Chemokine-independent Preference for T-helper-1 Cells in Transendothelial Migration*

We analyzed differences in the transendothelial migration (TEM) ability of T-helper (Th)-1 and Th2 cells across a murine endothelial cell line (F-2) under static conditions. The TEM abilities of Th1 cells from mice bearing autoimmune diseases and antigen-specific Th1 cell lines were severalfold higher than those of Th2 cells and lines of the same origin. These preferences were observed without exogenous chemoattractant and were insensitive to pertussis toxin, which completely blocks TEM induced by exogenous chemoattractants. Antibodies against LFA-1 and ICAM-1 as well as CD44 markedly blocked the TEM of Th1 cells. TEM ability was also blocked by pharmacological inhibitors of Src family protein-tyrosine kinases (PP2 and herbimycin A), phosphatidylinositol 3-kinase (wortmannin), and phosphatidylinositol-specific phospholipase C (U73122). Cross-linking of CD44 strongly induced highly elongated morphology in Th1 lines, but weakly in Th2 lines. The pharmacological inhibitors that blocked TEM also inhibited this morphological change, whereas pertussis toxin did not. These data indicate that there are signaling pathways for TEM independent of chemokine attraction, but through adhesion molecules including CD44, and that the preferential TEM ability of Th1 over Th2 cells is formed, at least in part, by intrinsic differences in these pathways.

T-helper (Th)1-1 cells are the key effector subset of CD4+ T lymphocytes; produce interferon-γ (IFN-γ); and mediate cytotoxic cellular immunity in situations such as parasitic infection, tumor eradication, allograft rejection, and autoimmune diseases. In contrast, Th2 cells produce interleukin (IL)-4 and regulate humoral immunity and allergic responses (1–5). As Th1 and Th2 cells inhibit the functions of each other, a balance of the two subsets influences disease pathology. Also, their differential localization may control the effectiveness of pathogen eradication. It has been shown that a preferential accumulation of Th1 cells occurs in lesions with few Th2 cells present (5–9). This accumulation may increase efficiency in eliminating allografts, pathogens, or pathogen-infected cells via a positive feedback loop. Once Th1-dominated infiltration occurs, the released cytokines and chemokines are thought to further selectively attract and support type 1 effectors, including Th1 cells, and the collected effectors may act more efficiently without inhibition by Th2 cells. However, if an immune response directed against self-components develops, a vicious circle develops, as accumulated effector cells destroy tissue, resulting in release of more autoantigen. Indeed, our previous studies indicated such a vicious circle in an experimental model for autoimmune gastritis (AIG) (6, 7), but it is not clear how it is triggered. We suggested that a preferential transendothelial migration of Th1 cells over Th2 cells in this model may be one of the mechanisms regulating the preferential accumulation of Th1 cells and type 1 effectors (6). Thus, understanding the detailed mechanisms of Th1 cell infiltration into tissues is an important issue for the immune regulation or therapeutic trials.

Migration of T cells into tissues occurs via a complex and sequential interaction between T cells and endothelial cells (10, 11). If T cells and the endothelium express pairs of tethering molecules such as L-, P-, and E-selectins and their sialylated carbohydrate ligands, T cells flowing through the blood stream become tethered to a vessel wall and can roll along the endothelium (10, 12). Subsequently, integrin activation and cell arrest in the endothelium are induced by chemokines, soluble or matrix-bound chemotactic factors produced by the surrounding tissue or endothelial cells and presented on the luminal surface of the vessel wall (10, 15). A large number of chemokines have been discovered in recent decades, and each chemokine acts upon a different set of immune cells, including Th1 and Th2 cells, according to their expression of specific receptors (9, 13, 14). Some chemokines such as RANTES (CCL5), MIP-1β (CCL4), and IP-10 (CXCL10) selectively attract Th1 cells, whereas MDC (CCL22) and TARC (CCL17) attract Th2 cells (14). Chemokines deliver downstream signals via heterotrigenic G-protein-coupled receptors and not only increase the affinity of integrins for their ligands, but also stimulate cell motility (13, 15). Several modes of integrin-mediated interaction between T cells and endothelial cells have been documented, e.g. between LFA-1 (αLβ2), VLA-4 (α4β1), and α4β7 on
T cells and their major receptors, ICAM-1, VCAM-1, and MadCAM-1, respectively, on endothelial cells (10, 11). Adhesion of CD44 to hyaluronan is also important for the extravasation of activated T cells into inflamed tissues (16, 17). Therefore, not only the combination of chemokine receptors, but also adhesion molecules expressed on immune cell subsets can determine their selective migration toward a particular site (10). Subsequent to arrest in the endothelium, T cells pass through the endothelial barrier and migrate into the tissue mesenchyme along a chemokine gradient. However, the details of the molecular mechanisms and signal mediators in this final “transendothelial migration” (TEM) step of each T cell subset are poorly understood.

