T cell interaction with ICAM-1-deficient endothelium *in vitro*: transendothelial migration of different T cell populations is mediated by endothelial ICAM-1 and ICAM-2

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Abstract

The trafficking of T lymphocytes is carefully regulated by adhesive interactions with the vascular endothelium. Depending on their maturation and activation stage, T lymphocytes exhibit distinctive patterns of homing and recirculation, which is at least partly due to the selective expression of cell adhesion molecules (CAM) on the T cell surface. In order to define whether the differential usage of CAM during the steps of transendothelial migration is involved in organ-specific recirculation of different T cell subsets we compared the interaction of three different T cell populations with mouse endothelioma cell lines *in vitro.* Using a novel approach, where we directly compared T cell interaction with ICAM-1-deficient endothelium to wild-type endothelium, we recently demonstrated that endothelial ICAM-1 and ICAM-2 play a key role in mediating the transendothelial migration of CD4+ memory T cells. Here we show that endothelial ICAM-1 and ICAM-2 are equally required for the transendothelial migration of other T cell populations such as thymocytes and T lymphoma cells, which differ from CD4+ memory T cells in their maturation and activation stage, as well as in their surface expression of adhesion molecules. Our data therefore demonstrate that transendothelial migration of different T cell populations is mediated by the same endothelial CAM, i.e. ICAM-1 and ICAM-2, and thus subset-specific interaction of T cells with endothelial cells must be regulated prior to transendothelial migration.

Introduction

One of the most characteristic features of the immune system is the continuous recirculation of lymphocytes through the body in order to ensure immunosurveillance. During this process naive lymphocytes preferentially migrate from blood into secondary lymphoid tissues, such as peripheral and mesenteric lymph nodes and Peyer's patches by extravasating through the endothelium of specialized post-capillary high endothelial venules (HEV) (1). Although memory/effector T lymphocytes also extravasate into secondary lymphoid organs (2), they display, compared to naive T cell populations, an increased capacity to migrate across inflamed and non-lymphoid endothelium (3). Lymphocyte extravasation into tissue is a multi-step process that is precisely regulated by the sequential interaction of different sets of cell adhesion molecules (CAM) on the lymphocyte surface with their respective counter-receptors on the endothelial cell surface (4). Depending on their maturation and activation stage, T lymphocyte populations express distinct arrays of CAM on their cell surface and this has been shown to influence their migratory behavior *in vivo.* At the same time endothelial cells display tissue-specific expression of CAM which recognize their respective ligands on the lymphocyte surface. During inflammation certain endothelial CAM such as E-selectin, VCAM-1 or ICAM-1 are induced on the endothelial cell surface and are involved in the recruitment of distinct T lymphocyte subsets into different inflamed tissues depending on the time point of inflammation or the organ involved (1,5). Although T lymphocytes present at sites of chronic inflammation are...
T lymphocytes must be regulated at earlier steps of the multi-step cascade. In vitro data show that adhesion to endothelium is restricted to CD4+ memory T cells. Therefore, we suggest that organ-specific homing of T lymphocytes must be regulated at earlier steps of the multi-step cascade.

**Methods**

**mAb**

The hybridomas 29G1 (anti-ICAM-1), MK2.7 (anti-VCAM-1), FD441.8 (anti-LFA-1), M1/70 (anti-Mac-1), KM201 (anti-CD44), PS/2 (anti-a4~7 integrin), 29G2 (anti-CD45RB), Mel-14 (anti-L-selectin), MJ 7/18 (anti-endoglin), DATK 32 (anti-a4~7 integrin), Fib 30.1.1 (anti-β7 integrin), M1/9 (anti-CD45) and Hermes-1 (anti-human-CD44; used as an isotype-matched control) were purchased from PharMingen (Hamburg, Germany). Mec 13.3 (anti-PECAM-1; 13) was provided by E. Dejana (Milano, Italy). The hybridomas YNI/1.7 (anti-ICAM-1; 0) and MECA-32 (pan-endothelium; 11) were provided by E. Butcher (Stanford, CA). UZ4 and UZ7 (anti-E-selectin; 12) were a gift from R. Hallmann (Erlangen, Germany). GC51 (anti-PECAM-1; 13) was a gift from B. Imhof (Geneva, Switzerland).

