

# Rap1 translates chemokine signals to integrin activation, cell polarization, and motility across vascular endothelium under flow

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Chemokines arrest circulating lymphocytes within the vasculature through the rapid up-regulation of leukocyte integrin adhesive activity, promoting subsequent lymphocyte transmigration. However, the key regulatory molecules regulating this process have remained elusive. Here, we demonstrate that Rap1 plays a pivotal role in chemokine-induced integrin activation and migration. Rap1 was activated by secondary lymphoid tissue chemokine (SLC; CCL21) and stromal-derived factor 1 (CXCL4) treatment in lymphocytes within seconds. Inhibition of Rap1 by Spa1, a Rap1-specific GTPase-activating protein, abrogated chemokine-stimulated lymphocyte rapid adhesion to endo-

thelial cells under flow via intercellular adhesion molecule 1. Expression of a dominant active Rap1V12 in lymphocytes stimulated shear-resistant adhesion, robust cell migration on immobilized intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, and transendothelial migration under flow. We also demonstrated that Rap1V12 expression in lymphocytes induced a polarized morphology, accompanied by the redistribution of CXCR4 and CD44 to the leading edge and uropod, respectively. Spa1 effectively suppressed this polarization after SLC treatment. This unique characteristic of Rap1 may control chemokine-induced lymphocyte extravasation.

## Introduction

Leukocyte emigration is multistep processes mediated by adhesive interactions with the vascular endothelium. Orchestrated by chemokines and adhesion molecules, these interactions sequentially result in rolling, firm adhesion, and transmigration through the endothelial layer (Springer, 1995; Butcher and Picker, 1996). Firm attachment to the

endothelium under flow is mediated by leukocyte integrins, typically the  $\beta 2$  and  $\alpha 4$  subtypes (Springer, 1995; Butcher and Picker, 1996). Chemokines presented on the endothelium in lymphoid and inflammatory tissues rapidly up-regulate the adhesive activity of leukocyte integrins, enabling integrin-dependent adhesion of circulating lymphocytes to the endothelium at these sites (Butcher et al., 1999). Chemokines also induce a distinct polarized cell morphology and cell surface receptor distribution, which also facilitates cell migration (Sanchez-Madrid and del Pozo, 1999). Adhering lymphocytes migrate over the endothelium under the control of chemokine promigratory signals and adhesion molecules, usually initiating emigration at the intercellular junction. In addition, fluid shear stresses are required for rapid and efficient transmigration of lymphocytes (Cinamon et al., 2001). Thus, the roles of chemokines in lymphocyte trafficking can be subdivided into the rapid activation of integrins, the induction of attached lymphocyte migration, and the facilitation of shear-dependent lymphocyte transmigration across the endothelium.

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\*Abbreviations used in this paper: HUVEC, human umbilical vascular endothelial cells; ICAM-1, intercellular adhesion molecule 1; LFA-1, lymphocyte function-associated antigen 1; LN, lymph node; PTX, pertussis toxin; SDF-1, stromal-derived factor 1; SLC, secondary lymphoid tissue chemokine; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late antigen 4.

Key words: chemokines; LFA-1; ICAM-1; lymphocyte; transmigration

Chemokines signal through heptahelical receptors that activate pertussis toxin (PTX)\*-sensitive Gi-type heterotrimeric G proteins. Gi protein-linked receptors trigger a diversified cascade of second messengers (Thelen, 2001). Although PI3K is important in neutrophil chemotaxis, lymphocytes did not show a significant dependence on this mediator (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000). Inhibition of PI3K activity has little effect on lymphocyte homing in vivo (Constantin et al., 2000) or on transendothelial migration in laminar flow chambers (Cinamon et al., 2001). These findings imply that chemokine receptors can induce lymphocyte chemotaxis through PI3K-independent pathways.

The small GTPase Rap1 is a potent inside-out signal that functions in a distinct manner from PI3K and PKC, and increases the adhesive activity of both lymphocyte function-associated antigen 1 (LFA-1) and very late antigen 4 (VLA-4; Katagiri et al., 2000; Reedquist et al., 2000). Rap1 activation occurs in response to a variety of external stimuli (Bos et al., 2001), including T cell receptor engagement (Katagiri et al., 2002), CD31 stimulation (Reedquist et al., 2000), and CD98 ligation (Suga et al., 2001). In this paper, we investigate the possibility that Rap1 is involved in lymphocyte adhesion and transmigration by chemokines. We show that rapid Rap1 activation by chemokines is required for adhesion and transmigration of lymphocytes through endothelium. Furthermore, Rap1 has the unique functions that stimulate robust cell migration and induce lymphocyte polarization. Our work suggests that Rap1 plays a pivotal role in lymphocyte trafficking.

