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Identification of Endothelial Cell Junctional Proteins and Lymphocyte Receptors Involved in Transendothelial Migration of Human Effector Memory CD4$^+$ T Cells

Thomas D. Manes* and Jordan S. Pober*,†,‡

Human effector memory (EM) CD4$^+$ T cells can rapidly transmigrate across an endothelial cell (EC) monolayer in response either to chemokine or to TCR-activating signals displayed by human dermal microvascular EC under conditions of venular shear stress. We previously reported that the TCR-stimulated transendothelial migration (TEM) depends on fractalkine (CX3CL1), PECAM-1 (CD31), and ICAM-1 (CD54) expression by the EC, whereas chemokine-stimulated TEM does not. In this study, we further analyze these responses using blocking mAb and small interfering RNA knockdown to show that TCR-stimulated TEM depends on CD99 on EC as well as on PECAM-1 and depends on nectin-2 (CD112) and poliovirus receptor (CD155) as well as EC ICAM-1. ICAM-1 is engaged by EM CD4$^+$ T cell LFA-1 (CD11a/CD18) but not Mac-1 (CD11b/CD18); nectin-2 and poliovirus receptor are engaged by both DNAX accessory molecule-1 (CD226) and Tactile (CD96). EC junctional adhesion molecule-1 (JAM-1), an alternative ligand for LFA-1, contributes exclusively to chemokine-stimulated TEM and ICAM-2 appears to be uninvolved in either pathway. These data further define and further highlight the differences in the two pathways of EM CD4$^+$ T cell recruitment into sites of peripheral inflammation. ***The Journal of Immunology*****, 2011, 186: 1763–1768.**

A cardinal feature of the adaptive immune system is memory; recall responses to an Ag are faster and stronger than are primary responses. In the T cell compartment, memory arises both from clonal expansion of naive T cells that are specific for a particular Ag and from developmental changes of naive T cells into memory cells that have reduced activation requirements from and more rapidly acquire effector functions than naive T cells. In the circulation, memory T cells may be further divided into central memory (CM) T cells that home to secondary lymphoid organs and effector memory (EM) T cells that can be directly recruited into sites of peripheral inflammation. We have previously reported that human EM CD4$^+$ T cells, but not naive CD4$^+$ T cells or CM CD4$^+$ T cells, can rapidly (within 10 min) transmigrate across cultured endothelial cell (EC) monolayers in response to the inflammatory chemokine inflammatory protein-10 (IP-10) (CXCL10) (1). Transendothelial migration (TEM) required that the ECs express either ICAM-1 or VCAM-1 and that the adherent T cells be subjected to venular levels of shear stress (1). The interactions with ICAM-1 (CD54) and VCAM-1 (CD106) are consistent with the observations that, compared with naive T cells, EM T cells upregulate expression of the counterreceptors for these molecules, namely LFA-1 (CD11a/CD18) and VLA-4 (CD49d/CD29), respectively.

In humans and most other mammals (rats and mice excepted), peripheral ECs in the microvasculature basally express high levels of both class I and II MHC molecules. The only well documented function of these molecules is to present peptide Ags to CD8$^+$ and CD4$^+$ T cells, respectively. Cultured human dermal microvascular (HDM)ECs that have been pretreated with IFN-γ to reinduce MHC class II molecules (which are lost during culture) are able to activate resting memory T cells to secrete cytokines and proliferate and are particularly adept at inducing EM CD4$^+$ T cells to secrete effector cytokines (2). We had wondered whether Ag presentation by ECs played any role in TEM and found that engagement of the TCR of CD4$^+$ T cells by superantigen or anti-CD3 mAb displayed on the surface of cultured HDMECs under conditions of venular shear stress can also trigger TEM of EM but not naive or CM CD4$^+$ T cells (3). Remarkably, TCR engagement actually blocks the response of EM CD4$^+$ T cells to IP-10 (3, 4). TEM by the TCR-activated pathway differs from the inflammatory chemokine response in that it depends upon EC expression of fractalkine (CX3CL1), PECAM-1 (CD31), and ICAM-1, the latter in a manner that cannot be replaced by VCAM-1 (3, 5). The role of fractalkine is consistent with the observation that EM T cells express high levels of the receptor for this molecule (6) and that HDMECs display high levels of fractalkine when activated by TNF (7).

The role of PECAM-1 and the selective requirement for ICAM-1 were not anticipated in the process of TCR-driven TEM by EM CD4$^+$ T cells, raising several new questions. In fact, PECAM-1 had been reported to be uninvolved in T cell TEM (8, 9), although previous experiments had focused on the chemokine pathway. The role of PECAM-1 in TEM of other leukocytes involves recruitment of this molecule from the lateral border recycling compartment (LBRC), which is the site of most PECAM-1 molecules

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Abbreviations used in this article: CM, central memory; DNAM-1, DNAX accessory molecule-1; EC, endothelial cell; EM, effector memory; HDMEC, human dermal microvascular endothelial cell; IP-10, inflammatory protein-10; JAM-1, junctional adhesion molecule-1; LBRC, lateral border recycling compartment; PVR, poliovirus receptor; siRNA, small interfering RNA; TEM, transendothelial migration; TSST-1, toxic shock syndrome toxin-1.