**Endothelial cells**

The ICAM-1-deficient endothelioma cell line bEnd11.1 was established by infection of primary endothelial cells from ICAM-1-deficient mice (14) with a recombinant retrovirus transducing the polyoma virus middle T oncogene (15,16) as previously described (17). The wild-type endothelioma line bEnd5 derived from mouse brain endothelium of BALB/c mice has been described before (17). Prior to the assay endothelial cells were preincubated with 20 μg/ml mAb for 30 min at 20°C. After removal of excess mAb endothelial cells were co-incubated with either 1×10^6 thymocytes, 1×10^5 TK-1 or 1×10^5 SJL/J.PPD2 for 30 min on a rocking platform. Addition of 1×10^6 SJL/J.PPD2 to the assays resulted in adherence rates which were too high to be analyzed. The lower cell number for SJL/J.PPD2 was chosen as this T cell population showed an ~10-fold higher adherence rate to bEnd5 when compared to thymocytes and TK-1. Assays were performed in triplicates for each value.

**Transmigration assay**

Transmigration assays were performed as previously described (17,18). Prior to the assay, endothelial cells were preincubated with 20 μg/ml mAb for 30 min at 37°C. After removal of excess mAb endothelial cells were co-incubated with either 1×10^6 thymocytes, 1×10^5 TK-1 or 1×10^5 SJL/J.PPD2 for 30 min on a rocking platform. Addition of 1×10^6 SJL/J.PPD2 to the assays resulted in adherence rates which were too high to be analyzed. The lower cell number for SJL/J.PPD2 was chosen as this T cell population showed an ~10-fold higher adherence rate to bEnd5 when compared to thymocytes and TK-1. Assays were performed in triplicates for each value.

**Flow cytometry**

Flow cytometric analysis was performed as previously described (6,17,19). Cells were live scatter gated and analyzed with a FACScan using CellQuest software (Becton Dickinson, Heidelberg, Germany).

**Adhesion assays**

Adhesion assays were carried out using 16-well glass chamber slides (Nunc, Wesbaden, Germany) and were performed as previously described (17). To analyze adherence rates which were too high to be analyzed. The lower cell number for SJL/J.PPD2 was chosen as this T cell population showed an ~10-fold higher adherence rate to bEnd5 when compared to thymocytes and TK-1. Assays were performed in triplicates for each value.
TK-1, SJL/J.PPo2 spontaneously migrated in much higher numbers across an endothelial monolayer. All assays were performed in triplicates for each value.

**Statistical analysis**

Parameters were tested in triplicate within each assay. The mean ± SD within one assay or over the sum of all assays were evaluated according to Student's t-test using the Macintosh software Instat.

**Results**

**Phenotype of T cell populations**

The phenotype of the different T cell populations employed in this study was determined by FACS analysis. Thymocytes were chosen as representative for a T lymphocyte population that did not previously encounter antigen and because mature thymocytes have been reported to specifically home from the thymus (primary immune organ) to secondary immune organs (1). The thymocyte populations were usually comprised of -65% CD4/CD8 double-positive, 5% CD4/CD8 double-negative thymocytes plus thymocytes single positive for CD4 (20%) or CD8 (10%) (Figs. 1 and 6). Thymocytes showed high surface expression of CD45RB, which in the case of CD4+ T cells is characteristic for naive/virgin T cells. Thymocytes expressed moderate surface levels of ICAM-1, LFA-1, Mac-1 and CD44, of the integrin subunits \( \alpha_4 \) and \( \beta_1 \), and of the integrin heterodimer \( \alpha_4 \beta_7 \). Surface expression levels for \( \beta_7 \) integrin were extremely heterogeneous (Fig. 1). Positive surface staining for PECAM-1 and ICAM-2 and the high surface levels of L-selectin were characteristic for the thymocyte population.

As a model for effector/memory T cells the CD4+ antigen-specific T1/1 cell line SJL/J.PPD2, which specifically recognizes PPD of *M. tuberculosis* in the context of MHC class II, was chosen. All T cells of this line exhibit the memory/effector phenotype CD44/ICAM-1/LFA-1 high/CD45RB low (Fig. 2) (17). They showed homogenous medium levels of surface expression for the integrin subunit \( \beta_1 \), whereas surface expression for the integrin subunits \( \alpha_4 \) and \( \beta_7 \), and the integrin heterodimer \( \alpha_4 \beta_7 \), was heterogeneous as detected by FACS analysis, suggesting that only subpopulations of T cells within the SJL/J.PPD2 line express \( \alpha_4 \beta_7 \) integrin (or \( \alpha_4 \beta_1 \)) integrin (Fig. 2). SJL/J.PPD2 showed low surface staining for Mac-1 and L-selectin, and moderate levels for PECAM-1 and ICAM-2 (Fig. 2).

The T lymphoma cell line TK-1 is a murine tumor cell line that exhibits a specific, mucosa-associated homing phenotype by virtue of expressing \( \alpha_4 \beta_7 \) integrin but not \( \alpha_4 \beta_1 \) integrin (Fig. 3). TK-1 contain CD8+ single-positive and CD4+/CD8+ double-positive T cells. TK-1 also showed positive surface staining for CD44, ICAM-1 and LFA-1, but heterogeneous CD45RB expression (Fig. 3). Their surface expression of L-selectin and Mac-1 was low, whereas they showed moderate to high surface expression for PECAM-1 and ICAM-2.

