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Functional Consequences of Noncognate Interactions Between $CD4^+$ Memory T Lymphocytes and the Endothelium[1]

Lutz-Peter Berg,[2†] Martha J. James,* Montserrat Alvarez-Iglesias,* Sarah Glennie,* Robert I. Lechler,* and Federica M. Marelli-Berg[3]*

The recruitment of Ag-specific T cells to sites of inflammation is a crucial step in immune surveillance. Although the molecular interactions controlling T cell extravasation are relatively well characterized, the effects of these events on T cell function are still poorly understood. Using an in vitro model of transendothelial migration of human $CD4^+$ memory T cells, we have investigated the molecular and functional changes induced in T cells that come into contact with the endothelium. First, we show that transendothelial migration is precluded by signals that lead to T cell division. In addition, activation of the transcription factor AP-1, without induction of NF-κB, is observed in T cells after noncognate interactions with endothelial cells (EC), a pattern of transcriptional regulation different from that observed in dividing T cells. Up-regulation of certain adhesion (CD11a, CD49d), activation (CD69), and costimulatory (CD86) receptors accompany these transcriptional events. Most importantly, recently migrated T cells display a faster rate of migration when reseeded onto an EC monolayer. Finally, T cells become hyperresponsive to antigenic challenge after noncognate interactions with the endothelium. These effects appear not to be due to the selection of preactivated T lymphocytes, because they occur also in clonal T cell populations and appear to be mediated by $\alpha_L\beta_2$ integrin-CD54 interactions. We conclude that $CD4^+$ memory T cell extravasation is accompanied by phenotypic and functional changes induced by the interactions with the EC, which favor tissue infiltration by T cells and their further activation once they reach the antigenic site. *The Journal of Immunology,* **2002, 168: 3227–3234.**

Lymphocyte recirculation and the localized recruitment of Ag-specific T cells to sites of inflammation are critical to the surveillance and effector functions of the immune system. The ability of T lymphocytes to reach inflamed tissue is regulated at different levels. First, promigratory modifications in T cell phenotype already occur during priming in the lymph nodes, where changes in the array of surface-expressed adhesion receptors (1, 2) are induced in response to Ag-initiated differentiation. These changes allow T cell access into nonlymphoid tissue (3). Activated Ag-specific T cells are then recruited by the endothelium to sites of inflammation. Receptor-mediated interactions between T cells and EC regulate this multistep process. T cells are first captured from the blood stream by fast reversible interactions mediated by selectins (1), integrins (1), and CD44 (4). Subsequent T cell adhesion and spreading on the endothelium are regulated by chemokine-dependent activation of integrins and possibly other molecules such as CD40-CD154 (5, 6). Following these initial interactions, T cells cross the endothelial barrier and eventually enter the tissue. Some endothelial cell (EC)[4] molecules such as CD31 appear to optimize the efficiency of this last step of the migratory process (7), and enrichment of T lymphocytes expressing surface molecules such as CD26 (dipeptidylpeptidase IV) in migratory T cell populations has been described (8). Together with intrinsic properties of the T cells (such as the high expression of certain adhesion molecules, as observed in memory T cells) (2), signals mediated by these relatively well-characterized receptors are likely to determine whether a T cell that is engaging in adhesive interactions with EC will eventually migrate into the underlying tissue.

Tissue infiltration is a slow and complex process, which involves T cell interactions with the basal membrane and the extracellular matrix. Thus, once a T cell has crossed the endothelial barrier and is committed to migration, further phenotypic and functional changes including cytoskeletal rearrangements (9) and responsiveness to tissue-derived chemotactic factors (10) are likely to mediate the subsequent interactions with basement membrane and extracellular matrix resulting in tissue invasion. It is likely that the endothelium itself might induce functional changes in transiting T cells, which favor T cell motility and further infiltration of the underlying tissue. In this context, up-regulation of certain molecules, like CD86 (11) and CD69 (12), has been observed in T cells after noncognate interactions with the EC. These changes are likely to help further activation of the T cells once they have reached their antigenic sites within the tissue (11).

The effects of noncognate interactions occurring between T lymphocytes and the endothelium during extravasation on subsequent T cell invasiveness and responsiveness to antigenic stimuli have not yet been explored.

In this study, using an in vitro model of transendothelial migration of human memory $CD4^+$ T cells, we have identified a pattern of transcriptional regulation induced in T cells after the interaction with the endothelium, which correlates with sustained changes in surface molecule expression and may lead to the acquisition of an highly mobile and reactive phenotype by migrated T lymphocytes.

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[4] Abbreviations used in this paper: EC, endothelial cell; HS, AB-pooled human serum; B-LCL, B-lymphoblastoid cell line.

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Furthermore, these changes appear to be mediated by $\alpha_L\beta_2$ integrin-CD54 interactions.

Materials and Methods

Monoclonal Abs

The following mAbs were used to purify $CD4^+CD45RO^+$ T cells: anti-CD56 (Leu 19; BD Biosciences, San Jose, CA), mouse anti-human Ig (Fab-specific; Sigma, Poole, U.K.), anti HLA-DRα (L243; American Type Culture Collection (ATCC), Manassas, VA), anti-CD8 (OKT8; ATCC), and anti-CD45RA (SN 130, gift of G. Janossy, Royal Free Hospital, London, U.K.) (13). For phenotypic analysis, the following Abs were used: anti-CD11a (LFA-1, clone 38; Serotec, Kidlington, U.K.); anti-CD49d (VLA-4, clone HP2.1; Serotec); anti-CD26 (M-A261; Serotec); anti-CD86 (Bu63; Caltag Laboratories, Buckingham, U.K.); anti-CD80 (BB-1; BD PharMingen, San Diego, CA); anti-CD69 (CH/4; Serotec); anti-CD25 (CD25-3G10; Caltag); anti-CD154 (TRAP-1; BD PharMingen). For T cell activation experiments, purified anti-CD3 (OKT3; ATCC) and anti-CD28 Abs (CD28.2; BD PharMingen) were used. The blocking mAb anti-CD54 (ICAM-1, clone 6.5B5; Ref. 14) and anti-CD106 (VCAM-1, clone 1G11; 15) were a gift from D. Haskard (Imperial College School of Medicine, London, U.K.).