in the resting EC, to the contact region between the EC plasma membrane and that of the leukocyte (10). The interaction with PECAM-1 has been shown to be coordinated with a subsequent interaction involving CD99, another protein resident within and mobilized from the LBRC. Is CD99 also involved in the TCR-dependent pathway of TEM by EM CD4$^+$ T cells? It is also unclear why VCAM-1 is unable to substitute for ICAM-1 in TCR-initiated TEM but can do so in chemokine-initiated TEM. One possible explanation is that we initially demonstrated a role for VCAM-1 in experiments using HUVECs, which express high levels of VCAM-1. However, to observe TCR-driven TEM, we switched to HDMECs, which express substantially higher levels of fractalkine but substantially lower levels of VCAM-1 than do HUVECs. Nevertheless, we wondered whether other components of the LFA-1/ICAM-1 interaction could be engaged in the TCR response that are not engaged in the chemokine response. For example, EM T cells express Mac-1 (CD11b/CD18), an alternative receptor for ICAM-1 that is lacking on naive and CM T cells (6). Could EM CD4$^+$ T cells be using engagement of Mac-1 to undergo TCR-dependent TEM? LFA-1 is thought to act coordinately with DNAX accessory molecule-1 (DNAM-1; CD226) on NK cells, a molecule that interacts with nectin-2 (CD112) and/or poliovirus receptor (PVR; CD155), two other proteins known to localize to EC junctions (11–13). Tactile (CD96) is an alternative counterreceptor for PVR. Are any of these molecules also involved in the TCR pathway of EM CD4$^+$ T cells? Finally, LFA-1 may also interact with junctional adhesion molecule-1 (JAM-1) and ICAM-2, both expressed at significant levels by ECs. What, if any, is the role of these molecules in either pathway of TEM? The present study was designed to answer these questions.

Materials and Methods

Cells and reagents

CIITA HDMECs were generated using a retroviral vector and characterized as described previously (3). Prior to flow experiments, CIITA HDMECs were incubated in the presence of 10 ng/ml recombinant human TNF-α (R&D Systems) for 18–28 h and toxic shock syndrome toxin-1 (TSST-1) for 30 min. For the blocking Ab experiments, ECs were incubated in the presence of 10 μg/ml blocking Abs to CD112 (clone R2.525; eBioscience), CD155 (clone D171; GeneTex), ICAM-2 (clone CBRIC2/2; eBioscience), or nonblocking Ab to VE-cadherin (clone 16B1; eBioscience) for 30 min prior to the flow assay. For small interfering RNA (siRNA) experiments, CIITA HDMEC were transfected with 20 nM siRNA targeting CD112 (Hs_PVRL2_5, Hs_PVRL2_6), CD155 (Hs_PVR_1, Hs_PVR_5), CD99 (Hs_CD99_1, Hs_CD99_5), JAM-1 (Hs_F11R_5, Hs_F11R_6), ICAM-2 (Hs_ICAM2_1, Hs_ICAM2_5), or negative control (Allstar negative control siRNA; all siRNAs are from Qiagen) using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturers' instructions ~72 h prior to flow assay.

CD4$^+$ T cells were isolated by positive selection with magnetic beads and released with Detachabead (Dynal Biotech) from PBMCs prepared by Ficoll gradient of blood collected from healthy donors. Memory (CD4$^+$ CD45RA$^-$) T cells were isolated by depletion of CD45RA$^+$ cells from CD4$^+$ T cells using anti-CD45RA mAb (eBioscience) and pan-mouse IgG beads (Dynal Biotech). EM cells were further enriched by depleting CD4$^+$ memory cells with anti-CCR7 mAb (R&D Systems) and anti-CD62L (eBioscience) and pan-mouse IgG beads. Isolated EM CD4$^+$ T cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, penicillin/streptomycin, and nonessential amino acids overnight prior to assays. For blocking Ab experiments, EM CD4$^+$ T cells were preincubated with 10 μg/ml control IgG or anti-CD226 (clone DX11; Serotec, Raleigh, NC), CD96 (clone NK92.39; BioLegend, San Diego, CA), CD11a (clone HI111; eBioscience), CD11b (clone CBRM1/5; eBioscience), or CD44 (clone IM7; BioLegend) 30 min before flow assay.

FACS analysis

Flow cytometry standard files of ECs harvested by incubation in 2 mM EDTA (for CD99 and JAM-1, which are trypsin sensitive) or by trypsin (nectin-2 and PVR, which are not trypsin sensitive) and stained for 30 min on ice with control IgG, anti-CD99 (hec2 mAb; a gift from W. Muller, Northwestern University, Chicago, IL), anti–JAM-1, anti-CD112, anti-CD155, and anti–ICAM-2, followed by Alexa Fluor 488-conjugated donkey anti-mouse IgG, or FITC-conjugated anti–PECAM-1, then washed twice with 1% BSA in PBS, were acquired using a LSRII flow cytometer with FACSDiva software and analyzed using FlowJo software. Total CD4$^+$ or CD45RA$^-$CD4$^+$ T cells were stained with control IgG either conjugated with PE or FITC or precomplexed with Zenon Alexa Fluor 647 reagent (Invitrogen) or PE-conjugated anti-CD11b and FITC-conjugated anti-CCR7 or PE-conjugated anti-CD226 and FITC-conjugated anti-CCR7 or Alexa Fluor 647-precomplexed CD96 and FITC-conjugated anti-CCR7, washed twice with 1% BSA in PBS, and acquired using LSRII flow cytometer with FACSDiva software and analyzed using FlowJo software.