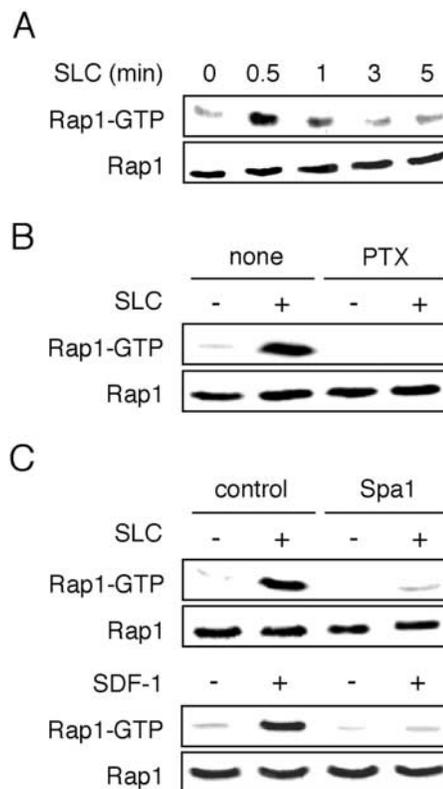
## Results

### SLC stimulates rapid Rap1 activation in lymphocytes

To examine the activation of Rap1 in primary lymphocytes, mouse lymph node (LN) cells were treated with secondary lymphoid tissue chemokine (SLC). Rap1 activation was subsequently measured with the pull-down assay using a GST-RalGDS-RBD fusion protein (Zwartkruis et al., 1998; Fig. 1 A). Rap1 was maximally activated by SLC at 30 s, the earliest time point measurable, but down-regulated to basal levels over the minutes after stimulation (Fig. 1 A). Rap1 activation by SLC was completely inhibited by PTX treatment, indicating that Rap1 activation is linked to Gi protein signaling (Fig. 1 B). Stromal-derived factor 1 (SDF-1) also induced rapid Rap1 activation in LN cells (Fig. 1 C).

### Rap1 triggers rapid LFA-1-mediated adhesion to ICAM-1 induced by chemokines

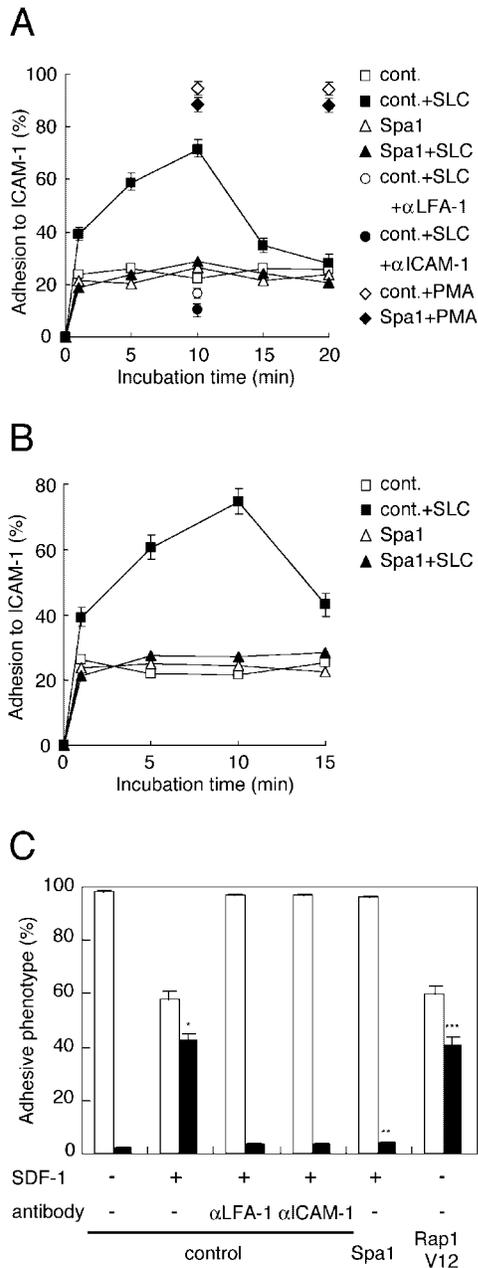
We examined the requirement of Rap1 activation for integrin-dependent adhesion. Soluble SLC induced the transient shear-resistant adhesion of LN cells to immobilized intercellular adhesion molecule 1 (ICAM-1; Fig. 2 A), an effect that was blocked by treatment with either anti-LFA-1 or anti-ICAM-1 antibodies. Increased adhesion was first detected at 1 min and peaked at 10 min, but was quickly down-regulated to basal levels after 15 min. To examine the induction of adhesion after Rap1 activation, we inhibited Rap1 activation by overexpressing Spa1, a Rap1-specific GTPase-activating protein (Kurachi et al., 1997), in lym-



