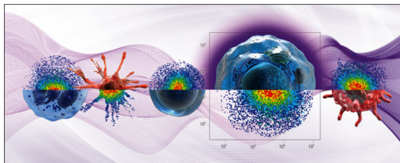

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Ly6C Induces Clustering of LFA-1 (CD11a/CD18) and Is Involved in Subtype-Specific Adhesion of CD8 T Cells[1]

Ilkka Jaakkola, Marika Merinen, Sirpa Jalkanen, and Arno Hänninen[2]

Ly6C is a hemopoietic cell differentiation Ag found on a subset of CD8 T cells in the periphery. It is involved in target cell killing by CTLs, augments TCR-mediated activation of IL-2 and IFN-γ production in CD8 T cells, and regulates CD8 T cell homing in vivo. In this study, we show that cross-linking of Ly6C causes clustering of LFA-1 (CD11a/CD18) on the surface of CD8 T cells via a mechanism dependent on reorganization of actin cytoskeleton and intracellular protease, calpain, but not the phosphatidylinositol 3-kinase pathway. In the capillary flow-adhesion assay, Ly6C cross-linking significantly augments lymphocyte adhesion to endothelium, and this is inhibited by an Ab that blocks LFA-1 function. Furthermore, upon in vitro cross-linking and during in vivo homing into lymph nodes, Ly6C is transiently lost from cell surface but becomes re-expressed on lymph node-resident CD8 T cells. The abilities of Ly6C to induce LFA-1 clustering and to be re-expressed after signaling-associated down-regulation may be important in regulating the homing of CD8 T cells into lymph nodes and in subsequent steps of CD8 T cell activation and effector function that again involve LFA-1. ***The Journal of Immunology,* 2003, 170: 1283–1290.**

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Ly6 is a multigene family of GPI-anchored cell surface glycoproteins mainly expressed on cells of hemopoietic origin (1). This family consists of several members that are mainly involved in cellular interactions and T cell activation. The most characterized member of this family, Ly6A/E, has a role in several distinct immunological processes such as thymocyte development, T cell activation, and cell-cell adhesion (2–6). Ly6C is expressed on ~50% of murine peripheral CD8 T cells and, in addition, bone marrow mononuclear cells and monocyte/macrophages express Ly6C to some extent (7). It has an accessory role in the cytolytic function of CTL (8) and it augments T cell proliferation and IL-2 production upon activation (9). IL-2 production is induced upon Ly6C cross-linking (10) and after T cell stimulation the expression of Ly6C is shown to correlate with IFN-γ production (11). Despite many reports on Ly6C expression, the functional characteristics of Ly6C are still incompletely understood.

LFA-1 (CD11a/CD18) is the major β_2 integrin on T cells and with its endothelial ligands ICAM-1 and 2, it forms important receptor-ligand pairs for lymphocyte-endothelial adhesion and transmigration. To prevent undesired, nonspecific aggregation of circulating leukocytes in the vasculature, LFA-1 generally exists in an inactive, nonadherent state (12). To become capable of binding to its ligands, LFA-1 must first be activated. Rapid activation is made possible at least by the chemokine system, as binding of an appropriate chemokine to its G protein-coupled receptor on the leukocyte surface is able to trigger immediate integrin affinity/avidity changes and, consequently, induce leukocyte arrest to endothelium (13, 14).

In addition to chemokines and their receptors, LFA-1 can be activated by other receptors on the cell membrane, including TCR/CD3 (15), CD24 (16), CD43 (17), CD28 (18), CD45 (19),and CD73 (20). In lymphocytes, increased binding activity is largely mediated by changes in avidity, also termed clustering (21). In the course of LFA-1 activation via avidity increase, it is released from the control of the actin cytoskeleton which enables lateral diffusion of LFA-1 on the cell surface (22). Clustering and affinity modulation are not totally exclusive from each other and they may also play complementary roles (23).

Previously we have shown that Ly6C is involved in endothelial adhesion and in vivo homing of CD8 T cells (24). Although cross-linking of Ly6C induced homotypic aggregation of lymphocytes in vitro via an integrin-dependent pathway, the exact mechanism of Ly6C involvement in lymphocyte-endothelial interaction remained unresolved. In this study, we show that cross-linking of Ly6C increases adherence of CD8 T cells to endothelium. This increase is due to effective clustering of LFA-1 that involves the Ca^{2+}-dependent protease calpain but not the phosphatidylinositol 3-kinase (PI3)[3] pathway involved in chemokine-induced clustering (13). We also show that upon cross-linking in vitro and during homing in vivo, there is a significant decrease in Ly6C surface expression. Our data suggest a novel, chemokine-independent pathway of LFA-1 activation in CD8 T cells that may be involved in several distinct actions of CD8 T cells, including their homing, activation, and target cell killing.

Materials and Methods

Mice

A local colony of BALB/c mice was used. The colony was housed and bred in the Central Animal Laboratory of the Turku University (Turku, Finland) under specific pathogen-free conditions. The quality of these conditions was regularly tested with microbial tests (BioDOC Microbial Laboratories,

MediCity Research Laboratory, University of Turku and National Public Health Institute, Department in Turku, Turku, Finland

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[2] Address correspondence and reprint requests to Dr. Arno Hänninen, MediCity Research Laboratory, University of Turku, Tykistökatu 6 A, FIN-20520 Turku, Finland. E-mail address: arno.hanninen@utu.fi

[3] Abbreviations used in this paper: PI3, phosphatidylinositol 3-kinase; CMFDA, 5-chloromethyl fluorescein diacetate; HEV, high endothelial venule; MFI, mean fluorescence intensity; PNAd, peripheral node addressin.