Thus, the investigated T cell populations differed not only in their activation and differentiation state but also in their CAM phenotype.

The role of ICAM-1 in the adhesion of different T cell populations

Wild-type endothelium cells (bEnd5), ICAM-1-deficient endothelium cells (bEnd11.1) and ICAM-1-deficient endothelium cells re-transfected with ICAM-1 (bEnd11.1-ICAM-1) were used as published (17) to investigate the role of ICAM-1 in the interaction of different T cell populations with endothelium. To qualitatively and quantitatively compare T cell adhesion to ICAM-1-deficient endothelium with T cell adhesion to wild-type endothelium, adhesion of each T cell population to the three different endothelium cell lines was always investigated within one experiment.

Thymocytes, SJL/J.PPo2 and TK-1 adhered to both unstimulated and tumor necrosis factor (TNF-\( \alpha \))-stimulated endothelium. Upon stimulation of the endothelium with TNF-\( \alpha \) the number of thymocytes and TK-1 adhering to all three endothelium cell lines significantly increased by 2.0 ± 0.1-fold (n = 4) and 1.3 ± 0.1-fold (n = 3), whereas adhesion of SJL/J.PPo2 was not significantly changed (1.2 ± 0.2-fold, n = 4). (See Fig. 4 for one representative assay.)

Endothelial ICAM-1 was involved in the adhesion of all investigated T cell populations to endothelium. Figure 4 shows the results of one representative assay for each T cell population. Over the sum of all assays performed the adhesion of thymocytes to unstimulated and TNF-\( \alpha \)-stimulated bEnd5 pre-treated with the anti-ICAM-1 mAb YN1/17 was reduced by 56.2 ± 12.9 and 57.4 ± 19.8% respectively (n = 5). Thymocyte adhesion to unstimulated and TNF-\( \alpha \)-stimulated bEnd11.1 when directly compared to their adhesion to bEnd5 was reduced to the same degree, i.e. by 51.3 ± 20.7 and 41.5 ± 21.6% (n = 4). Similarly, the adhesion of SJL/J.PPD2 to unstimulated and TNF-\( \alpha \)-stimulated bEnd5 pre-treated with YN1/17 was reduced by 44.6 ± 10.5 and 51.6 ± 6.2% (n = 5). SJL/J.PPD2 adhesion to unstimulated and TNF-\( \alpha \)-stimulated bEnd11.1 was reduced by 47.0 ± 14.0 and 47.6 ± 6.1% (n = 4) when directly compared to bEnd5. In the same way pretreatment of bEnd5 with the blocking mAb YN1/1.7 reduced adhesion of TK-1 to resting and activated bEnd5 by 57.5 ± 2.5 and 54.0 ± 6.7% respectively (n = 3). In the absence of ICAM-1, adhesion of TK-1 to unstimulated bEnd11.1 was reduced by 52.3 ± 10.1 and to stimulated bEnd11.1 by 42.3 ± 6.6 (n = 3) in direct comparison to their adhesion to bEnd5.

Re-expression of ICAM-1 in bEnd11.1 brought the adhesion levels of all three T cell populations back to wild-type levels. Additionally, YN1/1.7 reduced adhesion rates of all investigated T cells to bEnd11.1-ICAM-1 to the same degree as on bEnd5 (Fig. 4).

Taken together, direct comparison of T cell adhesion to endothelium expressing or lacking ICAM-1 revealed the same qualitative and quantitative role for endothelial ICAM-1 in the adhesion of all three T cell populations to endothelium.

VCAM-1 and E-selectin are absent on the surface of unstimulated endothelium cells, although readily up-regulated upon stimulation with TNF-\( \alpha \). In correlation to this, mAb directed against VCAM-1 and E-selectin did not reduce adhesion of the investigated T cell populations to unstimulated endothelium (Fig. 4). Upon stimulation of endothelium with TNF-\( \alpha \) all three T cell populations could be shown to adhere
Thymocytes

Fig. 1. Phenotype of thymocytes. The phenotype of thymocytes as determined by FACS analysis is shown. T cells are scatter gated on live cells.

via VCAM-1 and E-selectin to activated endothelium (Fig. 4). Pre-treatment of TNF-α-stimulated bEnd5 with anti-VCAM-1 mAb reduced the adhesion of thymocytes by 43.2 ± 11.7% (n = 5), of SJL/J.PPD2 by 42.8 ± 11.7% (n = 5) and of TK-1 by 24.9 ± 1.2% (n = 3). Pre-treatment of TNF-α-stimulated bEnd1.1 with anti-VCAM-1 mAb reduced adhesion of thymocytes by 61.3 ± 12.2% (n = 4), of SJL/J.PPD2 by 47.8 ± 7.5% (n = 4) and of TK-1 by 40.2 ± 11.5% (n = 3). Thus, in the absence of ICAM-1, VCAM-1 seemed to play a more important role in adhesion of thymocytes and TK-1 to stimulated endothelium, whereas this could not be observed for the adhesion of SJL/J.PPD2.

