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Antigen Recognition Influences Transendothelial Migration of CD4⁺ T Cells

Federica M. Marelli-Berg,† Loredana Frasca,* Ling Weng,† Giovanna Lombardi,† and Robert I. Lechler²†

The functional significance of MHC class II expression by vascular endothelial cells remains obscure. In this study the possibility that Ag presentation by endothelial cells (EC) influences T cell transmigration, facilitating the recruitment of Ag-specific T cells into tissues, was investigated. The frequencies of T cells with specificity for an HLA-DR alloantigen, or for the recall Ag tetanus toxoid (TT), were measured in peripheral blood CD45RO⁺ (memory) CD4⁺ T cells before and after transmigration through γ-IFN-treated EC monolayers. Frequencies of anti-DR17, IL-2-secreting T cells were fourfold higher in the T cells that transmigrated through a monolayer of DR17-expressing EC. Similar increases were seen in TT-specific, DR7-restricted T cells that transmigrated through TT-pulsed, DR7-expressing EC. To examine more directly the effects of cognate recognition of Ag presented by EC, T cell clones were used. For clones that proliferated in a costimulation-independent manner to Ag presented by EC, cognate recognition arrested transmigration. In contrast, Ag presentation by EC to B7-dependent T cell clones, which do not proliferate following cognate recognition of EC, enhanced the rate of transendothelial migration. These data suggest that Ag presentation by EC may serve to augment the recruitment of Ag-specific T cells into tissues and that proliferation and transmigration are mutually exclusive T cell responses. The Journal of Immunology, 1999, 162: 696–703.

The functional significance of MHC class II molecule expression by endothelial cells (EC),¹ which is often observed in vivo during inflammation (1–3), is to date a matter of debate. Several reports have assigned to EC a role in T cell activation, suggesting that EC can induce (4–6) or enhance (7–8) T cell proliferation. In contrast, we (9) and others (10–11) have found that EC are incapable of inducing primary T cell responses due to the lack of costimulatory molecule expression (9). However, in contrast with most situations in which recognition without activation leads to T cell unresponsiveness, neither memory T cells (9) or T cell clones (F. M. Marelli-Berg and R. I. Lechler, unpublished observation) were rendered unresponsive following specific recognition of EC.

EC also play a key role in the immune surveillance by mediating T cell recruitment into tissue, as a result of chemokine (12–13), selectin (14–15), and integrin (16–18) display.

It is well known that lymphocyte recirculation and the localization of Ag-specific T cells at sites of inflammation are not random events. Naive and memory T cells exhibit distinct patterns of recirculation (19). Naive T cells migrate from blood into lymphoid tissues via high endothelial venules. Memory and activated T cells, in contrast, can recirculate between blood and the tissues. The differences between these patterns of traffic are determined by the different cell surface arrays of adhesion molecules displayed by naive and memory T cells (20). Other T cell surface molecules, named addressins, can even determine the preferential homing of T cells in a particular body compartment (21–23). As a counterpart, a number of adhesion receptors have been identified on EC that participate in the interaction of T cells with the venular EC in secondary lymphoid tissues and with activated endothelium in inflamed tissues (16–18, 24–26).

Although these and other studies have helped to shed light on the Ag-independent molecular mechanisms underlying these events, very little is known of the effect, if any, that cognate recognition of endothelial cells has on T cell recruitment. Most studies have emphasized the importance of Ag-independent expression of certain surface molecules by T cells for their migratory ability (20) and localization preferences (21–23). However, indirect evidence exists that suggests that TCR engagement may, at least, facilitate T lymphocyte recruitment. For example, it is clear that the integrin interaction requires activation of LFA-1 and VLA-4 on the T cell, to induce the high affinity conformation of the integrin (27–30). However, the mechanism of integrin activation is not yet clear. One possibility is that chemokines present at the EC surface may contribute to this activation step. An alternative mean of inducing the high affinity integrin conformation is signaling through the TCR/CD3 complex (27–28). The problem posed by this finding is that the high affinity integrin conformation persists only for a short time, such that it would be lost by the time a T cell that was activated in a lymph node had recirculated to the site of inflammation.