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Hannover, Germany). All experiments were performed with 8- to 16-wk-old mice. All animal experiments were approved by the Ethical Committee of the University of Turku.

Antibodies

The following mAbs were used: anti- CD11a (TIB-217, TIB-237) and anti-IL-2Rα (TIB-222) hybridomas were purchased from the American Type Culture Collection (Manassas, VA); anti-L-selectin (anti-CD62-L, MEL-14), anti-peripheral node addressin (PNAd; MECA-79), and anti-human CD44 (Hermes-1, 9B5) hybridomas were kind gifts from E. C. Butcher (Stanford University, Palo Alto, CA); and anti-Ly6C (G10) and its isotype-matched control mAb (2E8) were from immunizations described in Ref. 24. All of the Abs were concentrated from cell hybridoma supernatants with ammonium sulfate precipitation and purified with protein G columns (Pharmacia, Peapeck, NJ). For some experiments, G10 and TIB-217 were FITC conjugated using FITC (Sigma-Aldrich, St. Louis, MO) and G10 was biotinylated using *N*-hydroxysuccinimide-biotin (Calbiochem, La Jolla, CA). FITC- and R-PE-conjugated anti-CD8 were purchased from Caltag Laboratories (Burlingame, CA).

Cross-linking

Cross-linking of Ly6C was performed for isolated lymphocytes by incubating the cells in cold RPMI 1640 first with the anti-Ly6C mAb (G10) at 10 μg/ml (either unconjugated or, for the detection of its surface expression, biotinylated), or with the isotype-matched control mAb (2E8) or anti-IL-2Rα (TIB-222) on ice. After washes, rabbit anti-rat IgG (DAKO, Glostrup, Germany) was added at 10 μg/ml (HRP-conjugated for dot blot analysis). The cells were then washed twice with cold RPMI 1640, followed by a 20-min incubation at room temperature to allow cells to be metabolically active. After this incubation, the cells were used for functional or phenotypic analyses.

Detection of LFA-1 clustering with confocal microscopy

For each experiment, lymphocytes were isolated from mesenteric and peripheral lymph nodes of two mice and pooled. CD4 T cells and B cells were depleted using L3T4 and B220 magnetic beads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of CD8 T cells was $\geq$98%. Ly6C cross-linking was performed as described above for purified CD8 T cells and the cells were then washed with cold PBS containing 2% FCS and 0.5% NaN_3 (FACS-PBS). The cells were stained in this medium with FITC-conjugated anti-LFA-1 (TIB-217) for 20 min on ice. The unbound mAb was washed away twice with FACS-PBS. Cells were then spun to glass slides in a cytocentrifuge, 5×10^5 cells/slide. Slides were mounted with Prolong Anti-fade (Molecular Probes) and analyzed under a confocal microscope (Leica TCS SP; Leica, Deerfield, IL). From each slide a representative area was chosen and each area was analyzed in 15 separate layers and a superimposition from these was produced. For experiments with inhibitors, the cells were pretreated before cross-linking as follows: 20 nM wortmannin (Sigma-Aldrich), 1 μM jasplakinolide (Molecular Probes), 100 μg/ml CBZ-Leu-Val-Gly diazomethyl ketone (Sigma-Aldrich), and 100 μg/ml calpeptin (Calbiochem-Novabiochem, San Diego, CA). The incubations were performed at +37°C for 30 min.

Dot blot analysis

Cross-linking of Ly6C on isolated CD8 T cells was performed as described above with the exception that HRP-conjugated rabbit anti-rat IgG was used as a second-step Ab. After extensive washes to remove unbound Abs, cells were incubated at +37°C in RPMI 1640 medium supplemented with 10% FCS L-glutamine, penicillin, and streptomycin (complete medium). Five hundred microliters of cell suspension was collected at six different time points between 0 and 120 min. Suspensions were centrifuged, supernatants were collected, and pellets were lysed in 500 μl of lysis buffer for 1 h at +7°C in a rotation wheel. Lysates and corresponding supernatants were filtered through nitrocellulose membrane (Hybond-ECL; Amersham, Arlington Heights, IL) with a vacuum pump. Ly6C-specific signal was detected by ECL (Amersham).

Capillary flow assay

The capillary flow assay was performed as previously described (25) with slight modifications. In brief, mouse endothelial cell line bEND3 (CRL2299; American Type Culture Collection) was grown as a monolayer into the lumen of the glass capillary and treated with 100 U/ml recombinant mouse TNF-α (BD PharMingen) for 20 h at +37°C. Lymphocytes collected from peripheral and mesenteric lymph nodes of BALB/c mice were pooled and used as either unfractionated lymphocytes or as purified CD8 T cells. The cells were pretreated with anti-Ly6C and a second-step Ab to cross-link Ly6C, after which they were incubated with a function-blocking anti-LFA-1 or control mAb for 20 min. The cells were resuspended in complete medium at 10^6 cells/ml and allowed to flow through a glass capillary precoated with bEND3 endothelial cells. Because the bEND3 cells are not able to support selectin-dependent rolling, the flow was stopped for 15 min to create optimal conditions for integrin-dependent lymphocyte adherence and then restarted. For calculating the bound cells, digital video shots of 15 separate, randomly chosen areas of each capillary were recorded.

Immunohistochemistry of peripheral lymph nodes

Frozen sections of peripheral lymph nodes were cut from several BALB/c mice and incubated with FITC-conjugated anti-Ly6C and simultaneously with biotinylated anti-PNAd (MECA-79) to detect high endothelial venules (HEV), and then with streptavidin-conjugated PE to detect the biotinylated mAb. The sections were analyzed and photographed with a fluorescence microscope (Olympus, Melville, NY) using a double filter.

