry used five subjects. Croton oil and IVM experiments contained three mice for each condition. Numerical data from experiments were averaged between experiments. TEM, TR, croton-oil and IVM show standard error of the mean in figures. P

values were calculated using the student's t test with a Bonferroni correction for multiple comparisons. Statistical analysis was performed using GraphPad Prism software. Statistical differences are denoted by asterisks and described in figure legends.

RESULTS

Genetic ablation of L2 arrests leukocytes partially through the endothelium and disrupts leukocyte TEM *in vivo*

Previous studies have shown the importance of L2 in leukocyte emigration *in vivo*. (4–8) However, the step of extravasation at which leukocytes are arrested has only been reported for mice of the C57BL/6 strain (4, 10). In FVB/n mice, PECAM regulates a step in PMN extravasation upstream of the CD99 regulated step similar to that seen in *in vitro* studies involving human cells (3, 9). Blockade or knockout of PECAM or CD99 arrests neutrophils before they completely cross the endothelium in FVB/n mice and almost all other strains (2, 9, 26, 27), however they impair PMN extravasation at the level of the basement membrane in the C57BL/6 strain (28), (10). Similarly, L2 has been reported to block PMN extravasation between the basal surface of the endothelium and the basement membrane in C57BL/6 mice (4, 10). Therefore, we wanted to examine where mouse neutrophils were arrested in L2-deficient FVB/n mice. To address this, we utilized the croton oil dermatitis model of inflammation in combination with immunofluorescence staining, as previously published(19). Briefly, croton oil was applied to one ear of wild-type or L2−/− mice and carrier to the contralateral ear. After 5 hours and the tissue harvested and fixed. Tissues were stained for PECAM, Collagen IV, and neutrophils. Neutrophil position was scored relative to endothelium and basement membrane in high resolution confocal stacks (Fig 1A).

Wild-type FVB/n mice showed a robust increase in the presence of neutrophils in post capillary venules and in surrounding tissue (Fig 1B upper right panel, 1C). L2 deficient mice showed an equivalent inflammatory response; however, the majority of neutrophils never transmigrated into the interstitium (Fig 1B lower right panel, 1C). Orthogonal projections of confocal z-stack images were examined to identify where leukocytes were arrested in relation to the endothelium and basement membrane. In croton oil treated mice, most leukocytes were blocked part way across the endothelial cell lining in L2 $^{-/-}$ mice (Fig 1B lower right panel, 1C), downstream of where anti-PECAM blocks (19).

The croton oil dermatitis studies provide a high-resolution view of transmigration; however, they are a static snapshot. To observe the effect of L2 deficiency on leukocyte/endothelial cell interactions in real time, we performed complementary intravital imaging experiments, utilizing the cremaster model of inflammation (9). For these studies, chimeric mice were created by adoptively transferring bone marrow from mice with myeloid cells expressing eGFP (LysM-eGFP) into lethally irradiated wildtype or L2 knockout mice. This generated mice with GFP-positive, wildtype L2 expressing neutrophils and either wildtype or L2−/− endothelium. Compared to sham control, wild type mice treated with IL-1β showed pronounced increase in recruited/adherent neutrophils and extravasation out of the vessel (Fig 2A–D, and Video 1). Ablation of endothelial L2 resulted in a significant reduction in TEM despite an equivalent recruitment of neutrophils to the vessel (Fig. 2A; 2D, Video 2). In contrast, there were no differences in leukocyte rolling or adhesion between wild type and

endothelial L2 deficient mice (Fig. 2B and C). These data collectively show that endothelial L2 is critical for leukocyte transendothelial migration *in vivo*.

L2 is expressed on Human leukocytes and along endothelial cell borders

L2 is expressed on mouse endothelial cells and leukocytes (5, 8). Whether L2 is expressed on primary human cells is not known. Due to the shared amino acid identity between CD99 and L2 we blotted for and selectively immunoprecipitated the respective proteins to ensure that the antibody against human L2 used in these studies does not recognize CD99. In fact, our antibody against L2 does not recognize CD99 and our monoclonal antibody against CD99 does not recognize L2 (Fig 3A). Additionally, we show that neither of these proteins recognize the other on intact cells (Fig 3B).

To determine expression of L2 on leukocytes, peripheral blood was collected from healthy donors and specific leukocyte populations identified using flow cytometry. Similar to PECAM, L2 was found to be most highly expressed on monocytes (CD14+). Other leukocyte subtypes expressed L2 (> 95% of cells), but at significantly lower levels (Fig. 3C–D). Because monocytes appeared to express L2 in a biphasic manner, we also characterized L2 expression on CD14+ vs. CD16+ monocytes. We observed no difference in L2 expression between $CD14^{hi}/CD16^{-}$ and $CD14^{lo}/CD16^{+}$ monocyte subsets (data not shown).

Next, expression of L2 on human endothelial cells was investigated by immunofluorescence (Fig 3E). Confluent HUVEC monolayers were fixed and stained for L2. Endothelial cells show positive immunofluorescence signal for L2 at endothelial cell borders (marked using VE-cadherin) similar to PECAM and CD99, supporting its role in transmigration (Fig 3E). Unlike molecules whose expression is upregulated during inflammation, such as ICAM-1, L2 expression was not increased upon stimulation with TNFα (Fig 3F). This is similar to the constitutive expression of PECAM and CD99 (3, 24), which also play a role in TEM. These data reveal that L2 is constitutively expressed on both primary human leukocytes and at endothelial cell borders.

Inhibition of L2 function reduces TEM of leukocytes across human endothelial cells

We next sought to elucidate the role of L2 in human inflammation and transmigration. To test this, we performed a series of TEM assays using antibody-mediated and genetic ablative approaches. Human neutrophils (Fig 4A) and monocytes (Fig. 4B) were added to human umbilical vein endothelial cells (HUVEC) monolayers in the presence of antibody against L2. Blocking L2 function inhibited neutrophil and monocyte transmigration to a comparable degree as anti-PECAM or anti-CD99 treatment. Furthermore, the combination of anti-L2 with anti-CD99 did not block better than either antibody alone, suggesting that they function on the same pathway (Fig. 4A, 4B). To validate this, we performed TEM assays with HUVEC monolayers transduced with shRNA targeting L2. Knockdown of L2 expression was confirmed using western blot (Fig 4C). Similar to antibody-mediated blockade, genetic ablation of endothelial L2 prevented TEM (Fig 4D). Furthermore, re-expression of L2 in endothelial cells restored TEM to control levels (Fig 4C, D). These studies showed that endothelial L2 is important for TEM in human cells.

Endothelial L2 and leukocyte L2 are both required to facilitate TEM

PECAM and CD99 on leukocytes rely on homophilic interaction with PECAM and CD99 on endothelial cells, respectively, to promote transmigration (3, 24). Schenkel et al showed that murine cells transfected with murine L2 adhere to each other in a homophilic manner (5). In C57Bl/6 mice, L2 is thought to have a heterophilic interaction with an unknown ligand on mouse neutrophils to promote TEM (4, 7). We next sought to determine whether human endothelial L2 and monocyte L2 were required to promote TEM. L2 function was selectively blocked on either the endothelium or monocytes using an antibody against L2. Inhibition of L2 on either the endothelium or the monocyte reduced TEM to ~30–40 %, similar to inhibition achieved by blocking both EC and monocytes (Fig 5A). This is consistent with a homophilic interaction between these molecules.

If a homophilic interaction were really occurring, we should be able to competitively inhibit the interaction by adding the extracellular domain of L2 to either the EC or leukocyte. To this end, we constructed a soluble L2-Fc chimera that contains the extracellular domain (required for ligand binding) of L2 fused to the Fc domain of human IgG1 modified to not bind leukocyte Fc receptors (DNAM-Fc served as negative control for western blot analysis; (22)) (Fig 5B). In monolayers treated with L2-Fc, we observed a significant reduction of TEM (Fig 5C). Leukocyte adhesion to endothelium, where binding is predominantly to ICAM-1, was not affected by presence of the soluble protein (Fig 5D).