**Figure 1. Chemokine-induced Rap1 activation in lymphocytes.** (A) Rap1 activation by SLC. LN cells were treated with 100 nM SLC for the indicated time (min). GTP-bound Rap1 was detected with pull-down assays using GST-RalGDS-RBD (top). Total Rap1 is shown (bottom). (B) PTX treatment prevented Rap1 activation. LN cells were cultured for 2 h in 50 ng/ml of PTX, then stimulated with 100 nM SLC for 30 s and analyzed for Rap1 activation as above (A). (C) Spa1-inhibited Rap1 activation. Lymphocytes infected with an adenovirus encoding GFP alone (control) or Spa1 (Spa1) were stimulated with either 100 nM SLC or 100 nM SDF-1 for 30 s and analyzed for Rap1 activation as above. Infection efficiencies as indicated by GFP expression were ~80%. Rap1 activation was reduced to 20% of the control levels, as estimated by an image analyzer (model LAS1000; Fuji).

phocytes. LN cells derived from transgenic mice in which the adenovirus receptor (coxsackie adenovirus receptor) is only expressed in T cells (Wan et al., 2000) were infected with adenovirus expressing either GFP alone or Spa1. Approximately 80% of the infected cells, primarily Thy-1-positive T cells, expressed GFP (unpublished data). Rap1 activation after a 30-s SLC stimulation in Spa1-expressing cells was reduced to 20% of the levels observed in control cells (Fig. 1 C). The degree of Rap1 suppression correlated with the infection efficiency, suggesting that Rap1 activation is markedly inhibited in Spa1-expressing cells. Then, we examined the effect of Spa1 expression on lymphocyte adhesion to ICAM-1 after SLC stimulation (Fig. 2 A). Spa1-expressing T cells did not adhere in response to soluble SLC treatment. PMA-stimulated adhesion was not affected in Spa1-expressing cells, indicating that Spa1 specifically inhibits chemokine-induced adhesion (Fig. 2 A).

We also examined the induction of lymphocyte attachment by immobilized SLC and the effect of Spa1 on this



**Figure 2. Lymphocyte adhesion under shear stress.** (A) SLC-stimulated adhesion to immobilized ICAM-1. Lymphocytes infected with the control or Spa1-encoding adenovirus were incubated in the absence or presence of either 100nM SLC or 10 ng/ml PMA, at 37°C for the indicated time on ICAM-1, and then washed with shear stress at 2 dyne/cm<sup>2</sup> for 1 min. Attachment of SLC-stimulated control cells treated with anti-LFA-1 or anti-ICAM-1 antibody is also indicated at the 10-min time point. Quantitated numbers of GFP-positive attached cells were counted and expressed as a percentage of the input GFP-positive cells. PMA-stimulated attachment is indicated for the 10- and 20-min time points. The data are representative of three independent experiments. (B) Spa1-inhibited lymphocyte adhesion triggered by immobilized SLC. ICAM-1-coated disks were pretreated with 100 nM SLC. Unbound SLC was removed by washing. Lymphocytes infected with either the control or Spa1 adenovirus were incubated on uncoated or SLC-coated ICAM-1 discs at 37°C for the indicated time and then washed with shear stress at 2 dyne/cm<sup>2</sup> for 1 min. GFP-positive attached cells were counted and expressed as a percentage of the input GFP-positive cells. The data are representative of three independent experiments. (C) Attachment

