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

J Immunol published online 29 December 2010
http://www.jimmunol.org/content/early/2010/12/29/jimmunol.1002835

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/12/29/jimmunol.1002835.5.DC1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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 (CD185) as well as 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 DNA 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: 000–000.
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 whether 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) (Toxin Technologies) 30 min prior to the flow assay. For 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; Biorad) for 30 min prior to the flow assay. For small interfering RNA (siRNA) experiments, CIITA HDMECs were transfected with 20 nM siRNA targeting CD112 (Hs_PVR.2_5, Hs_PVR.2_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 (Dyna Biotech) from PBMCs prepared by Ficoll gradient of blood collected from healthy donors. Memory (CD4+CD45RA−) T cells were stained with control IgG either conjugated to FITC or Alexa Fluor 488. Isolation of the nuclear fraction was achieved using a 37˚C heating surface, CD4+CD45RA−,CCR7low,CD62Llow (EM) human T cells (106 cells/500 μl) suspended in the same medium were loaded onto the EC monolayer at 0.75 dyne/cm2, followed by medium only at 1 dyne/cm2 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), 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-conjugated (Zenon; Invitrogen) NFAT mAb (BD Biosciences; Supplementary 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 Cy3 filter was used to detect T cells 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 followed by 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−,CCR7low,CD62Llow) and primary, nonimmortalized HDMEC that had been transduced 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, and 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-conjugated CD62 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 coverslips 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−,CCR7low,CD62Llow (EM) human T cells (106 cells/500 μl) suspended in the same medium were loaded onto the EC monolayer at 0.75 dyne/cm2, followed by medium only at 1 dyne/cm2 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), samples were incubated 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-conjugated (Zenon; Invitrogen) NFAT mAb (BD Biosciences; Supplementary 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 Cy3 filter was used to detect T cells 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.
with CIITA (to reinstate expression of MHC class II), activated by 
TNF (to induce the expression of adhesion molecules and che-
romines 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 su-
perantigen 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 Vβ2TCR+ 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 siR-
NAs (Fig. 1A) inhibited TCR-dependent but not chemokine-de-
pendent TEM of EM CD4+ T cells. 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 ex-
press two known receptors for ICAM-1, namely LFA-1 (CD11a/
CD18) and Mac-1 (CD11b/CD18) (Fig. 2A) (6). We reasoned that

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 in-
hibited both chemokine- and TCR-dependent TEM (Fig. 2B).

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. 3B). siRNA knock-
down 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
(i.e., EM) population. Strikingly, treating EM CD4+ T cells
with anti–DNAM-1 mAb inhibited both chemokine- and TCR-de-
pendent TEM (Fig. 4A). Anti–DNAM-1 mAb inhibited TEM
whether to either TNF-driven or chemokine-driven TEM, whereas anti–LFA-1 mAb
inhibited both chemokine- and TCR-dependent TEM (Fig. 2B).

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. 3B). siRNA knock-
down 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. 4A). Strikingly, treat-
ment of EM CD4+ T cells with anti–DNAM-1 mAb inhibited
TCR-dependent TEM but had no effect on chemokine-dependent
TEM (Fig. 4B); 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 lo-
cated at EC intercellular junctions (12). Blocking Abs to nectin-2

![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 V
β2 segment in their TCR; 5–20% in normal T cell populations). Class
II MHC induction is necessary to bind and present the super-
antigen 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 Vβ2TCR+ 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 siR-
NAs (Fig. 1A) inhibited TCR-dependent but not chemokine-de-
pendent TEM of EM CD4+ T cells (Fig. 1B). 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 ex-
press two known receptors for ICAM-1, namely LFA-1 (CD11a/
CD18) and Mac-1 (CD11b/CD18) (Fig. 2A) (6). We reasoned that

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 in-
hibited both chemokine- and TCR-dependent TEM (Fig. 2B).

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. 3B). siRNA knock-
down 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. 4A). Strikingly, treat-
ment of EM CD4+ T cells with anti–DNAM-1 mAb inhibited
TCR-dependent TEM but had no effect on chemokine-dependent
TEM (Fig. 4B); 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 lo-
cated at EC intercellular junctions (12). Blocking Abs to nectin-2
EM CD4+ T cells. The selectivity of LFA-1 in the LFA-1–DNAM-1 pathway prior to diapedesis (18, 19) but does not appear to effect TEM of EM CD4+ T cells. The requirement for nectin-2 and PVR can be mediated, 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-

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. 4B), played a role in TEM. Blocking Ab to Tactile also inhibited TCR-dependent TEM. 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–IgG), anti–Tactile (Tactile–IgG), and both anti–DNAM-1 and anti–Tactile blocking mAbs (DNAM-1–IgG+Tactile–IgG) 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.

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 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). In NK cells, LFA-1 and DNAM-1 imply 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 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 3. Knockdown of EC JAM-1 inhibits chemokine-dependent TEM of EM CD4+ T cells. CITA 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–IgG), anti–Tactile (Tactile–IgG), and both anti–DNAM-1 and anti–Tactile blocking mAbs (DNAM-1–IgG+Tactile–IgG) 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.
TEM (9, 21). This is not the case for EM CD4+ T cells, which do not require leukocyte PECAM-1 or CD177 to bind to EC PECAM-1 for CD4+ T cells (1, 3).


does not seem to be necessary for monocyte and neutrophil TEM, and T. Manes, unpublished observations). Although shear stress flow causes demecolcine-treated EC monolayers to detach (Ref. 22 static (nonshear) conditions, but the introduction of venular level of


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 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 protrusions” (3). We speculate that this difference in the organization of the cytoskeleton 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.

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.

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.
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.

Acknowledgments
We thank Louise Benson, Gwendoline Davis, and Lisa Gras for excellent assistance in cell culture.

Disclosures
The authors have no financial conflicts of interest.

References