The coordination of chemooattractant- or adhesion-induced signals, cell movement, and detachment from endothelial cells is thought to be crucial for lymphocyte transmigration across the endothelium. A number of studies have demonstrated that tyrosine phosphorylation of proteins and phosphatidylinositol metabolism play pivotal roles in cell adhesion as well as in cell motility (18–26). Chemokines are known to activate both pathways through G-protein-coupled receptors by heterotrimeric G-protein signaling (27–33). However, the roles of the enzymes mediating these pathways in the TEM of T cell subsets remain to be characterized.

Using CD4+ T cells from AIG mice containing substantial numbers of Th1 and Th2 cells, we have reported that IFN-γ (but not IL-4)-producing cells were markedly enriched after passing through the cell monolayer of the murine endothelial cell line F-2, without exogenous chemotactants (6). In this study, using this unique experimental model suitable for investigating the TEM of Th cells, we examined the chemokines, adhesion molecules, and signaling pathways involved. To understand the detailed mechanisms by analyzing the two subsets separately, we also used the Th1 and Th2 cell lines of a single known antigen specificity established from T cell receptor transgenic mice. We found that there is a Qα-independent signal for TEM mediated by adhesion through LFA-1 and CD44 probably connected to Src family protein-tyrosine kinases (Src-PTKs), phosphatidylinositol 3-kinase (PI3K), and phosphatidylinositol-specific phospholipase C (PI-PLC). There may be intrinsic differences in these pathways between the two subsets, resulting in the preferential TEM ability of Th1 cells over Th2 cells.

**EXPERIMENTAL PROCEDURES**

**Mice**—BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan). A transgenic mouse cell line carrying an ovalbumin (ovalbumin-m[232–239]-specific, I-Aβ-restricted T cell receptor gene on a BALB/c background (TG-d mice) (34) was provided by Dr. S. Habu (Tokai University School of Medicine, Kanagawa, Japan). Mice were maintained in the animal facility at the Center for Molecular Biology (Tokai University School of Medicine, Kanagawa, Japan). Mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and housed in the animal facility at the Center for Molecular Biology (Tokai University School of Medicine, Kanagawa, Japan). Mice were housed in the animal facility at the Center for Molecular Biology (Tokai University School of Medicine, Kanagawa, Japan).

**Production of IFN-γ and IL-4**—T cells from AIG mice containing substantial numbers of Th1 and Th2 cells were examined by an intracellular staining method using a Cytostain kit (Pharmingen). Cells were transiently stimulated with 50 ng/ml pertussis toxin (PTx; Sigma), 0.5 μg/ml anti-CD4 antibody (GK1.5) followed by biotin-labeled anti-rat IgG/alkaline phosphatase-conjugated avidin (both Immunotech, Marseilles, France), and phycoerythrin-labeled anti-IL-4 antibody (Pharmingen); VCAM-1 (MK2, Immunotech, Marseilles, France); and CD44 (KM201, IQ Products, Gottingen, Germany). Cells were then analyzed with a FACScalibur flow cytometer using Cell Quest software (BD Biosciences).

**Reverse Transcription-PCR**—Total RNA was extracted from cells using TRIzol reagent (Invitrogen). 2 μg of total RNA were reverse-transcribed with SuperScript II (Invitrogen) using oligo(dT)12–18 (Amershambiosciences, Tokyo) as a primer. Specific cDNAs were amplified by PCR with Taq polymerase (Takara Shuzou, Otsu, Japan) and primer sets, resulting in the preferential TEM ability of Th1 cells over Th2 cells.

**Flow Cytometry Analysis**—T cells or F-2 cells, a murine endothelial cell line (35, 36), were stained with the following rat monoclonal antibodies. For primary reagents, hybridoma supernatants against LFA-1α (BD4181), CD44 (KM201), ICAM-1 (YN1/7.4), and VCAM-1 (MR2) and purified antibodies against MadCAM-1 (Serotec, Oxford, England); P-selectin, E-selectin, and VE-cadherin (Pharmingen); and PE-CAM-1 (Caltag Laboratories, Burlingame, CA) were used. Biotin-labeled anti-CD16–17 IgG and alkaline phosphatase-labeled streptavidin (both from Caltag Laboratories) were used as secondary and tertiary reagents, respectively. For the staining performed using phycocerythrin-labeled anti-CD4 antibody (BD Biosciences) in combination with the antibodies described above. Cells were then analyzed with a FACScalibur flow cytometer using Cell Quest software (BD Biosciences).