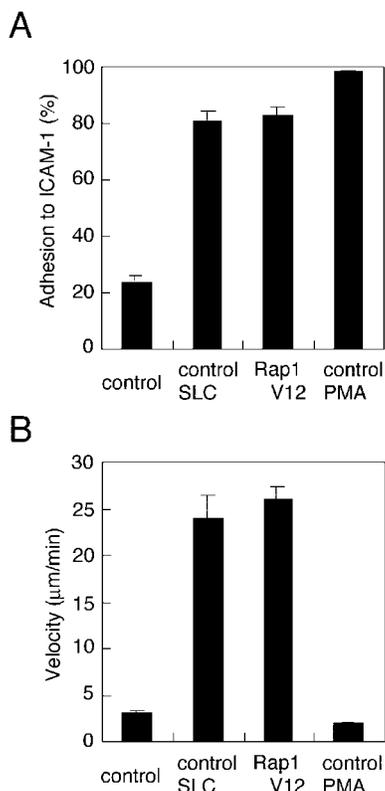
of lymphocytes to endothelial cells under flow. Lymphocytes infected with adenoviruses encoding GFP alone (control), Spa1, or Rap1V12 adenovirus were perfused over a BC1 endothelial monolayer overlaid with 100 nM SDF-1 under shear stress (0.1 dyne/cm<sup>2</sup>) for 10 min. The effect of antibody blocking of LFA-1 or ICAM-1 was shown for lymphocytes infected with the control adenovirus. Lymphocytes that rolled away or attached throughout the shear stress phase were considered as “roll-away” (open bar) and “firm attachment” (closed bar), respectively. These categories were expressed as a percentage of total interacting cells, including rolling, transiently attached, and firmly attached cells. Adenovirus-infected cells, suspended at  $2 \times 10^6$ /ml were perfused into the flow chamber. Approximately 60–70 GFP-positive cells were captured by the BC1 monolayer during a 10-min perfusion (per high power field,  $440 \times 440 \mu\text{m}$ , magnification 20). Data means and SE were determined from four independent experiments with SE. Statistical significance was determined by *t* test. \*,  $P < 0.002$ , compared with unstimulated lymphocytes. \*\*,  $P < 0.002$ , compared with SLC-stimulated control adenoviruses-infected lymphocytes. \*\*\*,  $P < 0.003$ , compared with unstimulated control lymphocytes.

process. ICAM-1-coated discs were pretreated with SLC; unbound SLC was removed by washing. Adenovirus-infected lymphocytes were loaded into the flow chamber and incubated for the time indicated before application of shear stress. Immobilized SLC also triggered rapid, transient adhesion to ICAM-1, comparable to that induced by soluble SLC (Fig. 2 B). Spa1 inhibited the adhesion to ICAM-1 induced by immobilized SLC. Thus, Spa1 expression inhibited adhesion triggered by both soluble and immobilized SLC. Furthermore, we examined the effect of Spa1 inhibition of Rap1 on the arrest of rolling lymphocytes in under-flow adhesion assays using a mouse endothelial cell line, BC1 (Tatsuta et al., 1992). Pretreatment of BC1 with SDF-1 drastically increased firm adhesion of rolling lymphocytes to the BC1 monolayer (Fig. 2 C), as seen with human umbilical vascular endothelial cells (HUVEC; Cinamon et al., 2001). Firm attachment was significantly inhibited by treatment with antibodies against either LFA-1 or ICAM-1. Spa1 expression abrogated firm adhesion of rolling T cells. Conversely, expression of a constitutively activated Rap1 (Rap1V12) in T cells increased adhesion to immobilized ICAM-1 (see following paragraph) and induced T cell arrest on endothelial cells in the absence of SDF-1 (Fig. 2 C). However, neither Spa1 nor Rap1V12 expression in T cells affected rolling or tethering adhesion on BC1 monolayers (unpublished data). These results suggest that Rap1 activation is both necessary and sufficient for chemokine-induced T cell arrest on endothelial layers via the LFA-1–ICAM-1 interaction.

### Activation of Rap1 induced robust cell migration on immobilized ICAM-1

Next, we examined the effect of Rap1V12 on T cell adhesion and migration. Rap1V12 expression in primary T cells increased adhesion to ICAM-1 (Fig. 3 A), as previously seen for T cell clones (Katagiri et al., 2002) or lymphocytes derived from Rap1V12-transgenic mice (Sebzda et al., 2002). Surprisingly, Rap1V12 also stimulated robust cell migration on ICAM-1, comparable to that seen after SLC stimulation (Fig. 3 B). Rap1V12-expressing cells moved on ICAM-1 as

of lymphocytes to endothelial cells under flow. Lymphocytes infected with adenoviruses encoding GFP alone (control), Spa1, or Rap1V12 adenovirus were perfused over a BC1 endothelial monolayer overlaid with 100 nM SDF-1 under shear stress (0.1 dyne/cm<sup>2</sup>) for 10 min. The effect of antibody blocking of LFA-1 or ICAM-1 was shown for lymphocytes infected with the control adenovirus. Lymphocytes that rolled away or attached throughout the shear stress phase were considered as “roll-away” (open bar) and “firm attachment” (closed bar), respectively. These categories were expressed as a percentage of total interacting cells, including rolling, transiently attached, and firmly attached cells. Adenovirus-infected cells, suspended at  $2 \times 10^6$ /ml were perfused into the flow chamber. Approximately 60–70 GFP-positive cells were captured by the BC1 monolayer during a 10-min perfusion (per high power field,  $440 \times 440 \mu\text{m}$ , magnification 20). Data means and SE were determined from four independent experiments with SE. Statistical significance was determined by *t* test. \*,  $P < 0.002$ , compared with unstimulated lymphocytes. \*\*,  $P < 0.002$ , compared with SLC-stimulated control adenoviruses-infected lymphocytes. \*\*\*,  $P < 0.003$ , compared with unstimulated control lymphocytes.