Short-term in vivo homing

Lymphocytes isolated from peripheral and mesenteric lymph nodes of donor mice were pooled, resuspended in complete medium (RPMI 1640 with 10% FCS and HEPES), and fluorescently labeled with 5-chloromethyl fluorescein diacetate (CMFDA; Molecular Probes) as described elsewhere (26). Briefly, 20×10^6 labeled cells were injected i.v. into each recipient and after a 30-min recirculation period, each recipient was sacrificed and lymphocytes were isolated from blood, lymph nodes, spleen, and liver. Isolated lymphocytes were then stained with biotinylated anti-Ly6C, anti-L-selectin, or control mAb (9B5) and with streptavidin-conjugated PE (BD Biosciences) as a second-step reagent. Surface expression of Ly6C and L-selectin was analyzed with flow cytometry (FACScan and CellQuest software; BD Biosciences) on CMFDA-labeled lymphocytes before injection into recipient mice and again after collection of blood and tissue samples from them at the end of the recirculation period.

Short-term in vivo homing of $Ly6C^+$ and $Ly6C^-$ T cells

Purified CD8 T cells prepared as above were split into two groups, one of which was subjected to further depletion of all $Ly6C^+$ cells. This was done with magnetic cell sorting using biotinylated anti-Ly6C mAb followed by incubation with streptavidin-conjugated magnetic beads (MACS; Miltenyi Biotec). The other group of purified CD8 T cells (of which one-half were $Ly6C^+$) was subjected to the same treatment without biotinylated anti-Ly6C mAb and used as "$Ly6C^+$ " cells. $Ly6C^-$ and $Ly6C^+$ CD8 T cells were then separately labeled with CMFDA and 6×10^6 labeled cells were injected i.v. into each recipient. After a 60-min recirculation period, recipient mice were killed and their lymph nodes were collected. To determine the homing efficiency of injected cells (either $Ly6C^+$ or $Ly6C^-$ cells), the percentage of CMFDA-labeled cells was analyzed by flow cytometry from 1×10^6 isolated peripheral and mesenteric lymph node lymphocytes from each recipient mouse.

Phenotype of homed $Ly6C^-$ T cells after 18-h recirculation

$Ly6C^-$ CD8 T cells, purified and labeled with CMFDA as above, were injected i.v. and allowed to recirculate for 18 h. The recipients' lymph nodes were then collected and lymphocytes were stained with biotinylated anti-Ly6C and streptavidin-conjugated PE (BD Biosciences). Surface expression of Ly6C was analyzed on CMFDA-labeled cells by flow cytometry.

Results

Cross-linking of Ly6C induces clustering of LFA-1

To test the effect of Ly6C cross-linking on LFA-1 distribution on the cell surface, cross-linking of Ly6C on isolated CD8 T cells was performed, after which the expression of LFA-1 was determined. Confocal microscopy analysis revealed that cross-linking induced the formation of massive, polarized clusters of LFA-1 on the cell surface (Fig. 1*A*). Clustering of LFA-1 could only be seen in half of the cells, consistent with the fact that Ly6C is expressed on ~50% of CD8 T cells. Clustering of LFA-1 was strictly dependent on cross-linking, because without a second-step Ab, LFA-1 remained evenly distributed on the cell surface (Fig. 1*B*). To further support the specificity of the phenomenon, cross-linking with anti-IL-2Rα mAb (TIB-222) and secondary Ab had no effect on LFA-1 surface expression (Fig. 1*C*). The effect of Ly6C cross-linking was

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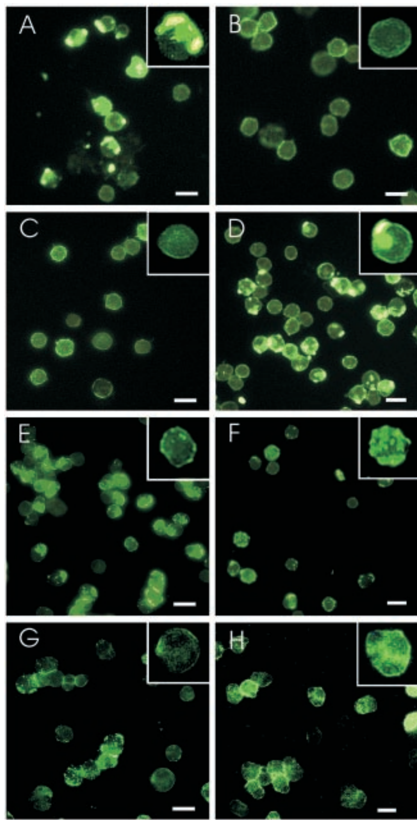

FIGURE 1. Cross-linking of Ly6C induces polarized clustering of LFA-1 on a subset of CD8 T cells via calpain-dependent but PI3-independent pathway. *A,* Ly6C cross-linking with anti- Ly6C mAb and secondary Ab. *B.* Anti- Ly6C mAb incubation without secondary Ab. *C,* Control cross-linking with anti-IL-2Rα mAb and secondary Ab. *D,* Pretreatment with PI3 inhibitor wortmannin followed by cross-linking of Ly6C. *E,* Pretreatment with actin stabilizer jasplakinolide followed by cross-linking of Ly6C. *F,* Pretreatment with calpain inhibitors calpeptin and CBZ-LVG followed by cross-linking of Ly6C. *G,* Pretreatment with a combination of wortmannin, jasplakinolide, and calpeptin followed by cross-linking of Ly6C. *H,* Pretreatment with a combination of wortmannin, jasplakinolide, and calpeptin without cross-linking. In each photograph, equal settings and exposure times were used. Bars, 10 μm. Original magnification, ×400)

also specific for LFA-1 since no clustering of CD8 or MHC I (H-2K^d) could be seen (data not shown).