To more directly support the hypothesis of homophilic interaction, we first performed aggregation assays with L-cells transfected to express human L2 and parental L-cells. Parental L-cells did not aggregate; L-cells expressing human L2 formed aggregates almost exclusively with other L-cells expressing human L2 but not with parental L-cells (data not shown). Even more directly, we performed a series of binding experiments in which L2 was the only substrate. We coated polystyrene plates with soluble L2 or BSA for 1 hour at RT. Primary leukocytes were then added to plates and allowed to bind. After washing we observed that leukocytes were able to bind L2-Fc coated wells, whereas essentially none bound to the wells coated with BSA. Adding L2-Fc to the leukocytes blocked their ability to bind the wells (Fig 5E; Fig 5F). Binding was not affected by pre-incubation with BSA or non-specific IgG1. These data further suggest that like PECAM and CD99, endothelial L2 promotes TEM through homophilic interaction with L2 expressed on leukocytes, even though leukocytes do not require this interaction to attach to endothelium.

L2 functions at a step in TEM between steps regulated by PECAM and CD99

Leukocyte transmigration is a multi-step process with PECAM and CD99 functioning sequentially to regulate TEM(3). We wanted to determine where L2 functions in relation to PECAM and CD99. To initially address this question, we performed TEM assays blocking PECAM, L2 or CD99 and stained cells for immunofluorescence. Using confocal microscopy, orthogonal images allowed us to see leukocytes blocked at distinct stages of passage across the monolayer. Cells treated with anti-PECAM show monocytes arrested on the apical surface of endothelial cells. However, inhibition of L2 or CD99 show monocytes arrested partway through the endothelial cell monolayer, arguing that these two molecules function downstream of PECAM (Fig 6A).

In order to elucidate if CD99 and L2 were acting in parallel or at separate, distinct steps we utilized a modified TEM assay, the “sequential block assay”, as previously published (3, 22), This method takes advantage of the fact that inhibition of TEM using antibodies against PECAM, CD99 or L2 is reversible once the added antibody has been cleared or digested below the level required to block TEM. Note that all of the incubations described for figures 6B, C, and D were performed at the same time on the same batches of leukocytes and endothelial cells. The data have been separated into three panels to facilitate description.

To demonstrate that anti-L2 blockade was reversible, we first added anti-PECAM mAb or anti-L2 to the TEM assay. TEM was blocked, as shown in earlier figures. We then chilled the monolayers to prevent further TEM, washed extensively on ice to remove unbound antibody, and returned the monolayers to the incubator in the presence of anti-VE-cadherin (non-blocking) antibody. After an hour, transmigration had returned to the levels observed in non-blocked controls (Fig 6B). To demonstrate that after this washing procedure, the monocytes were still capable of being blocked by the original antibodies, the monolayers were placed back in the original mAb and returned to the incubator. TEM remained blocked, as expected (Fig. 6C). If endothelial cell/leukocyte co-cultures were first exposed to anti-PECAM mAb, washed as above, and then returned to culture in the presence of either anti-CD99 or anti-L2, transmigration remained blocked (Fig 6D). This showed that L2 and CD99 both function “downstream” of PECAM. Consistent with this, when cells were first incubated with anti-CD99 mAb or anti-L2, washed on ice as above, and then returned to culture in the presence of anti-PECAM mAb, transmigration resumed (Fig 6D). This further confirmed the published data that CD99 functions downstream of PECAM (3, 9) and indicated that like CD99, L2 functions downstream of PECAM. Finally, to test the relationship of L2 to CD99, TEM was first blocked with anti-L2 mAb, washed, and re-incubated in the presence of anti-CD99. Transmigration remained blocked. (Fig 6D). However, when TEM was first blocked with anti-CD99, then washed and incubated in the presence of anti-L2 mAb, transmigration resumed to control levels (Fig 6D). This indicated that L2 functions at a step in transmigration upstream of the step regulated by CD99. These data collectively show that L2 regulates a step in transmigration between the PECAM and CD99 regulated steps.

L2 clustering promotes continued Targeted Recycling of the LBRC to sites of TEM

During transmigration, membrane from the lateral border recycling compartment (LBRC) is directed to the site of TEM in a process known as targeted recycling (TR). All junctional molecules that are required for TEM are found in the LBRC unless, like VE-cadherin, they are actively excluded (29). TR brings more membrane and unligated molecules like PECAM and CD99 from the LBRC to the leukocyte, to provide the migrating cell with sufficient membrane surface area and adhesion molecules required for transmigration. Inhibition of TR of the LBRC inhibits TEM and prevents leukocytes from getting into sites of inflammation. Targeted recycling of the LBRC is essential for TEM; anything that inhibits TR blocks TEM (2, 30, 31). Preventing homophilic PECAM interactions between the endothelium and the leukocyte blocks TR of the LBRC thus preventing the initiation of TEM (25). Blocking homophilic CD99 interaction allows initial PECAM-dependent TR but prevents further influx of the LBRC (2). The role of L2 in TR is not known.

Because L2 functions at a step downstream of PECAM (fig. 6), we hypothesized that, as with blockade of TEM by anti-CD99 (2), when we block TEM with anti-L2, TR will be initiated despite disruption of homophilic L2 interactions. To test this, we used our targeted recycling assay (2, 25, 30) (see Materials and Methods) to track the movement of the LBRC membrane during transmigration in the presence of anti-L2. (25) In samples treated with anti-L2, LBRC enrichment was observed around leukocytes even though they were arrested in the junction due to the block in TEM, like the appearance of control monolayers when monocytes were caught in the act of TEM (Fig. 7A–C). However, parallel co-cultures incubated for an hour demonstrated that TEM in the presence of anti-L2 was indeed blocked (Fig. 7C). Thus, L2 is not required for the initial TR of the LBRC. This is similar to what we have previously reported with CD99 (2) and consistent with L2 regulating a step in TEM downstream of the step regulated by PECAM.

Because L2 is not required for the initiation of TR, we next tested whether blocking L2 function disrupts further recruitment of the LBRC to the transmigrating leukocyte. To test this, we altered the TR assay as we had previously done to examine the role of CD99 in recruiting the LBRC (2). The targeted recycling (TR) assay was started after TEM had been blocked by antibody against L2. Samples in which endothelial cells had been treated with anti-L2 and were blocked in the process of TEM showed no subsequent LBRC enrichment around arrested leukocytes beyond the initial influx, while control samples showed continued LBRC enrichment around transmigrating leukocytes (Fig. 7D–F).

Blocking L2 with anti-L2 inhibits the homophilic interaction between endothelial L2 and leukocyte L2. We hypothesized that mimicking L2 homophilic interactions through artificial clustering of endothelial L2 could bypass the antibody blockade thus restoring TR and TEM, similar to what we reported with anti-CD99 (2). To test this hypothesis, we first blocked TEM selectively on EC using anti-L2 as above. Leukocytes were arrested, unable to transmigrate, as before. However, cross-linking L2 using an anti-L2 secondary antibody restored the subsequent delivery of the LBRC to the site of transmigration, thus restoring TEM. (Fig 7D–F Crosslinked samples). We conclude that L2 promotes transmigration by signaling for continued recruitment of the LBRC.

DISCUSSION

CD99L2 has been shown by our lab (5, 8) and others (4, 6, 10) to be important for efficient leukocyte extravasation in mice. Bixel et al showed that leukocytes were arrested at the basement membrane during leukocyte extravasation when blocking L2 using antibodies in C57BL/6 mice (4). Our lab demonstrated that the step in TEM inhibited by blockade or knockout of PECAM and CD99 in C57BL/6 and FVB/n mouse strains were different (9). Therefore, we wanted to determine the phenotype of L2 blockade in FVB/n mice. Our data revealed that L2 is required for transmigration of neutrophils, specifically migration across the endothelial cell border and not the basement membrane (Fig 1A, B). Intravital microscopy experiments further support the important role for L2 in transmigration, but not rolling or adhesion, during leukocyte recruitment (Fig 2A–D). Thus, as with PECAM and CD99, interfering with the function of CD99L2 has a different effect in FVB/n and C57BL/6 mice.

All previous studies on L2 have been performed in mice. The role of L2 in human inflammation had yet to be examined. Like mouse CD99, L2 is expressed on mouse leukocytes and endothelial cells (5, 8). Our data show that L2 is expressed on human leukocyte subsets and at human endothelial cell borders (Fig. 3). Similar to PECAM and CD99, two other endothelial cell border molecules that play roles in TEM, L2 is constitutively expressed (Fig. 3). The slightly granular appearance of CD99L2 seen in Fig. 3D and 3E was also seen when non-permeabilized cells were stained, and may be the consequence of using polyconal primary and secondary antibodies. The data in this study show that antibody inhibition or knockdown of the expression of L2 in human endothelial cells also arrests leukocytes partway through the endothelial cell junction (Fig. 6). Monocyte and neutrophil transmigration are substantially reduced in the presence of antibodies against L2, as they are in the presence of blocking antibodies against PECAM and CD99 (Fig 4). In addition, knockdown of endothelial L2 reduced monocyte transmigration to the same degree (Fig 4D); off-target effects were ruled out since re-expression of L2 restored TEM to baseline levels (Fig. 4D). These data are the first evidence of L2 playing a major functional role in human leukocyte transendothelial migration.

