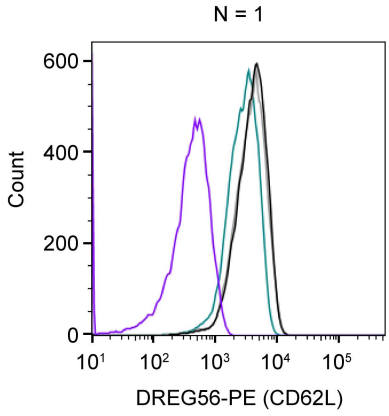
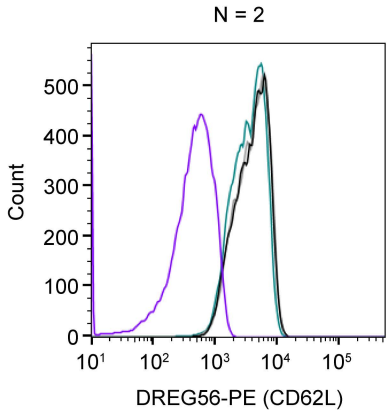
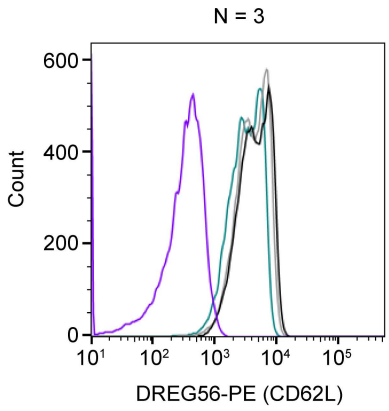


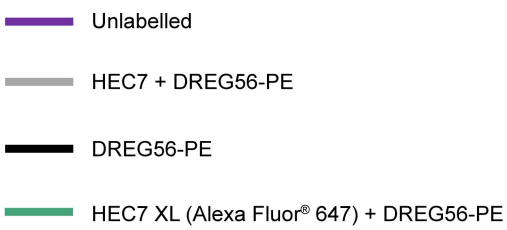
	Sample Name	Subset Name	Count	Median : FL2-A	Median : FL4-A
☐	C01 2Aug Cells Alone.fcs	Neutrophils	18180	400	244
☐	C02 2Aug Cells DREG56-PE.fcs	Neutrophils	18435	3928	243
☐	C04 2Aug HEC7 + D56-PE.fcs	Neutrophils	17964	3745	247
☐	C03 2Aug HEC7+2o 0`+D56-PE.fcs	Neutrophils	18502	2826	3854



	Sample Name	Subset Name	Count	Median : FL2-A	Median : FL4-A
☐	A01 3 Ago Cells alone.fcs	Neutrophils	19083	453	261
☐	A02 3 Ago Cells D56-PE.fcs	Neutrophils	17969	4099	284
☐	A03 3 Ago HEC7+ D56-PE.fcs	Neutrophils	18184	4110	285
☐	A04 3 Ago HEC7+2o 0`+ D56-PE.fcs	Neutrophils	19053	3561	4780



	Sample Name	Subset Name	Count	Median : FL2-A	Median : FL4-A
☐	G10 4Aug Cells Alone.fcs	Neutrophils	19335	330	217
☐	G11 4Aug Cells DREG56-PE.fcs	Neutrophils	19029	4380	223
☐	G12 4Aug HEC7+D56-PE.fcs	Neutrophils	19434	4066	226
☐	H01 4Aug HEC7+2o 0`+D56-PE.fcs	Neutrophils	19216	3185	3496

