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In Vitro Differentiation from Naive to Mature E-Selectin Binding CD4 T Cells: Acquisition of Skin-Homing Properties Occurs Independently of Cutaneous Lymphocyte Antigen Expression

Ryo Takahashi, Yoshiko Mizukawa, Yoshimi Yamazaki, Kazuhito Hayakawa, Jun Hayakawa, Akihiko Kudo, and Tetsuo Shiohara

We previously showed that skin-homing CD4 T cells in peripheral blood can be subdivided into three populations on the basis of the expression pattern of the cutaneous lymphocyte Ag (CLA) and fucosyltransferase VII (FucT-VII): FucT-VII+/CLA−, FucT-VII+/CLA+, and FucT-VII−/CLA−. In view of the known late appearance of CLA during T cell differentiation, T cells programmed to attain skin-homing properties may start to generate E-selectin-binding epitopes at early stages of differentiation before induction of CLA expression. To this end, the in vitro differentiation from naive to CLA+ memory T cells was followed after activation with anti-CD3 mAb. Here we demonstrate that naive skin-homing CD4 T cell precursors undergo a linear differentiation process from the FucT-VII+/CLA− phenotype to the FucT-VII+/CLA+ phenotype and eventually to the FucT-VII−/CLA+ phenotype. The appearance of the FucT-VII+/CLA+ subset coincided with or could be immediately followed by the generation of E-selectin binding epitopes, and even after E-selectin-binding epitopes were no longer detectable, CLA remained expressed for prolonged periods of time, suggesting that induction of functional E-selectin ligands depends primarily on the expression of FucT-VII, but not CLA. Immunofluorescence and confocal microscopy studies of these T cells confirm that most E-selectin ligands were found independently of CLA expression. The Journal of Immunology, 2003, 171: 5769–5777.

T cells display tissue-restricted migration to particular sites, such as skin, where they are most likely to exert their effector function: this process is called homing. The skin homing is controlled by multistep molecular interactions between circulating T cells and endothelium, mediated by a unique combination of cell adhesion molecules and chemokines. The central importance of adhesive interactions between E-selectin and its ligand expressed on inflamed superficial dermal postcapillary venule and T cells, respectively, in the first step of skin homing has been well documented (1–9). The prevailing dogma depicts cutaneous lymphocyte Ag (CLA)3 on T cells as a ligand for E-selectin and thus causally links CLA expression with skin-homing potential (5–8); CLA+ skin-homing T cells are generally thought to represent a subset that constantly patrols through the skin. Recent evidence, however, argues that this view may be oversimplified; it has become clear that the CLA epitope is a post-translational modification of the protein core of P-selectin glycoprotein-1 by the activity of α(1,3)-fucosyltransferase VII (FucT-VII), but does not necessarily represent the epitope required for binding to E-selectin (3, 10–12). The importance of FucT-VII in construction of ligands for E- and P-selectin on leukocytes in vivo has also been confirmed through the analysis of targeted disruption of the murine FucT-VII gene (13). The generation of E-selectin ligands on T cells undergoing naive-to-memory transition in skin-draining lymph nodes requires FucT-VII activity (10, 13, 14). These studies led us to consider the possibility that the generation of CLA epitope on T cells merely reflects previous or ongoing activation of FucT-VII. In view of the requirement for FucT-VII in the synthesis and regulation of E-selectin ligands on T cells, it is likely that the timing and levels of FucT-VII expression, rather than simply the expression of CLA, are critical determinants of the ability to interact with vascular selectins and subsequently migrate to the skin sites of inflammation. However, research on FucT-VII expression in T cell populations was hampered by the lack of mAb by which expression at the protein level can be detected. In this regard, we have recently established a reliable immunohistochemical assay that allows the identification of FucT-VII expression in cultured (15) or freshly prepared (16) T cells.

The skin homing is supposed to be imprinted on T cells during activation and eventual differentiation into effector and memory T cells under the influence of particular cytokines (12, 17). Each of the distinct phases involving activation, proliferation, and differentiation of these T cells is assumed to occur sequentially and/or simultaneously. It remains unknown, however, where, when, and how the commitment of T cells to the skin-homing phenotype is determined; we cannot be sure whether the induction of CLA expression on these T cells occurred in temporal correlation with the acquisition of skin-homing properties or whether the induction of CLA appeared afterward. In this regard, recent kinetic studies have demonstrated that optimal CLA induction on T cells is a relatively slow process even in serum-free medium highly permissive for...
CLA expression (18, 19), and we have also seen a similar requirement for 5 days or more to generate CLA-positive T cells from naive T cells in vitro (20, 21). Nevertheless, the acquisition of skin-homing properties has been thought to occur rapidly upon activation. This raises the possibility that skin-homing T cell precursors may begin to express E-selectin ligands at a relatively early stage of differentiation before the induction of CLA and thereby acquire skin-specific tropism.