Finally, the role of Ag in specific recruitment of T cells has also been emphasized by recent data that suggested that the presence of Ag in a tissue can attract and retain Ag-specific T cell clones in vivo (31), although the molecular basis of this phenomenon was not investigated.
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The purpose of this study was to reinvestigate the immunological role of Ag-specific interactions between MHC class II-expressing EC and CD4+ T cells. In particular, given the apparent “neutral” effect that Ag recognition of EC had on resting memory T cells in our hands, the possibility that such encounter might instead influence transendothelial migration of Ag-specific activated and resting memory T cells, thereby contributing to their recruitment, was analyzed.

Materials and Methods

Ags and mitogens

The synthetic influenza virus hemagglutinin (HA) peptides (HA 307–319 and HA 100–115) were synthesized by the Imperial Cancer Research Fund (ICRF) Peptide Unit and kindly provided by Dr. Hans Stauss. Tetanus toxoid (TT) was purchased from Evans Medical (Leatherhead, U.K.). Purified protein derivatize (PPD) was a kind gift of S. Wilson (The London Hospital, London, U.K.).

Monoclonal Abs

The following mAbs were used in purified form for preparation of CD4+ T cells: Leu-19 (anti-CD56; Becton Dickinson, Cowley, U.K.), mouse anti-human Ig (Fab-specific; Sigma, Poole, Dorset, U.K.), L243 (anti HLA DRa; American Type Culture Collection (ATCC), Manassas, VA), and OKT8 (anti-human CD8; ATCC). The OKT8 and L243 mAbs were purified from culture supernatant on protein A-Sepharose beads by standard methods. Eluted Ab was dialyzed against three changes of PBS. The mAb anti-CD45RA (SN 130, gift of G. Janossy, Royal Free Hospital, London) (32), was purified as described above. The mAb 24 (anti-LFA-1; Ref. 33) was a kind gift of N. Hogg (ICRF, London). The Anti-VCAM mAb was a kind gift of N. Hogg (ICRF, London). The Anti-VCAM mAb was a kind gift of N. Hogg (ICRF, London). The Anti-VCAM mAb was a kind gift of N. Hogg (ICRF, London). The Anti-VCAM mAb was a kind gift of N. Hogg (ICRF, London).

Separation and culture of HUVEC

Endothelial cells (HUVEC) were isolated from human umbilical cord veins by collagenase (Sigma) treatment according to a modification of the technique described by Jaffe et al. (34) and depleted of contaminating MHC II+ cells using the Dynabead technique (Dynal Ltd., Merseyside, U.K.) according to the manufacturer’s instructions. Recovered cells were serially subcultured at 37°C with 5% CO2 in Medium 199 (Sigma) supplemented with 20% heat inactivated FCS, 2 mM glutamine (Flow Labs., Irvine, U.K.), 150 mg/ml Endothelial Cell Growth Supplement (Sigma), 12 μg/ml heparin (Sigma), 100 IU/ml penicillin (Flow), 100 μg/ml streptomycin (Flow), and 2.5 μg/ml Fungizone (ICN Biomedicals, Costa Mesa, CA) in gelatin (Sigma)-coated tissue culture flask (Greiner Labortechnik, Dursley, U.K.). At confluence, HUVEC were detached from the culture flasks, using a solution of 0.125% trypsin in 0.2% EDTA (Life Technologies, Paisley, U.K.) and passaged. For functional assays, HUVEC were used in the assays at passage 4–10. Before use in assays, HUVEC were cultured in the presence of 500 U/ml of γ-IFN (kindly provided by T. Meager, National Institute for Biological Standards and Controls, U.K.) to induce expression of MHC class II molecules. Confirmation of the endothelial lineage of the cells obtained was achieved by staining with anti-von Willebrand factor (DAKO) and anti-CD31 (DAKO, Ely, U.K.) mAbs.

Cell lines

EBV-transformed B-lymphoblastoid cell lines (B-LCL), from the 10th International Histocompatibility Workshop, were cultured in RPMI 1640 tissue culture medium (Flow) supplemented with 10% FCS, 2 mM glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin.

The IL-2-dependent murine T cell line CTLL-2 (European Collection of Animal Cell Cultures, Salisbury, U.K.) was cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, 10 U/ml of human rIL-2 (Boehringer Mannheim, Mannheim, Germany), and 10% FCS. The cells were cultured in 25-cm² flasks and were subcultured every 3 days. Before use in a proliferation assay, the CTLL-2 cells were washed twice and cultured overnight in normal culture medium, but without added rIL-2.