Several signal transduction pathways and cytoskeletal molecules have been shown to be involved in LFA-1 clustering. Therefore, we investigated whether Ly6C-dependent signaling of LFA-1 activation uses any of the known pathways and involves cytoskeletal reorganization (16, 22, 27, 28). The possible involvement of these mechanisms was tested by pretreating CD8 T cells before Ly6C cross-linking with different known inhibitors of signaling pathways such as the PI3 inhibitor wortmannin, actin stabilizer jasplakinolide, or with a combination of calpeptin and CBZ-LVG, both inhibitors of the intracellular protease calpain. Blocking the PI3 alone had no effect on polarized clustering (Fig. 1*D*). However, treatment with either jasplakinolide (Fig. 1*E*) or the combination of two calpain inhibitors (Fig. 1*F*) clearly inhibited the formation of polarized clusters but was not completely able to prevent clustering, as smaller clusters could still be seen. These inhibitors had an additive effect because clustering of LFA-1 was further inhibited when cells were pretreated with a combination of all of the aforementioned inhibitors (Fig. 1*G*). Although this combination abolished the formation of clearly detectable clusters of LFA-1, it did not result in a completely homogenous distribution of LFA-1 on the cell surface, possibly implicating an additional pathway with minor importance in Ly6C-induced LFA-1 clustering. For comparison, LFA-1 was more homogenously distributed on the cell surface if Ly6C was not cross-linked after pretreatment with the combination of all aforementioned inhibitors (Fig. 1*H*). Thus, the inhibitors themselves had no effect on LFA-1 distribution on the cell surface.

Cross-linking of Ly6C increases adhesion of CD8 T cells to endothelium

In the adhesion cascade, LFA-1 is known to mediate firm adhesion of lymphocytes to endothelium. Since the clustering of LFA-1 increases its avidity to its ligands, we investigated whether Ly6C cross-linking was able to increase firm adhesion of lymphocytes to endothelium. For this purpose, a capillary flow assay was used. Even with unselected lymphocytes, a slight but significant increase (19%, $p < 0.001$) in adhesion could be seen in comparison to control-treated cells. Since in the periphery Ly6C expression is restricted to CD8 T cells (i.e., 50% of them are Ly6C$^+$), purified CD8 T cells were used in subsequent experiments. Cross-linking of Ly6C increased firm adhesion of CD8 T cells to endothelium by 32% in comparison to the adhesion of control-treated cells ($p < 0.01$). This increase was LFA-1 dependent since it could be abolished with an anti-LFA-1 mAb incubation after Ly6C cross-linking, whereas the control mAb did not abolish this increase (Fig. 2).

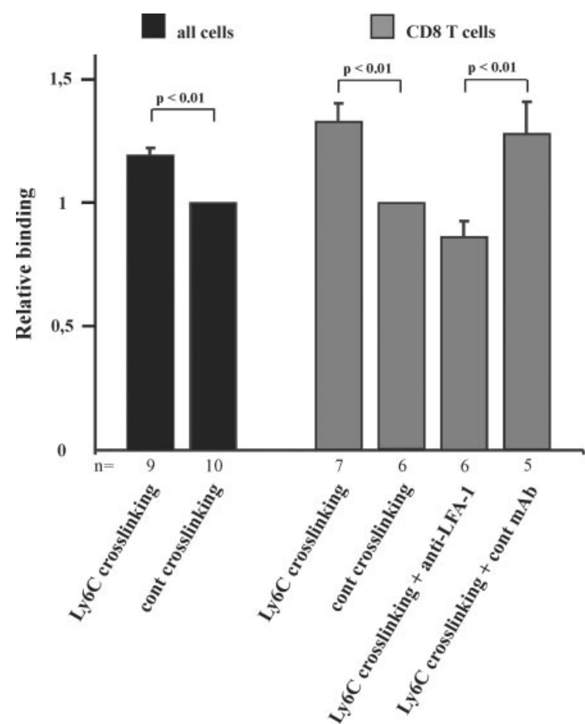

FIGURE 2. Cross-linking of Ly6C increases LFA-1-mediated binding to endothelium. Cross-linking of Ly6C with anti-Ly6C mAb and secondary Ab or control cross-linking was performed as described in *Materials and Methods*. Either nonselected (■) or purified CD8 T cells (▩) were used. With purified CD8 T cells, additional inhibition studies were done in which cells were pretreated after Ly6C cross-linking with anti-LFA-1 mAb or control mAb before the flow assay. The number of analyzed capillaries is presented under each bar. Within each capillary, 15 randomly chosen areas of equal size were recorded with a digital video camera for further analysis. On average, there were 78 bound cells/mm^2 (SEM, 6.4) within each field of the control capillaries analyzed and this was arbitrarily defined as a reference value for binding (relative binding, 1.0). The p values were calculated using the two-tailed, unpaired Student's t test.

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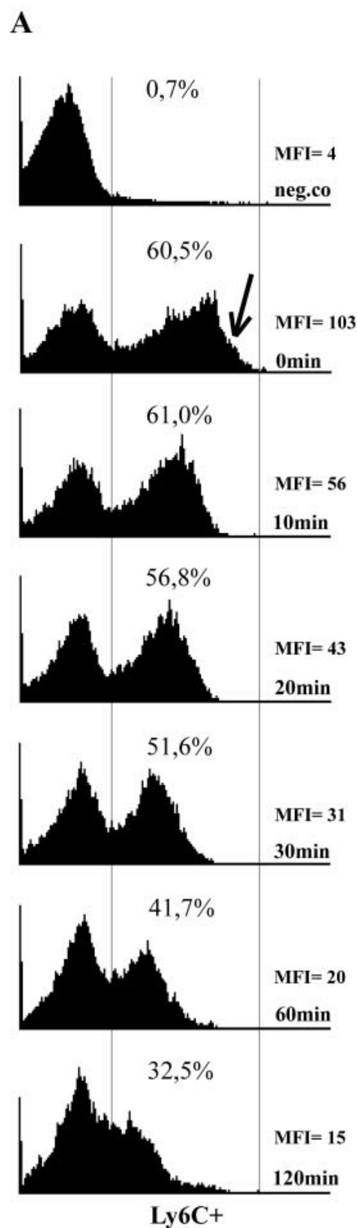