In our previous study of peripheral blood CD4+ and CD8+ T cells, we noted that skin-homing T cells can be subdivided into three populations on the basis of the expression pattern of CLA and FucT-VII. FucT-VII-CLA- and FucT-VII-CLA+ cells, we noted that skin-homing T cells can be subdivided into three populations on the basis of the expression pattern of CLA and FucT-VII. FucT-VII-CLA-, FucT-VII-CLA+, and FucT-VII-CLA+ (15, 16). In view of the known late appearance of CLA induction on memory T cells, this heterogeneity of the expression pattern in peripheral blood T cells appeared to imply that CLA, FucT-VII, and E-selectin ligand expression may be induced on the same T cell, but with different kinetics. To address the issue of whether the phenotypic changes could be associated with the acquisition of skin-homing properties, purified naive CD4+ T cells were stimulated with anti-CD3 mAb to sequentially analyze on the basis of the expression of CLA and FucT-VII, which included a morphological assessment of E-selectin ligand (ESL) expression.

In this study we have established the method to directly visualize the binding sites to E-selectin. Here we provide evidence to indicate that FucT-VII-CLA- T cells are at an early stage of differentiation. The appearance of this subset temporarily coincided with or immediately followed the generation of E-selectin binding epitope, reflecting the commitment to skin-homing T cells. Surprisingly, CLA expression was not detected in this subset. Our results indicated that FucT-VII-CLA- and FucT-VII-CLA+ subsets represent preterminally differentiated cells and terminally differentiated cells, respectively; the latter could lose the ability to localize within the skin. Immunofluorescence and confocal microscopy verified the difference in expression and distribution patterns of E-selectin ligand and CLA during T cell differentiation.

Materials and Methods

Human subjects

Peripheral blood (PB) was obtained from healthy adult volunteers with their informed consent.

Antibodies

For cell culture, anti-CD3ε mAb was purchased from R&D Systems (Minneapolis, MN). For FACS analysis, cell sorting, immunofluorescence, and confocal microscopic analysis, anti-CD4 mAb (allophycocyanin-labeled) was purchased from BD PharMingen (San Diego, CA), goat F(ab’2) anti-human IgG Fc mAb (PE-labeled) was purchased from Immunotech (Mariselles, France), and anti-CLA mAb (HECA-452, rat IgM, FITC-labeled) was a gift from L. Picker (5). For immunohistochemical staining, biotinylated horse anti-mouse IgG mAb was purchased from Vector Laboratories (Burlingame, CA), and anti-FucT-VII enzyme mAb (KM1939, mouse IgG) was prepared in our laboratory described previously (15, 16).

Isolation of normal human naive CD4 T cells

To enrich the naive CD4 T cells (CD4+CD45RA+CLA- T cell), PBMC were prepared by density gradient separation (Lymphoprep 1.077; Nycomed Pharma, Oslo, Norway) of PB from healthy donors. CD4+CLA- T cells were isolated by negative selection on magnetic separation using a CD4+ T cell isolation kit (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-CLA mAb (FITC-labeled) followed by MACS FITC-microbeads according to the manufacturer’s protocol. Subsequently, naive CD4+ T cells were isolated by positive selection using MACS CD45RA-microbeads from CD4+CLA- T cell. Isolated cells used in these studies were typically >98% naive CD4 T cells and virtually free of monocytes as evidenced by CD14 expression by FACS analysis (not shown).

Cultivation of cells to differentiate into memory cells

To differentiate naive to memory CD4 T cells, isolated naive CD4 T cells (0.5 × 10^5 cells/ml) were cultured in 24-well plates (Corning Glass, Corning, NY) coated with anti-CD3ε mAb in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FCS (HyClone, Logan, UT), and 4 mg/ml IL-2 (Invitrogen, Carlsbad, CA) for 5 days (activation culture). After 5 days, cells were harvested from the mAb-coated wells, washed, and recultured in non-mAb-coated plates (0.2 × 10^5 cells/ml) using T cell medium for 5 days (resting culture). The addition of a 5-day resting culture period in the absence of anti-CD3ε was necessary to induce the generation of CLA epitope on the T cells (20). The cultured cells were harvested from the plates for FACS analysis, confocal and conventional fluorescence microscopy analysis, and immunohistochemical staining.