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. (16). Recovered cells were serially subcultured at 37°C with 5% CO_2 in medium 199 (Sigma) supplemented with 20% heat-inactivated FCS, 2 mM glutamine (Flow Laboratories, Irvine, U.K.), 150 mg/ml endothelial cell growth supplement (Sigma), 12 U/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 flasks (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. In some experiments, HUVEC were cultured in the presence of either 300 U/ml IFN-γ (kindly provided by A. Meager, National Institute for Biological Standards and Controls, South Mimms, U.K.) or 10 ng/ml TNF-α (PeproTech EC, London, U.K.). Confirmation of the endothelial lineage of the cells obtained was achieved by staining with anti-von Willebrand factor and anti-CD31 mAbs (17).

Purification of $CD4^+CD45RO^+$ T cells

PBMC were obtained by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) centrifugation of heparinized blood, washed twice, and resuspended in RPMI 1640 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, Leu19, 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 magnetic microbeads (Miltenyi 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 Biotec).

T cell clone

The human T cell clone 7P.61, specific for HA 307–319 and restricted by DRB1*0701, was generated as described previously (17). T cells were maintained in culture by weekly stimulation with autologous PBMC, HA peptide (10 μg/ml), and rIL-2 (Roche, Mannheim, Germany) in RPMI 1640 supplemented with 10% heat-inactivated, AB-pooled human serum (HS), 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 $\times$ *g*, 5 min) before use, to exclude any contamination by accessory cells.

Lymphocyte transmigration assays

The transmigration experiments were conducted using HUVEC monolayers grown on Costar Transwell tissue culture well inserts that contained polycarbonate membranes with a 3-μm pore size (Costar, High Wycombe, U.K.), as previously described (18). EC (10^5 or 7×10^4) were seeded onto fibronectin (50 μg/ml; Sigma)-coated polycarbonate membranes overnight to form a monolayer. Purified resting $CD4^+CD45RO^+$ T cells ($2–4 \times 10^6$) or T cell clones ($3–7 \times 10^5$) in RPMI 1640 supplemented with 2% HS were added into each insert and left to migrate through the monolayer; the well volume was also replaced with fresh medium. T cells were then left to migrate. T cells were collected from the upper and lower chambers at different time points. In the experiments assessing lymphocyte motility, the number of migrated T cells was determined by counting the lymphocytes present in the well medium at different time points for the next 24–26 h. In these experiments, results are expressed as percentage of transmigrated cells.

Induction of activation leading to T cell proliferation

T cells were cocultured with plastic-bound anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml) mAb in 48-well plates (Costar). Coated plates were incubated at 37°C for a minimum of 2 h in a humidified incubator in air with 7% CO_2. The wells were then washed once with PBS, and the T cells were plated (10^4 clonal T cells or 5×10^4 purified $CD4^+CD45RO^+$ T cells/well) in 200 μl 2% HS-RPMI 1640. T cells were removed at different time points for use in transmigration assays or EMSA assays. The induction of proliferation was assessed in parallel by measuring [^{3}H]TdR (Amersham International, Amersham, U.K.) incorporation by 5×10^4 $CD4^+$ $CD45RO^+$ T cells 96 h later, by liquid scintillation spectroscopy on a beta plate counter (PerkinElmer Wallac, Gaithersburg, MD).

T cell proliferation assays

$CD45RO^+$ T cells (2×10^4/well) were stimulated with increasing numbers of sublethally irradiated allogeneic B-lymphoblastoid cell lines (B-LCL), in flat-bottom microtiter plates, in a total volume of 200 μl. After 3, 5, and 8 days, wells were pulsed with 1 μCi [^{3}H]TdR (Amersham International), and the cultures were harvested onto glass fiber filters 18 h later. The proliferative response to PHA by $CD45RO^+$ T cells (2×10^4/well) was measured in a 48-h assay. Proliferation was measured as [^{3}H]TdR incorporation by liquid scintillation spectroscopy.

Measurement of transcription factor activation

Activation of the transcription factors AP-1 and NF-κB was measured according to a modification of the technique described by Brunvand et al. (19). This procedure involved the following.

Isolation of nuclei. $CD4^+CD45RO^+$ T cells ($5–10 \times 10^6$) were washed in PBS, resuspended in 1 ml of PBS, and transferred to a microcentrifuge tube. The cells were then pelleted by centrifugation in a microfuge at 6500 rpm for 3 min. The pellet was resuspended in hypotonic buffer (10 mM HEPES (pH 7.9) at 4°C, 10 mM KCl, 0.1 mM EDTA; Sigma) at 70 μl/10^6 cells to which protease inhibitor mixture (5 μl/10^6 cells; Sigma) was added and left on ice. After 15 min, 25 μl of a 10% IGEPAL CA-630 solution (Sigma) were added. After vortexing for 10 s, the cell lysate was pelleted by centrifugation (at 8000 rpm in a microfuge for 2 min at 4°C). The supernatant was discarded, and the nuclear pellet was either used directly or stored at −70°C.

Extraction of nuclear proteins. The nuclei were resuspended in extraction buffer (10 mM HEPES (pH 7.9) at 4°C (Sigma), 400 mM KCl (Sigma), 0.2 mM EDTA (Sigma), 0.2 mM PMSF (Sigma), 0.5 mM DTT (Promega, Madison, WI)) at 5 μl/10^6 nuclei and left on ice for 15 min, occasionally vortexing at low speed. After centrifugation at 20,000 $\times$ *g* and 4°C for 7 min, the supernatant containing the nuclear proteins was collected and stored at −75°C until used. The protein concentration was quantified using the MicroBCA protein assay (Pierce, Rockford, IL).

Generation of radiolabeled, ds-oligonucleotide probes. Complementary oligonucleotides (Santa Cruz Biotechnology, Calne, U.K.) were denatured (96°C for 5 min) and left to cool to room temperature to generate ds-oligonucleotides. ds-oligonucleotides (100 ng) were incubated with 5 μl [γ-^{32}P]ATP (3000 Ci/mmol; DuPont, Stevenage, U.K.), 1 μl T4 polynucleotide kinase (Promega), and 2 μl 10$\times$ kinase buffer (700 mM Tris-HCl (pH 7.6), 100 mM $MgCl_2$, 50 mM DTT; all from Sigma) in a total volume of 10 μl at 37°C for 30 min. Unincorporated ATP were removed using spin columns (Life Technologies) according to manufacturer's instructions.