TEM assays

CIITA HDMECs grown to confluence on 35-mm fibronectin-coated coverglasses were incubated with 100 ng/ml TSST-1 (Toxin Technologies) 30 min prior to the flow assay, washed twice with RPMI/10% FBS, and assembled with a parallel plate flow chamber apparatus (Glycotech) using the 0.01-in-high, 5-mm-wide slit gasket provided by the manufacturer. On a 37°C heating surface, CD4$^+$CD45RA$^-$CCR7^{low}CD62L^{low} (EM) human T cells (10^6 cells/500 μl) suspended in the same medium were loaded onto the EC monolayer at 0.75 dyne/cm^2, followed by medium only at 1 dyne/cm^2 for 15 or 60 min. Samples were then fixed with 3.7% formaldehyde in PBS, stained with anti-Vβ2TCR mAb (Immunotech), followed by Alexa Fluor 594-or 488-conjugated donkey anti-mouse IgG (Invitrogen), mounted on slides using mounting medium containing DAPI (Invitrogen), and examined by microscopy. For blocking Ab experiments, or when samples were also stained with a mAb to detect targets of siRNAs (i.e., when another mAb was present on the samples that could also bind to anti-mouse IgG secondary Ab, thereby preventing the identification of Vβ2TCR$^+$ T cells), samples were stained sequentially with FITC-conjugated anti-Vβ2TCR mAb (Immunotech), Alexa Fluor 488-conjugated rabbit anti-FITC, and Alexa Fluor 488-conjugated goat anti-rabbit IgG (staining with FITC-conjugated anti-Vβ2TCR mAb alone was too weak to visualize manually). To ensure TSST-1 presentation by CIITA HDMECs was effective in the CD99, nectin-2, and PVR knockdown samples, NFAT translocation to the nucleus was determined in the Vβ2TCR$^+$ cells by staining with anti-Vβ2TCR mAb, Alexa Fluor 488-conjugated donkey anti-mouse IgG, followed by Alexa Fluor 647-preconjugated (Zenon; Invitrogen) NFAT mAb (BD Biosciences) (Supplemental Figs. 1, 4). An FITC filter was used to detect FITC or Alexa Fluor 488-stained cells, a tetramethylrhodamine isothiocyanate filter was used to detect Alexa Fluor 568- and 594-stained cells, a DAPI filter used to detect DAPI-stained nuclei, and a Cy5 filter was used to detect Alexa Fluor 647 stained cells. Using a ×40/0.60 korr Ph2 objective, phase contrast optics were used to determine whether CD4$^+$ T cells were either on top of or underneath the HDMEC monolayer. T cells that were captured and not spread were round and bright when viewed under phase contrast. CD4$^+$ T cells that were spread but still on top of the HDMEC monolayer were surrounded by a bright corona of light in contrast to those that had transmigrated. The percentage of transmigrated CD4$^+$ T cells were calculated for 100–200 Vβ2TCR$^+$ and 100–200 Vβ2TCR$^-$ cells per sample by analyzing 5–10 groups of 20 cells each (one cell at a time, covering the entire area of flow), calculating the percentage for each group, and calculating the mean and SEM for the groups.

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 posttest (Prism 4.0 for Macintosh). Statistical error is expressed as SEM. For experiments in which two groups were compared, a *t* test was used.

Results

Because we have shown previously that PECAM-1 was required for TCR-dependent, but not chemokine-dependent, TEM, we first examined whether TCR signals induce EM CD4$^+$ T cells to use CD99, another known component of the LBRC involved in TEM of other leukocyte cell types in conjunction with PECAM-1 (14, 15). We investigated the role of CD99 in TCR- and chemokine-driven TEM using an in vitro flow assay with freshly purified human EM CD4$^+$ T cells (CD4$^+$,CD45RA$^-$,CCR7^{low},CD62L^{low}) and primary, nonimmortalized HDMEC that had been transduced

with CIITA (to reinduce expression of MHC class II), activated by TNF (to induce the expression of adhesion molecules and chemokines necessary to capture flowing T cells), and overlaid with the superantigen TSST-1 (to activate the T cells that contain a Vβ2 segment in their TCR; 5–20% in normal T cell populations). Class II MHC induction is necessary to bind and present the superantigen to T cells. T cells that do not have the Vβ2TCR (referred to as $VB2^-$ cells in this study) respond to chemokines presented on the EC apical surface and transmigrate within 15 min, whereas the $V\beta2TCR^+$ cell response to chemokine is blocked, but instead undergo TCR-dependent TEM ~45 min later (3). We used siRNA knockdown to assess the function of CD99 in TEM of EM $CD4^+$ T cells. Effective knockdown of EC CD99 by two different siRNAs (Fig. 1*A*) inhibited TCR-dependent but not chemokine-dependent TEM of EM $CD4^+$ T cells (Fig. 1*B*). We conclude that EM $CD4^+$ T cells activated through their TCR use CD99 as well as PECAM-1 to undergo TEM.

Previously, we have shown that blocking Ab experiments identified ICAM-1 as an EC molecule required for TCR-driven TEM (3). Unlike other lymphocyte populations, EM T cells express two known receptors for ICAM-1, namely LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) (Fig. 2*A*) (6). We reasoned that