Detection of cytoplasmic FucT-VII enzyme and cell surface CLA expression on cultured individual cells

The detection of cytoplasmic FucT-VII enzyme and cell surface CLA expression was performed as previously described (15, 16). Briefly, 1 × 10^4 single-cell suspensions of cultured cells or purified PB CD4 T cells were centrifuged onto the microscope slides, air-dried, and fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at 4°C. The cells were exposed to the anti-FucT-VII enzyme mAb for 6 h at 4°C, followed by staining with biotinylated horse anti-mouse IgG mAb, HRP-conjugated streptavidin, and the 3-amin-9-ethyl-carbazole substrate system (Dako, Glostrup, Denmark). After washing, the cells were exposed to the puriﬁed CLA mAb for 30 min at 4°C, followed by staining with biotinylated goat anti-rat IgM mAb, avidin-biotin-alkaline phosphatase complex (Vectastain ABC-AP Mouse IgG kit; Vector Laboratories), and Vector Blue Alkaline Phosphatase Substrate kit III (Vector Laboratories). The number of FucT-VII and CLA positive cells was counted using a microscope.

FACS analysis

Four-color flow cytometry was performed by incubating purified or cultured CD4 T cells with three mAbs (anti-CLA-FITC, anti-human IgG Fc mAb-PE, and anti-CD4-allophycocyanin) and 7-aminomethylcoumarin D for 30 min at 4°C. Cells were fixed in 1% PFA and analyzed using a FACS-Calibur flow cytometer (BD Biosciences, Mountain View, CA). Total cell number and number of each subpopulation recovered from culture were evaluated at various time points. Live cell recoveries were determined by performing direct cell counts of total cells and extrapolating them from the percentages of each subpopulation present in the whole cultures.

Detection and visualization of ESL

In this study we define ESL as a site marked by rE-selectin-IgG Fc chimera. The detection of ESL was performed by FACS analysis with modifications (22). Briefly, 1 × 10^6 single-cell suspensions of cultured cells were incubated with 10 μg/ml rE-selectin-IgG Fc chimera (R&D Systems), or 10 μg/ml human IgG Fc (ICN Pharmaceuticals, Costa Mesa, CA; negative control) in an incubation medium (3% FCS in HBSS) for 30 min at 37°C. Subsequently, cells were washed and stained with goat F(ab’2) anti-human IgG Fc mAb (PE-labeled), anti-CLA mAb (FITC-labeled), and anti-CD4 mAb (APC-labeled) in a staining buffer (1% BSA in PBS) for 30 min at 4°C. The exclusion of nontiable cell was performed by VIA-PROBE (7-amino-actinomycin D; BD PharMingen) simultaneously. Stained cells were then fixed with 1% PFA in PBS and analyzed on a flow cytometer. Visualization of ESL and CLA was performed using a fluorescence microscope and a confocal microscope, as described below.

Conventional and confocal fluorescence microscopy analysis

Conventional fluorescence digital images were acquired using an ECLIPSE E600 fluorescence microscope (Nikon, Tokyo, Japan) with B-2E/C (FITC) and G-2A (PE) filter sets and a COOLPIX 990 CCD camera (Nikon). Confocal fluorescence digital images were performed using the MRC1024 laser-scanning microscope system (Bio-Rad, Hercules, CA) with krypton/argon laser. FITC and PE were imaged separately to avoid channel bleed-through. FITC was excited at 488 nm and read through the 522/32 nm bandpass filter. PE images were collected in separate channels at 0.5-μm optical slices. Images were acquired with LaserSharp 3.2 software (Bio-Rad) and converted with iProp software (Nikon Visual Science, Tokyo, Japan). Pseudo-coloring and overlaying were performed using Adobe Photoshop 6.0 software (Adobe Systems, San Jose, CA) with a PowerMac G4 (Apple Computer, Cupertino, CA). Areas of red and green overlapping fluorescence were represented with a yellow signal.
T cell preparations for sorting

PB CD4+ T cells (containing CLA- and CLA+ cells) were prepared in the same manner as the isolation of normal human naive CD4 T cells, except for anti-CLA mAb and MACS CD45RA-microbeads. Isolated cells used for sorting were typically >97% PB CD4+ T cells by FACS analysis (not shown). PB CD4+ T cells (1 x 10^6) were incubated with 10 μg/ml rE-selectin-IgG Fc chimera or 10 μg/ml human IgG Fc in an incubation medium. Subsequently, cells were stained with goat F(ab’), anti-human IgG Fc mAb (PE-labeled) and VLA-PROBE for 30 min at 4°C. Cell sorting was performed on the basis of ESL expression using a FACSVantage cell sorter (BD Biosciences). All sorted cell populations exhibited >95% purity as evidenced by back-analysis of sorted fractions analyzed using a FACS-Calibur flow cytometer. Sorted ESL+ and ESL− cells were stained with anti-FucT-VII enzyme mAb, followed by staining with biotinylated goat anti-rat IgM mAb and avidin-biotin-alkaline phosphatase complex, and then the number of FucT-VII positive cells was counted using a microscope.