EMSA. Nuclear protein extract (3 μg) was incubated with 0.5 ng radiolabeled oligonucleotide in the presence of 2 μg nonspecific competitor (poly(dI-dC)·poly(dI-dC); Pharmacia Biotech, St. Albans, U.K.), 2 μl 5$\times$ binding buffer (250 mM Tris-HCl (pH 7.5), 500 mM KCl, 5 mM DTT, 5 mM EDTA, 20% glycerol) and, if required, 1 μl unlabeled oligonucleotide serving as specific competitor in a total volume of 10 μl, at room temperature for 20–60 min. The reaction was loaded onto a 6% polyacrylamide gel and electrophoresed at 100 V for 45 min. The gel was dried (45 min at 80°C) and autoradiographed for 24–48 h.

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Flow cytofluorometric analysis

For flow cytofluorometric analysis, 10^5 T cells were incubated with the indicated mAb at 4°C for 30 min. As a control, T cells were incubated with an isotype-matched irrelevant Ab. After two washings in PBS with 2.5% FCS, the cells were incubated for a further 30 min at 4°C with 100 μl 1/50 dilution of fluoresceinated sheep anti-mouse Ig (Amersham, Amersham, U.K.). After two additional washes, stained cells were analyzed using a FACSCalibur (BD Biosciences) flow cytometer.

Results

Migration is abolished by signals leading to T cell proliferation

We have previously reported that transendothelial migration of costimulation-independent T cell clones, which proliferate in response to Ag presentation by EC, is completely abrogated by the presence of cognate peptide on the endothelium (18). This effect occurs immediately after cognate recognition of the endothelium and persists for at least 48 h (18). To further establish whether the functional status of the T cells determines their ability to migrate, we assessed the effect of signals leading to T cell division on the migratory ability of peripheral blood memory $CD4^+$ T cells.

Peripheral blood $CD4^+CD45RO^+$ T cells were incubated with either medium alone or immobilized anti-CD3 and anti-CD28 mAb, as described in *Materials and Methods*. Coligation of CD3 and CD28 induced T cell proliferation, as shown in Fig. 1*a*). To assess the effect of proliferative stimuli on T cell motility, T cells were removed after 30–36 h ($CD4^+CD45RO^+$ T cells), washed, seeded onto IFN-γ- or TNF-α-treated EC monolayers, and monitored for migration. As shown in Fig. 1*b,* T cell transmigration was completely abrogated after coligation of CD3 and CD28 in the $CD4^+CD45RO^+$ T cells (Fig. 1*b*). This effect persisted for at least 26 h.

Because only a small percentage of the T lymphocytes transmigrate and only a portion of the T cells is responsive to mitogenic stimuli at any time point as they are not synchronized with respect to the cell cycle, a kinetic study was performed to test whether the recruitment into the proliferating pool prevented T cell migration. T cells were incubated in the presence of immobilized anti-CD3 and anti-CD28 for 4, 8, 16, and 24 h and then harvested and washed; their migration through a TNF-α-treated (to optimize migration while excluding allorecognition of EC) EC monolayer was monitored for the next 24 h (Fig. 1*c*). As a control, T lymphocytes cultured in medium alone were used. Thymidine incorporation 96 h after the initial delivery of the proliferative stimulus was assessed in both migrated and nonmigrated T cell populations. As shown in Fig. 1*c*, the percentage of T cells capable of transendothelial migration was inversely proportional to the length of exposure to the proliferative stimulus. Most importantly, at any time point, thymidine incorporation was observed exclusively in the nonmigrated T cell population (Fig. 1, *d–h*), suggesting that the progressive recruitment into the proliferating pool of a greater percentage of the migratory T cell fraction prevented their migration through the EC monolayer. The alternative possibility that activated T cells might produce a factor that inhibits T cell migration was ruled out by the observation that migration of resting T cells in the presence of supernatant harvested from 48-h cultures of activated T cells was not affected (data not shown). These results suggest that cell division and migration are mutually exclusive cellular program.

Transcription factor expression in transmigrated T cells

The effects of EC on transiting T cells was further explored by assessing the activation of transcription factors expressed by T cells after contact with the endothelium. The proliferative T cell response induced by activating stimuli such as TCR and coreceptor engagement is known to involve the transcription factors AP-1 and NF-κB as key mediators. By contrast, the potential role of these factors in EC transmigration has not yet been investigated. To determine whether such transcriptional changes are induced by noncognate interactions with EC and how they might differ from those controlling T cell proliferation, we studied the nuclear levels of AP-1 and NF-κB using EMSA.

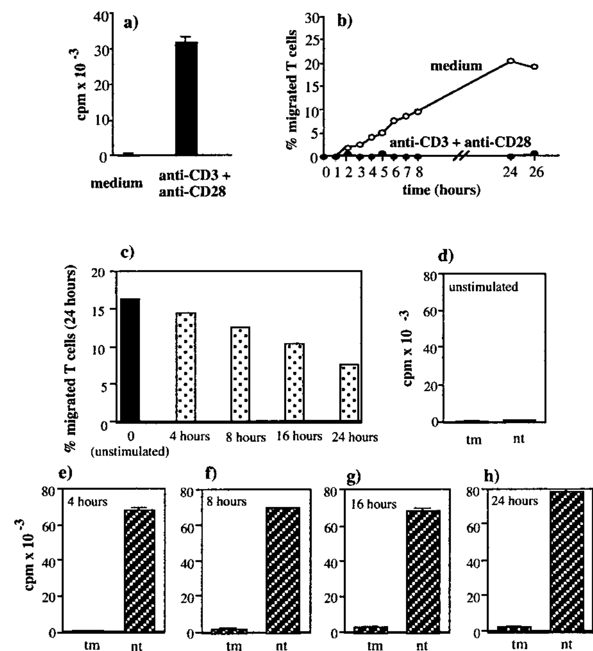

FIGURE 1. Delivery of mitogenic stimuli abrogates T cell motility. $CD4^+CD45RO^+$ T cells were activated with immobilized anti-CD3 and anti-CD28 mAbs, or incubated with medium alone. This treatment resulted T cell division 96 h later (*a*). T cell proliferation was measured as [^{3}H]TdR incorporation by liquid scintillation spectroscopy. The results are expressed as cpm (mean of triplicate cultures). After 36 h of incubation in the presence of immobilized Abs, T cells (*b*, ●) were harvested, seeded onto cytokine-treated EC monolayers (10^6/well), and left to transmigrate. As a control, migration of T cells incubated in medium alone was monitored in parallel (○). Data are expressed in percentage of migrated cells at the indicated time points. To assess the effect of this stimulus on T cell motility, $CD4^+CD45RO^+$ T cells were stimulated with immobilized anti-CD3 and anti-CD28 mAbs for 4, 8, 16, and 24 h and then washed and seeded onto an EC monolayer. Migration of T cells recovered from each culture was assessed after 24 h (*c*). As a control, migration of unstimulated T cells was measured (■). *d–h,* At the end of the transmigration assay (24 h after seeding onto the EC monolayer), T cells were sampled from the migrated (tm) and nonmigrated (nt) T cell populations and plated out at 5×10^4 cells/well in a flat-bottom 96-well plate; [^{3}H]TdR was added for the last 16 h of a 96-h interval from the initial exposure to immobilized anti-CD3 and anti-CD28, so that proliferation was measured at the same time point after stimulation for all the cultures. As a control, proliferation of unstimulated migrated and nonmigrated T cells was measured. Proliferation was measured and expressed as indicated above.