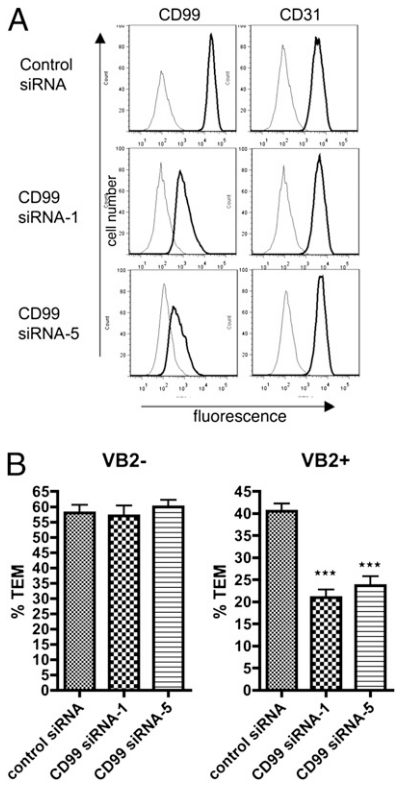

FIGURE 1. Knockdown of EC CD99 inhibits TCR-dependent TEM of EM $CD4^+$ T cells. CIITA HDMEC were transfected with control siRNA or two different siRNAs targeting CD99 (CD99 siRNA-2 and CD99 siRNA-5), treated with TNF, analyzed by FACS (*A*) or overlaid with TSST-1 superantigen (recognized by those T cells with the germline-encoded Vβ2 segment in the TCR), and used in flow TEM assays with EM $CD4^+$ T cells (*B*). *A*, FACS plots of HDMECs stained with isotype-matched control IgG (thin lines) or anti-CD99 or -CD31 (thick lines in *left panels* and *right panels*, respectively) demonstrating knockdown of CD99 without reduction of CD31 expression. *B*, TEM assays. Graph on the left ($VB2^-$) shows TEM of $V\beta2TCR^-$ cells (i.e., those T cells with TCR that are not activated by TSST-1) at 15 min. Graph on the right ($VB2^+$) shows TEM of $V\beta2TCR^+$ cells at 60 min. Graphs display data from one representative experiment of two ($VB2^-$) or three ($VB2^+$) separate experiments using T cells from different donors. $***p < 0.001$ compared with control.

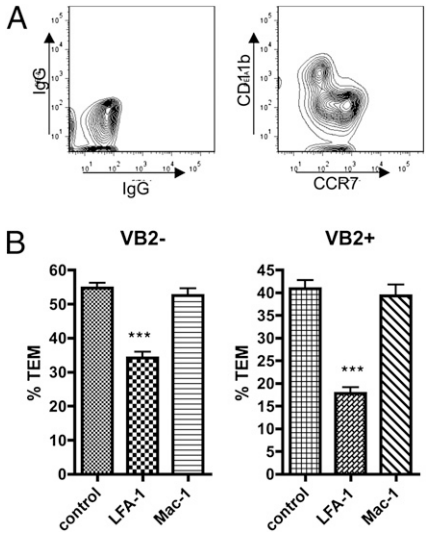

FIGURE 2. Blocking of T cell LFA-1 but not Mac-1 inhibits TCR-dependent TEM of EM $CD4^+$ T cells. *A*, Mac-1 is expressed on EM $CD4^+$ T cells. Contour plots of FACS analysis of $CD45RA^-CD4^+$ T cells stained with isotype-matched control PE- and FITC-conjugated IgG (*left plot*) or PE-conjugated anti-CD11b and FITC-conjugated anti-CCR7 (*right plot*). Note that there is a significant proportion of $CD11b^+$ cells in the CCR7 low (i.e., EM) population. *B*, TEM assays. EM $CD4^+$ T cells were preincubated with isotype control IgG (control), anti–LFA-1 (LFA-1), or anti–Mac-1 (Mac-1) blocking mAb 30 min prior to flow TEM on TNF-treated CIITA-transduced HDMECs overlaid with TSST-1. Panel on the left ($VB2^-$) shows TEM of $V\beta2TCR^-$ cells at 15 min. Panel on the right ($VB2^+$) shows TEM of $V\beta2TCR^+$ cells at 60 min. Graphs display data pooled from three separate experiments using T cells isolated from three different donors. $***p < 0.001$ compared with control.

Mac-1 may account for the dependence of TCR-driven TEM on ICAM-1. We therefore tested whether LFA-1 or Mac-1 mediate TCR-dependent TEM. Because these experiments are designed to study freshly isolated EM $CD4^+$ T cells, we are unable to use siRNAs to modify T cell molecules in this process. However, effective blocking mAbs to both human Mac-1 and LFA-1 are readily available. Anti–Mac-1 mAb had no effect on either TCR-driven or chemokine-driven TEM, whereas anti–LFA-1 mAb inhibited both chemokine- and TCR-dependent TEM (Fig. 2*B*).

LFA-1 binds to at least two other ligands on HDMECs besides ICAM-1, namely ICAM-2 and JAM-1. JAM-1 knockdown in ECs slightly but reproducibly impairs chemokine-dependent TEM (Fig. 3), in agreement with previous reports (16). Interestingly, JAM-1 knockdown has no effect on TCR-dependent TEM, thereby further differentiating these two modes of TEM (Fig. 3*B*). siRNA knockdown of ICAM-2 disrupted the EC monolayer so that it could not be used in TEM assays. An anti–ICAM-2 mAb had no effect on TEM (Supplemental Fig. 2).

We next examined the role of DNAM-1 (CD226), a molecule that is expressed on T cells as well as other leukocytes and that is known to be involved in the TEM of monocytes (13). Like many other T cell adhesion molecules that have ligands expressed on EC, expression of DNAM-1 is enhanced in EM $CD4^+$ T cells compared with CM and naive T cells (Fig. 4*A*). Strikingly, treatment of EM $CD4^+$ T cells with anti–DNAM-1 mAb inhibited TCR-dependent TEM but had no effect on chemokine-dependent TEM (Fig. 4*B*); several other Abs that bind to the surface of T cells, including anti-MHC class I and anti-CD44, had no effect (data not shown and Supplemental Fig. 3).