Cell sorting of ESL+ and ESL− populations from cultured CD4+ T cells

On day 5 of activation culture, CD4+ T cells were harvested from the mAb-coated wells, washed twice with HBSS, and stained with rE-selectin-IgG Fc chimera and anti-human IgG Fc mAb, followed by MACS PE-microbeads (Miltenyi Biotec). Cell isolation on the basis of ESL expression was performed according to the manufacturer’s protocol. ESL+ and ESL− fractions thus obtained were washed twice in HBSS and recultured in complete medium containing IL-2 for 1 day. We chose cell sorting using MACS microbeads for obtaining ESL+ and ESL− populations from cultured cells based on our preliminary observation that the observed viability of cells recovered 1 day after reculture was much less when a FACSVantage cell sorter was used.

Statistical analysis

Data were expressed as the mean ± SEM and were analyzed by ANOVA for repeated measures for within-group comparisons and by one-way ANOVA for comparisons among groups.

Results

Phenotypic changes during culture

We initially investigated whether CLA and FucT-VII expression could be induced with different kinetics in the differentiation process from naive T cells to memory/effector T cells. CD4+CD45RA−CLA− (naive CD4+) were isolated via mAb-coated magnetic beads from PBMC of healthy blood donors. The isolated CD4 T+ cells highly enriched for naive T cells were stimulated with anti-CD3ε mAb for up to 5 days (activation culture) and then recultured without anti-CD3ε stimulation for an additional 5 days (resting culture). Because immunofluorescence detection of cytoplasmic FucT-VII expression in PB CD4 T cells was not sensitive enough for reliable quantitation of this enzyme in FACS, simultaneous detection of FucT-VII and CLA expression in freshly prepared naive CD4+ T cells was performed by double immunohistochemical staining. Using double-label immunohistochemistry, ~4% of freshly naive CD4+ T cells were found to express FucT-VII, while CLA expression was barely detectable. At this time in the majority of FucT-VII+ T cells, small amounts of the staining were detected in a dot-like or punctate pattern close to the cell surface within the cytoplasmic components of the cells. When these CD4+ T cells were examined after 3 days of culture, up to 11% of the cells had acquired expression of FucT-VII, while CLA expression was not induced. Maximal expression of FucT-VII was observed 5 days after stimulation with anti-CD3ε mAb, whereas CLA expression remained undetectable; activation led to no induction of CLA expression at any time point in an activation culture period, although this may reflect some lack of sensitivity in available means of detecting CLA epitope expressed at lower levels. Cell numbers increased dramatically during the first 3 days, then reached a plateau (see Fig. 2). The proportion of FucT-VII+ cells increased linearly in relation to the number of recovered cells.

Thus, FucT-VII+ CLA− T cells are probably typical of cells at earlier stages of cell differentiation. On day 1 after transfer to resting culture, the cells began to down-regulate their expression of FucT-VII, whereas CLA expression inversely increased. By 3 days after transfer, the majority of the CD4+ T cells no longer expressed significant levels of FucT-VII. When the cells were examined after 3 days of resting culture, ~2% of the recovered CD4+ T cells expressed both CLA and FucT-VII (Fig. 1, B and D) and ~15% expressed CLA alone (Fig. 1, C and D). Interestingly, the frequency of FucT-VII+ CLA− cells on day 5 in activation culture indicated that most, if not all, FucT-VII+ cells acquired the FucT-VII+ CLA− phenotype by day 3 in resting culture. Because there was no evidence for expansion of either FucT-VII+ or CLA− cells during the period from day 5 in activation culture to day 1 in resting culture (Fig. 1D vs Fig. 2, A and B), a dramatic increase in the number of CLA− cells observed at 1 day in resting culture would result from a transition from the FucT-VII+ CLA− to the FucT-VII+/− CLA+ phenotype rather than the phoenix-like proliferation of remaining CLA+ T cells on day 5 in activation culture that were below the detection limit. The majority of CLA+ cells recovered and analyzed between day 5 in activation culture and day 1 in resting culture obviously represent in vitro differentiated cells rather than a replicating subset of input cells. Thus, these results indicate that CLA expression is a secondary event that requires and follows the prior activation of FucT-VII, and that the FucT-VII+ CLA− phenotype is at a terminal stage of differentiation.