Peripheral blood $CD4^+CD45RO^+$ T cells, which had transmigrated through EC monolayers in vitro, were harvested, and crude nuclear protein extracts were obtained from these cells as well as from resting T cells (negative control) and from T cells activated to proliferate using immobilized anti-CD3 and anti-CD28 mAb. Transcription factor levels were compared using ds-oligonucleotides corresponding to single-consensus binding sites for AP-1 and NF-κB. Because strong activation of both factors is generally observed 4–12 h after cell stimulation (19, 20), we tested AP-1 and NF-κB activation at 4, 8, and 16 h after seeding on EC monolayers

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or after T cell receptor and CD28 engagement. As shown in Fig. 2, transmigrated T cells exhibited increased nuclear levels of AP-1 throughout the time course (Fig. 2*c*) but showed no increase in NF-κB at any of the time points analyzed (Fig. 2*e*, data at 8 h after seeding are shown as an example). As expected, AP-1 (Fig. 2*b*) and NF- κB levels (Fig. 2*e*, data at 8 h are shown for comparison) were increased in activated T cells as compared with resting T cells at all time points. Interestingly, a similar pattern of transcription factor induction (presence of AP-1 but absence of NF-κB) was also observed in the nontransmigrated T cells harvested 16 h after contact with EC (Fig. 2, *c* and *f*), suggesting that induction of AP-1 transcription by EC may not be sufficient to promote migration. These results indicate that 1) the process of T cell migration through EC layers is associated with transcriptional activation and 2) the pattern of transcription factor activation differs from that observed in mitogen-activated cells.

Similar results were obtained when IFN-γ-treated or TNF-α-treated as well as resting EC were used (data not shown) to select migratory T cell populations.

Phenotypic changes are induced in T cells after noncognate interactions with the endothelium

Previous studies have demonstrated that noncognate interactions of T cells with EC result in changes in the expression of T cell surface molecules such as CD86 and CD69, irrespective of the occurrence of transmigration (11, 12). To establish whether the transcriptional events induced in T cells by EC-T cell interactions correlated with phenotypic changes, the expression of an extended array of T cell surface molecules (including CD26, CD11c, CD49d, CD80, CD86, CD69, CD25, and CD154) was studied in transmigrated $CD4^+CD45RO^+$ T cells harvested 4–24 h after seeding onto an EC monolayer by cytofluorometric analysis. As shown in Fig. 3*a*, noncognate interactions with the EC induced up-regulation of CD26, CD11a, CD49d, CD69, and CD86 molecules, as compared with the resting $CD4^+CD45RO^+$ T cell population (time 0). Increased expression of CD26 and CD49d occurred within 4 h of exposure to the EC, with CD26 levels remaining elevated for the next 24 h. T cell CD49d expression by 16–24 h but remained higher than that of resting T lymphocytes. Up-regulation of CD11a, CD86 and CD69 was induced by 8–16 h after contact with the EC, and increased (CD11a, CD86) or remained elevated (CD69) for at least 24 h. In contrast, no changes were observed in the expression of CD80, CD25, and CD154 molecules at any of the time points analyzed. As shown in Fig. 3*b*, these changes occurred in both migrated and nonmigrated T cells, although the up-regulation of these molecules was reproducibly more marked in the transmigrated T cell population. This experiment is representative of a series of at least three independent experiments. As in our observations with the induction of transcription factors, the pattern of surface molecule up-regulation in T cells was similar irrespective of EC activation (data not shown).

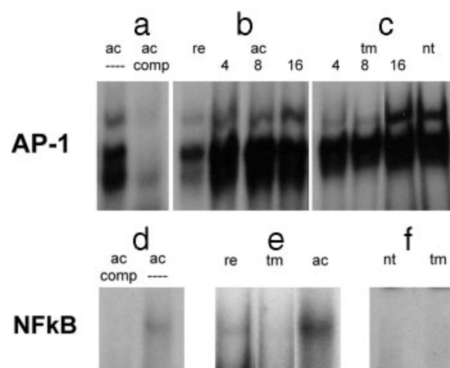

FIGURE 2. Activation of the transcription factor AP-1 occurs in both transmigrated and mitogen-activated T cells, whereas sustained NF-κB activation is induced only by proliferative stimuli. Resting (re), transmigrated (tm), nontransmigrated (nt), and mitogen-activated (ac) $CD4^+CD45RO^+$ T cells were harvested 4, 8, and 16 h after the beginning of the treatment. Nuclear proteins were extracted from T cell samples, analyzed, and visualized as described in *Materials and Methods*; equal loading of all lanes was demonstrated by the equal signal intensity from free probe at the bottom of the gel (data not shown). Nuclear levels of the transcription factor AP-1 in resting T cells were compared with those in transmigrated and activated T cells at 4, 8, and 16 h after T cell encounter (*b* and *c*). *e,* Comparison of NF-κB levels at a representative time point (8 h). *c* and *f,* Comparison of AP-1 and NF-κB levels in transmigrated (tm) and nontransmigrated (nt) T cells. In the presence of a 100-fold molar excess of unlabeled AP-1 or NF-κB oligonucleotides acting as specific competitors (ac comp, *a* and *d*), the bands representing the DNA-protein complexes were abolished, thus confirming the specificity of the binding reactions.

Migrated T cells display increased motility

Having established that phenotypic changes were induced in the T cells by noncognate interactions with the endothelium, we assessed whether T cell motility was affected by interaction with EC. A lymphocyte migration assay was set up to compare the migration of resting (i.e., cultured in medium alone), recently migrated, and nonmigrated $CD4^+CD45RO^+$ T cells. As shown in Fig. 3*c*), transmigrated T cells migrated more efficiently and in larger proportion than resting T cells. In contrast, the rate of transmigration was only slightly increased in the nontransmigrated T cell population. This suggests that the phenotypic changes we have identified (which occur in both migrated and nonmigrated T cells) do not necessarily correlate with the enhancement of motility observed in the transmigrated T cell population. In addition, these functional changes could not be explained by the activation of integrins during transmigration, given that migrated T cells did not react to Abs specific for conformationally activated integrins, such as the mAb 24, which recognizes activated LFA-1 (kind gift of N. Hogg, ICRF, London, U.K.; data not shown). The increased migratory ability was maintained for at least 48 h after the initial contact with the EC and was induced by resting, IFN-γ-treated, or TNF-α-treated EC (data not shown).