Just like the leukocyte adhesion to the vessel wall, TEM is a multistep process that relies on protein interactions that occur in sequential order. Previous data from our lab showed that TEM is sequentially regulated on the endothelial side by PECAM-1 and CD99 (3) interacting with PECAM and CD99 on the leukocyte, respectively. Previous studies have suggested that mouse CD99 and mouse L2 function at the same step but independent of PECAM (10). Our in vitro studies with human cells, where we can control the environment more precisely than in vivo, show that human CD99 and L2 do not function at the same step in TEM but rather L2 regulates a step in TEM before the one regulated by CD99 and after the one regulated by PECAM (Fig. 6). The differences between these studies could be due to the different species.

Both human PECAM and CD99 function homophilically to support leukocyte transmigration. Inhibition of these interactions disrupts TEM (3, 24, 27, 32). Previous studies in C57BL/6 mice show that endothelial L2 interacted heterophilically with an unknown ligand on PMN to support transmigration (6, 7). We show that disruption of L2 function on either human leukocytes or endothelial cells attenuated transmigration (Fig 5A). Our data also show that leukocyte transmigration is reduced in the presence of soluble L2-Fc (Fig 5C). Additionally, L2 extracellular domain interfered with the ability of leukocytes to bind wells coated with soluble L2-Fc (Fig 5E; 5F). These data, collectively, strongly suggest that like PECAM and CD99, human L2 functions homophilically to support transmigration in human cells. We do not know whether the heterophilic interaction reported in mice (7) is relevant for all mice or limited to the C57BL/6 mouse strain.

Targeted recycling of the LBRC is critical for successful leukocyte extravasation. Homophilic PECAM leukocyte-endothelial interaction is required for activating the cation channel TRPC6, which allows entry of calcium that is required for TEM (31). The calcium signaling proceeds through IQGAP1 (18) and CaMKIIδ (33) to initiate recruitment of the LBRC to the site of transmigration. Blocking PECAM disrupts the initial delivery of the LBRC, thus preventing TEM (25). Homophilic CD99 interaction, which also recruits the

LBRC, but functions through activation of protein kinase A (2) is not required for the initiation of targeted recycling of the LBRC. Watson et al. showed initial LBRC enrichment surrounding arrested leukocytes despite blocking the CD99 facilitated step of TEM. Their data revealed that CD99 is required for continued recycling of the LBRC membrane to the site of transmigrating leukocytes. By restoring CD99 signaling, they restored targeted recycling and thus allowed TEM to continue to completion (2). In this study, we too, observed the initial LBRC enrichment around arrested leukocytes in the presence of L2 inhibition of TEM (Fig 7A–C). Further influx of LBRC did not occur once the leukocytes were blocked at the L2-dependent step. However, LBRC targeted recycling and TEM were restored upon restoration of L2 signaling in EC (Fig 7D–F) by mimicking engagement with leukocyte L2. These data indicate that, as with CD99, blocking L2 function disrupts further recruitment of the LBRC to the site of migrating leukocytes, thus preventing transmigration. These data further support the hypothesis that L2 facilitates a step in TEM that occurs between PECAM and CD99 dependent steps.

In conclusion, we show for the first time a novel and important role for L2 in human leukocyte transmigration. Leukocyte L2 and endothelial L2 are both required for the continued recruitment of the LBRC to the site of transmigration and the forward progression of migrating leukocytes in TEM. The molecular signaling pathway that L2 uses to promote TEM is a focus of future research. L2 is a relatively understudied molecule. Indeed, there have been very few reports of potential (indirect) L2 signaling partners in mice (34) and none in humans. Previous studies in mice show the promise of L2 as a potential therapeutic target for several inflammatory disorders such as peritonitis, dermatitis, and neuroinflammation (5–8). This report is the first to demonstrate that human L2 plays a significant role in TEM, and thus makes these studies clinically relevant. Gaining a better understanding of how L2 facilitates transmigration will allow for development of potential therapeutics for inflammatory diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Suh YH, Shin YK, Kook MC, Oh KI, Park WS, Kim SH, Lee IS, Park HJ, Huh TL, and Park SH 2003. Cloning, genomic organization, alternative transcripts and expression analysis of CD99L2, a novel paralog of human CD99, and identification of evolutionary conserved motifs. Gene 307: 63–76. [PubMed: 12706889]
2. Watson RL, Buck J, Levin LR, Winger RC, Wang J, Arase H, and Muller WA 2015. Endothelial CD99 signals through soluble adenylyl cyclase and PKA to regulate leukocyte transendothelial migration. J. Exp. Med. 212: 1021–1041. [PubMed: 26101266]

3. Schenkel AR, Mamdouh Z, Chen X, Liebman RM, and Muller WA 2002. CD99 plays a major role in the migration of monocytes through endothelial junctions. Nat. Immunol. 3: 143–150. [PubMed: 11812991]
4. Bixel MG, Petri B, Khandoga AG, Khandoga A, Wolburg-Buchholz K, Wolburg H, Marz S, Krombach F, and Vestweber D 2007. A CD99-related antigen on endothelial cells mediates neutrophil, but not lymphocyte extravasation in vivo. Blood 109: 5327–5336. [PubMed: 17344467]
5. Schenkel AR, Dufour EM, Chew TW, Sorg E, and Muller WA 2007. The Murine CD99-Related Molecule CD99-Like 2 (CD99L2) Is an Adhesion Molecule Involved in the Inflammatory Response. Cell Commun Adhes 14: 227–237. [PubMed: 18163232]
6. Samus M, Seelige R, Schafer K, Sorokin L, and Vestweber D 2018. CD99L2 deficiency inhibits leukocyte entry into the central nervous system and ameliorates neuroinflammation. J Leukoc Biol 104: 787–797. [PubMed: 29791026]
7. Seelige R, Natsch C, Marz S, Jing D, Frye M, Butz S, and Vestweber D 2013. Cutting edge: Endothelial-specific gene ablation of CD99L2 impairs leukocyte extravasation in vivo. J Immunol 190: 892–896. [PubMed: 23293350]
8. Rutledge NS, Weber EW, Winger R, Tourtellotte WG, Park SH, and Muller WA 2015. CD99-like 2 (CD99L2)-deficient mice are defective in the acute inflammatory response. Exp Mol Pathol 99: 455–459. [PubMed: 26321243]
9. Sullivan DP, Watson RL, and Muller WA 2016. 4D intravital microscopy uncovers critical strain differences for the roles of PECAM and CD99 in leukocyte diapedesis. Am J Physiol Heart Circ Physiol 311: H621–632. [PubMed: 27422987]
10. Bixel MG, Li H, Petri B, Khandoga AG, Khandoga A, Zarbock A, Wolburg-Buchholz K, Wolburg H, Sorokin L, Zeuschner D, Maerz S, Butz S, Krombach F, and Vestweber D 2010. CD99 and CD99L2 act at the same site as, but independently of, PECAM-1 during leukocyte diapedesis. Blood 116: 1172–1184. [PubMed: 20479283]
11. Wakelin MW, Sanz M-J, Dewar A, Albelda SM, Larkin SW, Boughton-Smith N, Williams TJ, and Nourshargh S 1996. An anti-platelet/endothelial cell adhesion molecule-1 antibody inhibits leukocyte extravasation from mesenteric microvessels in vivo by blocking the passage through basement membrane. Journal of Experimental Medicine 184: 229–239. [PubMed: 8691137]
12. Thompson RD, Noble KE, Larbi KY, Dewar A, Duncan GS, Mak TW, and Nourshargh S 2001. Platelet-endothelial cell adhesion molecule-1 (PECAM-1)-deficient mice demonstrate a transient and cytokine-specific role for PECAM-1 in leukocyte migration through the perivascular basement membrane. Blood 97: 1854–1860. [PubMed: 11238129]
13. Muller WA, Ratti CM, McDonnell SL, and Cohn ZA 1989. A human endothelial cell-restricted, externally disposed plasmalemmal protein enriched in intercellular junctions. J. Exp. Med. 170: 399–414. [PubMed: 2666561]
14. Ali J, Liao F, Martens E, and Muller WA 1997. Vascular endothelial cadherin (VE-Cadherin): Cloning and role in endothelial cell-cell adhesion. Microcirculation 4: 267–277. [PubMed: 9219219]
15. Wright SD, Rao PE, Van Voorhis WC, Craigmyle LS, Lida K, Talle MA, Westberg EF, Goldstein G, and Silverstein SC 1983. Identification of the C3bi receptor of human monocytes and macrophages by using monoclonal antibodies. Proceedings of the National Academy of Science 80: 5699–5703.
16. Bogen S, Pak J, Garifallou M, Deng X, and Muller WA 1994. Monoclonal antibody to murine PECAM-1 [CD31] blocks acute inflammation in vivo. J Exp Med 179: 1059–1064. [PubMed: 8113674]
17. Liao F, Huynh HK, Eiroa A, Greene T, Polizzi E, and Muller WA 1995. Migration of monocytes across endothelium and passage through extracellular matrix involve separate molecular domains of PECAM-1. J Exp Med 182: 1337–1343. [PubMed: 7595204]
18. Sullivan DP, Dalal PJ, Jaulin F, Sacks DB, Kreitzer G, and Muller WA 2019. Endothelial IQGAP1 regulates leukocyte transmigration by directing the LBRC to the site of diapedesis. J Exp Med 316.
19. Schenkel AR, Chew TW, and Muller WA 2004. Platelet endothelial cell adhesion molecule deficiency or blockade significantly reduces leukocyte emigration in a majority of mouse strains. J Immunol 173: 6403–6408. [PubMed: 15528380]