FIGURE 1. Expression of cytoplasmic FucT-VII (red) and surface CLA (blue) on cultured and freshly isolated CD4 T cells. Isolated naive CD4 T cells (CD4+CD45RA−CLA− T cells) were stimulated with anti-CD3ε mAb for 5 days (activation culture), and rested for 5 days (resting culture). The cultured cells were harvested at the indicated time points and stained with anti-FucT-VII mAb and anti-CLA mAb as described in Materials and Methods. A, FucT-VII+ CLA− cells. B, FucT-VII+ CLA− cells. C, FucT-VII+ CLA− cells (bar = 10 μm). D, Expression kinetics of FucT-VII and CLA in cultured cells. The stained cells were counted using a FACS Calibur flow cytometer. Sorted ESL+ and ESL− cells were stained with anti-FucT-VII enzyme mAb, followed by staining with biotinylated goat anti-rat IgM mAb and avidin-biotin-alkaline phosphatase complex, and then the number of FucT-VII+positive cells was counted using a microscope.

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We next tested whether PB CD4⁺ T cells isolated from healthy volunteers contained a variable ratio of the three populations, FucT-VII⁺ CLA⁻, FucT-VII⁺ CLA⁺, and FucT-VII⁺ CLA⁻ cells, as demonstrated in culture, because PB CD4⁺ T cells are assumed to be a heterogeneous population of T cells at different stages of activation/differentiation. As shown in Fig. 1E, although the ratio of the three populations varied considerably among individuals, FucT-VII⁺ CLA⁻ cells were the most abundantly identifiable population, followed by FucT-VII⁺ CLA⁺ cells in the CD4⁺ T cell population.

Functional alterations during culture

Considering the established requirement for FucT-VII expression in the construction of selectin ligands (9, 10, 13, 14, 23) and a discordance between E-selectin binding and expression of the CLA epitope (10–12), the timing and levels of this enzyme expression, rather than simply surface expression of CLA, are probably critical determinants of T cell’s ability to adhere to E-selectin. We therefore attempted to define the exact point in the differentiation process at which functional selectin ligands appear on the cell surface and to determine how the expression could be related to the expression of CLA. To explore the dynamic change in the expression of ESL that occurs during in vitro culture, we used an rE-selectin-IgG Fc chimera as described previously (20, 22–24). Freshly isolated naive CD4⁺ CD45RA⁺ CLA⁻ T cells were stimulated as described above, and the frequencies of CLA⁺ and ESL⁺ positive cells were determined by flow cytometry at different time points after stimulation. Fig. 3 shows a representative experiment, comparing CLA⁺ and ESL expression. No significant binding ability to E-selectin was detected in freshly isolated naive CD4⁺ T cells, as shown on day 0 in Fig. 3A. Concomitant with the observed phenotypic changes, profound functional alterations with respect to the ability to bind E-selectin emerged upon culture. The first change observed upon stimulation was the induction of functional ESL expression. The expression of ESL was detected by day 3 of culture, and the frequency of positive cells constantly increased on days 3 and 5 in activation culture. This increase, however, was not associated with the expression of CLA. Compared with the increase in the frequency of FucT-VII⁺ cells at an early stage of culture (Fig. 1D vs Fig. 3A), the increase in the frequency of ESL⁺ cells appeared to occur in parallel to a steady increase in FucT-VII⁺ cells. The increase in ESL expression as well as FucT-VII expression correlated with an increased number of cells recovered (Fig. 2). One day after transfer to resting culture, the cultured cells were rapidly induced to express CLA, as shown in Fig. 3B; they had acquired the characteristic surface phenotype of memory T cells (i.e., CLA⁺ CD45RO⁺; not shown). The proportion of CLA⁺ cells, either ESL⁺ or ESL⁻, increased dramatically on day 1 in resting culture, and the CLA⁺ ESL⁻ subset declined gradually thereafter (Fig. 3B). Because a dramatic increase in the numbers of recovered cells was not observed during the same period (from day 5 in activation culture to day 1 in resting culture; Fig. 2), differentiation into the CLA⁺ phenotype would occur independently of cell proliferation. Proliferation is not required for their differentiation into the CLA⁺ phenotype. These findings show that the increase in the ESL⁺ CLA⁺ phenotype, like that of the FucT-VII⁺ CLA⁺ phenotype, is not the result of selective expansion of a few ESL⁺ CLA⁺ cells already present in the purified naive cells, but indeed is derived from ESL⁺ CLA⁺ cells. In contrast, under these conditions the frequency of the ESL⁺ CLA⁻ phenotype steadily decreased, a finding reminiscent of the FucT-VII⁺ CLA⁺ phenotype. Upon further cultivation, the frequency of the ESL⁺ CLA⁺ phenotype increased and reached a maximum level on day 3 in resting culture, again correlating with the frequency of the FucT-VII⁺ CLA⁺ phenotype. These cells remained abundantly detectable even after a prolonged culture period (day 5 in resting culture), indicating that they are at very advanced stages of maturation, even after E-selectin binding sites were no longer detectable, CLA continued to be expressed on the cell surface for a prolonged period of time. Fig. 4 shows a summary of the expression kinetics during a culture period (day 0 in activation culture, about day 5 in resting culture) that are relevant to this study to facilitate the understanding of relationships between FucT-VII/CLA expression and ESL expression in vitro differentiated CD4⁺ T cells. These data suggest that induction of functional ESL expression would depend primarily on the expression of FucT-VII, but not CLA expression.