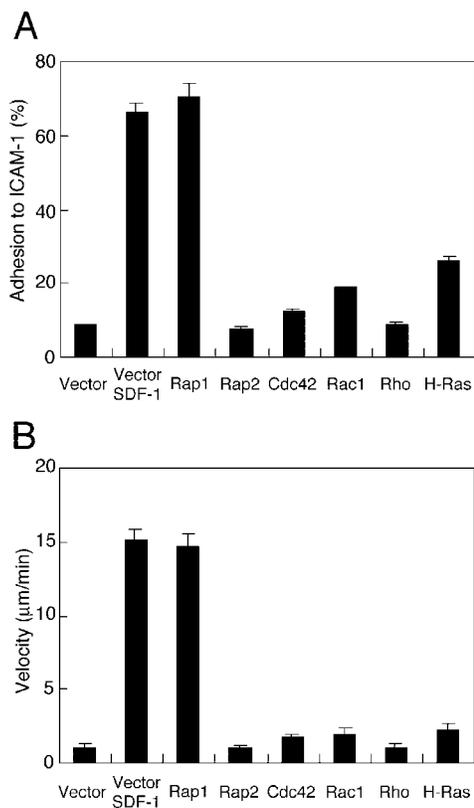
**Figure 3. Random cell migration on ICAM-1.** (A) Adhesion of T cells on immobilized mouse ICAM-1-Fc. The adhesion of T cells infected with control adenovirus (control) stimulated with either 100 nM SLC or 10 ng/ml PMA and Rap1V12-expressing T cells (Rap1V12) are shown. Adhesion to ICAM-1 was <10% in the presence of anti-LFA-1 or anti-ICAM-1 antibodies. The mean and SE of triplicate determinations are shown. (B) Migration velocity of T cells on immobilized mouse ICAM-1-Fc. Migration velocity of T cells infected with control adenovirus (control) stimulated with either 100 nM SLC or 10 ng/ml PMA and Rap1V12-expressing T cells (Rap1V12) are shown. The mean velocity of GFP-positive cells ( $n = 20$ ) was calculated and indicated with SE.

fast as 25  $\mu\text{m}/\text{min}$  (Videos 1 and 2, available at <http://www.jcb.org/cgi/content/full/jcb.200301133/DC1>). PMA treatment increased adhesion levels, but did not stimulate migration (Fig. 3, A and B), in contrast to the stimulatory effect of Rap1 on both adhesion and migration.

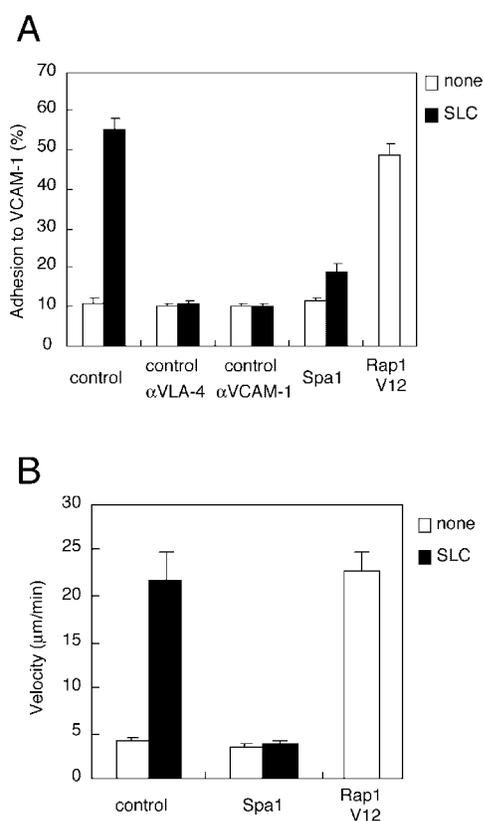
Then, we compared the Rap1V12 promigratory effect with other Ras/Rho family GTPases. We used a proB cell line, BAF/3, reconstituted with human LFA-1 (BAF/LFA-1; Katagiri et al., 2000), which endogenously expresses CXCR4 and migrates on immobilized ICAM-1 in response to SDF-1 stimulation (Fig. 4, A and B). Rap1V12 expression in BAF/LFA-1 stimulated both adhesion and migration (Fig. 4, A and B) at a migratory speed of 15  $\mu\text{m}/\text{min}$ , comparable to that induced by SDF-1. Introduction of constitutively activated Rap2A, Rac, Cdc42, Rho, or H-Ras had little or small stimulatory effect on adhesion. Previously, we demonstrated that mild stimulation of adhesion by Rac1V12 and H-RasV12 required PI3K activity (Katagiri et al., 2000). However, none of these GTPases, with the exception of Rap1V12, enhanced cell migration. Thus, Rap1 has unique characteristics facilitating both adhesion and migration.