20. Muller WA, and Weigl S 1992. Monocyte-selective transendothelial migration: Dissection of the binding and transmigration phases by an in vitro assay. J Exp Med 176: 819–828. [PubMed: 1512545]
21. Muller WA, and Luscinskas FW 2008. Assays of transendothelial migration in vitro. Methods Enzymol. 443: 155–176. [PubMed: 18772016]
22. Sullivan DP, Seidman MA, and Muller WA 2013. Poliovirus receptor (CD155) regulates a step in transendothelial migration between PECAM and CD99. Am J Pathol 182: 1031–1042. [PubMed: 23333754]
23. Falco M, Marcenaro E, Romeo E, Bellora F, Marras D, Vely F, Ferracci G, Moretta L, Moretta A, and Bottino C 2004. Homophilic interaction of NTBA, a member of the CD2 molecular family: induction of cytotoxicity and cytokine release in human NK cells. Eur J Immunol 34: 1663–1672. [PubMed: 15162436]
24. Muller WA, Weigl SA, Deng X, and Phillips DM 1993. PECAM-1 is required for transendothelial migration of leukocytes. J Exp Med 178: 449–460. [PubMed: 8340753]
25. Mamdouh Z, Chen X, Pierini LM, Maxfield FR, and Muller WA 2003. Targeted recycling of PECAM from endothelial cell surface-connected compartments during diapedesis. Nature 421: 748–753. [PubMed: 12610627]
26. Liao F, Ali J, Greene T, and Muller WA 1997. Soluble domain 1 of platelet-endothelial cell adhesion molecule (PECAM) is sufficient to block transendothelial migration in vitro and in vivo. J Exp Med 185: 1349–1357. [PubMed: 9104821]
27. Dufour EM, Deroche A, Bae Y, and Muller WA 2008. CD99 is essential for leukocyte diapedesis in vivo. Cell Commun Adhes 15: 351–363. [PubMed: 18923973]
28. Nourshargh S, Krombach F, and Dejana E 2006. The role of JAM-A and PECAM-1 in modulating leukocyte infiltration in inflamed and ischemic tissues. J Leukoc Biol 80: 714–718. [PubMed: 16857733]
29. Feng G, Sullivan DP, Han F, and Muller WA 2015. Segregation of VE-cadherin from the LBRC depends on the ectodomain sequence required for homophilic adhesion. J Cell Sci 128: 576–588. [PubMed: 25501813]
30. Mamdouh Z, Kreitzer GE, and Muller WA 2008. Leukocyte transmigration requires kinesin-mediated microtubule-dependent membrane trafficking from the lateral border recycling compartment. J Exp Med 205: 951–966. [PubMed: 18378793]
31. Weber EW, Han F, Tauseef M, Birnbaumer L, Mehta D, and Muller WA 2015. TRPC6 is the endothelial calcium channel that regulates leukocyte transendothelial migration during the inflammatory response. J. Exp. Med. 212: 1883–1899. [PubMed: 26392222]
32. Muller WA 2009. Mechanisms of transendothelial migration of leukocytes. Circ. Res. 105: 223–230. [PubMed: 19644057]
33. Dalal PJ, Sullivan DP, Weber EW, Sacks DB, Gunzer M, Grumbach IM, Heller Brown J, and Muller WA 2020. Spatiotemporal Restriction of Endothelial Cell Calcium Signaling is Required during Leukocyte Transmigration. The Journal of Experimental Medicine In press.
34. Song J, Zhang X, Buscher K, Wang Y, Wang H, Di Russo J, Li L, Lutke-Enking S, Zarbock A, Stadtmann A, Striewski P, Wirth B, Kuzmanov I, Wiendl H, Schulte D, Vestweber D, and Sorokin L 2017. Endothelial Basement Membrane Laminin 511 Contributes to Endothelial Junctional Tightness and Thereby Inhibits Leukocyte Transmigration. Cell Rep 18: 1256–1269. [PubMed: 28147279]

Key Points:

1. Human CD99L2 is expressed on human leukocytes and human endothelial cells.
2. Human CD99L2 regulates TEM of leukocytes between PECAM and CD99 dependent steps.
3. Human CD99L2 is required for continuous targeted recycling of the LBRC.