Purification of CD4+ CD45RO+ T cells

PBMC were obtained by Ficoll-Hypaque (Pharmacia, Uppala, Sweden) centrifugation of heparinized blood, washed twice, and resuspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. The cell preparation was then depleted of adherent cells by two 45-min rounds of adherence to plastic on tissue culture dishes at 37°C. The nonadherent cells were subsequently collected and incubated with a mixture of purified mAbs (L243, OKT8, Leu-19, mouse anti-human Ig, and SN130) at saturating concentrations for 30 min at 4°C. The cells were then washed twice to remove excess Ab and further enriched by magnetic immunodepletion. Briefly, mAb-treated cells were incubated with magnetocarbon microparticles (Milenyi Biotec, Bergisch Gladbach, Germany) coated with sheep anti-mouse Ig for 15 min at 4°C, and bead/mAb-coated cells were removed by passage through a magnetic column (miniMAC system; Miltenyi). The purified cells were resuspended in medium ready for the proliferation assay, and accessory cell contamination was assessed by culture with 1 μg/ml PHA in a 48-h assay. T cell clones

Clones HC3, HC6, and NF4, specific for HA 307–319 and restricted by DRB1*0101, were generated as described previously (35–36). Clones LR34 and LR47, specific for HA 307–319 and restricted by DRB1*0401, were derived from PBMC isolated from a DR 4,15 individual by stimulating PBMC with purified influenza hemagglutinin (HA 5 μg/ml). The clones were maintained in culture by weekly stimulation with autologous PBMC, HA peptide, and rIL-2 (Boehringer Mannheim) in RPMI 1640 medium supplemented with 10% human serum, 2 mM glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. For use in experiments, the T cells were purified by isolation on a Ficoll-Paque gradient 7 days after restimulation and washed five times by low speed centrifugation (210 g, 5 min) before use, to exclude any contamination by accessory cells.

T cell proliferation assays

T cell clones (10⁴ cells/well) were cultured in the presence of B-LCL (2 × 10⁵/well), treated with 120 Gy x-irradiation, or EC (2 × 10⁵/well), treated with 30 Gy x-irradiation, in flat-bottom microtiter plates, in a total volume of 200 μl. The stimulator cells were prewashed overnight with peptide and then washed to remove any soluble peptide. Wells were pulsed with 1 μCi of [³²P]Thydr (Amersham International, Amersham, U.K.) after 48 h, and the cultures were harvested onto glass fiber filters (Wallac, Turku, Finland) 18 h later. Proliferation was measured as [³²P]Thydr incorporation by liquid scintillation spectroscopy.

Lymphocyte transmigration assays

The transmigration experiments were conducted using HUVEC monolayers grown on Costar Transwell tissue culture well inserts (diameter 24.5 mm), which contained polycarbonate membranes with a 3-μm pore size (Costar, High Wycombe, U.K.). EC (10⁴) were seeded onto fibronectin-coated (50 μg/ml Sigma) polycarbonate membranes overnight to form a monolayer. In some experiments, Ag was added for 16–18 h before the transmigration assay. Purified resting CD4+ CD45RO+ T cells (4 × 10⁴) in RPMI 1640 supplemented with 10% HS were added into each insert and left to migrate through the monolayer; the well volume was also replaced with fresh media. T cells were left to migrate overnight; then each insert was removed in turn and the base was thoroughly washed with media from the well to detach any transmigrated cell still loosely attached. On average, 10–20% of the seeded T cells were recovered from the lower chamber. The transmigrated cells were then counted and used for the limiting dilution analysis assay. In other experiments using T cell clones, T cells (2 × 10⁶) were placed into each insert and left to migrate through the EC monolayer. After 1 h, the number of migrated T cells was determined by counting the lymphocytes present in the well media. This was done at different time points for the next 48 h. In these experiments, results are expressed as percentage of transmigrated cells.

Limiting dilution analysis assays

Responder T cells were diluted into seven serial twofold dilutions, and twenty-four replicate wells of each dilution were plated out, in 50 μl, in U-bottomed 96-well plates (Costar), with responder cell number decreasing from 2 × 10⁶ to 0.03125 × 10⁶ per well. The stimulator cells (either B-LCL or PBMC) were γ-irradiated (192 Gy for the B-LCL or 50 Gy for the PBMC), and 1 × 10⁵ PBMC or 5 × 10⁵ B-LCL were added to each well. After 72 h incubation the culture plates were γ-irradiated (25 Gy) to prevent further proliferation of responder cells, and 1 × 10⁵ indicator CTLL-2 cells were added to each well. After 8 h, the plates were labeled with [³²P]Thydr (1 μCi/well), and proliferation of the CTLL-2 was assayed by [³²P]Thydr incorporation after a further 18 h incubation. Background control wells contained stimulator cells and CTLL-2 cells, but no responder cells. Assay wells were considered positive if proliferation exceeded the
average, plus three SD, of control wells. In all experiments, CTLL-2 proliferation to a range of rIL-2 concentrations was measured to ensure that the CTLL-2 cells gave a dose-dependent response to IL-2.