### Activation of Rap1 also induced VLA-4-dependent adhesion and migration on immobilized VCAM-1

We also examined the involvement of Rap1 activation in chemokine-induced VLA-4-dependent adhesion to vascular cell adhesion molecule 1 (VCAM-1). Soluble SLC induced shear-resistant adhesion of lymphocytes to immobilized VCAM-1, which was blocked by treatment with either anti-VLA-4 or anti-VCAM-1 antibodies (Fig. 5 A). Spa1 expression greatly reduced SLC-stimulated adhesion to VCAM-1 (Fig. 5 A). Conversely, Rap1V12 expression in T cells increased adhesion to VCAM-1 (Fig. 5 A), as reported in Jurkat cells (Reedquist et al., 2000). As is the case of LFA-1/ICAM-1, Rap1V12 stimulated lymphocyte motility on VCAM-1, the velocity of which was comparable to that stimulated with SLC on VCAM-1 (Fig. 5 B; Videos 3 and 4). The migration velocity of Rap1V12-expressing lymphocytes on VCAM-1 was similar to that on ICAM-1 (Fig. 3 B). The cell motility on VCAM-1 stimulated by SLC was



**Figure 4. Effects of the Ras/Rho family of GTPases on adhesion and migration on immobilized ICAM-1.** (A) Effects of the Ras/Rho family on the adhesion of BAF/LFA-1 to ICAM-1. BAF/LFA-1 cells expressing constitutively activated small GTPases were measured for adhesion on immobilized human ICAM-1-Fc. BAF/LFA-1 transfected with vector alone (vector) were stimulated with 20 nM SDF-1 and measured as in Fig. 3. The mean and SE of triplicate determinations are shown. (B) Effects of the Ras/Rho family on the migration of BAF/LFA-1 on ICAM-1. BAF/LFA-1 cells expressing constitutively activated small GTPases were measured for migration on immobilized human ICAM-1-Fc as described in Materials and methods. BAF/LFA-1 transfected with vector alone (vector) were stimulated with 20 nM SDF-1.



**Figure 5. Cell adhesion and migration on VCAM-1.** (A) SLC-stimulated adhesion to immobilized VCAM-1-Fc. Lymphocytes infected with the control, Spa1-, and Rap1V12-encoding adenovirus were incubated in the absence or presence of 100 nM SLC at 37°C for 10 min on VCAM-1, and then washed with shear stress at 2 dyne/cm<sup>2</sup> for 1 min. Attachment of SLC-stimulated control cells treated with anti-VLA-4 or anti-VCAM-1 antibody is also indicated. The numbers of GFP-positive attached cells were counted and expressed as a percentage of the input GFP-positive cells. The mean and SE of triplicate determinations are shown. (B) Migration velocity of T cells on immobilized VCAM-1-Fc. Migration velocity of T cells infected with control or Spa-1 adenovirus stimulated with or without 100 nM SLC and Rap1V12-expressing T cells are shown. The mean velocity of GFP-positive cells ( $n = 20$ ) was calculated and indicated with SE.

abrogated by Spa-1 expression (Fig. 5 B). Together, these results indicate that Rap1 mediates proadhesive and promigratory effects of SLC through VLA-4 /VCAM-1.

