Materials and Methods

Cells and reagents

All human materials were obtained from de-identified blood or tissue donors under protocols approved by the Yale Human Investigation Committee. CIITA-transduced human dermal microvascular EC (CIITA HDMEC) were generated using a retroviral vector and characterized as described [1]. Prior to flow experiments, CIITA HDMEC were incubated in the presence of 10 ng/ml recombinant human TNF (rhTNFα, R&D Systems) for 20-26 hours to upregulate adhesion molecules, and with 100 ng/ml recombinant TSST-1 (R-TT 606, Toxin Technology, Inc.) for 30 minutes on CIITA HDMEC to allow TCR-mediated activation of all T cells utilizing Vβ2 gene segment to form their TCR, approximately 5-20% of the circulating T cells in most donors. For antibody blocking experiments, EC were treated with 20 µg/ml anti-ICAM-1 (CD54, clone BBIG-I1, R&D Systems), VCAM-1 (clone P8B1, Millipore or clone B-N8, eBioscience), anti-PVR (CD112, clone R2.525, BD Pharmingen), and anti-nectin 2 (CD155, clone D171, GeneTex) 40 min prior to flow. For siRNA knockdown of PECAM-1 (CD31), ICAM-1, VCAM-1, JAM-A, and JAM-B cells were transfected 72 h prior to flow with 10 nM siRNA (PECAM-1_1, ICAM-1_7, VCAM-1_1, F11R_5 Qiagen, JAM-2-6, JAM-2-7, Ambion), as described [2 3]. To inhibit soluble adenylyl cyclase, EC were incubated with 50 µM KH7 (Cayman Chemical) 30 min prior to flow assays. For alloantigen experiments, HDMEC were treated with 50 ng/ml IFN-γ 72 h and 10 ng/ml TNF 20 h before flow assay.

Leukapheresis was performed on healthy volunteer adult donors and PBMCs were enriched by Ficoll-Hypaque density gradient centrifugation prior to cryopreservation of aliquotted cells. Total peripheral blood CD4 and CD8 T cells were isolated from the cryopreserved samples by positive selection with CD4 or CD8 Dynabeads magnetic beads and released with Detachabead (Dynal) according to the manufacturer's protocol. CD4 and CD8 T_{EM} were enriched by depletion of naïve and central memory cells with anti-CCR7 mAb (BioLegend) and pan-mouse IgG beads. Approximately 80-90% (CD4) or 60-70% (CD8) of the initial T cell population as well as essentially all other leukocyte types were removed by these manipulations. The remaining T cells, highly enriched for T_{EM}, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin overnight prior to assays. For pertussis toxin sensitivity, T cells were treated overnight with 100 ng/ml pertussis toxin (Sigma). To inhibit myosin IIA, T_{EM} were treated with 50 µM (-)-blebbistatin (Sigma), or vehicle (DMSO) 30 min and washed prior to flow TEM assays. For granule inhibition, T cells were treated for 2 h with 0.1 µM concanamycin A (Sigma), or vehicle (DMSO) and washed before flow assay. For serine protease inhibition, 100 µM AEBSF (Pefabloc, Roche) was included in the flow medium during the flow assay. For CD96 blocking, T cells were incubated with 20 µg/ml anti CD96 mAb (clone NK92.39, BioLegend) 30 min before flow. For integrin blocking, T cells were incubated with saturating levels of antibody 30 min before flow, as well as during flow. Blocking mAbs included anti-VLA-4/CD49d/α4 integrin clone PS/2 (LifeSpan Biosciences), anti-LFA-1/CD11a/αL integrin mAb TS1/22 (ATCC), anti-LFA-1, Mac-1/CD18/β2 integrin mAb TS1/18 (BioLegend), and anti-Mac-1/CD11b/αM integrin mAb ICRF44 (BioLegend).