**Statistical analysis**

Frequencies of alloreactive or Ag-specific CD4+ T cells were calculated using a maximum likelihood statistical program, based on the method of Finney (37). The proportion of negative wells at each sample size of responder cells, according to the Poisson distribution, $-\log P_{neg} = fX$, where $P_{neg}$ is the proportion of negative wells, $f$ is the frequency of responder cells, and $X$ is the sample size of responder cells/well.

In addition, 95% confidence limits of the frequencies and $\chi^2$ estimates of probability were calculated. Frequencies are regarded as different if their 95% confidence limits (approximately two SD) do not overlap.

**Results**

**Cognate recognition on EC favors the recruitment of Ag-specific, resting CD45RO+ T cells.**

Given that the central role of EC during an immune response is to facilitate selective access of leukocytes to inflamed tissues, one attractive possibility is that TCR engagement by EC-presented Ags augments the recruitment of Ag-specific T cells into the tissue. Consistent with this hypothesis, it has been recently shown that TCR engagement delivers a "stop" signal to ICAM-1-triggered T cell locomotion on planar phospholipid bilayers (38).

The effect of cognate recognition on transmigration of CD4+ T cells was investigated by analyzing the frequencies of allo-specific and Ag-specific purified CD45RO+ T cells before and after overnight migration through a monolayer of EC expressing the relevant ligand. CD45RO+ T cells were selected for these experiments in relation to our previous finding that they are neither activated nor rendered unresponsive by cognate recognition of EC (9). An attempt to use CD45RA+ T cells as a control was unsuccessful, in that less than 1% of the total input of T cells migrated.

Following overnight incubation on allogeneic EC monolayers, the frequencies of specific T cells were found to be two- to fourfold increased following transendothelial migration. The results are summarized in Tables I and II. The highly purified CD45RO+ T cells used in the transmigration assays were not activated by Ag-pulsed EC, as is illustrated by a proliferation assay performed in parallel and reported below each panel. As shown in Table I, exp. 1, the frequency of DR17-specific alloreactive T cells was twofold higher in the cells that had migrated through a DR17-expressing EC monolayer, compared with the frequency in the starting population. No change in the frequency of T cells specific for a third party alloantigen (DR1) was seen. Nontreated EC did not cause preferential migration of allospecific T cells. A similar increase in frequency of T cells specific for the recall Ag TT was

| Table I. Antigen recognition on EC enhances the transmigration of Ag-specific T cellsa |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Exp. No.        | Condition       | Stimulator Cells | 1/Frequency     | 95% Confidence Limits | $\chi^2$ |
| 1               | Before TM       | DR17-EC          | 486             | 359–656           | 0.95   |
|                 | DR17-EC        | 232              | 172–314         | 4.8               |
|                 | EC (DR17)      | 401              | 303–532         | 1.9               |
|                 | Before TM      | DR1             | 435              | 320–592           | 2.6    |
|                 | DR17-EC        | (3rd party)      | 418              | 315–554           | 2.5    |
|                 | EC (DR17)      | 402              | 303–539         | 22                |
| 2               | Before TM      | DR17-EC + TT    | 2452             | 1881–3197        | 5.6    |
|                 | DR17-EC + TT  | 990              | 754–1302        | 6.7               |
|                 | DR17-EC + TT  | 2876             | 2208–3747       | 5.5               |
|                 | DR17-EC + TT  | 2870             | 2203–3739       | 3.9               |
| 3               | Before TM      | DR7-EC          | 3064             | 2349–3995        | 2.5    |
|                 | DR7-EC        | 862              | 651–1141        | 3.6               |
|                 | DR7-EC + L243 | 1956             | 1495–2559       | 1.3               |
|                 | Before TM      | DR15             | 2142             | 1639–2800        | 25.2   |
|                 | DR7-EC        | (3rd party)      | 2343             | 1948–3319        | 110    |
|                 | DR7-EC + L243 | 2625             | 1984–3472       | 5.4               |
| 4               | Before TM      | DR17              | 6618            | 5028–8771        | 8.3    |
|                 | DR17-EC       | 1566             | 1167–2102       | 8.6               |
|                 | DR17-EC (–24) | 3514             | 2664–4636       | 6.4               |
|                 | Before TM      | DR7              | 1426             | 1060–1918        | 10.2   |
|                 | DR17-EC       | (3rd party)      | 1397             | 1038–1886        | 7.8    |
|                 | DR17-EC (–24) | 978              | 721–1329        | 8.6               |