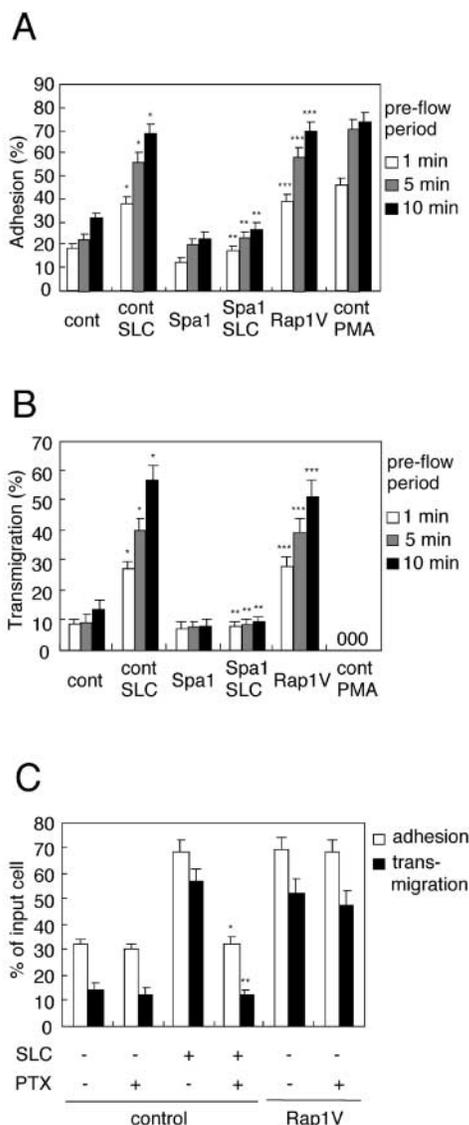
### Rap1 stimulates transmigration of lymphocytes under shear flow

Next, we examined the role of the promigratory effect of Rap1 on transendothelial migration under shear stress using an MBEC4 endothelial cell line. LN cells infected with either control or Spa1-encoding adenovirus were incubated with MBEC4 monolayers in the presence or absence of SLC for the indicated times (1, 5, and 10 min). 20 min of shear stress (2 dyne/cm<sup>2</sup>) was then applied. SLC stimulated shear-resistant adhesion of T cells infected with control adenovirus, enhancing transmigration as early as 1 min (Fig. 6, A and B). The adhesion and transmigration levels were augmented by increasing the period of SLC incubation, with the maximal transmigration level (55% of input cells) at the

10-min time point. The transmigration efficiency reached ~70% of the attached cells under these conditions (Fig. 6, A and B). However, in the absence of shear flow, no lymphocytes transmigrated through the MBEC4 monolayer, demonstrating shear-stress dependency of lymphocyte transmigration, as seen for HUVECs (Cinamon et al., 2001). Experiments using soluble or immobilized SDF-1 stimulation demonstrated similar results, but possessed very low efficiencies of adhesion and transmigration through the MBEC4 monolayer (unpublished data). Spa1 expression in lymphocytes reduced SLC-induced adhesion and transmigration to basal levels (Fig. 6, A and B). Conversely, Rap1V12 expression in T cells augmented both adhesion and transmigration under flow in the absence of SLC (Fig. 6, A and B). The rate of transmigration without shear flow was <10% at any time points measured (unpublished data), indicating that Rap1V12-expressing T cells still requires shear stress for efficient transmigration. The time course and efficiency of Rap1V12-expressing T cell transmigration was similar to those of SLC-stimulated cells (Fig. 6, A and B). These results indicate that Rap1 rapidly induces firm attachment and enhances transmigration, which is consistent with the Rap1 effect on integrin-dependent adhesion and migration (Fig. 3 and Fig. 5). PMA stimulated attachment to endothelial cells, but failed to induce transmigration under flow (Fig. 6, A and B). Although treatment with PTX reduced SLC-stimulated adhesion and transmigration to basal levels, the transmigration induced by Rap1V12 was unaffected (Fig. 6 C). Time-lapse images exhibit the active migration of SLC-stimulated or Rap1V12-expressing lymphocytes over the endothelium before transmigration under shear stress (Fig. 7). In contrast, PMA-stimulated lymphocytes adhered to the endothelium were not motile (Fig. 7). These results paralleled those obtained for adhesion and migration on immobilized ICAM-1 and VCAM-1 (Fig. 3 and Fig. 5), suggesting that cell migration enhancement by Rap1 is crucial for transmigration.

### Rap1 activation triggers cell polarization

Chemokines induce a polarized cell morphology and induce the redistribution of chemokine receptors to the leading edge (Nieto et al., 1997; Gomez-Mouton et al., 2001) and intercellular adhesion molecules, including CD44 (del Pozo et al., 1995), to the uropod. As leukocyte polarization plays an important role in rapid directional movement, we examined the effect of Rap1 activation on this process. Rap1V12 expressing BAF/LFA-1 cells, fixed in suspension, demonstrated a polarized cell shape with a morphologically-defined leading edge and a uropod; control cells were round in shape (Fig. 8). The polarized cell shape was examined with CXCR4 and CD44, leading edge and uropod markers, respectively. Compared with homogenous or intermingled distribution in nonpolarized control cells, CXCR4 and CD44 were segregated, concentrating at the leading edge and uropod, respectively. A similar polarization phenotype concurrent with the redistribution of cell surface receptors was also seen on stimulation with SDF-1 (Fig. 8). Spa1 expression completely suppressed the polarized phenotype induced by SDF-1. These results suggest that Rap1 activation by chemokines induces cell polarization.