Besides VCAM-1, E-selectin was involved in the adhesion of all three T cell populations to TNF-α-stimulated bEnd5, as antibodies directed against E-selectin significantly reduced adhesion rates for all three T cell populations to stimulated bEnd5 (Fig. 4). Anti-E-selectin mAb inhibited adhesion of thymocytes to activated bEnd5 by 47.3 ± 18.0% (n = 3), of SJL/J.PPD2 by 30.3 ± 8.1% (n = 3) and of TK-1 by 21.5 ± 10.4% (n = 3). A similar involvement of E-selectin in the adhesion of all three T cell populations to stimulated ICAM-1-deficient bEnd1.1 was demonstrated as a mAb directed against E-selectin reduced the adhesion of thymocytes to activated bEnd1.1 by 46.0 ± 9.9% (n = 2), of SJL/J.PPD2 by 41.0 ± 12.7% (n = 2) and of TK-1 by 39.3 ± 18.5% (n = 3). Investigation of T cell adhesion to the ICAM-1-retransfectants bEnd1.1-ICAM-1 revealed adhesion levels for all three T cell populations as obtained with the wild-type endothelium cell line bEnd5 (Fig. 4).

Although ICAM-2 and PECAM-1 are expressed on the surface of resting and activated endotheliomas, mAb against ICAM-2 or PECAM-1 did not interfere with the adhesion of thymocytes and SJL/J.PPD2 to unstimulated or TNF-α-stimulated endothelium cell lines (data not shown). However, endothelial ICAM-2 was involved in TK-1 adhesion to ICAM-1-deficient bEnd1.1 as the anti-ICAM-2 mAb inhibited the
**SJL/J.PPD2**

<table>
<thead>
<tr>
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<th>CD45</th>
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</tr>
<tr>
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<td>CD44</td>
<td>LFA-1</td>
<td>CD45RB</td>
</tr>
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<td>PECAM-1</td>
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<td>Counts</td>
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</tr>
</tbody>
</table>

**Fig. 2.** Phenotype of SJL/J.PPD2. The phenotype of SJL/J.PPD2 as determined by FACS analysis is shown. T cells are scatter gated on live cells.

Transendothelial migration of different T cell populations is mediated by ICAM-1 and ICAM-2.

To qualitatively and quantitatively compare transendothelial migration of T cells across ICAM-1-deficient endothelium with T cell migration across wild-type endothelium, transendothelial migration of each T cell population across the three different endothelial cell lines was always investigated within one experiment.

During a 4 h time period thymocytes, SJL/J.PPD2 and TK-1 spontaneously migrated across a monolayer of unstimulated and stimulated bEnd5 (see Fig. 5 for one representative assay for each T cell population). Migration rates were characteristic for each T cell population. In the case of unstimulated bEnd5 2.7 ± 0.8% \((n = 8)\) of the added thymocytes and 4.2 ± 1.2% \((n = 8)\) of TK-1 spontaneously migrated across the endothelial monolayer. The migration rate of SJL/J.PPD2 was significantly higher, i.e. 27.8 ± 7.5% \((n = 7)\). The number of thymocytes transmigrating across a monolayer of stimulated bEnd5...
Increased by 3.7 ± 1.9% (n = 8) and of TK-1 to 5.0 ± 3.0 (n = 8). However, when calculated over the sum of all assays both increases in transmigration rates were not significant when compared to the migration rates across unstimulated bEnd5. Surprisingly, when calculated over the sum of all assays, migration rates of SJL/J.PPD2 across stimulated bEnd5 did not significantly change (24.1 ± 9.6%; n = 7) compared to their migration rates across unstimulated bEnd5.