**Transendothelial Migration Assay**—F-2 cells (2 × 10^5) were cultured on an FCS-precultured Transwell insert (6.5-mm diameter, 5-μm pore size, Corning Costar, Tokyo) with 10% FCS-containing Dulbecco’s modiﬁed Eagle’s medium for 38–40 h to make a confluent monolayer. Cells were removed by Lympholight-M from the cultured CD4+ T cells at 6 days after stimulation. Then, 5 × 10^6 cells in 100 μl of FCS/RPMI were applied to the F-2 cell monolayer, and the lower chamber was ﬁlled with 0.5 ml of FCS/RPMI. After the proper孵育期, the migrated cells were collected from the lower compartments and counted with a FACScalibur flow cytometer as the flow-through cell number over a constant time period (60 s). Most of the TEM assays were carried out over a 4-h incubation of cells on the F-2 cell monolayer because the rate of TEM was almost constant up to 5 h, but the stability of the fast growing F-2 cell monolayer was disturbed after a longer time period (see Fig. 2D). Purified rat monoclonal antibodies against adhesion molecules such as LFA-1α (M17/4), ICAM-1 (3E2), VLA-4α (R1-2), P-selectin (RB40.34), and E-selectin (10E9.6) (Pharmingen); VCAM-1 (MK2, Immunotech, Marseilles, France); and CD44 (KM201, IQ Products, Groningen, The Netherlands) were added to the upper chamber for the TEM assay. Normal rat IgG (Caltag Laboratories; 5 × 10^6) was used as a control, and all antibodies were used at 10 μg/ml. Alternatively, T cells were preincubated with 1 μg/ml pertussis toxin (PTx; Sigma), 0.5 μg/ml wortmannin (Nakalai Tesque), 30 μM PP2, 10 μM herbimycin A, 2 μM U73122, 20 μM D609 (Calbiochem, Darmstadt, Germany), or 0.5 μg/ml anti-CD4 antibody (GK1.5) followed by biotin-labeled anti-rat IgG/alkaline phosphatase-labeled streptavidin, cells were fixed and permeablized with Cytofix/Cytoperm solution (Pharmingen). Cells were then stained with fluorescein isothiocyanate-labeled anti-IFN-γ antibody (Caltag Laboratories) and phycocerythrin-labeled anti-IL-4 antibody (Pharmingen) and analyzed with a FACScalibur flow cytometer.

**Experimental Animals**—Preparation of SuperScript II (Invitrogen) using oligo(dT)12–18 (Amershambiosciences, Tokyo) as a primer. Specific cDNAs were amplified by PCR with Taq polymerase (Takara Shuzou, Otsu, Japan) and primer sets, resulting in the preferential TEM ability of Th1 cells over Th2 cells.

**Preparation of F-2 Cells**—Grown F-2 cell monolayer was disturbed after a longer time period because the rate of TEM was almost constant up to 5 h, but the stability of the fast growing F-2 cell monolayer was disturbed after a longer time period. Most of the TEM assays were carried out over a 4-h incubation of cells on the F-2 cell monolayer because the rate of TEM was almost constant up to 5 h, but the stability of the fast growing F-2 cell monolayer was disturbed after a longer time period.

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AGAAGGACTCTATATGGG-3’ and 5’-TCTTTTATGTCGCTGACG-CAGC-3’.

Cross-linking Assay—After labeling of T cells with the fluorescent dye CMFDA (20 μM; Molecular Probes, Inc., Eugene, OR) at 37 °C for 20 min, cells were washed and stained with antibodies against cell-surface molecules such as LFA-1, CD44, VLA-4, CD4, or control rat IgG for 20 min at 4 °C. Cells were then placed onto a culture plate precoated with 100 μg/ml goat anti-rat IgG and incubated for 30 min at 37 °C. Cells were fixed with paraformaldehyde and examined under a Nikon TND380 fluorescence microscope. Digital images obtained with a 3CCD camera (C5810, Hamamatsu Photonics, Hamamatsu, Japan) were prepared using Adobe Photoshop software. Higher magnification views stained with anti-CD44 antibody and Alexa Fluor 546-phalloidin (Molecular Probes, Inc.) were obtained with a confocal laser scanning microscope (MRC-1024, Bio-Rad, Osaka, Japan). Cells with roughly >2.5 of ellipticity (the ratio between longer and shorter axes) were considered to bear the “elongated morphology.” To measure the effect of inhibitors, cells were treated for 30 min at 37 °C before antibody staining.

Adhesion Assay—CMFDA-labeled, inhibitor-treated or -untreated T cells (5 × 10⁵) were placed onto F-2 cell monolayers on 24-well culture plates and incubated for 1 h at 37 °C. After removing unbound cells and washing once with phosphate-buffered saline, bound cells and F-2 cells were trypsinized to a single cell suspension and analyzed with a FACScalibur flow cytometer over a constant time period (60 s) to count labeled T cells. Digital images were obtained with a fluorescence microscope and a 3CCD camera after washing unbound cells and fixation and were prepared using Adobe Photoshop software.