TEM assays

CIITA HDMEC were grown to confluence on 20 μg/ml human plasma fibronectin-coated 35 mm coverglasses, treated with TNF and loaded with TSST-1 and as described (7), washed twice with RPMI/10% FBS, and assembled with a parallel plate flow chamber apparatus (Glycotech) using the 0.01 inch height, 5 mm wide slit gasket provided by the manufacturer. On a 37°C heating surface, $CCR7^{low}$ human CD4 or CD8 T_{EM} (1-2 x 10^6 cells/500 μl) suspended in the same medium were loaded onto the EC monolayer at 0.75 dyne/cm^2 for 2 minutes, followed by washing with medium only at 1 dyne/cm^2 for 5, 15 or 30 minutes. Samples were then fixed with 3.7% formaldehyde in PBS, stained with anti-Vβ2TCR mAb (Beckman Coulter), followed by Alexafluor 488 or 546-conjugated goat or donkey anti-mouse IgG, mounted on slides using mounting medium containing DAPI (Prolong Gold, Invitrogen). Alternatively, FITC conjugated anti-Vβ2TCR mAb (Beckman Coulter), AlexaFluor 488-conjugated rabbit anti-FITC, and AlexaFluor 488-conjugated goat anti-rabbit IgG were used for staining samples that have been treated with blocking mAb. Samples were then examined by microscopy with a Zeiss Axiovert 200M microscope. A FITC filter was used to detect FITC or Alexafluor 488-stained cells, a TRITC filter was used to detect Alexafluor 546-stained cells, and a DAPI filter used to detect DAPI-stained nuclei. Using a 40X/0.60 korr Ph2 objective, phase contrast optics were used to determine whether CD8 T cells were either on top or underneath the HDMEC monolayer. The percentage of transmigrated T cells were calculated for 100-200 cells per sample by analyzing five to ten groups of 20 cells each, calculating the percentage for each group, and calculating the mean and s.e.m. for the groups. For total adhesion, T cells in ten fields using a 10X objective were counted for each sample. For antigen-induced adhesion, the percentage of Vβ2TCR+ cells from a total of more than 300 total cells counted was calculated for each sample. This percentage was divided by the percentage of Vβ2TCR+ cells of the input as determined by FACS to obtain the fold enrichment.

Staining quantification

For P-ZAP70, samples were stained with anti-Vβ2TCR mAb (Beckman Coulter) and AlexaFluor 488-conjugated donkey anti-mouse IgG, permeabilized with 0.2% Triton X-100, blocked with 50% FBS/PBS, and stained with Phospho-Zap-70 (Tyr319) antibody (2701, Cell Signaling Technology). For granzyme A, samples were permeabilized with 0.2% Triton X-100, blocked with 50% FBS/PBS, stained with anti-human Granzyme A mAb (clone 2D9, BioLegend) and AlexaFluor 546-conjugated donkey anti-mouse IgG, incubated with mouse IgG (MOPC21) followed by FITC conjugated anti-Vβ2TCR mAb (Beckman Coulter), AlexaFluor 488-conjugated rabbit anti-FITC, and AlexaFluor 488-conjugated goat anti-rabbit IgG. Fluorescent images were acquired using Volocity, exported as TIFF files, and analyzed with Image J. In Image J, cells were outlined, measured, and the identical outline used to measure adjacent background. Background was subtracted from the cell measurement, and this value was multiplied by the area to obtain a number that represents the granzyme A content/cell. For P-ZAP70, all samples were acquired using the same exposure time. For granzyme A, the exposure time for CD4 cells was 16 times longer than for CD8 cells.