a Summary of four experiments in which the frequencies of T cells specific for HLA-DR molecules expressed by a γ-IFN-treated EC monolayer and for third party DR molecules (DR1, DR15, and DR7) or Ag (PPD) not expressed by the responder T cells and the EC were measured before and after transmigration (TM) through the monolayer. In order to verify T cell purification, a proliferation experiment was performed in parallel by coculturing the EC used for the relevant monolayer (10^5/well) and a B-LCL (10^4/well) expressing the same DR molecule (or Ag) as the EC. After 6 days, the wells were pulsed with 1 μCi [3H]Tdr (Amersham) and the cultures were harvested onto glass fiber filters (Wallac) 18 h later. Proliferation was measured as [3H]Tdr incorporation by liquid scintillation spectroscopy. The data are expressed as mean of triplicate cultures, corrected for background proliferation of both T cells and stimulators alone (Δcpm). The results are reported below each panel. In expt. 1, γ-IFN-un-treated EC (EC(DR17)) were used in the transmigration assay as a specificity control. In expt. 2, frequencies against nominal Ag were measured and compared. In expt. 3, the effect of blockade of cognate recognition on the specific recruitment was analyzed by treating the EC monolayer with anti-DR mAb (L243) prior to the transmigration experiments. Finally, in expt. 4, frequencies were measured in transmigrated T cells depleted of mAb 24-positive T lymphocytes.

b DR7,13 CD45RO+ T cell transmigration through γ-IFN-treated or untreated DR17-EC monolayer. T cell (10^5) proliferation (Δcpm): DR17-EC (10^5); 1513 ± 192; DR17-B-LCL (10^3); 3.383 ± 3.311.

c DR17 CD45RO+ T cell transmigration through γ-IFN-treated DR17-EC monolayer prepulsed with TT. T cell (10^5) proliferation (Δcpm): DR17-EC (10^5) + TT: 428 ± 52; DR17-PBMC (5 × 10^6) + TT: 20898 ± 1546.

d DR16,11 CD45RO+ T cell transmigration through γ-IFN-treated DR7-EC monolayer. T cell (10^5) proliferation (Δcpm): DR7-EC (10^5): 1676 ± 544; DR7-B-LCL (10^3); 29,830 ± 7414.


f p = 0.95; DR17-EC (3rd party) 418 315–554 2.5

g p = 0.95; DR7-EC (3rd party) 2343 1948–3319 110

h p = 0.95; DR7-EC 862 651–1141 3.6

i p = 0.95; DR7-EC 2343 1948–3319 110

j p = 0.95; DR7-EC 2142 1639–2800 25.2

k p = 0.95; DR7-EC 2343 1948–3319 110

l p = 0.95; DR7-EC 1426 1060–1918 10.2
The transmigration of Ag-specific T cells is decreased in the presence of contaminating autologous APC. The frequencies of T cells specific for HLA-DR molecules expressed by a γ-IFN-treated EC monolayer and for third party DR molecules (DR 4, 15) were measured before and after transmigration (TM) through the monolayer. In order to assess T cell purity, a proliferation experiment was performed in parallel by coculturing the EC used for the relevant monolayer (10⁵/well) and a B-LCL (10⁴/well) expressing the same DR molecule (or Ag) as the EC. The results are reported below the panel. T cells, which remained PHAresponsive due to poor autologous APC depletion, proliferated to DR17-expressing allogeneic EC. In contrast to what was observed when highly purified T cells were used, the frequency of DR17-specific T cells was found to be decreased following transmigration. The frequency of T cells specific for third party DR molecules was not modified following transmigration. T cell (10⁵) proliferation (Δ cpm): DR17-EC (10⁴):19,941 ± 1768; DR17-B-LCL (10⁴):29,967 ± 4239.