Pre-incubation of bEnd5 with the ICAM-1 blocking mAb YNI/1.7 reduced transmigration of thymocytes across unstimulated and TNF-α-stimulated bEnd5 by 48.6 ± 14.1 and 41.7 ± 14.7% (n = 7), of SJL/J.PPD2 by 45.9 ± 14.6 and 52.9 ± 10.8% (n = 7) and of TK-1 by 36.7 ± 7.4 and 40.2 ± 8.0% (n = 8) respectively (see Fig. 5 for one representative assay). These amounts of reduced transendothelial migration for each T cell population closely matched the amount of reduced adhesion observed after pre-treatment of bEnd5 with YNI/1.7. Therefore the reduced transendothelial migration of thymocytes, SJL/J.PPD2 and TK-1 appeared to simply be a direct consequence of their reduced adhesion to bEnd5 observed after pre-treatment with YNI/1.7. However, when we compared transendothelial migration of T cells across ICAM-1-deficient bEnd1.1 the results were substantially different (Fig. 5). Migration of thymocytes across unstimulated and TNF-α-stimulated bEnd1.1 was reduced by 73.0 ± 4.6 and 64.7 ± 18.1% (n = 8) respectively, when directly compared to their migration across bEnd5 (Table 1). Similar results were obtained when comparing the transendothelial migration of SJL/J.PPD2 across bEnd5 and bEnd1.1 (Fig. 5). In direct comparison to their transendothelial migration across bEnd5 transendothelial migration of SJL/J.PPD2 across unstimulated and stimulated ICAM-1-deficient endothelium was reduced by 64.1 ± 14.3 and 51.8 ± 11.6% (n = 7; Table 2) and the migration of TK-1 by 59.5 ± 12.9 and 61.0 ± 12.4% (n = 8; Table 3). Therefore, in the absence of ICAM-1, transendothelial migration of all three investigated T cell
Fig. 4. Comparison of the adhesion of thymocytes, SJL/J.PPD2 and TK-1 to bEnd5, bEnd1.1 and bEnd1.1.ICAM-1. For each T cell population one representative experiment comparing their adhesion to unstimulated and TNF-α-stimulated bEnd5 in direct comparison to unstimulated and TNF-α-stimulated bEnd1.1 and bEnd1.1-ICAM-1 is shown. Only those parameters which resulted in significant differences of T cell adhesion to endothelium are included. Bars represent mean ± SD (n = 3). Assays were repeated 3–4 times.

populations was reduced to a significantly larger extent than transendothelial migration across bEnd5 pre-incubated with a blocking antibody against ICAM-1 (Tables 1–3). Thus, only the absence of endothelial ICAM-1 allowed us to define its role in transendothelial migration of T cells, which was clearly distinct from its role in T cell adhesion. Furthermore, direct comparison of transendothelial migration of T cells across wild-type and ICAM-1-deficient endothelium clearly demonstrated that endothelial ICAM-1 was involved in transendothelial migration of all three T cell populations investigated, despite their differences in phenotype and maturation state.

In order to investigate whether there is a differential involvement of endothelial ICAM-1 in the transendothelial migration of T cell populations differing in their CD4/CD8 phenotype, we compared the transendothelial migration of these subpopulations amongst thymocytes and TK-1 cells. As determined by FACS analysis, all four thymocyte subpopulations, i.e. CD4⁺CD8⁻, CD4⁺CD8⁺, CD4⁺CD8⁻ and CD4⁺CD8⁺, migrated across unstimulated and stimulated bEnd5 and bEnd1.1 (Fig. 6). The transmigrated thymocyte population showed a slight shift in its relative composition of the four subpopulations such that the transmigrated population showed a lower percentage of CD4⁺CD8⁺ cells and a higher percentage of CD4⁺CD8⁻ cells (Fig. 6). This indicates that the CD4⁺CD8⁺ double-positive thymocytes had a lower migratory capacity, whereas the CD4⁺ single-positive thymocytes had a higher migratory capacity compared to the other thymocyte subpopulations. Transendothelial migration of thymocytes across ICAM-1-deficient bEnd1.1 showed exactly the same shift in the composition of thymocyte subpopulations after transendothelial migration, indicating that the lack of ICAM-1 affected all four thymocyte subpopulations to the same degree (Fig. 6). The same was true for the two TK 1 subpopulations, where both, CD4⁺CD8⁺ and CD4⁺CD8⁺ migrated with equal efficiency across unstimulated and stimulated bEnd5 and bEnd1.1. There was no significant difference in transendothelial migration of the two TK-1 subpopulations (Fig. 6). Thus lack of ICAM-1 equally affected the transendothelial migration of both CD4⁺CD8⁺ and CD4⁺CD8⁺ TK 1 cells.

Re-expression of ICAM-1 in bEnd1.1 was sufficient to reconstitute transendothelial migration of thymocytes, SJL/J.PPD2 and TK-1 to levels which were identical to those
Fig. 5. Comparison of thymocyte, SJL.J.PPD2 and TK-1 migration across bEnd5 and bEnd11.1. One representative experiment comparing transmigration of T cells across unstimulated and TNF-α-stimulated bEnd5 and bEnd11.1 is shown. Only parameters which resulted in significant differences in transendothelial migration of thymocytes, SJL.J.PPD2 or TK-1 are included. Bars represent mean ± SD (n = 3). Assays were repeated 7–8 times.