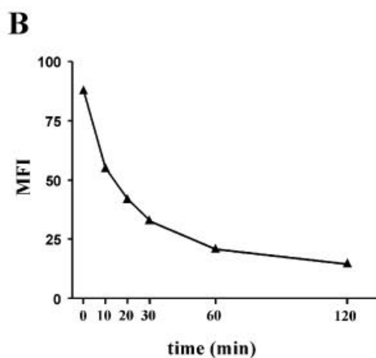

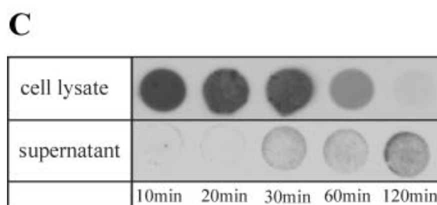

FIGURE 3. Ly6C is shed from the cell surface after cross-linking in vitro. *A,* Isolated lymphocytes were incubated with biotinylated anti- Ly6C followed by cross-linking with anti-rat IgG. Cells were collected from the incubation medium in the given time points and stained for flow cytometry with streptavidin-conjugated PE for detection of biotinylated anti-Ly6C and with FITC-conjugated anti-CD8. The $Ly6C^{high}$ T cell population (arrow) is lost during the first minute after cross-linking. Histograms are representative of three separate experiments. *B,* The MFI of $Ly6C^+$ in the given time points after cross-linking. The values are the average MFI values of three separate experiments. SD values in each time point are <3.0 and are therefore not displayed in the diagram. *C,* A dot blot analysis of Ly6C from cell lysates and corresponding supernatants in the given time points after cross-linking of Ly6C. A representative of three separate experiments.

Surface expression of Ly6C decreases after cross-linking

To investigate the surface expression of Ly6C after cross-linking, we stained lymphocytes for flow cytometry at six different time points between 0 and 120 min subsequently to its cross-linking. During this follow-up time, Ly6C surface expression reduced sharply, i.e., the percentage of $Ly6C^+$ cells among CD8 T cells declined from 61 to 33% (Fig. 3*A*). Also, in three consecutive experiments the mean fluorescence intensity (MFI) dropped 83% during the follow-up time (Fig. 3*B*). This drop was due to cross-linking, because without cross-linking no reduction in Ly6C surface expression could be seen in any medium or temperature tested (data not shown). The drop in surface expression was rapid, because during the first 10 min the MFI value declined from 103 to 56, although the percentage of $Ly6C^+$ cells had yet not declined.

Ly6C is shed from the cell surface upon cross-linking

To test whether the reduction in the surface expression of Ly6C after cross-linking was due to shedding or internalization of Ly6C, a dot blot analysis was performed. For this purpose, the purified CD8 T cells and the corresponding medium were collected at the time points of 10, 20, 30, 60, and 120 min after Ly6C cross-linking, and the Ly6C-specific signal was detected with dot blotting. A clear time-dependent increase in Ly6C-specific signal in the medium could be seen whereas the signal in the corresponding cell lysates decreased, indicating that cross-linking of Ly6C resulted in shedding of Ly6C rather than internalization (Fig. 3*C*).

Ly6C is temporarily lost from the cell surface upon homing to lymph nodes in vivo

As cross-linking induced shedding and consequently a decrease in Ly6C surface expression in vitro, we wished to determine whether Ly6C was lost from the cell surface also under in vivo conditions. First, a standard immunofluorescence staining was performed on frozen sections of lymph nodes of unmanipulated mice. To visualize the areas of active lymphocyte homing, sections were stained for PNAd and simultaneously for Ly6C. The staining showed that areas surrounding HEV were almost devoid of $Ly6C^+$ cells. However, $Ly6C^+$ cells could readily be detected elsewhere in T cell areas, suggesting that recently emigrated cells do not express Ly6C and that it becomes re-expressed later within the lymph node as the cells move out from HEV (Fig. 4). To investigate this observation in more detail, we injected fluorescently labeled (unselected) lymphocytes into unmanipulated recipients and followed their homing into different organs during a 30-min circulation period. Flow cytometric analyses showed that the number of $Ly6C^+$ cells among injected cells still circulating was unaltered, whereas the number of $Ly6C^+$ cells among the cells that had homed to lymph nodes had markedly reduced when compared with injected cells (Fig. 5*A*).

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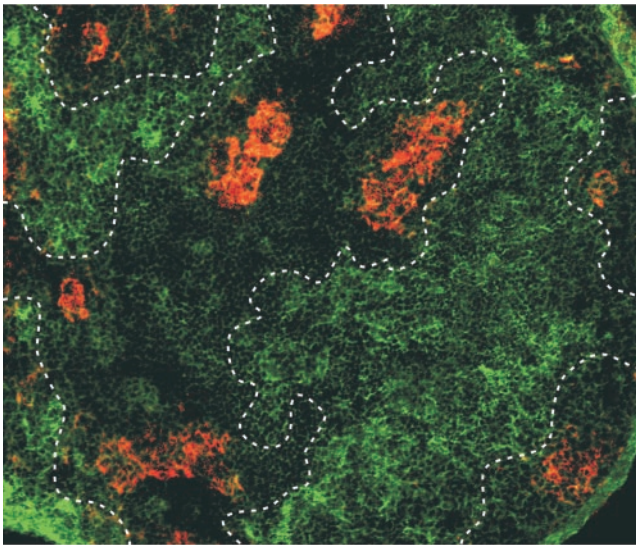