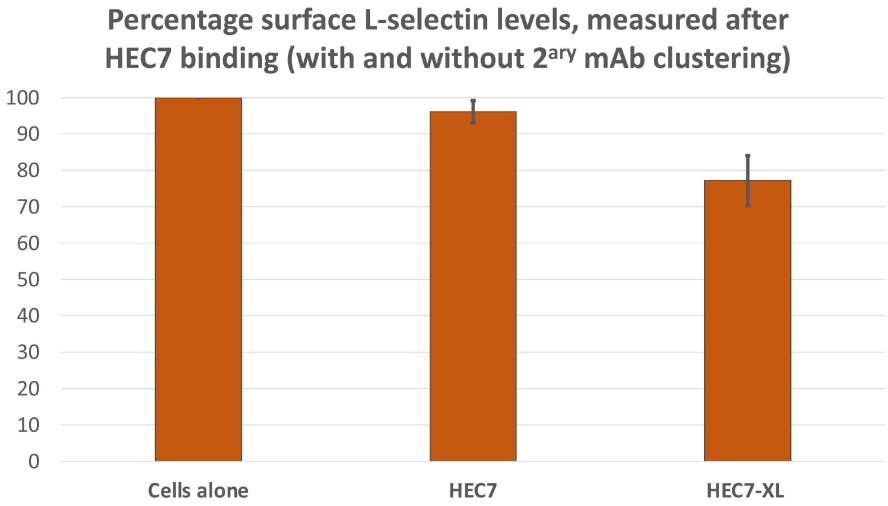


Supplementary Figure 1

Fig. S1

The effect of clustering PECAM-1 on surface L-selectin levels.

Flow cytometric analysis of primary human neutrophils treated with the anti-PECAM-1 monoclonal antibody, HEC7. Neutrophils were treated with HEC7 alone (grey line) or HEC7 plus a secondary clustering antibody conjugated to Alexa Fluor® 647 (green line). DREG56 directly conjugated to phycoerythrin was used to monitor L-selectin expression in untreated cells (black line), and cells with HEC7 alone (grey line) or with HEC7 plus clustering secondary antibody (green line). Purple line represents unstained neutrophils.

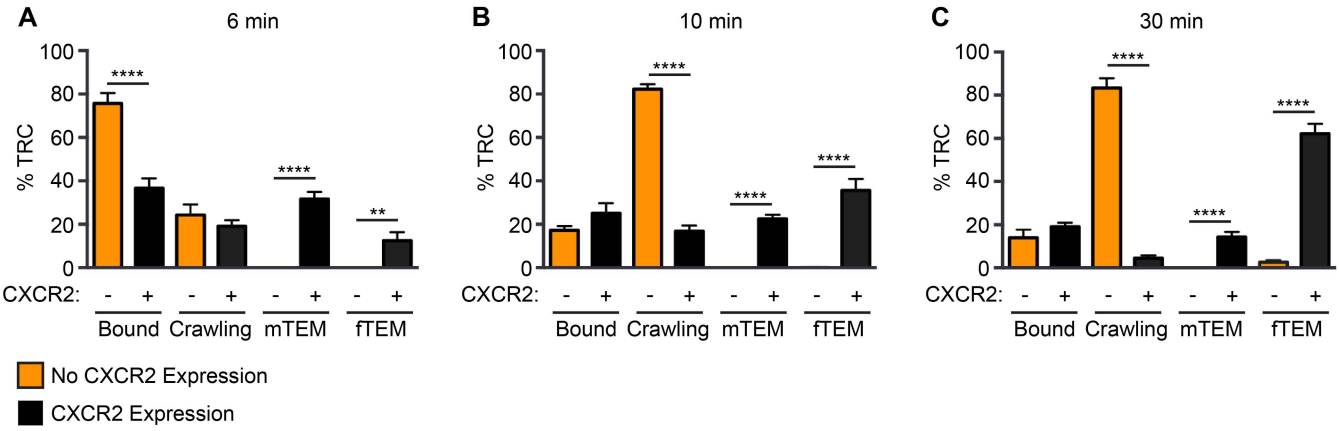


Supplementary Figure 2

Fig. S2

Summary of the data presented in Fig. S1

Bar graphs showing the impact of clustering PECAM-1 with HEC7 and secondary cross-linking monoclonal antibody can lead to a 22% reduction in L-selectin levels. Value of each bar: Cells alone = 100%; HEC7 = 96.15%; HEC7-XL = 77.18%

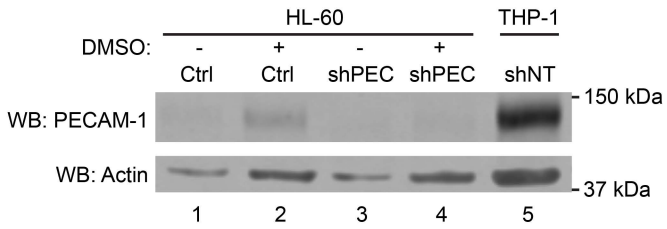


Supplementary Figure 3

Fig. S3

CXCR2 is essential for HL-60 TEM.

dHL-60 cells (orange bars) or dHL-60 cells stably expressing CXCR2 (black bars) were perfused over HUVEC monolayers, which were stimulated overnight with TNF-α. Flow assays were recorded as described in materials and methods (and see Movie 3 and stills in Fig. 5A). Transendothelial migration was scored by scoring for phase contrast. Cells were scored at 6 min, 15 min and 30 min. At each time point cells were scored as "Bound", "Crawling", in "mid-TEM" (see still images in panels 6 – 9 as an example of mid-TEM in Fig. 5A) or "full TEM" (see still image in panel 12 as an example of full TEM in Figure 5A). Error bars represent standard error of the mean. Statistical significance was assessed using an unpaired Student t-test, comparing the different cell lines at each separate stage of the adhesion cascade. **p<0.01, ***p<0.001 and ****p<0.0001.