Table 1. Comparison of thymocyte migration across bEnd5 and bEnd11.1

<table>
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<tr>
<th>Inhibition of migration across</th>
<th>No. of experiments</th>
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<tr>
<td></td>
<td>Unstimulated bEnds (%)</td>
</tr>
<tr>
<td></td>
<td>bEnd5 + 0</td>
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<tr>
<td></td>
<td>48.6 ± 14.1</td>
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<td></td>
<td>bEnd11.1 + α-ICAM-1</td>
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<td>66.3 ± 8.6..</td>
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<td></td>
<td>bEnd11.1 + α-ICAM-2</td>
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</table>

All values were calculated in relation to transmigration across bEnd5 + isotype control (0) = 100% migration = 0% inhibition. Compared to the inhibition of migration across bEnd5 (italic) the difference of the values below are: *significant (0.005 < P < 0.05); **very significant (0.0001 < P < 0.005); ***extremely significant (P < 0.0001); ****not quite significant (0.05 < P < 0.1) or *****not significant (P > 0.1) according to Student’s t-test.
Table 2. Comparison of SJL.PPD2 migration across bEnd5 and bEnd1.1

<table>
<thead>
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<th>Unstimulated bEnds (%)</th>
<th>TNF-α-stimulated bEnds (%)</th>
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<tr>
<td>bEnd5 + Ø</td>
<td>0</td>
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<tr>
<td>bEnd5 + α-ICAM-1</td>
<td>45.9 ± 14.6</td>
<td>52.9 ± 10.8</td>
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<tr>
<td>bEnd1.1 + Ø</td>
<td>64.1 ± 14.3&quot;</td>
<td>51.8 ± 11.6&quot;**</td>
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<tr>
<td>bEnd1.1 + α-ICAM-1</td>
<td>73.7 ± 8.5</td>
<td>60.4 ± 10.0&quot;***</td>
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<tr>
<td>bEnd5 + α-ICAM-1+α-ICAM-2</td>
<td>60.0 ± 16.1</td>
<td>57.0 ± 19.8</td>
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<td>bEnd1.1 + α-ICAM-2</td>
<td>84.7 ± 7.1</td>
<td>74.1 ± 13.2&quot;**</td>
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<td>bEnd1.1 + α-ICAM-1+α-ICAM-2</td>
<td>86.3 ± 6.9&quot;</td>
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All values were calculated in relation to transmigration across bEnd5 + isotype control and show the arithmetic mean ± SD calculated over the sum of the indicated number of individual experiments. (Ø) = 100% migration = 0% inhibition. Compared to the inhibition of migration across bEnd5 (italic) the difference of the values below are: "significant (0.005 < P < 0.05); "very significant (0.0001 < P < 0.005); "not quite significant (0.05 < P < 0.1) or ""not significant (P > 0.1) according to Student’s t-test.

Table 3. Comparison of TK-1 migration across bEnd5 and bEnd1.1

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<tr>
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<th>Unstimulated bEnds (%)</th>
<th>TNF-α-stimulated bEnds (%)</th>
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<td>36.7 ± 7.4</td>
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<td>bEnd1.1 + Ø</td>
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<td>61.0 ± 12.4&quot;**</td>
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<td>bEnd1.1 + α-ICAM-1</td>
<td>58.4 ± 11.8&quot;</td>
<td>63.7 ± 9.7&quot;***</td>
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<tr>
<td>bEnd5 + α-ICAM-1+α-ICAM-2</td>
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<td>bEnd1.1 + α-ICAM-2</td>
<td>88.8 ± 5.5&quot;***</td>
<td>84.0 ± 7.0&quot;***</td>
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<tr>
<td>bEnd1.1 + α-ICAM-1+α-ICAM-2</td>
<td>86.6 ± 2.9&quot;***</td>
<td>82.8 ± 5.6&quot;***</td>
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</table>

All values were calculated in relation to transmigration across bEnd5 + isotype control (Ø) = 100% migration = 0% inhibition. Compared to the inhibition of migration across bEnd5 (italic) the difference of the values below are: "very significant (0.0001 < P < 0.005) and ""extremely significant (P < 0.0001) according to Student’s t-test.

Fig. 6. CD4/CD8 phenotype of thymocytes and TK-1 before and after transendothelial migration. One representative experiment comparing the CD4/CD8 phenotype of thymocytes and TK-1 before and after transendothelial migration across unstimulated (Ø) and stimulated (TNF-α) bEnd5 and bEnd1.1 as determined by FACS analysis is shown. The T cell populations shown in the dot blots are scatter gated on live cells. Numbers in the quadrants indicate the relative percentage of the gated cells. This analysis was repeated 3 times with the same results.
observed for their respective transmigration across wild-type bEnd5 (Fig. 7). Thus, the reduced transendothelial migration of T cells observed across bEnd1.1 was solely due to the lack of ICAM-1 on the surface of bEnd1.1.