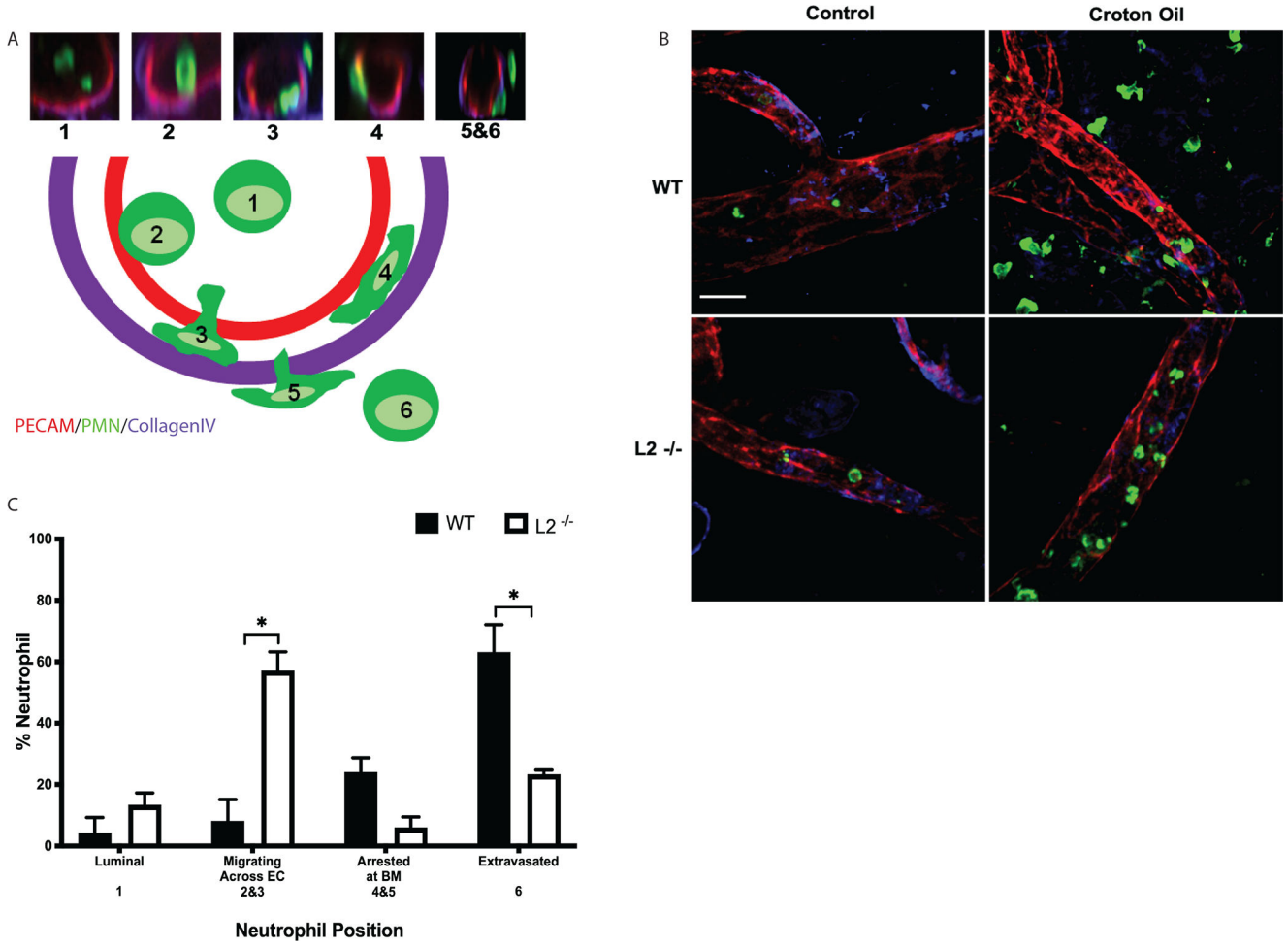

Figure 1. Genetic ablation of L2 arrests leukocytes partially through the endothelium in vivo.

The ears of wild-type (WT) and L2 knockout (L2 −/−) mice were treated with croton oil or carrier control. Five hours post stimulation, mice were euthanized, and ears were harvested. Wholemount sections were stained with antibody against PECAM, Collagen IV and Neutrophils. Sections were examined by confocal microscopy. **(A)** Neutrophil arrest position was ascertained by examination of orthogonally oriented confocal image stacks rotated to give a cross-sectional view. Positions were scored relative to the vessel and basement membrane, as shown in the Schematic. Position 1 (luminal), position 2 (apical), position 3 (Migrating Across EC), position 4 (Arrested at basement membrane), position 5 (Migrating through basement membrane), and position 6 (extravasated). **(B)** Representative images of WT and L2 $^{-/-}$ mouse ears with and without inflammatory stimulus. PMN are green, vessels are red and basement membrane is purple. Images are representative of 3 individual experiments. Scale bar= 50 μm **(C)** Neutrophil position of arrest was quantified in wild type and CD99L2 knockout mice post inflammatory stimulus. At least 8 fields per sample were imaged and quantified. Quantified data shows the average percent of neutrophil arrest site of 3 individual experiments based on the diagram in (A). Error bars show standard error of mean (SEM) *, P<0.001Student t-test.

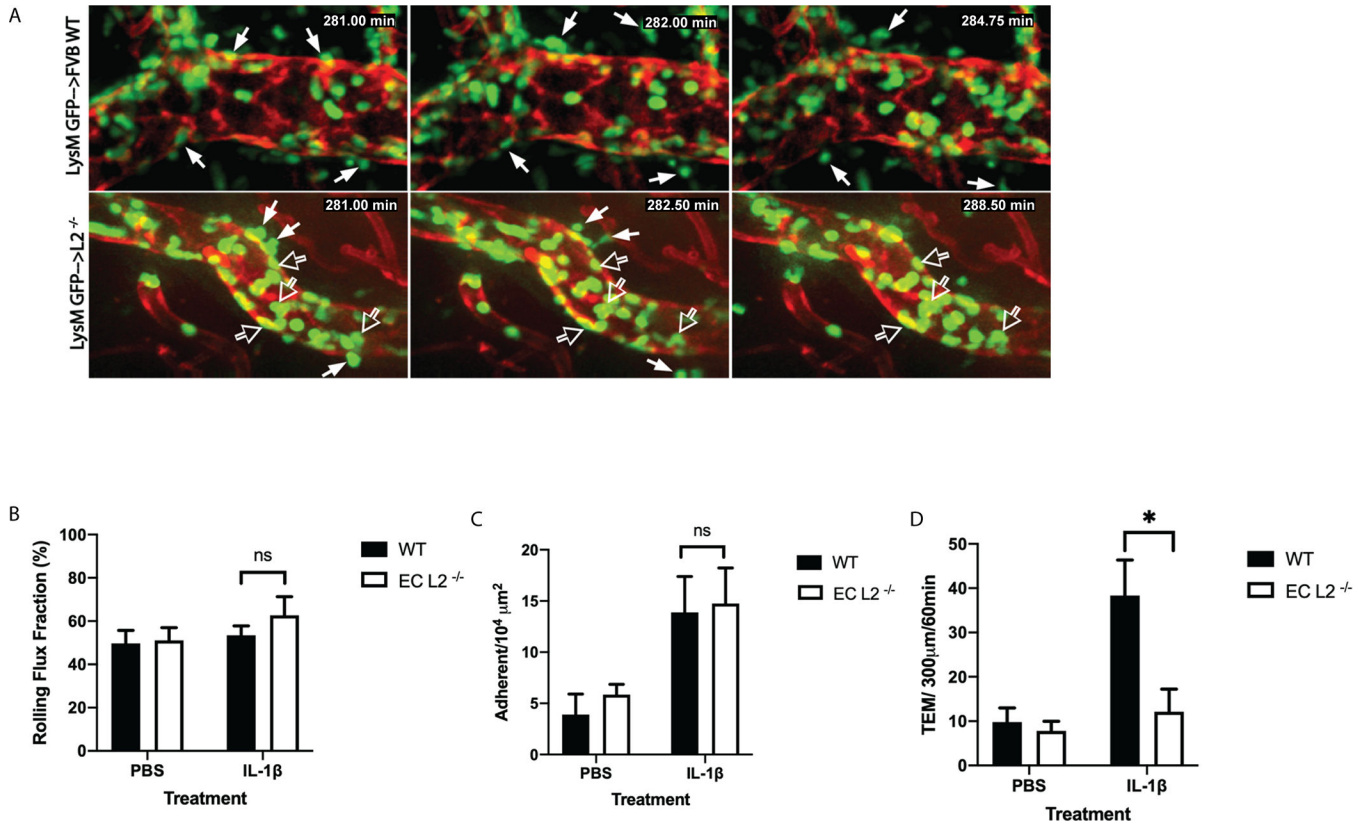

Figure 2. Genetic ablation of endothelial L2 disrupts leukocyte TEM but not leukocyte adherence or rolling in vivo.

FVB/n WT and L2 −/− mice received bone marrow transfers from FVB/n LysM-GFP. **(A)** WT mice and mice deficient in endothelial L2, respectively, were administered IL-1β intrascrotally and imaged live using intravital microscopy. Still images are sequential frames from a typical video. See videos 1 and 2. Solid white arrows label neutrophils undergoing TEM over time. Open arrows show neutrophils that remain arrested over time. Time stamp shows elapsed time since IL-1β minutes. Scale bar = 25 μm **(B-D)** Quantification of rolling flux, adhesion, and TEM events in IL-1β stimulated control and endothelial L2 knockout mice. Error bars show SEM. Sequential frames and quantified data are representative of 3 mice per group. *P<0.0001 Student's t-test.