CD112 (nectin-2) and CD155 (PVR) are DNAM-1 ligands located at EC intercellular junctions (12). Blocking Abs to nectin-2

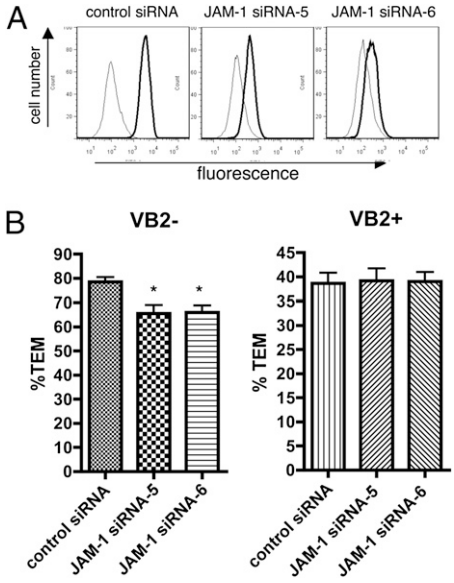

FIGURE 3. Knockdown of EC JAM-1 inhibits chemokine-dependent TEM of EM CD4$^+$ T cells. CIITA HDMECs were transfected with control siRNA or two different siRNAs targeting JAM-1 (JAM-1 siRNA-5 and JAM-1 siRNA-6), treated with TNF, either harvested for FACS analysis (*A*) or overlaid with TSST-1 superantigen, and used in flow TEM assays with EM CD4$^+$ T cells (*B*). *A*, Histograms show FACS analysis of cells stained with isotype control IgG (thin line) or anti–JAM-1 (thick lines) demonstrating effective knockdown. *B*, *Left lower panel* (VB2$^-$) shows TEM of Vβ2TCR$^-$ cells at 15 min. *Right lower panel* on (VB2$^+$) shows TEM of Vβ2TCR$^+$ cells at 60 min. Graphs display data from one representative experiment of three (VB2$^-$) and two (VB2$^+$) separate experiments using T cells from different donors. $*p < 0.05$ compared with control.

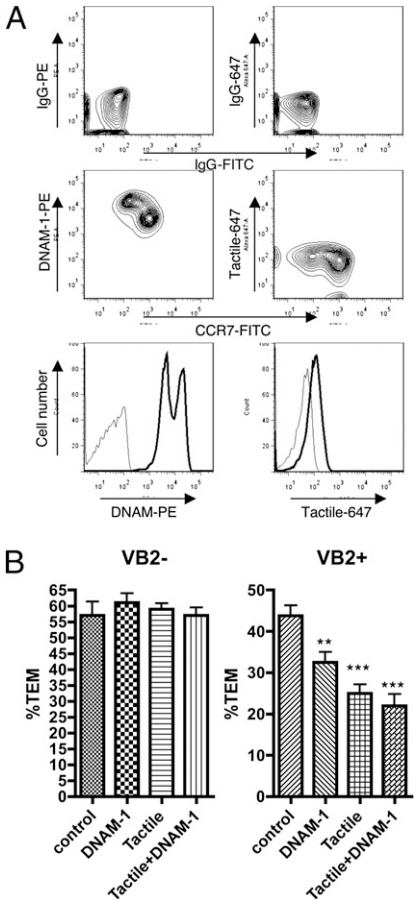

FIGURE 4. Blocking of T cell DNAM-1 and/or Tactile inhibits TCR-dependent TEM of EM CD4$^+$ T cells. *A*, Contour plots of FACS analysis of total CD4$^+$ T cells stained with FITC-conjugated IgG (IgG–FITC) and PE-conjugated IgG (IgG–PE, *upper left*) or FITC-conjugated anti-CCR7 (CCR7–FITC) and PE-conjugated anti–DNAM-1 (DNAM-1–PE, *lower left*) or FITC-conjugated anti-CCR7 and Alexa Fluor 647-complexed IgG (IgG-647, *upper right*) or FITC-conjugated anti-CCR7 and Alexa Fluor 647-complexed anti-Tactile (Tactile-647, *lower right*). Note the distinct population of cells that are DNAM-1 high, CCR7 low or Tactile high, CCR7 low in the *middle*, *left*, and *right plots*, respectively. Lower histograms show overlays of the isotype-matched control IgG and anti–DNAM-1 (*left*) or anti-Tactile (*right*) plots. *B*, EM CD4$^+$ T cells were preincubated with isotype-matched control IgG (control), anti–DNAM-1 (DNAM-1), anti-Tactile (Tactile), and both anti–DNAM-1 and anti-Tactile blocking mAbs (DNAM-1+Tactile) prior to flow TEM. *Left panel* shows TEM of Vβ2TCR$^-$ cells at 15 min. *Right panel* shows TEM of Vβ2TCR$^+$ cells at 60 min. Graphs display data from one representative experiment of two (VB2$^-$) and four (VB2$^+$) separate experiments using T cells isolated from different donors. $**p < 0.01$; $***p < 0.001$ compared with control.

and to PVR, as well as siRNA knockdown of these molecules, inhibited TCR-, but not chemokine-, dependent TEM (Figs. 5, 6). We also investigated whether CD96 (Tactile), another receptor for CD155 that is slightly elevated on EM CD4$^+$ T cells (Fig. 4*A*) and that also promotes adhesion and activation of NK cells (17), played a role in TEM. Blocking Ab to Tactile also inhibited TCR-dependent but not chemokine-dependent TEM (Fig. 4*B*). Thus, we conclude that the DNAM-1–Tactile complex on CD4$^+$ EM T cells, through interactions with nectin-2 and PVR on ECs, play a role in the TCR-driven pathway of TEM, perhaps in conjunction with LFA-1 but not Mac-1 recognition of ICAM-1.