FIGURE 4. Newly transmigrated T cells are Ly6C$^-$. Immunohistological staining of peripheral lymph node section shows diminished expression of Ly6C in close proximity (dashed lines) of HEV. HEV were stained with biotinylated anti-PNAd and detected with PE-conjugated streptavidin (red). For detection of the Ly6C$^+$ cells, anti-Ly6C mAb was used with FITC-conjugated goat anti-rat IgG secondary Ab (green). Original magnification, ×200)

Expression of L-selectin, which is shed during transmigration and rapidly gained back in the lymph node after homing (29), among the transferred, labeled T cells was the same before and after the homing to lymph nodes. A comparison of Ly6C expression among the labeled cells that were found in various organs indicated that they homed equally well to lymph nodes, liver, and spleen (Fig. 5*B*).

These data suggest that Ly6C is down-regulated during homing and later re-expressed within the lymph node. However, to substantiate this conclusion it was necessary to address two other possibilities. First, that Ly6C$^+$cells found in lymph nodes would represent cells that gained Ly6C after their entry and, second, that Ly6C$^+$ cells would home with slower kinetics, which could account for the decline in Ly6C expression observed immediately after homing. To examine the possibility that Ly6C$^+$ cells in lymph nodes would have homed as Ly6C$^-$ cells and acquired Ly6C expression after their homing, we transferred purified CM-FDA-labeled Ly6C$^-$ cells into mice and allowed them to accumulate in lymph nodes for 18 h. Even after this period, transferred Ly6C$^-$ CD8 T cells had remained Ly6C$^-$ in the recipients' lymph nodes and demonstrated no induction of Ly6C expression (5% on purified cells before transfer vs 5.2 ± 2.0% on transferred cells found in the recipients' lymph nodes after 18 h, respectively). This suggests that after homing to lymph nodes originally Ly6C$^-$ T cells do not acquire expression of Ly6C subsequently in the lymph node.

Ly6C$^+$ and Ly6C $^-$ cells home equally well to lymph nodes in vivo

To test the possibility that the low number of Ly6C$^+$ cells on recently homed lymphocytes is due to their slower homing kinetics, we compared the relative homing efficiency of CD8 T cells depleted of Ly6C cells with that of nondepleted CD8 T cells, 50% of which are Ly6C$^+$ (Fig. 6*A*). The total number of injected cells was equal in each case, meaning that twice as many Ly6C$^-$ cells were injected among T cells depleted of Ly6C$^+$ cells as among nondepleted CD8 T cells. Yet, accumulation of injected T cells

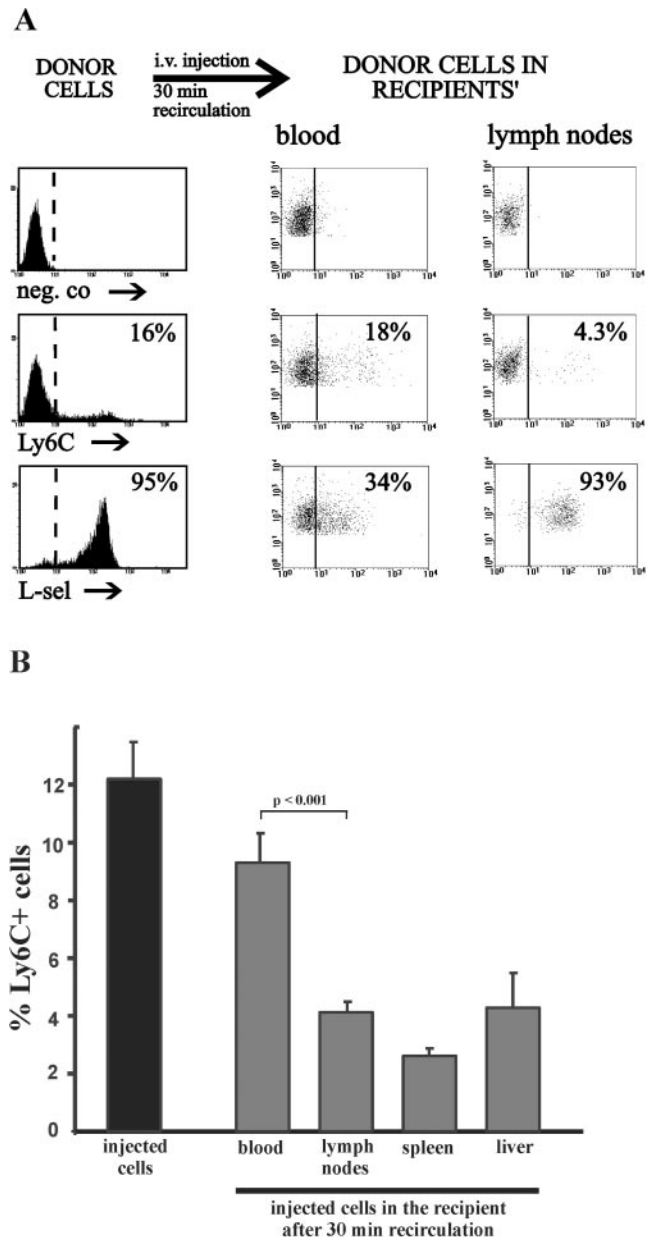

FIGURE 5. Ly6C is lost from the cell surface during in vivo homing. *A,* A flow cytometric analysis of the adoptively transferred lymphocytes. Fluorescently labeled cells were stained for negative control mAb (*top*), Ly6C (*middle*), and L-selectin (*bottom*) before the transfer (histograms) and after a 30-min recirculation in the recipients (dot plots). Lymphocytes isolated from lymph nodes and blood were analyzed. Percentages are indicative of positively stained cells in an individual experiment. The results are representative of three experiments. *B,* The percentage of Ly6C$^+$ cells in the transferred cells before injection (black bar) and the distribution of the transferred Ly6C$^+$ cells within the recipient after a 30-min recirculation (gray bars). The results are an average of another set of three experiments (than in *A*). The *p* value indicates the difference between cells in peripheral blood and peripheral lymph nodes and was calculated using the two-tailed, unpaired Student's *t* test.