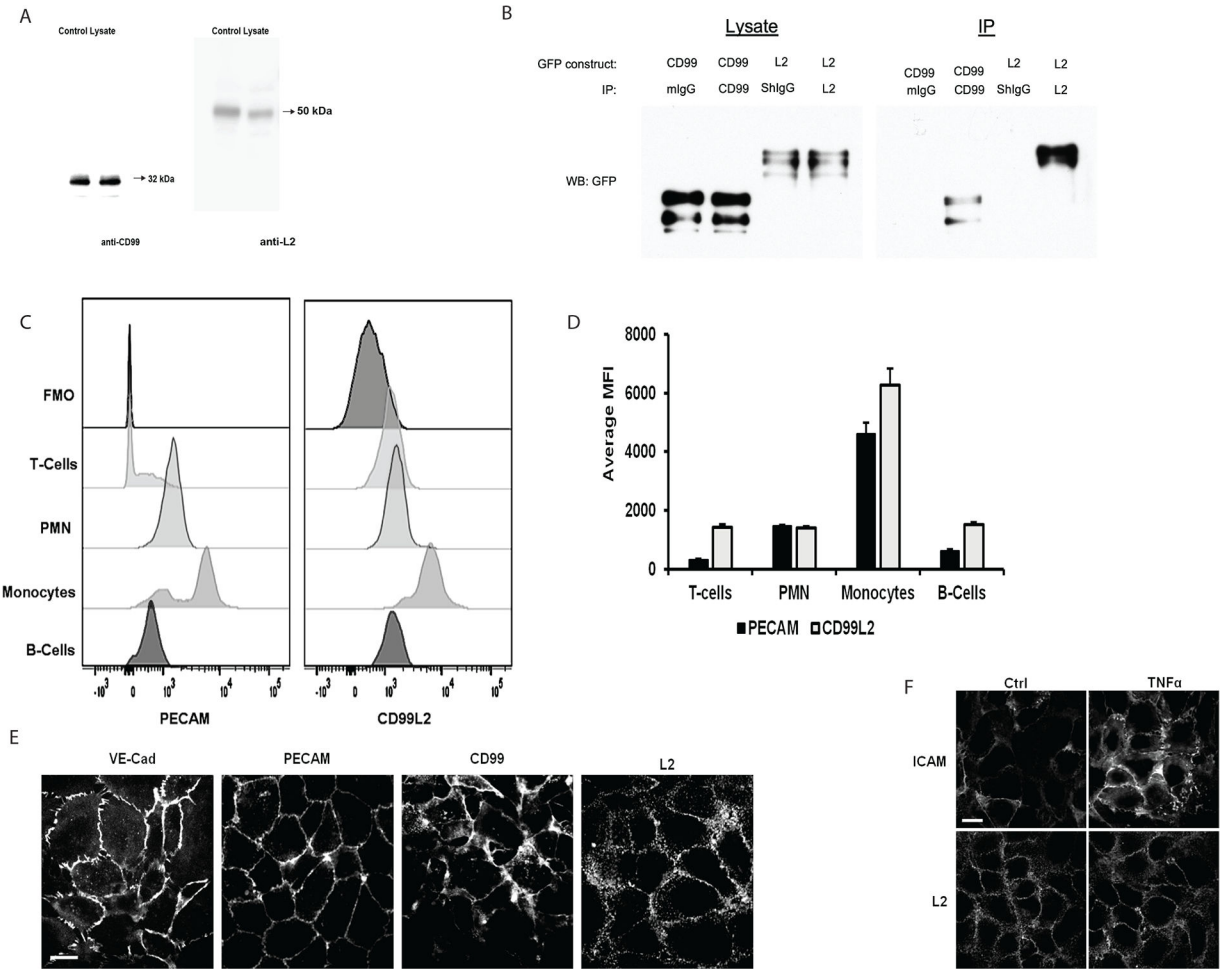

Figure 3. L2 is expressed on human leukocytes and along endothelial cell borders.

Leukocytes were prepared from peripheral blood collected from six healthy donors. (**A**) Lysates of primary human endothelial cells were analyzed using western blot analysis. Antibodies against CD99 and CD99L2 were used to probe for CD99 and CD99L2 respectively. Antibody against CD99 did not recognize CD99L2. Antibody against CD99L2 did not recognize CD99. (**B**) Antibodies against CD99 and CD99L2 were separately pre-bound to intact HUVEC, washed, and used to immunoprecipitate their respective antigens from the lysates. Anti-CD99 did not pull down CD99L2 and anti-CD99L2 did not pull down CD99. **(C)** Leukocytes were prepared from peripheral blood collected from six healthy donors. Histograms showing expression of CD99L2 and PECAM on leukocyte subsets. Histograms are representative of the 6 donors. FMO = Fluorescence minus one control. **(D)** Averaged mean fluorescence intensity (MFI) of leukocyte subsets expressing PECAM and CD99L2 among the 6 donors. **(E)** Confluent HUVECs were labeled with antibodies against VE-cadherin, PECAM, CD99 or CD99L2. Panels show positive junctional staining for each protein. (**F)** Confluent HUVEC were activated with TNFα (20 ng/mL in culture media) for four hours and stained for ICAM or L2. ICAM showed increased expression upon TNFα activation while L2 expression did not change. These panels are representative of 3 experiments. Scale bar = 50 μm.

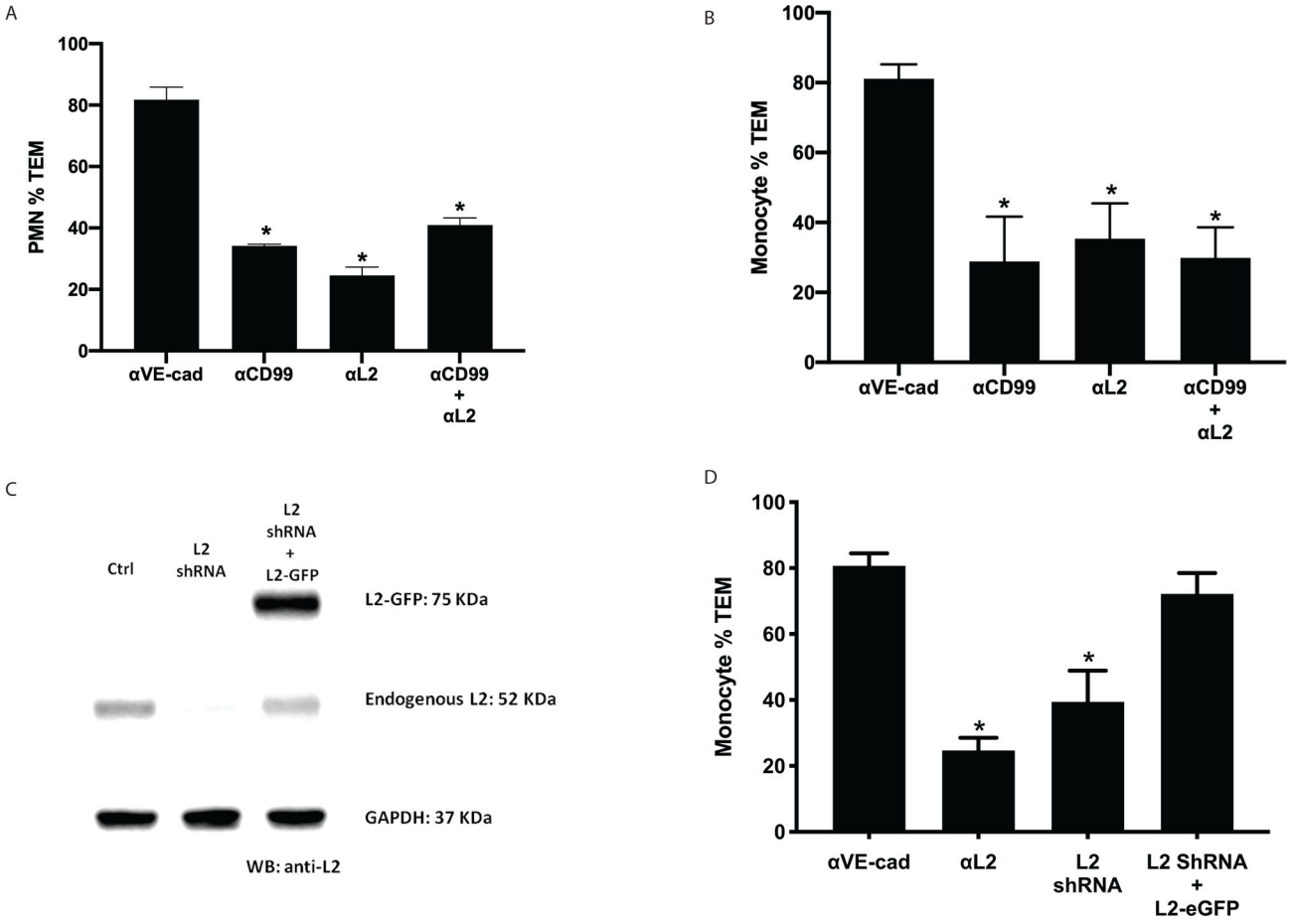

Figure 4. Inhibition of L2 function reduces TEM of human monocytes and neutrophils across human endothelial cells.