Discussion

In this study, we describe the requirement of three new EC molecules, namely CD99, nectin-2, and PVR, for TCR-driven TEM of EM CD4$^+$ T cells. The requirement for nectin-2 and PVR can be explained by the activation of their receptor on T cells, namely DNAM-1, by the TCR, in a manner that induces DNAM-1 and LFA-1 to form a complex and that depends on engagement of both receptors to be functional (11). The requirement for DNAM-1 ligands and ICAM-1 implies that ICAM-1, but not other LFA-1 ligands, such as JAM-1 and ICAM-2, may be the sole LFA-1 ligand necessary for TCR-driven TEM. This is not because JAM-1 and ICAM-2 cannot participate in TEM. On the contrary, JAM-1 does play a role in chemokine-driven TEM (16), and ICAM-2 has been shown to mediate crawling of leukocytes on the EC surface prior to diapedesis (18, 19) but does not appear to effect TEM of EM CD4$^+$ T cells. The selectivity of LFA-1 in the LFA-1–DNAM-1 complex for ICAM-1 is not presently understood.

DNAM-1 has been studied most intensively in NK cells and cytotoxic T lymphocytes, where it has been shown to work as a functional complex with LFA-1 to mediate cytotoxic responses (11). In NK cells, LFA-1 and DNAM-1 are constitutively associated, whereas T cells require TCR signaling to induce the physical and functional association of LFA-1 with DNAM-1 (11). Intriguingly, activation of CD8$^+$ T cells requires costimulation through DNAM-1 only when the Ag is presented by nonprofessional APCs (20); human ECs are nonprofessional APCs. The requirement for Tactile in TCR-driven TEM was not expected. Indeed, it was initially investigated as a formality, given that a blocking Ab to PVR, a ligand of Tactile as well as DNAM-1, inhibited TEM. However, Tactile and DNAM-1 have been shown to play similar, perhaps redundant, functions in NK cells, and it has been suggested that NK cells have evolved a dual receptor system because of the importance of their role in immunosurveil-

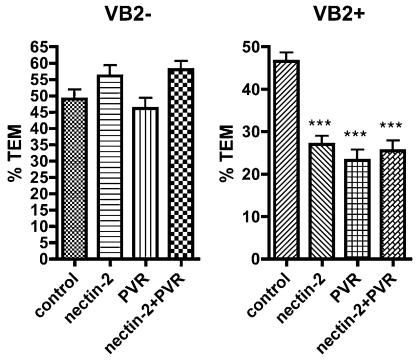

FIGURE 5. Blocking of EC nectin-2 and/or PVR inhibits TCR-dependent TEM of EM CD4$^+$ T cells. TNF-treated CIITA-transduced HDMECs overlaid with TSST-1 were preincubated with isotype control (control), anti–nectin-2 (nectin-2), anti-PVR (PVR), and both blocking Abs (nectin-2+PVR) prior to flow TEM. Panel on the left shows TEM of Vβ2TCR$^-$ cells at 15 min. Panel on the right shows TEM of Vβ2TCR$^+$ cells at 60 min. Graphs display data from one representative of eight different experiments, testing each condition with T cells from at least three different donors. ***$p < 0.001$ compared with control.

lance (17). This may apply to EM T cells as well, because they are unique among T cell subsets in the circulation in their ability to respond to Ag presented by ECs in the vasculature.

The finding that CD99 is required for TCR-driven TEM of EM CD4$^+$ T cells would seem to be obvious, given our previous finding that PECAM-1 is required for TCR-driven TEM (5), and both CD99 and PECAM-1 are required for TEM of other leukocytes (9). However, leukocytes other than EM CD4$^+$ T cells require leukocyte PECAM-1 or CD177 to bind to EC PECAM-1 for TEM (9, 21). This is not the case for EM CD4$^+$ T cells, which do not express any of the known counterreceptors for PECAM-1 (5), and it is not clear how EM CD4$^+$ T cells use EC PECAM-1 for TEM. However, because it is clear that EM CD4$^+$ T cells do use EC CD99 as well as PECAM-1, it is likely that TCR-driven TEM of EM CD4$^+$ T cells is following a path taken by other leukocytes (i.e., via interactions with the LBRC).

Various techniques, such as treating the EC with demecolcine, have been used to test the involvement of the LBRC in TEM under static (nonshear) conditions, but the introduction of venular level of flow causes demecolcine-treated EC monolayers to detach (Ref. 22 and T. Manes, unpublished observations). Although shear stress does not seem to be necessary for monocyte and neutrophil TEM, it is required for both chemokine- and TCR-driven TEM of EM CD4$^+$ T cells (1, 3).

Our in vitro studies support the role of Ag presentation by ECs in the process of EM T cell recruitment to inflammatory sites. Several animal models have suggested the importance of Ag-driven recruitment of T cells into peripheral tissue and the capacity of EC to present Ag as a trigger for this response (reviewed in Ref. 23). Often, however, it has been difficult to distinguish between Ag-specific effects and the effects of the inflammatory milieu, depending on the model used. However, a recent study has provided evidence that may clarify some of the confusion, showing that Ag-specific T cells act as "pioneers"; a small number of Ag-specific T cells are required to condition the tissue for recruitment of a large number of Ag-nonspecific effector T cells (24). Interestingly, this report identified TNF and IFN-γ as mediators that contribute to the tissue conditioning. These mediators may well be produced from the transmigrating Ag-specific T cells themselves as a result of TCR activation by the EC, which activates transcriptional pathways (3). It remains to be seen whether findings from these animal studies can be extrapolated to