Phenotypic and functional modifications are induced in a clonal population of T cells by noncognate interactions with EC

The observation that AP-1 mobilization and phenotypic changes are induced also in nonmigrated T cells suggest that EC-derived signals, rather than selection, are likely to induce these events. To further test this hypothesis, we analyzed the effect of noncognate interactions with the EC on a T cell clone. T cells were seeded onto an EC monolayer and migrated and nonmigrated T cells were collected after 16 h. T cells cultured in medium alone were used as a control. As shown in Fig. 4, *a–e*, CD26, CD11a, CD49d, CD86, and CD69 were up-regulated on both migrated and nonmigrated T cells. In contrast, the expression of CD80, CD25, and CD154 molecules remained unchanged.

In parallel, the migratory ability of resting, migrated, and nonmigrated T cells was assessed in a transendothelial migration assay. As shown in Fig. 4*i*), only migrated T cells displayed enhanced motility after the interactions with the EC. These results are virtually identical with those obtained with the $CD4^+CD45RO^+$ T cells and suggest that EC-derived signals can modify transiting T lymphocytes.

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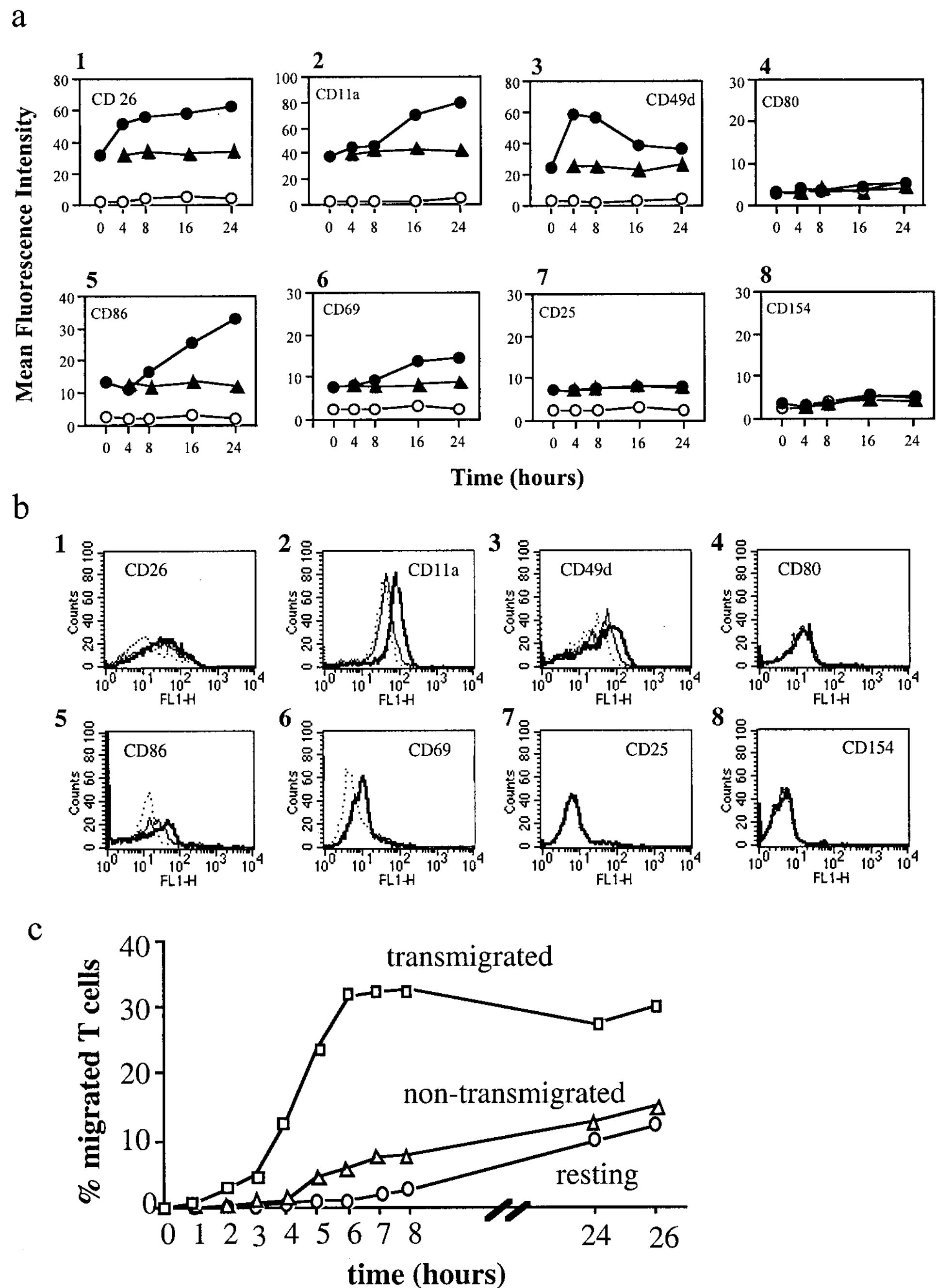

FIGURE 3. Phenotypic and functional changes occur in $CD4^+CD45RO^+$ T cells after interactions with the EC. *a, 1–8,* Purified $CD4^+CD45RO^+$ T cells (4×10^6) were seeded onto cytokine-treated EC monolayers and left to migrate. Migrated T cells were harvested after 4, 8, 16, and 24 h, and the expression of the surface molecules CD26, CD11c, CD49d, CD80, CD86, CD69, CD25, and CD154 was analyzed by cytofluorometric analysis (●). Freshly isolated T cells (time 0) and T cells cultured in medium alone (▲) were used for comparison. As a control, T cells were incubated with an irrelevant isotype-matched mAb (○). Data are expressed as mean fluorescence intensity. *b, 1–8,* Nontransmigrated (thin line) and transmigrated (thick line) $CD4^+CD45RO^+$ T cells were single-stained with mAbs for the surface molecules indicated in each panel. As a control, T cells incubated in tissue culture medium were used as a control (dotted line). Cells were then analyzed by flow cytometry. The panels show the profiles obtained from T cells harvested at the optimal time points indicated by kinetic studies (i.e., 8 h for CD26 and CD49d and 24 h for all the other molecules). *c,* Migrated (□), nontransmigrated (△), and resting (○) T cells were collected and washed as described above. T cells (10^6/well) were then seeded onto a freshly prepared EC monolayer. Data are expressed as percentage of transmigrated cells at the specified time points. Similar results were obtained when migrated T cells were seeded onto a fresh EC monolayer after being maintained in tissue culture medium for up to 72 h (data not shown). FL1-H, Fluorescence.