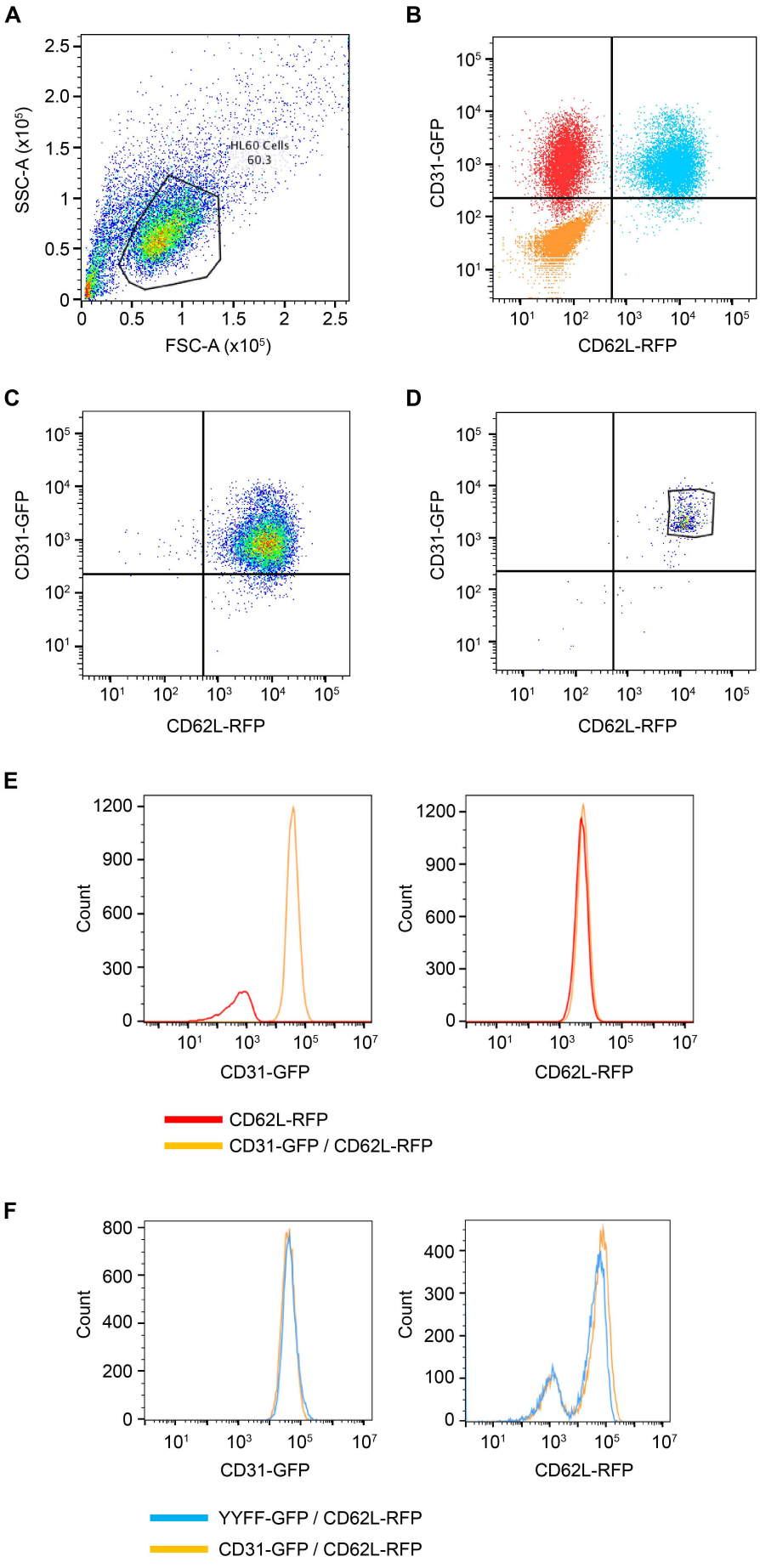


Supplementary Figure 4

Fig. S4

HL-60 cells express negligible levels of PECAM-1

Western blotting of whole cell lysates from THP-1 cells and HL-60 cells (stably expressing CXCR2) reveal that endogenous PECAM-1 is lowly expressed in HL-60 cells (lane 1), but highly expressed in THP-1 cells (lane 5). Low level PECAM-1 is expressed in HL-60 cells when differentiated in 1.3% DMSO (lane 2). This signal was deemed specific, as its expression could be reduced using lentiviral short hairpin (sh) RNA targeting PECAM-1. Given the low level of endogenous PECAM-1 in HL-60 cells, shRNA was not used to knock-down endogenous PECAM-1 in experiments requiring the expression of GFP-tagged PECAM-1.



Supplementary Figure 5

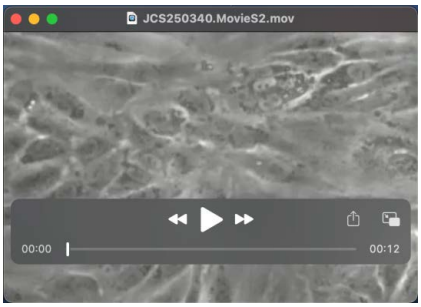
Fig. S5

Sorting of HL-60 cells after lentiviral transduction

HL-60 cells stably expressing CXCR2 were transduced with lentiviral particles possessing the open reading frames for PECAM-1-GFP and/or L-selectin-RFP (with a multiplicity of infection (MOI) of 10 for each construct). Cells were subsequently sorted prior to flow assays and FRET/FLIM experiments. (A) Lentivirally transduced cells were gated for the live cell population (black polygon). (B) Another gating event created quadrants based on expression: CD31-GFP$^-$ CD62L-RFP$^-$ (lower left, orange scatter); CD31-GFP$^+$ CD62L-RFP$^-$ (upper left scatter red scatter); CD31-GFP$^+$ CD62L-RFP$^+$ (upper right blue scatter); and CD31-GFP$^-$ CD62L$^+$ (lower right). (C) In an example of sorting for double-expressing cell lines, of the CD31-GFP$^+$ CD62L-RFP$^+$ population of live cells, (D) the top 5% were sorted and isolated from the sample. These cells were then cultured and cryopreserved for use in