The proliferative response of T cell clones to Ag presented by EC is heterogeneous and is determined by the requirement of the clone for B7-mediated costimulation (F. M. Marelli-Berg and R. I. Lechler, unpublished observations). Proliferation by the T cell clones chosen for the transmigration experiments in response to peptide presented by EC and B-LCL (2 × 10⁵ cells/well), prepuled with the cognate HA peptide (30 μg/ml). After 48 h, proliferation was measured as [3H]TdR incorporation by liquid scintillation spectroscopy. The results are expressed as the mean of triplicate cultures.

Table II. Transmigration of APC-contaminated DR13⁺ CD45RO⁺ T cells through a monolayer of γ-IFN-treated DR17-EC

<table>
<thead>
<tr>
<th>Condition</th>
<th>Stimulator Cells</th>
<th>95% Confidence Limits</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before TM</td>
<td>DR17</td>
<td>4152</td>
<td>3188–5408</td>
</tr>
<tr>
<td>DR17-EC</td>
<td>6154</td>
<td>4716–8031</td>
<td>8.2</td>
</tr>
<tr>
<td>Before TM</td>
<td>DR4, 15</td>
<td>1168</td>
<td>887–1538</td>
</tr>
<tr>
<td>DR17-EC (3rd party)</td>
<td>1419</td>
<td>1081–1864</td>
<td>3.6</td>
</tr>
</tbody>
</table>

The heterogeneity of clonal T cell responses to Ag presented by EC. T cell clones (10⁵ cells/well) were cultured in the presence of DR1- or DR4-expressing EC and B-LCL (2 × 10⁵ cells/well), prepuled with the cognate HA peptide (30 μg/ml). After 48 h, proliferation was measured as [3H]TdR incorporation by liquid scintillation spectroscopy. The results are expressed as the mean of triplicate cultures.

One possible explanation for the effect of cognate recognition on transmigration was that TCR ligation induced the high affinity conformation of T cell surface integrins. This possibility was explored by depleting the transmigrated T cells of cells expressing the high affinity conformation of LFA-1 using the mAb 24, which is specific for an epitope selectively displayed by activated LFA-1 (29, 33). As shown in Table I, exppt. 4, this led to a 50% reduction in the frequency of DR17-reactive T cells in the transmigrated population. In contrast, no diminution in the frequency of T cells specific for third party HLA-DR7 molecules, not expressed by the EC monolayer, was seen. These observations suggest that the mechanism by which Ag recognition enhances T cell migration is, at least in part, the activation of integrins following TCR ligation or coligation with other relevant molecules. Transmigrated T cells did not proliferate, but remained responsive to the DR molecules expressed by the EC, as it is shown by the limiting dilution assays. Indirect evidence for this is provided by the observation that when the T cell preparation was contaminated with autologous APC, T cell proliferation to the EC was accompanied by a decreased frequency against the relevant DR molecule after transmigration (Table II).

Cognate recognition on EC can affect the transmigration of T cell clones

One possible interpretation of the above results is that migration through an EC monolayer displaying specific ligand in some way preactivates the T cell, such that subsequent Ag reactivity is enhanced. To address this point we analyzed the influence of cognate recognition on the transmigration of T cell clones.

FIGURE 1. The heterogeneity of clonal T cell responses to Ag presented by EC. T cell clones (10⁵ cells/well) were cultured in the presence of DR1- or DR4-expressing EC and B-LCL (2 × 10⁵ cells/well), prepuled with the cognate HA peptide (30 μg/ml). After 48 h, proliferation was measured as [3H]TdR incorporation by liquid scintillation spectroscopy. The results are expressed as the mean of triplicate cultures.

The heterogeneity of clonal T cell responses to Ag presented by EC is heterogeneous and is determined by the requirement of the clone for B7-mediated costimulation (F. M. Marelli-Berg and R. I. Lechler, unpublished observations). Proliferation by the T cell clones chosen for the transmigration experiments in response to peptide presented by EC and B-LCL is shown in Fig. 1. Although all the T cell clones proliferated in response to B cell Ag presentation, HC3 and LR34 did not divide in response to the EC.

The transendothelial migration assays were then conducted by seeding T cells on a monolayer of MHC class II-expressing EC, with or without Ag, and monitoring the number of transmigrated T cells during the following 6 h. As shown in Fig. 2, cognate recognition accelerated transmigration by the B7-dependent clones, HC3 and LR34. Given that it has been estimated that T cell migration in vivo occurs within an hour (39), this phenomenon might be of particular relevance during the physiological recruitment of T cells into tissues.