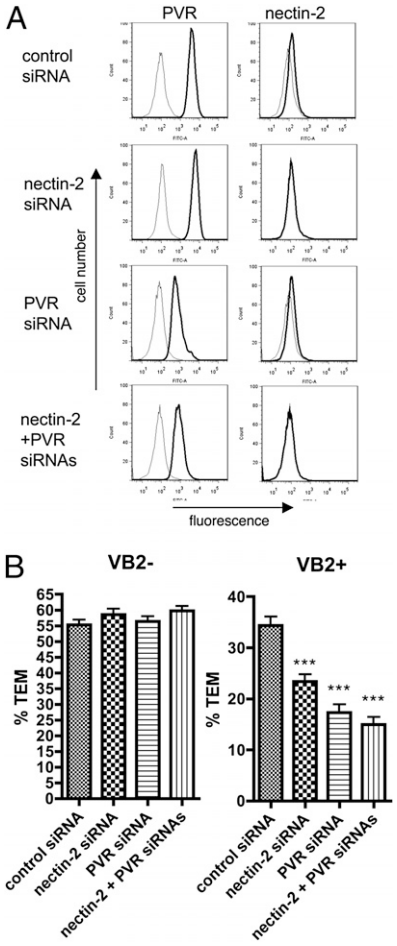

FIGURE 6. Knockdown of EC nectin-2 and PVR inhibits TCR-dependent TEM of EM CD4$^+$ T cells. CIITA HDMECs were transfected with control siRNA or siRNA targeting nectin-2 (nectin-2 siRNA), PVR (PVR siRNA), or both (nectin-2+PVR siRNA), treated with TNF, either harvested for FACS analysis (*A*) or overlaid with TSST-1 superantigen and used in flow TEM assays with EM CD4$^+$ T cells (*B*). *A*, Histograms show FACS analysis of cells stained with isotype control IgG (thin line) or anti–nectin-2 or anti-PVR (thick lines). *B*, Graphs display data combined from three separate experiments using T cells from different donors. ***$p <$ 0.001 compared with control.

humans, but our in vitro experiments are strongly consistent with this idea.

A key question raised by our study is the relationship between chemokine-initiated and Ag-initiated recruitment of EM T cells in general and why TCR signals inhibit the response to IP-10 in particular. We have noted that T cells undergoing TEM by these pathways appear to be different; only TCR signals invoke a cytoplasmic projection we have referred to as "transendothelial protusions" (3). We speculate that this difference in the organization and mobilization of the cytosol is based on distinct mechanisms for reorganizing the cytoskeleton by different small G proteins. Consistent with this hypothesis, it has previously been reported that chemokine recruitment of T cells uses cdc42 and that TCR-triggered, but not chemokine-triggered, recruitment of mouse T cells is dependent on the TCR-activated G protein exchange factor vav (25, 26). If vav leads to the activation of a cdc42-independent reorganization of the cytoskeleton and if TCR signals also turn on a cdc42-specific GTPase activating protein that blocks a cdc42-mediated response, these events could explain how chemokine recruitment is blocked, yet TEM is activated by engagement of the TCR.