RESULTS

Th1 Cells Preferentially Transmigrate across the Monolayer of Endothelial Cells Compared with Th2 Cells—To test whether there are any differences between Th1 and Th2 cells in their ability to transmigrate across endothelial cells, we performed the TEM assay under static conditions using a monolayer of F-2 cells as the separator. Th1 and Th2 cells were identified as IFN-γ and IL-4 producers, respectively, as determined by intracellular staining. As reported previously, neonatal thymectomized BALB/c mice develop AIG at a high frequency, and the spleen cells of the disease-bearing mice (AIG mice) contain a substantial number of both Th1 and Th2 cells (6). The splenocytes of AIG mice were loaded onto the F-2 cell monolayer in a Transwell chamber without exogenous chemoattractant. 4 h later, transmigrated cells in the lower compartment were examined regarding surface CD4 and intracellular cytokine staining to assess the Th1/2 ratio. Compared with the input cells, the percentage of IFN-γ- or IL-4+ cells in the CD4+ fraction was elevated in transmigrated cells, whereas IL-4+ cells were decreased (Fig. 1A); therefore, the Th1/2 ratio was enhanced 3.5-fold after TEM. Similar results were obtained using memory CD4+ T cells from Tg-d mice activated in vitro (B), and a Th1/2-mixed line established from Tg-d mice (C). Intracellular IFN-γ and IL-4 were stained and examined by FACS analysis. CD4+ gated cells (A) or all cells (B and C) are shown in dot plots, and Th1 or Th2 cells are represented by percentages in the upper left or lower right gates, respectively. Fold increments in Th1/2 ratios are shown to the right of each FACS profile. Data shown are representative of two or three independent experiments.

To investigate the TEM ability of the subsets independently, we established multiple lines of each type of cell from Tg-d mice by culturing and maintaining them in vitro for a long time period under biased conditions for Th1 or Th2 cells. The cell lines might be heterogeneous because we did not clone them. However, each cell line consisted almost entirely of Th1 or Th2 cells as assessed by staining of IFN-γ and IL-4 (Fig. 2A). When a mixture of the Th1 and Th2 cell lines was subjected to the TEM assay, Th1 cells transmigrated preferentially compared with Th2 cells, in agreement with the findings above (Fig. 2B). In separate experiments with each cell line, the number of cells that transmigrated into the lower chamber revealed that Th1 cells have 3–10-fold greater ability for transmigration than Th2 cells (Fig. 2C). Because F-2 cells fixed with paraformaldehyde, which showed no apparent change in morphology, did not pass any T cells, the TEM of T cells in our system is not due to leakage through an incompletely formed monolayer, but is an active process depending on the live cell monolayer. Time courses of TEM in each cell type were nearly linear for as long as 8 h, with severalfold higher counts of Th1 cells over Th2 cells (Fig. 2D), indicating that the Th1 cell line has a higher TEM ability per any time period than the Th2 cell line. We occasionally found a decrease in TEM ability in Th1 cell lines when maintained without IL-12 (data not shown). The above results show that Th1 cells not only from autoimmune disease-bearing mice but also in general have a potentially higher ability for TEM than Th2 cells, and our experimental system is useful to study the behavior of Th cells during TEM.

The Chemokine or Gαi Signaling Pathway Only Partially Contributes to the Th1 Cell Preference in TEM—As no exogenous chemoattractant was added to our system, there are two major possibilities to account for the preference for Th1 cells over Th2 cells in TEM ability. First, the separator endothelial cells (F-2) themselves may produce the chemokines that selectively attract Th1 cells, and the preference resides in the exogenous environment. Second, there may be some intrinsic differences other than chemokine signaling for transmigration per se in Th cells. We first checked the expression of several chemokines possessing the differential effect in the two subsets by reverse transcription-PCR. mRNAs for typical Th1 attractants, including RANTES, MIP-1β, and IP-10, were detectable in F-2 cells, whereas mRNAs for Th2 attractants such as MDC and TARC
were barely detectable (Fig. 3A). We also detected CCR5 (a receptor for RANTES and MIP-1α) as well as CXCR3 (a receptor for IP-10) and a low level of CCR4 (a receptor for MDC and TARC) in the Th1 cell lines (Fig. 3B). In contrast, the Th2 cell lines showed an opposite profile: a strong band for CCR4, but little or undetectable levels of CCR5 and CXCR3. These findings support the first possibility that the chemokines produced by F-2 cells as well as the receptor profiles of T cells may determine the TEM selectivity of our system. If the majority of the signals that drive the TEM of T cells are from chemokines, the blockage of G protein-coupled signals of chemokine receptors by pretreating the T cells with PTx should abolish the TEM ability and Th1 cell preference in our system. Surprisingly, however, PTx-treated Th1 cells still had a significant TEM ability (30–80% of the ability of untreated Th1 cells, depending on the experiments and cell lines), and clear preferences over PTx-treated Th2 cells were retained (Fig. 3C). In contrast, PTx completely abolished the increased selective TEM activity induced by exogenous recombinant RANTES and MDC, which are selective for Th1 and Th2 cells, respectively (Fig. 3D), indicating that the signaling through Gαi-coupled chemokine receptors was completely blocked. These results of TEM induction by the exogenous chemoattractants and blockage by PTx confirmed that the cell lines used in this study retained the responsiveness to appropriate chemokine stimuli and that the signals from such stimuli were mediated in a Gαi-coupled process. More importantly, these results strongly support the second possibility that there are differences between Th1 and Th2 cells in pathways driving TEM other than those regulated by chemokines.