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Interaction with the EC enhances T cell responsiveness to antigenic challenge

The induction of expression of transcription factors and activation markers that are observed also in response to proliferative stimuli may suggest that the interactions with EC during extravasation may render the T cells better prepared to respond to such stimuli once they have reached the site of inflammation (11). To test this possibility, the effect of interaction with EC on proliferative responses by T lymphocytes was analyzed. Nontransmigrated and transmigrated $CD4^+CD45RO^+$ T cells were obtained using an EC-coated Transwell and were challenged with an allogeneic B-LCL (Fig. 5, *a–c*) for 3, 5, and 8 days. As a control, T cells incubated in medium alone were used. As shown in Fig. 5, *a–c*, the proliferative responses of T cells that had interacted with the EC were enhanced. This effect was particularly marked in the transmigrated T cell population, even at later time points. In contrast, the response to PHA by T cells that had interacted with the EC was only slightly increased (Fig. 5*d*). The enhancement of T cell reactivity to antigenic stimuli was induced by resting, γ-IFN-treated, or TNF-α -treated EC (data not shown).

The phenotypic and functional changes induced in T cells by EC are mediated by β_1 integrin-CD54 interactions

Signals mediated by β_1 and β_2 integrins have long been known to favor T cell activation as well as mediate T cell extravasation (21). For this reason, the relative contribution of β_1 integrin-CD106 and β_2 integrin-CD54 interactions to the functional and phenotypic changes induced in T cells by contact with EC was analyzed. In these experiments, TNF-α-treated EC monolayers (which expressed high levels of both CD54 and CD106; data not shown) were incubated in the presence of anti-CD54 mAb (5 μg/ml) or anti-CD106 mAb (5 μg/ml) for 60 min at 37°C before use. The Transwells were then washed and seeded with purified $CD4^+CD45RO^+$ T cells (2×10^6/well). After

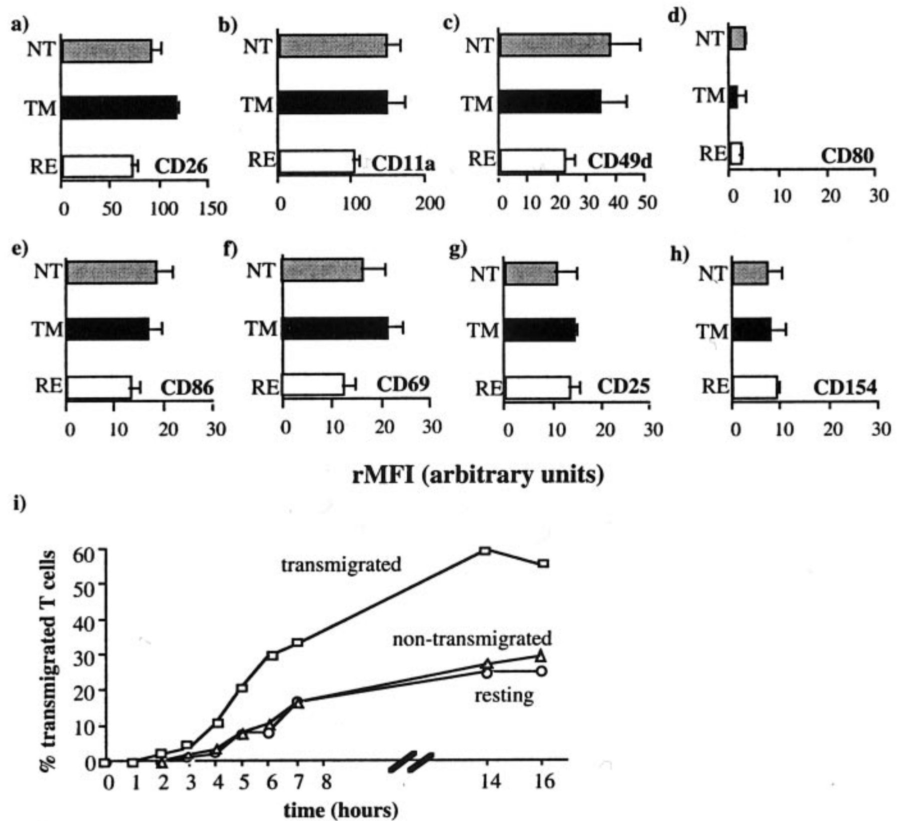

FIGURE 4. Phenotypic and functional changes are induced in clonal CD4$^+$ T cells after interaction with EC. *a–h,* Purified CD4$^+$ T cell clone (7 × 10^5) were seeded on a cytokine-treated EC monolayer and left to migrate for 16 h. As a control, T cells were incubated in culture medium for 16 h. Nontransmigrated (NT, ▨), transmigrated (TM, ■), and resting (RE, □) T cells were then harvested, and surface expression of CD26, CD11c, CD49d, CD80, CD86, CD69, CD 25, and CD154 molecule was analyzed (*a–h*). As a control, an irrelevant isotype-matched mAb was used. Data are expressed as relative mean fluorescence intensity (rMFI; MFI experimental to MFI control ratio). The mean + SE of three experiments are shown. *i,* Transmigrated (□), nontransmigrated (△), and resting (○) T cells were collected and washed as described above. After a resting period in tissue culture medium (0–72 h), T cells (2 × 10^5 T cell clone) were seeded onto a freshly prepared EC monolayer. Data are expressed as percentage of transmigrated cells at the specified time points.

overnight incubation, transmigrated T cells were collected and analyzed. In line with previous reports (12, 22), pretreatment of the EC with anti-CD54 mAb partly inhibited T cell migration (~20% inhibition on average), whereas anti-CD106 did not affect the rate of migration (data not shown).