To test whether ESL⁺ CLA⁺ cells could arise directly from ESL⁺ CLA⁻ cells, the ESL⁺ and ESL⁻ populations were isolated from CD4⁺ T cells harvested on day 5 in activation culture by cell sorting, and subsequently, they were recultured under resting conditions for 1 day. The ESL⁺ and ESL⁻ populations before and after 1 day of culture were phenotypically compared with each other. Before isolation, the percentage of ESL⁺ cells in the CD4⁺ T cells harvested on day 5 in activation culture was 14.5% (Fig. 2).
5A). After isolation, the ESL\(^{+}\) fraction contained \(\approx 37\%\) ESL\(^{+}\) cells, whereas the ESL\(^{-}\) fraction contained \(\approx 4.6\%\) ESL\(^{-}\) cells (Fig. 5B). On day 1 in resting culture, the frequencies of ESL\(^{+}\) cells increased in both ESL\(^{-}\) and ESL\(^{+}\) populations (78 vs 24%; Fig. 5C). Nevertheless, the ESL\(^{-}\) CLA\(^{-}\) phenotype was generated only from the ESL\(^{+}\) CLA\(^{-}\) population, not from the ESL\(^{-}\) CLA\(^{-}\) population. These results strongly suggest that ESL\(^{-}\) CLA\(^{-}\) cells function as precursors for ESL\(^{-}\) CLA\(^{-}\) cells. Interestingly, the low percentage (2.5%) of ESL\(^{-}\) CLA\(^{-}\) cells was generated from the ESL\(^{-}\) population, suggesting the alternative possibility that some CLA\(^{-}\) CD4\(^{+}\) T cells may arise directly from ESL\(^{-}\) populations.

We next examined the relationship between CLA expression and E-selectin binding function in PB CD4\(^{+}\) T cells isolated from healthy volunteers. Representative double-staining profiles of PB CD4\(^{+}\) T cells with HECA-452 and an rE-selectin-IgG Fc chimera are shown in Figs. 3C and 4B. Not consistent with a previous observation that the ability of T cells to bind E-selectin was exclusively detected within the CLA\(^{+}\) fraction (6–8, 20), an E-selectin binding function was detected within both CLA\(^{+}\) and CLA\(^{-}\) cells, and CLA\(^{+}\) cells lacking the ability to bind E-selectin were definitely identified.

Direct visualization of ESL in relation to expression of CLA
To verify the difference in the expression and distribution patterns of ESL and CLA on the cells after activation, we attempted to visualize both epitopes using conventional and confocal immunofluorescence microscopy. This approach allowed direct visualization of the changes in membrane dynamics that have been demonstrated by flow cytometry. Fig. 6 shows the conventional fluorescence digital images of the cells on day 5 in activation culture (A) and on day 1 in resting culture (B). A strikingly different staining pattern for ESL and CLA was observed. In activation culture, ESL expression showed a speckled pattern and was never associated with CLA expression. In the cells from the resting culture, most ESL were not colocalized with CLA, although its partial overlap with CLA was detectable. Apparently, however, cell surface staining for ESL in the CLA\(^{+}\) subset was more intense than that in the CLA\(^{-}\) subset in activation culture. To further confirm the differential expression of both epitopes, we obtained dual confocal digital images of the cultured cells stained with a rE-selectin-IgG Fc chimera and anti-CLA mAb. Fig. 7 displays optical slice
detection of both epitopes on the cells on day 1 in resting culture. Although only some overlap of both epitopes was observed, ESL epitope was found to be scattered on the cell surface independently of expression of CLA epitope. The lack of colocalization with CLA in ESL expression is further evidence to indicate that the CLA epitope is not directly involved in the binding to E-selectin.