In addition to ICAM-1, we could demonstrate a role for endothelial ICAM-2 in mediating transendothelial migration of all three investigated T cell populations (Fig. 5). Pre-incubation of bEnd5 with an anti-ICAM-2 mAb significantly inhibited the migration of thymocytes across unstimulated bEnd5 by 37.7 ± 13.7% and across stimulated bEnd5 by 37.71 ± 18.14% (n = 7). Transendothelial migration of SJL/J.PPD2 across unstimulated and stimulated bEnd5 was inhibited by 26.4 ± 9.2 and 22.1 ± 15.7% (n = 7) and of TK-1 by 28.0 ± 8.3 and 32.2 ± 13.0% (n = 8) respectively. The involvement of ICAM-2 in mediating transendothelial migration of all three T cell subsets was confirmed by investigating the reduced T cell migration across bEnd1.1 (Fig. 5). Pre-treatment of unstimulated and TNF-α-stimulated bEnd1.1 with anti-ICAM-2 mAb reduced the residual transendothelial migration of thymocytes by an additional 46.8 ± 15.4 and 44.4 ± 14.1% (n = 7), of SJL/J.PPD2 by an additional 50.1 ± 18.8 and 44.0 ± 16.7% (n = 7), and of TK-1 by an additional 69.7 ± 10.5 and 58.3 ± 14.0% (n = 8) respectively. Thus, in the absence of ICAM-1, blocking of endothelial ICAM-2 resulted in almost complete inhibition of transendothelial migration of all three T cell populations investigated (Fig. 5). In direct comparison to transendothelial migration across bEnd5 (Tables 1–3), pre-incubation of bEnd1.1 with anti-ICAM-2 resulted in a significantly larger inhibition of transendothelial migration of thymocytes, SJL/J.PPD2 and TK-1 than did pre-treatment of bEnd5 with a cocktail of anti-ICAM-1 and ICAM-2 antibodies (Tables 1–3). Therefore, in the absence of ICAM-1, ICAM-2 seems to play a more important role in mediating transendothelial migration of T cells in general.

Pre-incubation of endothelial cells with mAb directed against VCAM-1, E-selectin or PECAM-1 did not inhibit transendothelial migration of any investigated T cell population (data not shown). Therefore, although VCAM-1 and E-selectin were involved in T cell adhesion of all three T cell populations to activated endothelium, we could not define a role for VCAM-1 and E-selectin in transendothelial migration of any of the investigated T cell populations.
Discussion

Our laboratory has recently established a novel in vitro assay which demonstrated that only the direct comparison of T cell interactions with wild-type and ICAM-1-deficient endothelium allows the definition of a role for endothelial ICAM-1 in transendothelial migration of CD4+ memory T cells, which is different from its role in mediating T cell adhesion (17). Here we extend these studies, and by using the very same approach demonstrate that endothelial ICAM-1 and ICAM-2 are requisite in mediating transendothelial migration of T cell subpopulations that differ in their maturation stage and their in vivo recirculation pattern.

Naive lymphocytes have been shown to be the primary population to circulate from blood to the lymph nodes via the specialized HEV (1). Thymocytes express moderate to high levels of the adhesion molecules called homing receptors, which specifically recognize their respective ligands expressed in a tissue specific manner on the surface of the HEV. Mature thymocytes have been shown to use the peripheral homing receptor L-selectin to specifically recognize the peripheral addressin (PNAd) on HEV in peripheral lymph nodes (1). Upon antigen-specific stimulation of naive T lymphocytes or malignant transformation of T lymphocytes, expression of certain adhesion molecules is either lost or upregulated on their surface. The T lymphoma TK-1 expresses high levels of the mucosal homing receptor α4β7 integrin, and has been shown to specifically bind to HEV in mesenteric lymph nodes and Peyer’s patches, and to preferentially migrate into these organs in vivo (20). Previous studies have also shown that CD4+ T cells in chronic inflammatory infiltrates in different anatomical locations are enriched for memory T cells. However, these T cells are distinguishable on the basis of their expression of adhesion molecules. Additionally, in some cases the surface phenotype of the CD4+ T cells in chronic inflammatory infiltrates can be correlated to a specific subpopulation with respect to their CD4/CD8 phenotype. However, we observed that all four different thymocyte subpopulations (CD4+CD8+, CD4-CD8+, CD4+CD8- and CD4+CD8+) and the two different TK-1 subpopulations (CD4+CD8+ and CD4+CD8+) migrated across an endothelial monolayer and were equally affected in their transendothelial migration by the lack of ICAM-1.