**Chemokine-independent TEM Preference for Th1 cells from AIG Mice**—To confirm that the preference for Th1 cells in the chemokine-independent TEM process is not specific to the cell lines established in vitro, but is general to cells activated in vivo, we tested the PTx sensitivity of splenic CD4+ T cells from AIG mice in our TEM assay system. Th1 cell dominance after transmigration was not abolished at all, but was instead enhanced by PTx treatment compared with untreated controls; and as expected, the number of transmigrated cells was partially reduced (Fig. 4, A and B). These results indicated that almost half of migrating Th cells from AIG mice that had been activated in vivo were driven by chemokine-independent signaling pathways.

To identify the signaling pathways that drive TEM and that contribute to the Th1 cell preference, we tested whether pharmacological inhibitors that block intracellular signaling and antibodies against cell adhesion molecules have any effects on the TEM of T cells from AIG mice. Among them, anti-CD44 antibody inhibited the TEM of these cells more effectively than PTx. Inhibitors of the phosphatidylinositol signaling pathway (wortmannin and U73122) markedly inhibited the migration of AIG CD4+ T cells (Fig. 4C). Therefore, signals through these molecules and pathways are good candidates for contributing to the Th1 cell preference in TEM, but it is difficult to assess...
the effects of these treatments separately on each cell type using the mixture of cells activated in vivo.

LFA-1 and CD44 Mediate the TEM of Th1 Cells—Using the Th cell lines, we next explored what kinds of adhesion molecules are involved. F-2 cells showed a typical endothelial character, expressing PECAM-1 and VE-cadherin and, in addition, expressing the adhesion receptors for lymphocytes such as ICAM-1, VCAM-1, CD44, P-selectin, and E-selectin, and were negative for MAdCAM-1 (Fig. 5A). A panel of antibodies were tested for inhibition of TEM. Among them, anti-LFA-1/ICAM-1 and anti-CD44 antibodies marked inhibition the TEM of Th1 cells (Fig. 5C). We also found a marked reduction of TEM by anti-CD44 antibody (Fig. 5D). Antibodies against VLA-4α, VCAM-1, P-selectin, and E-selectin had no inhibitory effect (Fig. 5E). As both Th1 and Th2 cell lines expressed comparable levels of LFA-1 and CD44, respectively (Fig. 5F), the preferential TEM ability found for Th1 cells is not explained simply by the level of expression of these molecules.

Single blockade by LFA-1/ICAM-1 or CD44 seemed to be more efficient than PTx treatment. In addition, treatment with a combination of anti-LFA-1/ICAM-1 antibody, anti-CD44 antibody, and PTx resulted in almost complete inhibition of the TEM of Th1 cells below the basal level of Th2 cells (Fig. 5F).

Src-PTKs, PI3K, and PI-PLC Mediate the TEM of Th1 Cells—To evaluate the contribution of Src-PTKs, PI3K, or
It is possible that adhesion itself can transduce the chemokine-independent motility signals to Th1 cells. We therefore examined the cross-linking of cell-surface receptors using a specific antibody and a secondary antibody-coated plate, which can mimic adhesion-induced molecular assembly (Fig. 7A). We tested several molecules, including LFA-1, CD44, VLA-4, and CD4. Among these, the cross-linking of CD44 induced a highly elongated morphology or ~30–40% of the Th1 1L2 cells, but had no effect on the Th2 2L1 cells (Fig. 7, B–D). The elongated cells showed a clear polarity with morphologically distinct cell portions such as an antibody-sticking rear section, a long rod-like stem region, and a leading edge of the cell body containing the nucleus (Fig. 7E). Thus, it was thought to be an enhanced shape of the migrating T cells that also have polarity. Only the combination of anti-CD44 antibody and an anti-rat IgG antibody-coated plate induced this morphological change, whereas the soluble secondary antibody did not (data not shown), indicating that the attachment of cells to the plate through concentrated CD44 molecules on the cell surface forced the change in cell shape. This change might have occurred because it was the signal moving through the cell and not signaling in Th1 cells, but poor elongation for Th2 cells. Although the extent of the shape change of Th1 cells induced by CD44 cross-linking varied among the Th1 cell lines we tested (for instance, 1L1 showed a weaker ability for elongation), all Th2 cell lines were less inducible regarding shape change compared with Th1 cell lines. Elongation was blocked by pretreatment of T cells with PP2, herbimycin A, wortmannin, and U73122, whereas PTx showed no effect (Fig. 8, A and B). The cross-linking of T cells in the presence of CytD abolished cell elongation, although pretreatment did not block it, indicating that actin remodeling is required during the elongation (Fig. 8D). These findings suggest that a proportion of Th1 cells respond to CD44-induced motility signals and that this adhesion-driven motility seems to be higher in Th1 cells than in Th2 cells.