FIGURE 5. Differentiation from ESL⁺ CLA⁻ to ESL⁺ CLA⁺ cells. The ESL⁺ and ESL⁻ populations were isolated from CD4⁺ T cells harvested on day 5 in activation culture by cell sorting, and subsequently, they were recultured under resting conditions for 1 day. The ESL⁺ and ESL⁻ populations before and after 1-day culture were phenotypically compared with each other. A, CD4⁺ T cells harvested on day 5 in activation culture. B, The ESL⁺ (upper) and ESL⁻ (lower) populations thus isolated before 1-day resting culture. C, CD4⁺ T cells differentiated from the ESL⁺ (upper) and ESL⁻ (lower) populations after 1-day resting culture.

FIGURE 6. Immunolocalization of ESL and CLA in cultured CD4⁺ T cells. Single-cell suspensions of cultured cells were incubated with rE-selectin-IgG Fc chimera or 10 µg/ml human IgG Fc and stained with goat F(ab')₂ anti-human IgG Fc and anti-CLA mAb (FITC-labeled). They were visualized on glass coverslips and photographed by ECLIPSE E600 fluorescence microscope with a COOLPIX 990 CCD camera (Materials and Methods). The red channel is ESL, and the green channel is CLA. The merged image of the respective pictures is given in the left column. These cells in the photographs correspond to FACS data. Human IgG Fc did not react to the cells (not shown). A, ESL⁺ CLA⁻ cells on day 5 in activation culture. B, ESL⁺ CLA⁺ cells (upper) and ESL⁻ CLA⁺ (lower) cells on day 1 in resting cultures. Bar = 10 µm.
although we could not totally exclude the possibility that CLA may serve as a minor T cell ligand for E-selectin.

**Discussion**

Although the acquisition of skin-homing properties has been supposed to occur immediately upon activation, previous kinetic studies on the induction of CLA expression by T cells demonstrated that its induction only occurs after extended culture times (12, 18–21). Indeed, our studies also show that induction of CLA at high levels represents a late event during a differentiation process into skin-homing memory T cells. To achieve high levels of CLA expression, activated T cells had to be rested in culture without stimulation. Assuming that CLA per se is indeed a skin-homing receptor, the requirement for this resting culture period to induce CLA expression appears to conflict with the following ideas. T cells, upon activation, display a dominant trafficking pattern associated with reduced recirculation through lymphoid tissue and acquisition of homing properties to peripheral tissues such as skin, and the capacity to recirculate is restored when activated T cells revert to a resting state. Also in conflict with the delayed expression of CLA is the idea that activated T cells are preferentially extracted from the circulation into the inflammatory skin sites by their increased expression of the skin-homing receptor, CLA. One possible explanation of these seemingly contradictory results is that T cells committed to migrate into the skin can attain the skin-homing potential before the induction of CLA expression. Here we demonstrate that upon activation naive CD4⁺CD45RA⁺CLA⁻ T cells can differentiate into FucT-VII⁺CLA⁻ cells, which is the first phenotype to appear in activation culture, and that the appearance of this subset coincides with or could be immediately followed by the generation of E-selectin binding epitopes. Sorting experiments clearly indicate that the vast majority of FucT-VII⁺ cells are present in the ESL⁺ population of PB, but not in the ESL⁻ population. Our data, therefore, can be interpreted as showing that the induction of FucT-VII expression enables these cells to selectively acquire properties to bind E-selectin, preceding the induction of CLA expression. Thus, the appearance of FucT-VII⁺CLA⁻ T cells on day 3 in activation culture would represent the commitment of activated T cells to the skin-homing phenotype, and the induction of FucT-VII expression may be the decision-making factor determining whether these T cells will migrate into the skin. Nevertheless, it should also be pointed out that there would theoretically be a lag time from FucT-VII expression to the generation of CLA, because, despite its relatively early appearance, the generation of ESL on the cell surface is a secondary event that requires and follows the prior induction of FucT-VII expression as recognized by this mAb. In view of the finding that the extent of ESL expression in the FucT-VII⁺CLA⁻ subset was not as intense as that in the CLA⁺ subsets, this FucT-VII⁺CLA⁻ subset may represent skin-homing T cells that can only be arrested at the vessel wall expressing high levels of E-selectin in inflammatory sites.

Upon transfer to the resting culture, a substantial proportion of differentiated T cells acquired the CLA⁺ phenotype, either FucT-VII⁺CLA⁺ or FucT-VII⁺CLA⁺, on day 1; the decrease in the former occurred virtually in parallel with a steady increase in the latter on day 3 in resting culture. These results strongly suggest that the acquisition of high levels of CLA on FucT-VII⁺ T cells (ESL⁻) subpopulations; the former included both ESL⁺CLA⁻ and ESL⁺CLA⁺, while the latter included both ESL⁺CLA⁺ and ESL⁻CLA⁻. The percentage of FucT-VII⁺ cells in each sorted fraction was determined by immunohistochemistry. As shown in Fig. 8C, FucT-VII⁺ cells were present exclusively within the ESL⁺ fraction, although the frequency of FucT-VII⁺ cells (19.5%) was lower than the expected frequency. No detectable FucT-VII expression was noted in the ESL⁻ fraction, indicating that the vast majority of FucT-VII⁺ T cells have the ability to bind E-selectin.