As shown in Fig. 6, *a–e*), CD54 blockade prevented the phenotypic changes induced by the interactions with the EC, including the up-regulation of CD26 (Fig. 6*a*), CD11a (Fig. 6*b*), CD49d (Fig. 6*c*), CD69 (Fig. 6*d*), and CD86 (Fig. 6*e*) molecules. In contrast, CD106 blockade did not have any effect. In addition, disruption of β_2 integrin-CD54 but not β_1 integrin-CD106 interactions during transendothelial migration prevented the enhancement of motility in transmigrated T cells (Fig. 6*f*). Finally, T cells migrated through anti-CD54-treated, but not anti-CD106-treated, EC monolayers did not display hyperresponsiveness to antigenic challenge (Fig. 6*g*).

These data suggest that $\alpha_L\beta_2$ integrin-derived signals can account for the changes induced in T cells after interaction with EC.

Discussion

The present study analyzed the functional consequences of non cognate EC-T cell interactions at the molecular and cellular level. We have established that signals leading to T cell division lead to the abrogation of T cell motility. We have further observed that EC-T cell interactions are associated with the activation of transcription factors in T cells and that this activation pattern is different to that observed in dividing T cells. These transcriptional events correlated with changes in the expression levels of surface molecules, some of which are shared with the response to stimuli leading to proliferation. In addition, interactions with the EC leading to migration enhanced the ability of T lymphocytes to further migrate and to respond to antigenic stimuli. These events appear to require $\alpha_L\beta_2$ integrin-derived signals.

The molecular mechanisms that lead to immobility following the delivery of proliferative stimuli are unclear, although they are likely to involve signaling events linking the TCR and the cytoskeleton. This possibility is currently under investigation. Previous studies have shown that TCR-derived signals can abolish T cell motility on planar lipid membranes (23). The shutdown of motility by signals leading to proliferation may well be necessary for the T cells sampling the environment for cognate Ag to focus on the APC and form stable synapses (9, 24). In support of this interpretation, it has been shown that naive T cells are sequestered in the peripheral lymphoid tissues after encounter with specific Ag, with an immediate detection of reduction in the output through the efferent lymph (3). Finally, this mechanism may contribute to the retention of Ag-specific memory T cells upon re-encounter with Ag in nonlymphoid organs (25).

Noncognate interactions with the EC induced a specific pattern of transcription that differs from that induced in T cells by proliferative stimuli. The up-regulation of AP-1 on EC-T cell interactions is not unexpected because this transcription factor is induced by numerous stimuli and plays an important role in activation of a wide variety of genes (26). The main extracellular stimuli inducing AP-1 are mediated by growth factors, but many others signals including those initiated by ligation of β_1 and β_2 integrins, have been implicated in activation of AP-1 expression (27–29). These studies used either immobilized recombinant molecules or fibronectin to stimulate integrins on the T cell surface. In addition, interactions with EC have been shown to enhance the mobilization of AP-1 in mitogen-activated T cells (30). In the present study, we provide the first evidence that exposure to EC can induce a specific pattern of transcription factor expression in transmigrated resting T cells, in the absence of either Ag-dependent or other mitogenic stimuli. AP-1 induction is observed

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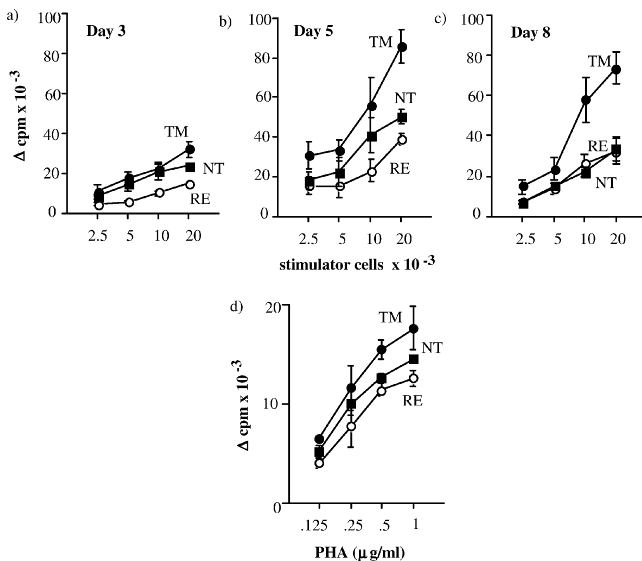

FIGURE 5. Proliferative responses are enhanced in recently migrated $CD4^+$ T cells. Purified $CD4^+$ $CD45RO^+$ T cells (4×10^6) were seeded on a cytokine-treated EC monolayer and left to migrate. As a control, T cells were incubated in culture medium. After16 h, transmigrated (TM, ●), nontransmigrated (NT, ■), and resting (RE, ○) T cells were collected, washed, and stimulated with either an allogeneic B-LCL (*a–c*) or PHA (*d*), in the presence of autologous irradiated PBMC (5×10^4). T cell proliferation to the B-LCL was assessed at days 3, 5, and 8, and response to PHA was measured after 48 h incubation. T cell division was measured as [^{3}H]TdR incorporation by liquid scintillation spectroscopy. The results are expressed as the mean of triplicate cultures, corrected for background proliferation of both T cells and stimulator cells alone (Δ cpm).