into lymph nodes was equal in both groups, ruling out the possibility that Ly6C$^+$ cells would represent cells that home with slower kinetics. Accordingly, the frequency of labeled cells in lymph nodes was 0.19 ± 0.04% and 0.17 ± 0.04% following injection of nondepleted and Ly6C-depleted CD8 T cells, respectively (Fig. 6*B*).

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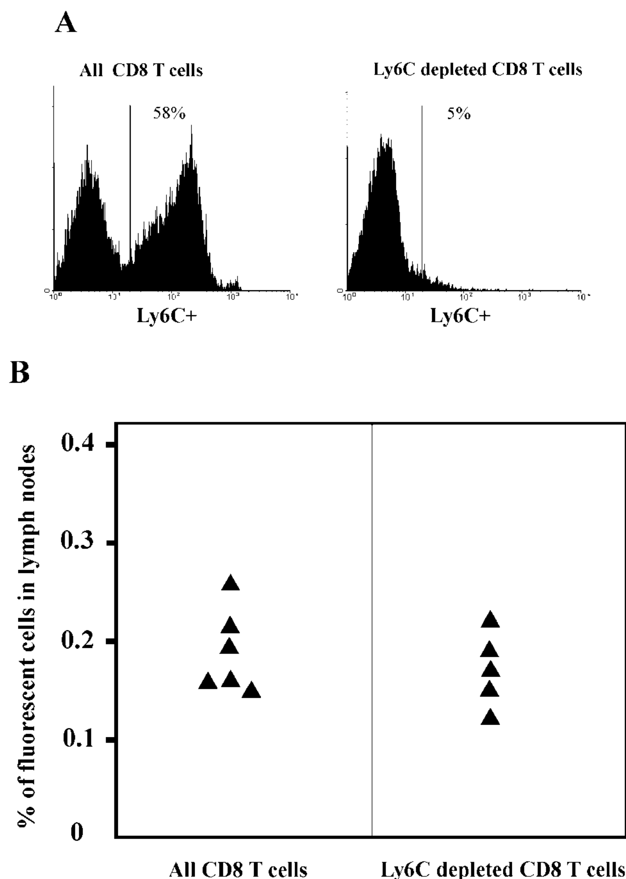

FIGURE 6. $Ly6C^+$ and $Ly6C^-$ CD8 T cells home equally well into lymph nodes in vivo. *A,* A flow cytometric analysis showing Ly6C expression on purified CD8 T cells before (*left*) and after (*right*) Ly6C depletion. *B,* Comparison of the homing capability of Ly6C-depleted CD8 T cells and nondepleted CD8 T cells into the recipients' lymph nodes after a 1-h recirculation.

Discussion

In this study, we have characterized the mechanisms by which Ly6C regulates endothelial adhesion and homing of CD8 T cells and determined the behavior of Ly6C expression on cell surface during receptor cross-linking in vitro and lymphocyte homing in vivo. We demonstrate that Ly6C cross-linking causes clustering of LFA-1 on the surface of CD8 T cells and increases their adhesion to endothelium via LFA-1. We also show that Ly6C expression is down-regulated during lymphocyte homing in vivo and after cross-linking in vitro and that this is due to shedding. Thus, Ly6C appears able to regulate endothelial adhesion of CD8 T cells via activation of LFA-1 in a manner that involves its down-regulation on the cell surface.

In the "life" of a CD8 T cell, LFA-1 provides the adhesion necessary also for events other than egress from vasculature, i.e., interaction with APC and target cell killing. It could be envisioned that a receptor able to repeatedly signal activation of LFA-1 would be suitable to ensure proper function of LFA-1 during these consecutive steps, all critical for the CD8 T cell. Ly6C appears as a receptor that could be able to function in that manner, because activation of LFA-1 via Ly6C cross-linking and lymphocyte homing in vivo are associated with Ly6C down-regulation, and because the distribution of Ly6C expression in lymph nodes suggests that there is a gradient from negative to positive when recently homed cells move further away from areas surrounding HEV. Thus, it appears that Ly6C can be re-expressed after signaling-associated down-regulation which could endow it with a capacity to signal LFA-1 activation repeatedly at temporally distinct steps of CD8 T cell responses. With that capacity, Ly6C could complement the role of chemokines in LFA-1 activation in CD8 T cells.

According to our confocal microscopy analysis, Ly6C cross-linking induces clustering of LFA-1 on CD8 T cells via a mechanism that involves reorganization of actin cytoskeleton. Clustering of LFA-1 also happens in parallel with aggregation of cell surface microdomains or lipid rafts (16), important in the formation of immunological synapses between T cells and APC. These rafts are enriched for GPI-linked molecules, which fits with the idea that a GPI-linked molecule such as Ly6C could be involved in LFA-1 activation via cytoskeletal reorganization (30).