**FIGURE 7.** Representative fields of optical continuous slices of the stained cells. Single-cell suspensions of cultured cells (day 1 in resting culture) were incubated with rE-selectin-IgG Fc chimera or 10 μg/ml human IgG Fc and stained with goat F(ab')₂ anti-human IgG Fc (PE-labeled) and anti-CLA mAb (FITC-labeled) and were confocal scanned by the Bio-Rad MRC1024 laser-scanning microscopy system with krypton/argon laser (Materials and Methods). Optical slices were taken at 0.5-μm intervals to ensure that the whole T cell was within the section. Human IgG Fc did not react to the cells (not shown). The colocalization of ESL (red) and CLA (green) resulted in a yellow color. A, Reconstruction image of optical slices. A1, Merged image of 605/32-nm channel PMT (A2, ESL) and 522/32-nm channel PMT (A3, CLA). Bar = 10 μm. B, Optical continuous slices (B9 is closest to the plane of the slide glass).
represents an ordered progression to more differentiated populations; the FucT-VII−CLA− phenotype could be assumed to be the immediate precursor of the FucT-VII+CLA− phenotype. It is unlikely that the transition to the CLA+ phenotype is due to the outgrowth of a minute population that had already existed in the activation culture, accompanied by the massive death of FucT-VII−CLA− phenotype T cells, because the transition occurs at a time when the total number of recovered cells is increasing only minimally. A likely explanation for the late appearance of CLA on these in vitro-differentiated T cells is that the CLA epitope might be generated only when FucT-VII and other fucosyltransferases, such as FucT-IV, are up-regulated in a sequential manner. Considering the intense staining of ESL in the CLA+ subset, the acquisition of high levels of CLA on FucT-VII+T cells may be a specific marker for the skin-homing T cells that can migrate into the skin, where endothelium expresses E-selectin at low levels. Interestingly, this subset has been the most abundantly identifiable population in PB CD4+ cells of healthy individuals (15). In view of our unpublished observation that this subset dramatically increases upon resolution of inflammatory responses in various skin diseases, the increase in this subset most likely represents the release of sequestrated CD4+ T cells back into the circulation. The FucT-VII+CLA−CD4+ T cells we observed in PB may represent T cells that are in the process of being purged from the skin and are simply losing the ability to be sequestrated in the skin. Thus, on the basis of the expression of FucT-VII and CLA, at least three subsets of skin-homing T cells at various stages of differentiation are identified: naive skin-homing CD4 T cell precursors would undergo a linear differentiation process from the FucT-VII−CLA− phenotype to the FucT-VII+CLA− phenotype and eventually to the FucT-VII+CLA+ phenotype, which may lose the ability to localize within the skin and migrate out of the skin.

The widely held belief that CLA is a ligand for E-selectin was first challenged by previous studies reported by Wagers et al. (10, 11); they clearly showed that the expression of CLA epitopes and E-selectin binding activity do not correlate on cultured T lymphoblasts, because the majority of the CLA epitopes was cleaved or

**FIGURE 8.** FACS sorting of PB CD4+ ESL+ and ESL− cells and FucT-VII expression on the sorted cells. Isolated PB CD4+ T cells (containing CLA+ and CLA− cells) were stained with goat F(ab′)2 anti-human IgG Fc (PE-labeled) and VIA-PROBE and were sorted on the basis of ESL expression with a FACSVantage (Materials and Methods). A, ESL and CLA expression in purified PB CD4+ T cells analyzed by FACSCalibur (four-color analysis using FITC-labeled anti-CLA mAb and allophycocyanin-labeled anti-CD4 mAb). B, ESL expression of purified PB CD4+ T cells analyzed by FACSVantage (two-color analysis). The sort gates for ESL+ VIA-PROBE+ and ESL− VIA-PROBE− cells are shown. The purity of sorted fractions (C, ESL+; D, ESL− cells) was analyzed using a FACSComp flow cytometer by two-color back-analysis. The sorted cells were stained with anti-FucT-VII mAb, and FucT-VII-positive cells were counted under the microscope. The percentage of FucT-VII+ cells in the sorted fractions is shown.
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References