Precisely the opposite was seen for clones NF4 and LR47, in that transmigration was arrested in the presence of Ag. These results suggest that T cell proliferation and T cell transmigration are two alternative, and mutually exclusive, responses to cognate recognition.

Adhesion molecule expression by B7-dependent and -independent T cell clones.

Lymphocyte strong adhesion to EC monolayers and possibly subsequent migration are likely to be effected by integrin-mediated interactions (16, 24–26). The expression of LFA-1 and VLA-4 molecules on the T cell clones used in the transmigration assay
FIGURE 2. Cognate recognition enhances transendothelial migration by B7-dependent T cell clones, while migration by B7-independent T cell clones is blocked. Two distinct sets of T cell clones, HA-specific and DR1-(HC3 and NF4, $4 \times 10^5$/well (a)) or DR4-restricted (LR34 and LR47, $7 \times 10^5$/well (b)) were seeded onto MHC class II-expressing EC monolayers with (filled symbols) or without (empty symbols) Ag pulsing. T cell migration was monitored for the following 6 h and is expressed as percentage of migrated cells at the specified time points.

was analyzed by cytofluorometric analysis. The clones were examined 7 days after Ag stimulation, before being used in a transmigration assay. As shown in Fig. 3, the B7-independent T cell clones LR47 and NF4 expressed higher levels of both LFA-1 and VLA-4 as compared with the B7-dependent T cell clones LR34 and HC3. None of the clones constitutively expressed the activated

FIGURE 3. Expression of adhesion molecules by the B7-dependent and -independent T cell clones. T cell clones HC3 and NF4 (a) and LR34 and LR47 (b) were stained with anti-LFA-1 mAb, anti-VLA-4 mAb, and mAb 24 before use in a transmigration assay (day 7 after Ag stimulation), as described in Materials and Methods. The mAb used is indicated above each graph, and the T cell clones are indicated within the graph.
LFA-1 reporter epitope recognized by the mAb 24. These results raise the possibility that the degree of B7 dependence of a T cell clone relates to its expression of adhesion molecules, such as LFA-1 and VLA-4, and that differences in the levels of these molecules may have contributed to the different characteristics of these clones in the transmigration assays.

Transendothelial migration of anergic T cells is severely impaired.

The above data demonstrate that cognate recognition on EC, while not inducing full T cell activation of costimulation-dependent T cell clones, did increase the rate of transmigration. To further explore the role of T cell activation in transendothelial migration, we examined the effect of inducing anergy in T cells on their capacity to transmigrate. The T cell clone HC3 was rendered nonresponsive by exposure to DR1-expressing thyroid epithelial cells (TFC) pre-pulsed with the relevant peptide, as previously described (40). The loss of Ag reactivity following overnight culture with TFC is illustrated in Fig. 4a). After overnight coculture, the anergic T cells and T cells incubated in medium alone were seeded onto γ-IFN-treated, DR1+ EC monolayers, either prepulsed or not with the cognate peptide, and the number of transmigrated T cells was then monitored. Anergic T cells retained the ability to proliferate in response to exogenously added IL-2. As seen in Fig. 4b, anergic T cells completely lost the ability to migrate through the EC monolayer. This was also true in the absence of cognate Ag (data not shown). This impairment appeared to be persistent, as judged by the complete absence of migration even after 26 h. The expression of VLA-4 and LFA-1 on T cells was not altered by the induction of anergy (data not shown), while CD40 expression was down-regulated, as previously described (41). Anergic T cells did not bind the mAb 24 before or after seeding onto the EC monolayer (data not shown), although this cannot rule out transient expression of the epitope. Coculture with the EC did not result in the recovery of responsiveness by anergic T cells while the reactivity of T cells cultured in medium alone was unaffected, independently of the occurrence of nonspecific or peptide-enhanced transendothelial migration (Fig. 4c).

Discussion

During an immune response, activated T cells recirculate and localize at sites of inflammation following priming in the draining lymph nodes. T cell transmigration from the vascular lumen into tissues will inevitably be accompanied by cognate recognition of peptide:MHC complexes displayed by the cytokine-activated EC for T cells with appropriate specificities. The purpose of this study was to determine whether cognate recognition by CD4+ T cells of Ag presented by MHC class II EC+ influenced transendothelial cell migration.