**Th1 Cells Preferentially Adhere to Endothelial Cells with Polarized Morphology**—Th1 cells nearly 60% of the Th1 1L2 cells had a long rod-like stem region, and a leading edge of the cell body containing the nucleus (Fig. 7E). This suggests a differential role of Src-PTKs and PI3K in adhesion and migration steps, especially that PI3K might play a major role in cell motility driving TEM in our system.

**DISCUSSION**

It is well known that type 1 reactions dominate over type 2 reactions in lesions of chronic inflammation including organ-specific autoimmune diseases (5–9). We also confirmed such inflammatory status in AIG, a useful experimental model of organ-specific autoimmunity (6, 7). This suggested that there is a vicious circle formed at the autoimmune lesion; the microenvironment formed by the infiltrated type 1 effector cells including Th1 cells further enabled the same effectors to infiltrate into the lesion.

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**Fig. 5.** LFA-1/ICAM-1 and CD44 are involved in the TEM of Th1 cells. A and B, surface expression of adhesion molecules on F-2 cells (A) and Th1 (1L2) and Th2 (2L1) cells (B). Cells were stained with various antibodies and examined by FACS. C–E, inhibitory effects of anti-LFA-1a and anti-ICAM-1 antibodies (C) and anti-CD44 antibody (Ab) (D) and the absence of effect of other antibodies (E) on the TEM of Th1 (1L2) and Th2 (2L1) cells. F, synergistic activity of antibodies and PTx effects on the TEM of the Th1 cell line (1L2). The basal TEM ability of the Th2 cell line (2L1) is also shown. P-selectin and E-selectin, P-selectin and E-selectin, respectively.

**CD44 Cross-linking Induces a Morphological Change in Th1 Cells**—It is possible that adhesion itself can transduce the chemokine-independent motility signals to Th1 cells. We therefore examined the cross-linking of cell-surface receptors using a specific antibody and a secondary antibody-coated plate, which can mimic adhesion-induced molecular assembly (Fig. 7A). We tested several molecules, including LFA-1, CD44, VLA-4, and CD4. Among these, the cross-linking of CD44 induced a highly elongated morphology or ~30–40% of the Th1 1L2 cells, but had no effect on the Th2 2L1 cells (Fig. 7, B–D). The elongated cells showed a clear polarity with morphologically distinct cell portions such as an antibody-sticking rear section, a long rod-like stem region, and a leading edge of the cell body containing the nucleus (Fig. 7E). Thus, it was thought to be an enhanced shape of the migrating T cells that also have polarity. Only the combination of anti-CD44 antibody and an anti-rat IgG antibody-coated plate induced this morphological change, whereas the soluble secondary antibody did not (data not shown), indicating that the attachment of cells to the plate through concentrated CD44 molecules on the cell surface forced the change in cell shape. This change might have occurred because one end of the cell started moving by the signal through CD44, but the other end of the cell where CD44 molecules were cross-linked was fixed on the plate. The cross-linking of LFA-1 and CD4 (but not VLA-4) also induced a small but significant elongation of Th1 cells, but poor elongation for Th2 cells. Although the extent of the shape change of Th1 cells induced by CD44 cross-linking varied among the Th1 cell lines we tested (for instance, 1L1 showed a weaker ability for elongation), all Th2 cell lines were less inducible regarding shape change compared with Th1 cell lines. Elongation was blocked by pretreatment of T cells with PP2, herbimycin A, wortmannin, and U73122, whereas PTx showed no effect (Fig. 8, A and B). The cross-linking of T cells in the presence of CytD abolished cell elongation, although pretreatment did not block it, indicating that actin remodeling is required during the elongation (Fig. 8D). These findings suggest that a proportion of Th1 cells respond to CD44-induced motility signals and that this adhesion-driven motility seems to be higher in Th1 cells than in Th2 cells.

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In this study, we characterized the preferential TEM of Th1 cells observed in our in vitro system using a murine endothelial cell line and several types of Th cells. F-2 cells, the endothelial cell line used here, show characteristics typical of endothelial cells (35, 36). However, these cells are thought to be in some activated state, as they constitutively express P-selectin and E-selectin on their surface as well as chemokines such as RANTES and IP-10, all of which are normally induced by inflammatory stimuli (12, 38, 39). Therefore, F-2 cells could serve as a model for inflammation-activated endothelial cells. The Th cells used in this study were 1) directly taken from mice with AIG or 2) independently established cell lines from a T cell receptor transgenic mouse and maintained in vitro as a heterogeneous population without cloning. With both cell types used, we observed a preference in TEM for Th1 cells over Th2 cells, suggesting that such a preference is not a specific character of autoimmune-derived Th cells, but is a common property of Th cells in some activation/differentiation stages. The Th cells differentiated from naive CD4+ T cells in a primary culture peeled off the monolayer of F-2 cells from polycarbonate membrane; and thus, we could not assess whether there were any differences in TEM ability between the two subsets in this case. In contrast, we consistently observed the Th1-dominated TEM of cytokine-producing, memory CD4+ T cells directly taken from mice in the previous study (6) and this study. Therefore, the Th cell lines showing a similar preference in TEM are thought to be of more fixed phenotypes that resemble the memory cells in vivo rather than transiently induced Th cell subsets. As we employed a static condition without shear flow, the phenomenon that we observed could simply mimic the passing process across the endothelial wall, without the rolling or flow-induced mechanical effect. Therefore, despite several differences from physiological conditions, our system is thought to represent some aspect of “trans-motility” of Th cell subsets.