Lateral diffusion of integrins (clustering) has been suggested to be important under conditions of low ligand density and therefore its role is thought to be critical in rapid lymphocyte arrest in physiological conditions. This was recently shown to be effected by chemokines and to require activation of the PI3 pathway (13). In this report, we have shown that also Ly6C induces lateral diffusion of LFA-1 and increases lymphocyte adhesion to endothelium. Lateral diffusion of LFA-1 under these conditions was not inhibited by the PI3 inhibitor wortmannin. Therefore, in our view Ly6C appears as an alternative PI3-independent signaling mechanism for regulating lymphocyte arrest.

The natural ligands for Ly6 family are poorly known and so far only a ligand for Ly6D has been identified (31). To study the role of these molecules in biological processes, mAbs have been used as artificial ligands to mimic the binding of the natural ligand. Several mAbs recognizing different epitopes of Ly6C with either inhibiting or activating capabilities have been identified (8–10, 32). Also cross-linking with a secondary Ab is a commonly used method for artificial activation (33), previously demonstrated also for Ly6C (9). The anti-Ly6C mAb (G10) used in this and our previous study is in itself function inhibiting (24). However, cross-linking with a secondary Ab triggers an opposite effect and an increase in lymphocyte binding. Based on our data, it is conceivable that cross-linking could mimic the effects induced by the binding of the natural ligand, and trigger a signal for cytoskeletal reorganization and clustering of LFA-1. These polarized and very intense clusters may then serve as an area for strong focal adhesion with their endothelial molecular counterparts and provide the means for extravasation.

In another system, internalization of Ly6C after cross-linking was reported to be a prerequisite for signaling, i.e., augmentation of T cell proliferation (34). However, our immunohistological stainings on frozen sections and stainings of permeabilized T cells failed to show any Ly6C intracellularly (our unpublished observations), suggesting that Ly6C was not internalized in our experiments. Since different mAbs that recognize Ly6C either antagonize (8) or stimulate (9, 10) function, it is conceivable that the behavior of the receptor after cross-linking may also vary.

In our capillary flow-adhesion experiments, we first used unselected lymph node lymphocytes and in additional experiments, purified CD8 T cells. Using unselected lymphocytes, Ly6C cross-linking increased adhesion to endothelium by one-fifth (19%), and after purification of CD8 T cells by one-third (32%). According to the literature (35) and our own in vitro experiments, CD8 T cells are two times more adherent to peripheral lymph node HEV than CD4 T cells. Therefore, the effect of Ly6C cross-linking on the adhesion of unselected lymphocytes can be more pronounced than expected on the basis of the proportion of $Ly6C^+$ cells (≈12–16%) among them. This fact also narrows the difference in increased adhesion between unselected lymphocytes and purified CD8 T cells.

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In our short-term in vivo-homing experiments, Ly6C was expressed on unexpectedly few lymphocytes that had homed to lymph nodes. This could be caused by two different phenomena: either Ly6C$^+$ cells are inferior to other lymphocytes in their capacity to home to lymph nodes or their homing capacity is normal but the expression of Ly6C on these cells is (partially) lost during homing. We believe that the latter alternative is true for the following reasons: First, if Ly6C$^+$ cells homed inferiorly, they would need to be overrepresented in blood and/or other organs. However, the numbers of Ly6C$^+$ cells in the spleen, liver, and blood were not elevated, suggesting that they were not accumulating in these sites. Second, pretreatment of lymphocytes with a function-inhibiting anti-Ly6C mAb before injection in the tail vein inhibits lymphocyte homing to lymph nodes significantly (24). This effect would be unlikely if cells that express Ly6C were unable to home efficiently. Third, our in vitro experiments indicated that cross-linking induces shedding of Ly6C from the cell surface, suggesting that the receptor can be lost from the cell surface. Fourth, in immunostainings of lymph node sections, Ly6C reactivity is detected in T cell areas excluding perivascular areas surrounding HEV, suggesting that Ly6C cells are readily trafficking through lymph nodes but that lymphocytes which have recently emigrated are Ly6C$^-$. Fifth, the number of cells that accumulated in lymph nodes during 1 h following the injection of equal numbers of either uniformly Ly6C$^-$ cells (obtained by depletion of Ly6C$^+$ cells) or of a 1:1 mixture of Ly6C$^-$ and Ly6C$^+$ cells (as occurs naturally among CD8 T cells) was the same, ruling out a major difference in the homing efficiency of Ly6C$^+$ and Ly6C$^-$ cells. Therefore, we propose that Ly6C becomes engaged by its natural ligand during lymphocyte homing, that this engagement causes shedding of Ly6C, and that later on in the tissue, cells re-acquire Ly6C on their surface. Interestingly, Ly6C is one of the markers found preferentially on memory T cells (11), many of which accumulate in lymph nodes via afferent lymphatics and thus with slower kinetics. However, "central" memory cells that remain positive for L-selectin and CCR7 expression (36) home through HEV (37) and thus accumulate in lymph nodes with kinetics similar to those of naive T cells. Therefore, efficient homing of Ly6C$^+$ cells as reported here would be consistent with the idea that many of the Ly6C$^+$ cells represent central memory cells. Some of the Ly6C$^+$ cells could also be naive, because naive CD8 T cells undergoing homeostatic proliferation in the absence of specific Ag recognition (as in lymphopenic hosts) gradually acquire Ly6C expression (38).

Although the group of molecules, other than chemokine receptors, that are able to induce LFA-1 activation under in vitro conditions is relatively large, evidence of their involvement in LFA-1 activation in vivo is sparse. According to our results, Ly6C could have significance as a signaling molecule of LFA-1 activation also under in vivo conditions. This notion is supported by the fact that Ly6C and LFA-1 both play a role in T cell proliferation, cytokine production, and cytolytic functions of cytotoxic T lymphocytes. Taken together, these facts suggest that Ly6C may repeatedly function as an activator of LFA-1 during distinct steps of immune responses involving CD8 T cells.

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