Despite the differences in their capacity to adhere to endothelium in vitro, by using blocking antibodies or by direct comparison of T cell adhesion to wild-type versus ICAM-1-deficient endothelium we could demonstrate that ICAM-1 is involved in the adhesion of all three investigated T cell populations to unstimulated endothelium. Using the same approach, ICAM-1, VCAM-1 and E-selectin were found to be involved in the adhesion of all three T cell populations to stimulated endothelium as expected in agreement with previous studies (17,24-26).

In the absence of ICAM-1, TK-1 cells were unique in their capacity to use endothelial ICAM-2 to bind to stimulated and to a lesser degree to stimulated bEnd1.1. This cannot be explained by a high surface expression of LFA-1 on TK-1 cells as LFA-1 levels on SJL/J.PPD2 were equally high as on TK-1. Therefore, LFA-1 might be present in a different activation stage on the surface of TK-1 cells, which in the absence of endothelial ICAM-1 can bind to ICAM-2 on endothelial cells more efficiently. The fact that ICAM-2-mediated adhesion of TK-1 to endothelium could only be observed in the absence of ICAM-1 points to a minor role of ICAM-2 in the binding to LFA-1 when compared to ICAM-1.

Besides its role in mediating T cell adhesion, the interaction of LFA-1 with endothelial ICAM-1 has been implicated in transendothelial migration of T cells before (27). However, until recently it has not been addressed whether the observed reductions in transendothelial migration of T cells were simply achieved by reducing T cell adhesion. By employing a novel approach we could demonstrate that only the direct comparison of T cell interaction with wild-type and ICAM-1-deficient endothelium allows us to define a role for endothelial ICAM-1 in the transendothelial migration of a specific T cell subset (17). Here we show that these findings can be extended to T cell populations as different as thymocytes, CD4+ memory T cells and T lymphoma cells. Besides ICAM-1, ICAM-2 could be shown to be involved in transendothelial migration of all investigated T cell populations. In concordance with other findings, neither VCAM-1, E-selectin or PECAM-1 could be assigned a role in mediating transendothelial migration of any of the investigated T cell populations (17,28).

Thus ICAM-1 is unique in mediating both adhesion and transendothelial migration of different T cell subsets. In contrast, ICAM-2 is only involved in adhesion of CD4+ T cells to ICAM-1-deficient endothelium, but mediates transendothelial migration of all T cell populations investigated. Furthermore, in the absence of ICAM-1, ICAM-2 seems to be more involved in mediating T cell migration of the three T cell populations across endothelium. This suggests a certain redundancy in the functions of endothelial ICAM-1 and ICAM-2 in transendothelial migration of T cells, because only if ICAM-1 was...
lacking, endothelial ICAM-2 could then partially take over its function. These in vitro findings are supported by the observations made in the respective ICAM-1- and ICAM-2-deficient mice. Whereas ICAM-1 deficiency leads to reduced lymphocyte recruitment into different tissues (14,29), lymphocyte recirculation is not affected in ICAM-2-deficient mice (30). Conversely, in mice deficient for LFA-1, the ligand for both ICAM, lymphocyte recruitment into lymph nodes as well as into inflammatory sites is significantly impaired (27,31). Taken together these data show that although different molecules can be involved in T cell adhesion to endothelium, transendothelial migration of T lymphocytes seems to be specifically dependent on the interaction of LFA-1 with endothelial ICAM-1 and ICAM-2.

PECAM-1, although it has originally been reported to be involved in transendothelial migration and motility of T cells (13,32), neither in our assays (17) nor in a separate study (27) could be assigned a role in transendothelial migration of thymocytes, antigen-specific CD4+ T cells or T lymphoma cells.

In summary, this study shows that endothelial ICAM-1 and ICAM-2 are the requisite adhesion molecules involved in transendothelial migration of three different T cell populations, which differ in their activation and differentiation stage, their adhesion and transmigration rates as well as their CAM phenotype. In addition, the three investigated T lymphocyte populations have been shown to display different recirculation patterns in vivo. Thus, subset-specific interaction of T cell subpopulations with endothelium must be regulated prior to their transendothelial migration, which is restricted to the interaction with endothelial ICAM-1 and ICAM-2. In this respect it is interesting to note that recent studies showed that engagement of ICAM-1 on endothelial cells leads to the phosphorylation of the actin-binding protein cortactin (33) and to the activation of the small GTPase rho (34). Thus the engagement of ICAM-1 and ICAM-2, but not of other CAM, on the surface of endothelial cells might induce signaling cascades leading to changes in the endothelial cytoskeleton which are instrumental for transendothelial migration of T cells.

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Abbreviations
CAM cell adhesion molecule
HEV high endothelial venule
PPD purified protein derivative
TNF tumor necrosis factor

References