One possible mechanism for the differential TEM ability of Th1 and Th2 cells in our system is attraction via chemokines produced by F-2 cells. Among a large number of chemokines, several kinds have been shown to affect each subset differently; therefore, it is widely believed that differential expression of those chemokines at lesions is involved in many types of Th1- or Th2-biased pathologies (9, 14). The Th1-dominated TEM without exogenous attractant in this study could also be explained by the action of those Th1 tropic chemokines derived from F-2 cells. Indeed, we could detect mRNAs for typical Th1 tropic chemokines such as RANTES, MIP-1β, and IP-10, but not Th2 tropic chemokines such as MDC and TARC in F-2 cells. Furthermore, each Th cell line appeared to express appropriate receptors. Addition of exogenous chemokines such as RANTES and MDC to the lower compartment augmented the TEM of Th1 cells in a subset-selective, Gαi-dependent manner; there is little doubt that chemokines exert powerful selectivity upon Th cell subsets during extravasation, and our system can reproduce such selectivity. In contrast to previous belief, PTx treatment of input cells, which completely blocked Gαi-coupled chemokine receptors, only partly inhibited the TEM of Th1 cells and did not abolish Th1 cell preference in the TEM of in vivo cells or of lines established in vitro. Therefore, the selectivity is only partially due to the F-2 cell-derived chemokines preferentially affecting Th1 cells. These results also indicate that there are signaling pathways for TEM mediated by chemokine-independent mechanisms and strongly suggest some intrinsic differences in these pathways between Th1 and Th2 cells.

We demonstrated that both LFA-1- and CD44-dependent adhesion is involved in the TEM of Th1 cells. LFA-1 binds to ICAM-1 (10), whereas CD44 binds to many kinds of extracellular matrix, including hyaluronan (16). Both adhesion pathways play important roles in T cell activation and function (16, 40, 41). The affinity for the ligands of each molecule is regulated by the activation states of the cells in an “inside-out” manner (15, 16, 42). Especially, chemokine signaling induces a rapid conformational change in LFA-1 to convert its low affinity state into a high one (10, 15). We actually showed that PTx treatment partially reduced Th1 cell adhesion to F-2 cells as well as TEM. However, because blocking LFA-1 or CD44 function inhibited Th1 cell TEM more effectively than PTx treatment, Gαi-independent pathways using both molecules are thought to be more important for interaction be-

**Fig. 6.** Src-PTKs, PI3K, PI-PLC, and actin remodeling are involved in the TEM of Th1 cells. A and B, effect of inhibitors of Src-PTKs (A) and phosphoinositide metabolism (B) on the TEM of Th1 (1L2) and Th2 (2L1) cell lines. C, effect of CytD treatment on the TEM of the Th1 cell line (1L2). T cells (T), F-2 cells (E), or both (T,E) were pretreated with CytD for 4 h followed by the TEM assay, or the TEM assay was performed in medium containing CytD. Wort., wortmannin.
between Th1 and endothelial cells than a chemokine-induced event. Both LFA-1 and CD44 can transmit adhesion-induced signals to cells via an "outside-in" manner (41, 43–50). We thought that the GaR-independent TEM of Th1 cells might be due to the adhesion-induced activation of Th1 cells after interaction with endothelial cells and that differences in this process could contribute to the Th1 cell preference in TEM. Indeed, the cross-linking of CD44 and, to a lesser extent, LFA-1 induced the elongated morphology of Th1 cells. Moreover, this induction of cell shape change was blocked by several signaling inhibitors that also inhibit TEM, but not by P Tx, indicating the link between cell adhesion signals and TEM in a chemokine signal-independent manner. CD44 transduces motility signals in fibroblasts or tumor cells (45,
In lymphocytes, CD44 associates with a Src-PTK (Lck) and transduces signals by tyrosine phosphorylation (47–49). Our findings strongly suggest that CD44 can transmit a migration signal to Th1 cells through Src-PTKs, although we still do not know the fine molecular mechanisms that activate T cells by the signals through CD44 during the interaction with the endothelium.

We also showed a higher binding activity of Th1 cells compared with Th2 cells for endothelial cells. LFA-1/ICAM-1-dependent homotypic aggregation in Th1 cells was reported to be much stronger than that in Th2 cells (4). Furthermore, osteopontin, a ligand of CD44, is reported to be necessary for type 1 immunity (51). Thus, the affinity for ligands or the activity for signal transduction of LFA-1 and/or CD44 might be intrinsically augmented more preferentially in Th1 cells than in Th2 cells.