In conclusion, our in vitro experiments demonstrate that human EM CD4$^+$ T cells use different T cell and EC molecules to transmigrate in response to chemokines or Ag presented by the EC. A similar phenomenon of TCR-driven TEM has been described in the mouse (23, 26), although it is not clear whether the same sets of molecules are used. It will be important to see whether these molecules in humans or mice can serve to reduce EM T cell recruitment to peripheral sites of inflammation, and if so, what are the consequences? As inflammatory disorders may arise from inappropriate responses of EM T cells, selectively targeting one pathway or another may be beneficial in various therapeutic settings. The work presented in this study has described some potential molecular targets for this therapeutic approach.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Manes, T. D., J. S. Pober, and M. S. Kluger. 2006. Endothelial cell-T lymphocyte interactions: IP[corrected]-10 stimulates rapid transendothelial migration of human effort but not central memory CD4$^+$ T cells—requirements for shear stress and adhesion molecules. [Published erratum appears in 2006 *Transplantation* 82: 721.] *Transplantation* 82(Suppl. 1): S9–S14.
2. Shiao, S. L., N. C. Kirkiles-Smith, B. R. Shepherd, J. M. McNiff, E. J. Carr, and J. S. Pober. 2007. Human effector memory CD4$^+$ T cells directly recognize allogeneic endothelial cells in vitro and in vivo. *J. Immunol.* 179: 4397–4404.
3. Manes, T. D., and J. S. Pober. 2008. 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. *J. Immunol.* 180: 8386–8392.
4. Manes, T. D., S. L. Shiao, T. J. Dengler, and J. S. Pober. 2007. TCR signaling antagonizes rapid IP-10–mediated transendothelial migration of effector memory CD4$^+$ T cells. *J. Immunol.* 178: 3237–3243.
5. Manes, T. D., S. Hoer, W. A. Muller, P. J. Lehner, and J. S. Pober. 2010. 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. *J. Immunol.* 184: 5186–5192.
6. Chtanova, T., R. Newton, S. M. Liu, L. Weininger, T. R. Young, D. G. Silva, F. Bertoni, A. Rinaldi, S. Chappaz, F. Sallusto, et al. 2005. Identification of T cell-restricted genes, and signatures for different T cell responses, using a comprehensive collection of microarray datasets. *J. Immunol.* 175: 7837–7847.
7. Beck, G. Ch., F. Ludwig, J. Schulte, K. van Ackern, F. J. van der Woude, and B. A. Yard. 2003. Fractalkine is not a major chemoattractant for the migration of neutrophils across microvascular endothelium. *Scand. J. Immunol.* 58: 180–187.
8. Bird, I. N., J. H. Spragg, A. Ager, and N. Matthews. 1993. Studies of lymphocyte transendothelial migration: analysis of migrated cell phenotypes with regard to CD31 (PECAM-1), CD45RA and CD45RO. *Immunology* 80: 553–560.
9. Muller, W. A. 2009. Mechanisms of transendothelial migration of leukocytes. *Circ. Res.* 105: 223–230.
10. Mamdouh, Z., X. Chen, L. M. Pierini, F. R. Maxfield, and W. A. Muller. 2003. Targeted recycling of PECAM from endothelial surface-connected compartments during diapedesis. *Nature* 421: 748–753.
11. Shibuya, K., L. L. Lanier, J. H. Phillips, H. D. Ochs, K. Shimizu, E. Nakayama, H. Nakauchi, and A. Shibuya. 1999. Physical and functional association of LFA-1 with DNAM-1 adhesion molecule. *Immunity* 11: 615–623.
12. Bottino, C., R. Castriconi, D. Pende, P. Rivera, M. Nanni, B. Carnemolla, C. Cantoni, J. Grassi, S. Marcenaro, N. Reymond, et al. 2003. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J. Exp. Med.* 198: 557–567.
13. Reymond, N., A. M. Imbert, E. Devilard, S. Fabre, C. Chabannon, L. Xerri, C. Farnarier, C. Cantoni, C. Bottino, A. Moretta, et al. 2004. DNAM-1 and PVR regulate monocyte migration through endothelial junctions. *J. Exp. Med.* 199: 1331–1341.
14. Schenkel, A. R., Z. Mamdouh, X. Chen, R. M. Liebman, and W. A. Muller. 2002. CD99 plays a major role in the migration of monocytes through endothelial junctions. *Nat. Immunol.* 3: 143–150.
15. Lou, O., P. Alcaide, F. W. Luscinskas, and W. A. Muller. 2007. CD99 is a key mediator of the transendothelial migration of neutrophils. *J. Immunol.* 178: 1136–1143.
16. Ostermann, G., K. S. Weber, A. Zernecke, A. Schröder, and C. Weber. 2002. JAM-1 is a ligand of the β_2 integrin LFA-1 involved in transendothelial migration of leukocytes. *Nat. Immunol.* 3: 151–158.
17. Fuchs, A., M. Cella, E. Giurisato, A. S. Shaw, and M. Colonna. 2004. Cutting edge: CD96 (tactile) promotes NK cell-target cell adhesion by interacting with the poliovirus receptor (CD155). *J. Immunol.* 172: 3994–3998.
18. Schenkel, A. R., Z. Mamdouh, and W. A. Muller. 2004. Locomotion of monocytes on endothelium is a critical step during extravasation. *Nat. Immunol.* 5: 393–400.
19. Steiner, O., C. Coisne, R. Cecchelli, R. Boscacci, U. Deutsch, B. Engelhardt, and R. Lyck. 2010. Differential roles for endothelial ICAM-1, ICAM-2, and VCAM-1 in shear-resistant T cell arrest, polarization, and directed crawling on blood-brain barrier endothelium. *J. Immunol.* 185: 4846–4855.
20. Gilfillan, S., C. J. Chan, M. Cella, N. M. Haynes, A. S. Rapaport, K. S. Boles, D. M. Andrews, M. J. Smyth, and M. Colonna. 2008. DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors. *J. Exp. Med.* 205: 2965–2973.
21. Sachs, U. J., C. L. Andrei-Selmer, A. Maniar, T. Weiss, C. Paddock, V. V. Orlova, E. Y. Choi, P. J. Newman, K. T. Preissner, T. Chavakis, and S. Santoso. 2007. The neutrophil-specific antigen CD177 is a counter-receptor for platelet endothelial cell adhesion molecule-1 (CD31). *J. Biol. Chem.* 282: 23603–23612.
22. Mamdouh, Z., G. E. Kreitzer, and W. A. Muller. 2008. Leukocyte transmigration requires kinesin-mediated microtubule-dependent membrane trafficking from the lateral border recycling compartment. *J. Exp. Med.* 205: 951–966.
23. Marelli-Berg, F. M., H. Fu, F. Vianello, K. Tokoyoda, and A. Hamann. 2010. Memory T-cell trafficking: new directions for busy commuters. *Immunology* 130: 158–165.
24. Ghani, S., M. Feuerer, C. Doebis, U. Lauer, C. Loddenkemper, J. Huehn, A. Hamann, and U. Syrbe. 2009. T cells as pioneers: antigen-specific T cells condition inflamed sites for high-rate antigen-non-specific effector cell recruitment. *Immunology* 128(Suppl. 1): e870–e880.
25. Shulman, Z., V. Shinder, E. Klein, V. Grabovsky, O. Yeger, E. Geron, A. Montresor, M. Bolomini-Vittori, S. W. Feigelson, T. Kirchhausen, et al. 2009. Lymphocyte crawling and transendothelial migration require chemokine triggering of high-affinity LFA-1 integrin. *Immunity* 30: 384–396.
26. David, R., L. Ma, A. Ivetic, A. Takesono, A. J. Ridley, J. G. Chai, V. L. Tybulewicz, and F. M. Marelli-Berg. 2009. T-cell receptor- and CD28-induced Vav1 activity is required for the accumulation of primed T cells into antigenic tissue. *Blood* 113: 3696–3705.