future assays. (E) FACS histograms provided are examples of similar expression levels between sorted single expressing L-selectin-RFP (red line) and the double expressing PECAM-1-GFP and L-selectin-RFP cell lines. The left histogram represents fluorescence intensity measurements of PECAM-1-GFP and the right histogram shows fluorescence intensity measurements of L-selectin-RFP. (F) Histogram plots of relative expression levels of PECAM-1 (left hand histogram) and L-selectin (right hand histogram) in the double-expressing cell lines: YYFF-PECAM-1-GFP/L-selectin-RFP and PECAM-1-GFP and L-selectin-RFP. It is noteworthy to mention that the FACS profiles of (E) and (F) were acquired on separate occasions.

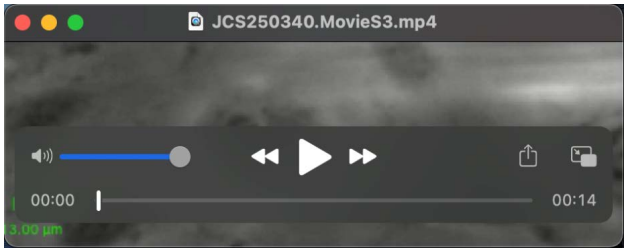
Movie 1

3D reconstituted image of a primary human neutrophil produced from iSIM analysis as described in Figure 1D of the main manuscript. Details of image acquisition are provided in the legend of Figure 1 in the main manuscript and in the Materials and Methods section. Cyan = PECAM-1 clusters. Green = L-selectin. Signal overlap is in white.



Movie 2

Primary human neutrophils were isolated from the whole blood of healthy volunteers and perfused over TNF-α-activated monolayers at a density of 1 x 10^6 cells per mL. The video represents how efficiently neutrophils undergo full TEM in our assays (over 90% efficiency). Phase bright cells are deemed adherent, whereas phase dark cells are deemed fully transmigrated. Time code in red represents minutes, seconds and milliseconds.



Movie 3

A representative video showing a dHL-60 cell undergoing adhesion, crawling and TEM. Stills taken from this movie are provided in Figure 5A of the main manuscript. Time code in red represents minutes, seconds and milliseconds.