The observations described in this study have in vivo relevance to the recruitment of either resting memory T cells or recently activated T cells into inflamed tissues. Naive T cells, in contrast, do not recirculate into extralymphoid tissue and are selectively activated in the lymph node (19, 39). The transmigration experiments revealed that cognate recognition of Ag presented by EC influenced the extent of transmigration for all the T cell populations studied. For the CD45RO+ T cells, it appeared that migration across EC monolayers expressing the relevant Ag over a period of 16 h enhanced two- to fourfold the frequency of Ag-specific T cells in the migrated T cell population. Had it been technically possible to measure such frequencies at an earlier time, the observed enhancement might have been more pronounced. This prediction is suggested by the results obtained when the influence of

![Figure 4](image_url)

**FIGURE 4.** Anergic T cells lose their transmigration capacity. **a**, Clone HC3 was rendered unresponsive by overnight coculture with γ-IFN-treated, peptide-prepulsed TFC. As a control, the same number of T cells cultured overnight in medium alone. T cells (10⁴ cells/well) were cultured in the presence of DR1-expressing B-LCL (2 × 10⁴ cells/well), prepulsed with the cognate HA 100 peptide (10 μg/ml). After 48 h, proliferation was measured as [3H]Thd incorporation by liquid scintillation spectroscopy. The results are expressed as the mean of triplicate cultures. A control for viability, T cells (10⁴ cells/well) were also cultured in the presence of 20 U/ml IL-2. Proliferation was measured and expressed as described above. **b**, Anergic (filled circles) and responsive (empty circles) T cells (4 × 10⁴/well) were subsequently seeded onto Ag-prepulsed, MHC class II-expressing EC monolayers. T cell migration was monitored for the following 26 h and is expressed as percentage of migrated cells at the specified time points. **c**, After the transmigration assay, anergic T cells were recovered from the upper well and responsive T cells from the upper (nontransmigrated, NT) and the lower (transmigrated, T) chambers after 26 h incubation in transwells containing peptide-pretreated EC monolayers. T cells (10⁴ cells/well) were then cultured in the presence of DR1-expressing B-LCL (2 × 10⁴ cells/well), prepulsed with the cognate HA 100 peptide (10 μg/ml). Proliferation was measured and expressed as described above.

\[ \text{NT, non-transmigrated T cells; T, transmigrated T cells} \]
cognate recognition on B7-dependent T cell clones was analyzed. In these experiments, the migration rate of T cells through EC monolayers was greatly enhanced in the first few hours following seeding onto the Ag-pulsed EC monolayers. Given that T cell migration in vivo is a rapid process, the physiological relevance of this observation might be of particular importance during T cell recruitment in vivo. Although it is clear that the majority of T cell trafficking into tissues is regulated by Ag-nonspecific mechanisms (16, 20, 39), these data suggest that Ag display by EC can facilitate this process. A recent study analyzing T cell locomotion on phospholipid layers has suggested that TCR engagement might in fact initiate this process by delivering an arrest signal to T cells (38).

In contrast with the enhanced migration that was observed with the B7-dependent T cell populations, transmigration was markedly inhibited for the clones that proliferated in response to EC Ag presentation. These findings have relevance to the debate surrounding whether γ-IFN-treated EC can initiate T cell proliferation. The results obtained here suggest that proliferation and transmigration are two alternative, and mutually exclusive, responses of T cells to Ag recognition. Given that the function of EC is to facilitate T cell entry into inflamed tissues, it would be undesirable for EC to induce T cells to proliferate.

The mechanism whereby cognate recognition promoted transmigration has yet to be fully defined. However, the finding that the frequency of Ag-specific T cells that had transmigrated was significantly reduced by removal of cells expressing the high affinity form of the integrin LFA-1 suggests that activation of integrins significantly reduced by removal of cells expressing the high affinity form of the integrin LFA-1. The need of a T cell for EC to induce T cells to proliferate, it would be undesirable for EC to induce T cells to proliferate.

In conclusion, the physiological relevance of these findings to T cell recruitment during an inflammatory response depends upon the ability of EC to process and present Ags that are sequestered in the underlying tissue. There is in vitro evidence that suggests that this occurs (45). Clearly, in the context of allotransplantation, EC display donor MHC alloantigens, and this could contribute to the recruitment of allospecific T cells into the graft. Further investigation of the issues raised by these data will necessarily involve the use of in vivo models.

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References


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