Confocal Microscopy

To visualize MTOC of CD8 T_{EM} transmigrating on CIITA HDMEC plus TSST-1, samples were stained with anti-Vβ2TCR mAb (Beckman Coulter), Alexa Fluor 546-conjugated donkey anti-mouse IgG (Invitrogen), permeabilized with 0.5% Triton/PBS, re-fixed with methanol/acetone (50/50), stained with rabbit anti-γ-tubulin (Sigma), Alexa Fluor 647-conjugated chicken anti-rabbit IgG and Alexa Fluor 488-conjugated phalloidin, and mounted on slides using mounting medium containing DAPI (Prolong Gold, Invitrogen). To visualize MTOC and lytic granules, samples were stained with anti-Vβ2TCR mAb (Beckman Coulter), Alexa Fluor 488-conjugated donkey anti-mouse IgG (Invitrogen), blocked with mouse IgG, permeabilized with 0.5% Triton/PBS, re-fixed with methanol/acetone (50/50), stained with rabbit anti-γ-tubulin (Sigma T3559), Alexa Fluor 546-conjugated donkey anti-rabbit IgG and Alexa Fluor 647-conjugated anti-granzyme B (clone GB11 BD Pharmingen), and mounted on slides using mounting medium containing DAPI (Prolong Gold, Invitrogen). Alternatively, samples were stained with anti-Vβ2TCR mAb (Beckman Coulter), Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen), permeabilized with 0.5% Triton/PBS, blocked with mouse IgG, stained with Zenon-Alexa Fluor 555 (Invitrogen)-labeled anti-granzyme A mAb (clone CB9 BioLegend), re-fixed with methanol/acetone (50/50), stained with rabbit anti-γ-tubulin (Sigma T3559), Alexa Fluor 647-conjugated chicken anti-rabbit IgG, and mounted on slides using mounting medium containing DAPI (Prolong Gold, Invitrogen). For alloantigen samples, samples were permeabilized with Triton, stained with NFAT-1 (BD Transduction Laboratories), Alexa Fluor 647conjugated goat anti-mouse IgG, blocked with 30 μg/ml MOPC, stained with FITC-conjugated anti-CD45 (Immunotech), re-fixed with methanol/acetone (50/50), stained with rabbit anti-γ-tubulin (Sigma), Alexa Fluor 546-conjugated donkey anti-rabbit IgG, and mounted on slides using mounting medium containing DAPI (Prolong Gold, Invitrogen).

Images were captured with a Leica TCS SP5 Spectral Confocal Microscope, 405UV using a 63X oil immersion objective and sequential scanning with 405 Diode, argon and He/Ne laser excitation lines of 405 nm, 488 nm, 543 nm, and 633 nm. Typically, 6-12 Z slices spanning portions of or the entire T cell were captured.

FACS analysis

To assess siRNA knockdown efficiency, Flow Cytometry Standard (FCS) files of CIITA HDMEC siRNA transfectants treated with TNF 18 h and stained with PE-conjugated anti-ICAM-1 mAb or control IgG (Beckman Coulter) were acquired using an LSRII flow cytometer (Becton Dickenson) with FACSDiva software (Becton Dickenson) and analyzed using Flowjo software (Tree Star). To assess Vβ2TCR percentage, PBMC were stained with FITC conjugated anti-Vβ2TCR (Beckman-Coulter), AlexaFluor 647-conjugated anti-CCR7 (Biolegend), and either eFluor 450 conjugated anti-CD4 or eFluor 450 conjugated anti-CD8 (eBioscience). Cells were gated according to CD4 or CD8, CCR7 low, and percent FITC positive were determined.

Statistics

For experiments in which more than two groups were compared, statistical significance was determined by one-way ANOVA using a 95% confidence interval and the Tukey post-test (Prism 6.0 for Macintosh). Statistical error is expressed as s.e.m. For experiments in which two groups were compared, a t-test was used. P values are designated as: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

References

1. Manes TD, Pober JS. Antigen presentation by human microvascular endothelial cells triggers ICAM-1-dependent transendothelial protrusion by, and fractalkine-dependent transendothelial migration of, effector memory CD4+ T cells. *Journal of immunology.* 2008;180:8386-8392.
2. Manes TD, Hoer S, Muller WA, Lehner PJ, Pober JS. Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins block distinct steps in transendothelial migration of effector memory CD4+ T cells by targeting different endothelial proteins. *Journal of immunology.* 2010;184:5186-5192.
3. Manes TD, Pober JS. Identification of endothelial cell junctional proteins and lymphocyte receptors involved in transendothelial migration of human effector memory CD4+ T cells. *Journal of immunology.* 2011;186:1763-1768.