(A) Quantitative TEM assay on TNFα activated HUVECs cultured on collagen in 96-well plates. Antibodies against CD99 and/or L2 were used to block transmigration. Percent transmigration was quantified by counting number of neutrophils beneath the endothelial cell monolayer and dividing by total neutrophils (above and below). **(B)** Quantification of transmigration assay showing reduction in transmigration of monocytes when blocking CD99 and L2 simultaneously similarly to blocking CD99 and L2 individually. **(C)** HUVECs were pretreated with adenovirus expressing shRNA against the non-coding region of human L2. Some cultures later received adenovirus expressing full-length L2-eGFP cDNA to re-express it. Western blot shows successful knock down of endothelial human L2 (lane 2) and re-expression of L2 (lane 3). GAPDH was used as loading control. **(D)** Quantification of TEM assay showed reduced TEM of human monocytes across human endothelial cells when expression of human endothelial L2 is knocked down by shRNA (**L2 shRNA)** similar to inhibition of L2 function using antibodies against L2 (**αL2)**. TEM is restored upon re-expression of human endothelial L2 (L2**shRNA+L2-eGFP**). Negative control in all panels is non-blocking anti-VE-Cadherin mAb **(αVE-cad**). All assays are average transmigration of 3 individual experiments. Error bars show SEM. *P<0.0001 student t-test compared to control.

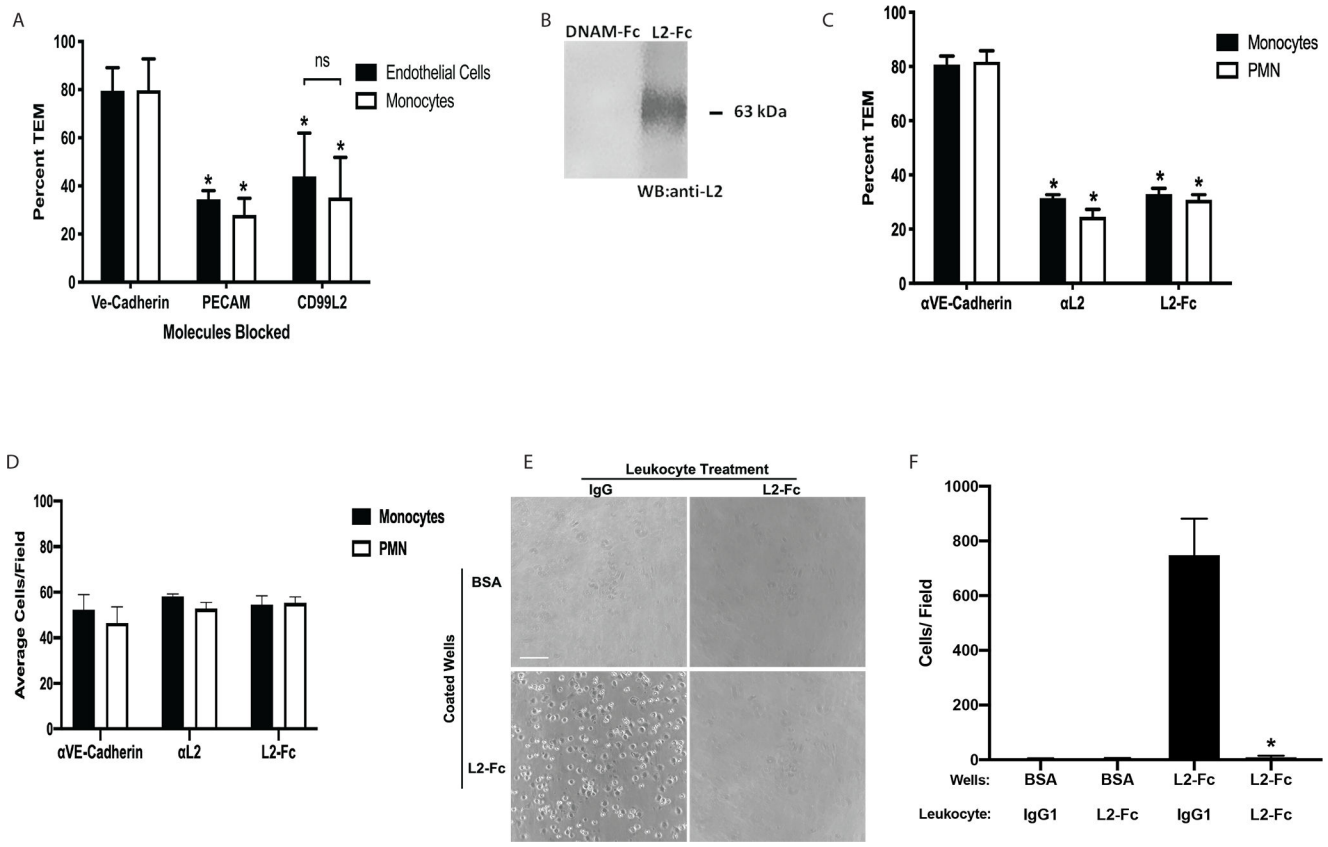

Figure 5. Endothelial L2 interacts homophilically with leukocyte L2 to facilitate TEM.

(A) Quantitative TEM assays were run as in figure 4. Antibody against L2 or PECAM and non-blocking control anti-VE-cadherin were preincubated with either EC (closed bar) or monocytes (open bar) and non-bound antibody was washed away before combining the cells. **(B)** Soluble L2-Fc was generated by expression of a fusion construct of the extracellular domain of L2 to human Fc. Fc portion was confirmed using ELISA. DNAM-Fc served as positive control for ELISA. Soluble L2-Fc was analyzed by western blot to confirm that L2-Fc runs at the appropriate size. DNAM-Fc served as negative control for anti-L2 blot. **(C)** Quantification of TEM assay showing reduced TEM of both monocytes and PMN in the presence of soluble L2-Fc is comparable to that of blocking TEM with antibodies against L2. **(D)** Quantification of adhesion in the presence of anti-L2 or soluble L2-Fc. (**E**) Images of binding assays showing that primary peripheral blood mononuclear cells can bind to wells coated with soluble CD99L2. Blocking CD99L2 on leukocytes prevented binging. Scale bar= 25μm (**F**) Cells/field were used to measure the binding of leukocytes to the coated wells. Error bars show SEM or 3 independent experiments; *p<0.0001. In (**C**) and (**D**), **Control** was anti-VE-cadherin mAb. In all panels the concentration of mAb or L2-Fc was 20 μg/ml. All TEM assays are averaged percent transmigration of 3 individual experiments. Errors bars show SEM. *P<0.01 (5A; student t-test) relative to control.; *p<0.0001 (5C student t-test) relative to control.