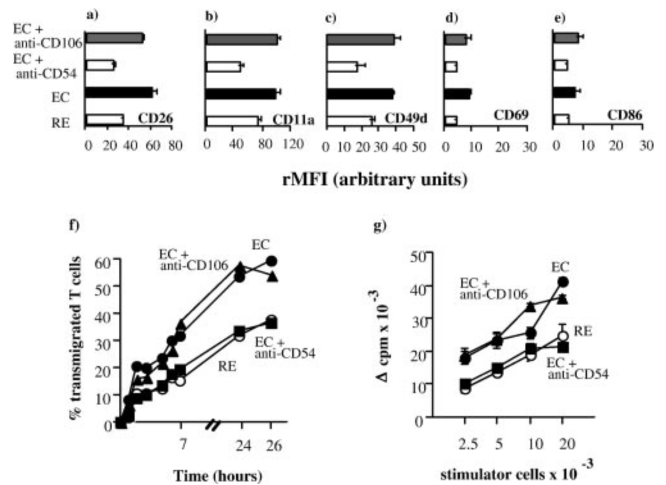

FIGURE 6. $\alpha_L\beta_2$ integrin-derived signals mediate phenotypic and functional changes induced in migrated T cells. TNF-α-treated EC monolayers were incubated either in medium alone or in the presence of anti-CD54 mAb (5 μg/ml) or anti-CD106 mAb (5 μg/ml) for 60 min at 37°C before use. The Transwells were then washed and seeded with purified $CD4^+$ $CD45RO^+$ T cells (2×10^6/well). After overnight incubation, transmigrated T cells were collected and analyzed. As a control, T cells that had not come into contact with EC were used (RE). In *a–e,* the effect of EC CD54 and CD106 blockade on the phenotypic changes induced by the interactions with the EC, including the up-regulation of CD26 (*a*), CD11a (*b*), CD49d (*c*), CD69 (*d*), and CD86 (*e*) molecules are shown. The columns represent the following experimental conditions: control resting T cells (RE); T cells transmigrated through TNF-α-activated EC monolayers either untreated (EC) or treated with anti-CD54 (EC+ anti-CD54), or anti CD106 (EC + CD106) mAbs. Data are expressed as relative mean fluorescence intensity (rMFI; MFI experimental to MFI control ratio). The mean + SE of three experiments are shown. *f,* T cells migrated through TNF-α-activated EC monolayers either untreated (EC, ●) or pretreated with anti-CD54 (■) or CD106 (▲) mAbs were collected and seeded onto fresh EC-coated Transwells. As a control, resting T cells that had not come into contact with EC (RE) were used. Transendothelial migration was monitored as described in *Materials and Methods*. Data are expressed as percentage of transmigrated cells at the specified time points. *g,* T cells migrated through TNF-α-activated EC monolayer either untreated (EC tm, ●) or pretreated with anti-CD54 (■) or CD106 (▲) mAbs were collected and stimulated with increasing numbers of an allogeneic B-LCL. As a control, resting T cells that had not come into contact with EC were used. Proliferation was measured and expressed as described above.

as early as 4 h after initial EC contact and is still found in cells analyzed 8–16 h after EC contact. Although we cannot rule out the possibility of an immediate and short-lived NF-κB response preceding our analysis, the sustained transcriptional activation does not appear to involve NF-κB because there is no detectable increase of this transcription factor 4 h after EC contact. The failure to detect prolonged NF-κB activation may suggest that in a more physiological context, excessive T cell activation is avoided, perhaps thus maintaining the ability of T cells to migrate.

Induction of specific transcriptional regulation in T cells after interactions with EC correlates with the up-regulation of a specific pattern of surface molecules including LFA-1 and VLA-4 integrins; CD26 molecules, which regulate T cell responses to chemotactic factors (31); the B7 family member CD86; and the activation marker CD69. Many of these phenotypic changes are shared with those induced by activation leading to proliferation. However, in contrast to proliferative stimuli, EC contact did not result in the up-regulation of CD25, CD80, and CD154 molecules. These phenotypic changes are unlikely to be merely due to selection of highly mobile T cell subsets (8), in that they occurred also in nonmigrated and cloned T lymphocytes and are likely to be induced by EC-derived signals, because they were abolished by ICAM-1 blockade. Our results confirm and extend other reports describing the induction of CD86 in $CD4^+$ T cells and monocytes (also at the transcriptional level and with similar kinetics) (11) and the up-regulation of the activation-induced molecule, CD69, in $CD8^+$ T cells (12) after interaction with EC.

The phenotypic changes induced in T cells by the interactions with EC cannot fully account for the dramatic and prolonged increase in motility, which occurred almost exclusively in migrated T cells. It is likely that also these events are influenced by EC-derived signals, as it is suggested by their abrogation by blockade of $\alpha_L\beta_2$ integrin-CD54 interactions, and that they may involve activation/induction/up-regulation of other motility-related molecules, such as chemokine receptors and intracellular regulators of cytoskeletal rearrangements. It is also possible that migrated T cells use a different pattern of molecules than those used in the initial migration, as has been observed in reversed transmigration of monocytes (32). A more systematic analysis is required to identify these factors and establish their relative contribution to the phenomenon observed. In a physiological context, the increased motility of migrated T cells may be important in subsequent tissue infiltration and localization after extravasation.

Our data confirm previous suggestions that exposure to EC during extravasation may amplify mitogenic stimuli favoring T lymphocyte activation during immune responses (30) as well as enhance T cell sensitivity to antigenic stimuli by interactions with the EC during extravasation (11, 12). It has been proposed that this enhancement is due to the induction of CD86 expression by T cells, which acquire the ability to trans-costimulate each other (11). In addition, migrated T cells displayed not only enhanced but also sustained proliferation, suggesting that they become less sensitive to homeostatic mechanisms containing T cell expansion. It is possible that the up-regulation of CD86 by the T cells, besides providing a costimulatory signal, might sequester CTLA-4 from being engaged at the sites of Ag recognition,

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thus preventing the decrease in proliferation. The increased responsiveness induced by the interaction with the EC may have relevance in vivo in the direct recruitment of resting memory T cells from the blood stream into the site of inflammation, given that the preactivation occurring during extravasation may favor the expansion of Ag-specific T cells once they have reached the inflammatory site.

Finally, all the phenotypic and functional changes induced in T cells by the contact with EC, including those exclusive of transmigrated T cells, appear to be mediate by $\alpha_L\beta_2$ integrin-derived signals. These findings confirm and extend those reported by other groups (12). The contribution of this interaction in Ag-dependent activation of T cells has long been known, and its central role in the immunological synapse has recently been recognized (24). However, the costimulatory nature of $\alpha_L\beta_2$ integrin-derived signals is still controversial (21, 33, 34). Our observation that these signals result in hyperresponsiveness and expression of activation-related surface molecules by T cells suggests that they may play a role in the amplification of TCR-derived stimuli by inducing a state of preactivation in resting T cells. In this context, it has recently been reported that triggering of β_7 and β_1 integrins results in increased T cell responsiveness to B7-mediated costimulatory signals (35).

Other groups have reported that EC amplify transcriptional events (30) and induction of activation markers such as CD154 (36) and IFN-γ secretion (37) in mitogen-stimulated $CD4^+$ T cells via the CD2:LFA-3 interaction, whereas $\alpha_L\beta_2$ integrin-derived signals do not appear to be functional in this effect. In addition, we have previously reported that this interaction is crucial in the activation of costimulation-independent T cells by Ag-presenting EC. These observations raise the interesting possibility that different interactions are functional in the EC-mediated amplification of T cell activation depending on the functional status of the T cells (i.e., resting vs recently activated T cells) and the type of interactions (i.e., cognate vs noncognate).

In conclusion, our observations suggest that the endothelium is not simply a passive filter barrier for infiltrating lymphocytes but also, by means of the signals that it delivers to extravasating T cells, it can actively influence the development of an inflammatory response.

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