PI3K is well known to play a pivotal role in cell motility, not only in hematopoietic cells, but also in many other lineages (13, 15, 20, 21). Activated PI3K produces phosphatidylinositol 3,4,5-trisphosphate from phosphatidylinositol 4,5-bisphosphate, which further activates the guanine nucleotide exchange factor for the Rho family of small G-proteins containing pleckstrin homology domains, which bind to phosphatidylinositol 3,4,5-trisphosphate and induce actin remodeling (20). Moreover, PI3K activates \( \gamma \)-subtypes of PLC that have pleckstrin homology domains (52). PLCs are also involved in a broad spectrum of cellular phenomena, including cell motility, by producing diacylglycerol and inositol 1,4,5-triphosphate from phosphatidylinositol 4,5-bisphosphate (23, 24, 28, 29). It is of importance that both PI3K and PLC are probably involved in the signaling for TEM of Th1 cells. PI3K may be coupled with PLC for this signaling because selective inhibitors of PI3K and PLC effectively blocked TEM in our model. Both enzymes can be activated by the \( \beta \gamma \)-subunit of heterotrimeric G-protein released from the chemokine receptor or tyrosine phosphorylation through Src-PTKs (18, 19, 27–33). In addition, PI3K or

![Fig. 8. Src-PTKs, PI3K, PI-PLC, and actin remodeling (but not chemokine signals) are involved in the elongation of Th1 cells upon CD44 cross-linking. A and B, wortmannin (Wort.) and U73122 (but not PTx) inhibited the elongated morphology of Th1 cells upon CD44 cross-linking (X-L). Th1 (1L2) and Th2 (2L1) cells were CMFDA-labeled and treated with inhibitors followed by CD44 cross-linking. Control experiments in which cells were stained with anti-CD44 antibody with (X-L (+)) or without (X-L (-)) a secondary antibody-coated plate are shown. C, shown are the inhibitory effects of Src-PTK inhibitors on the elongated morphology of Th1 cells. D, CytD treatment during CD44 cross-linking (CytD in.), but not CytD pretreatment (CytD pre.), inhibited the elongated morphology of Th1 cells. The control was CD44 cross-linking without drug treatment (C and D). HerA, herbimycin A.](http://www.jbc.org/doi/appendices/)
PLC is involved in several LFA-1- and CD44-mediated cellular events (43–45, 49, 50). Therefore, not only Gαi-dependent chemokine signals, but also LFA-1- and/or CD44-mediated adhesion stimuli may activate the cascade of Src-PTKs, PI3K, and PLC in Th1 cells.

From our recent findings, we would like to propose a model of the molecular signaling pathways for Th1 cell TEM, as shown in Fig. 9E, which were poorly understood so far. Th cells interacting with endothelial cells are activated by the chemokines presented on the luminal surface of the vessel as well as by the Gαi-independent adhesion-induced signals via LFA-1 and/or CD44. Signals are then integrated into actin rearrangement and cell movement for transmigration through Src-PTKs and phosphatidylinositol 4,5-bisphosphate-based phosphoinositide metabolism. It is strongly suggested that there are some differences in the adhesion and migration signaling between Th1 and Th2 cells through the above pathways, and detailed analysis is now ongoing. Similar intrinsic differences in the intracellular signaling between two subsets have also pointed out by several groups (53–56).

It has been thought that cell rolling using P-selectin ligands and firm adhesion induced by Th1 tropic chemokines and their appropriate receptors mainly contribute to the selective accumulation of Th1 cells in chronic inflamed tissue (8, 9, 14). It is clear that the selectin system and chemokine stimulus work as key players in the initial T cell/endothelial cell interaction under shear flow (12, 15, 57–59) and that they may be sources of Th cell subset selectivity. However, in this study, we found another possible mechanism: higher TEM or tissue invasive activity of Th1 cells per se in the later steps of extravasation compared with that of Th2 cells. Thus, the differences at several steps of extravasation between the two subsets may bring further disparity to their final tissue distribution. It is probable that differentiated Th1 pools in the periphery contain cells with higher basal mobility than Th2 cells resulting from intrinsic differences in the Gαi-independent signaling pathways. This difference gives us new insight into the establishment of Th1 cell dominance in inflamed lesions especially at early stages of lymphocytic infiltration, when the concentration of specific chemokines around the target tissue is still low. The chemokine-independent, high extravasation, and tissue-invasive potential of Th1 cells possibly can trigger a type 1 inflammatory loop, i.e. IFN-γ from Th1 cells induces Th1 tropic chemokine production from surrounding cells and further attracts Th1 cells. Therefore, Th1-primed immune responses tend to create a vicious circle of selective accumulation of type 1 effector cells at the lesion and following excessive type 1 immunity with tissue injury, especially in autoimmune diseases.

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