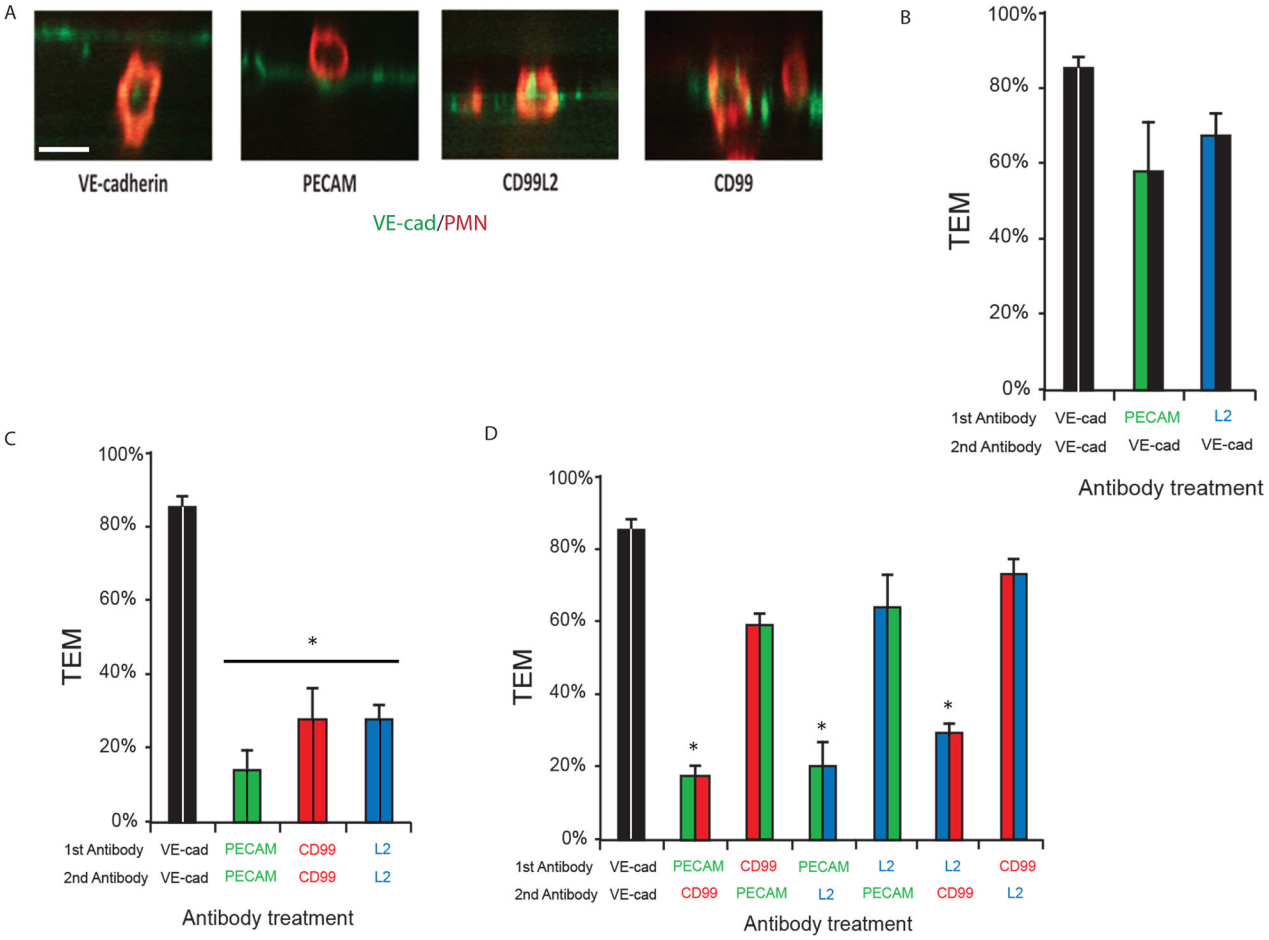

Figure 6. CD99L2 functions at a step in TEM between steps regulated by PECAM and CD99.

(A) Representative orthogonal images of TEM blocked by antibodies against PECAM, L2, and CD99. VE-cadherin (green) marks endothelial monolayer; PMN stained red by anti-CD18 **(B – D)** Quantification of sequential TEM assay. Transmigration was inhibited by blocking protein interactions sequentially. The color of the left half of the bar represents the first antibody incubation and color of the right half of the bar represents the second antibody incubation. The heights of the bars represent the final TEM count after the second incubation. **(B)** TEM resumes to control levels after washing away blocking antibodies and treating with non-blocking anti VE-cadherin. **(C)** Continued inhibition of TEM was seen when blocking PECAM, CD99 or L2 antibody was washed away as in (B) but added back again. **(D)** Overall TEM of monocytes when first blocking with the antibody against the indicated molecule, then washing it away and blocking with antibody against the indicated second molecule. Note that all of the incubations described for figures B, C, and D were performed at the same time on the same batches of leukocytes and endothelial cells. The data have been separated into three panels to facilitate description. All data are mean +/− SEM of 3 independent experiments. *$p<0.0001$ vs. VE-Cad/VE-Cad control; ANOVA. Scale bar= 10 μm

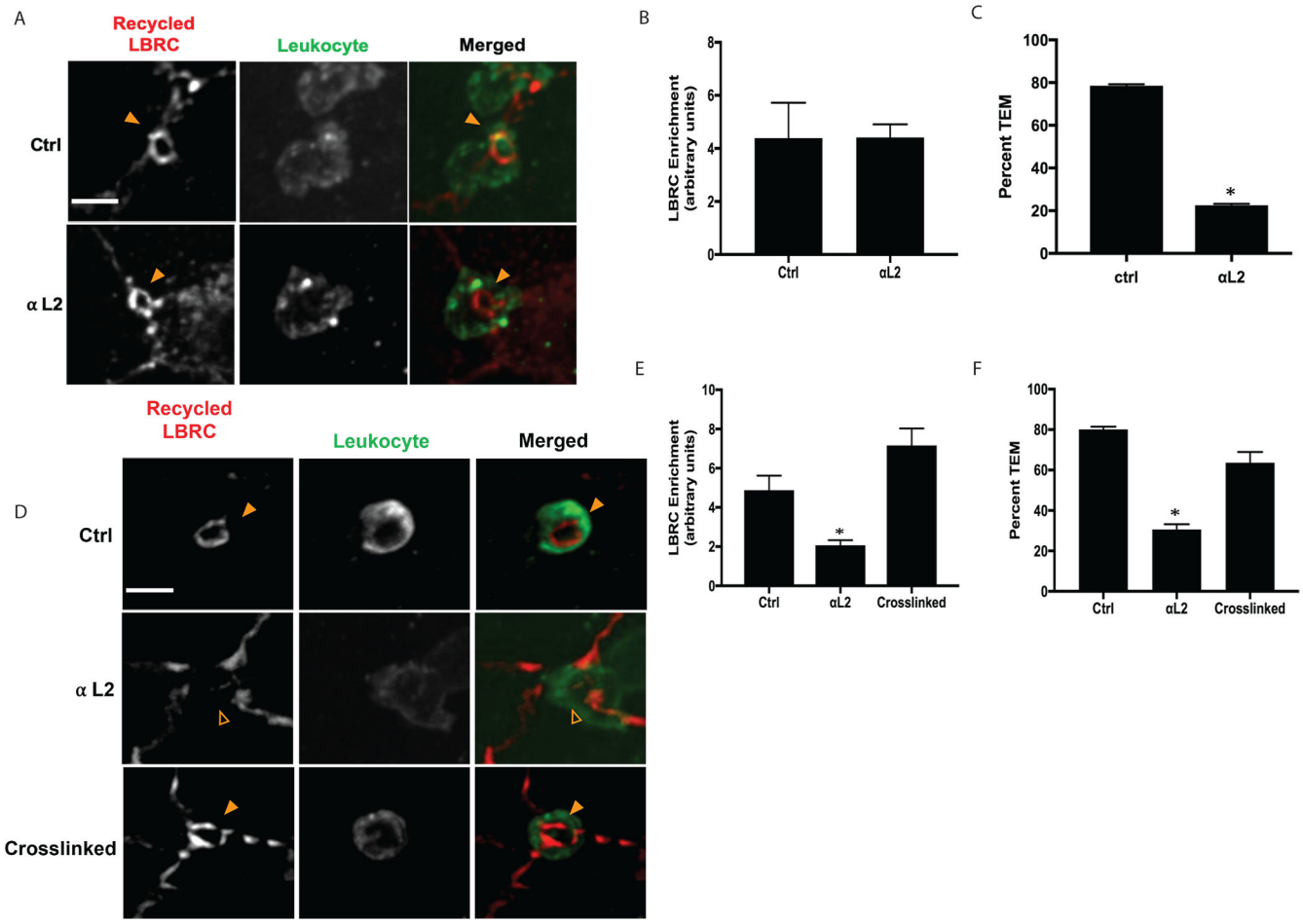

Figure 7. L2 clustering promotes continued TR to sites of TEM.

(A) Targeted recycling assay was performed on HUVEC monolayers pretreated with control antibody or anti-L2. Monolayers were fixed after 8 – 10 min allowing recruitment of enriched LBRC (red) around migrating leukocyte (green). **(B)** Quantification of initial LBRC enrichment around migrating leukocytes blocked by anti-L2 is similar to that of control leukocytes transmigrating for the same time. **(C)** Quantification of TEM assay run in parallel to the targeted recycling assay shows TEM of monocytes was truly blocked. **(D-F)** Targeted recycling assay run as in A-C but visualizing LBRC movement downstream of L2 blockade. Blocking L2 interaction prevented subsequent targeted recycling of LBRC. Cross-linking L2 (**Crosslinked**) restores the signal to recruit the LBRC and TEM goes to completion. (See text for details.) Solid arrowheads indicate recruitment of enriched LBRC around migrating monocyte. Open arrowheads indicate lack of recruitment of enriched LBRC. **A** and **D** are representative of 3 experiments. Data are mean +/− SEM for 3 independent experiments. *p<0.001 vs. Control; student t-test. Scale bar= 25 μm.