**Figure 6. Attachment and transmigration of T cells.** (A) Attachment of T cells to MBEC4 endothelial cells under flow. T cells infected with adenoviruses encoding either GFP alone (cont), Spa1, or Rap1V12 were incubated with or without either 100 nM SLC or 10 ng/ml PMA for 1, 5, and 10 min on MBEC4 monolayers pretreated with TNF $\alpha$  for 24 h. Shear stress was then applied at 2 dyne/cm<sup>2</sup> for 20 min. In the absence of TNF $\alpha$  pretreatment, lymphocyte adherence to MBEC4 endothelial cells was <10% of the input cells (not depicted). Loaded cell numbers were  $2 \times 10^6$  and  $\sim 100$ –120 GFP-positive cells per microscopic field ( $440 \times 440 \mu\text{m}$ ) were accumulated on the MBEC monolayers at each time point. Shear-resistant attachment to the endothelial cells was reduced to less than a half of normal levels after blocking with mAb specific for LFA-1 or VLA-4. Treatment with both antibodies further reduced cell numbers to  $\sim 15\%$  of normal levels. The mean and SE of triplicate experiments are shown. \*,  $P < 0.005$  (1 min), 0.002 (5 min), and 0.003 (10 min), compared with unstimulated control adenovirus-infected lymphocytes. \*\*,  $P < 0.006$  (1 min), 0.003 (5 min), and 0.002 (10 min), compared with SLC-stimulated control lymphocytes. \*\*\*,  $P < 0.001$  (1 min), 0.001 (5 min), and 0.001 (10 min), compared with unstimulated control lymphocytes. (B) Transmigration through MBEC4 endothelial cells under flow. T cells infected with the control, Spa1, or Rap1V12 adenovirus were incubated with or without 100 nM SLC, or with 10 ng/ml PMA as indicated, for 1, 5, and 10 min on the MBEC4 monolayers. Shear stress was then applied at 2 dyne/cm<sup>2</sup> for 20 min. The mean and SE of triplicate experiments are shown.

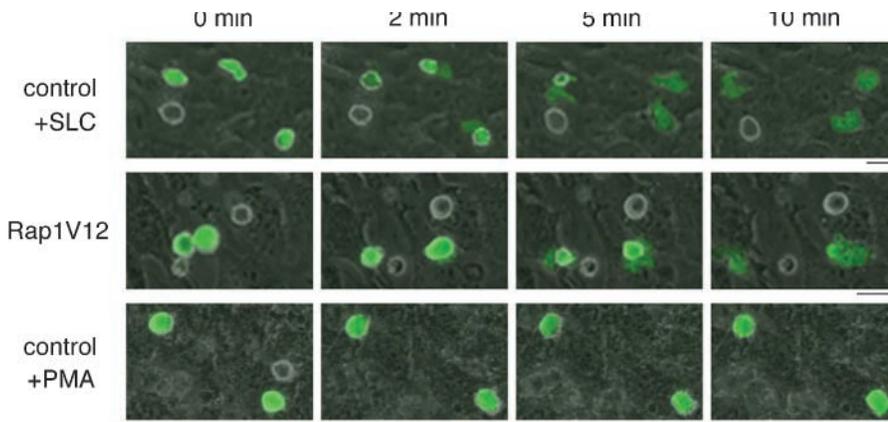
To confirm whether Rap1 is also involved in lymphocyte polarization, we examined the effects of Rap1V12 and Spa1 after their introduction into lymphocytes via adenoviral infection. When stimulated with SLC or SDF-1, lymphocytes were transformed into polarized cell shapes with well-developed leading edges and uropods marked by CXCR4 and CD44, respectively (Fig. 9 A). Control adenovirus-infected lymphocytes, indicated by GFP expression, were similarly transformed by SLC into a polarized cell shape with the development of CD44-marked uropods (Fig. 9 C). Quantitative analysis indicated that  $\sim 60\%$  of stimulated cells exhibited this polarized phenotype (Fig. 9 B). Rap1V12 expression, also indicated by GFP expression, markedly increased the number of polarized lymphocytes with CD44-marked uropods (Fig. 9, B and C), comparable to the levels induced by SLC. Conversely, Spa1 expression reduced the number of SLC-stimulated polarized lymphocytes exhibiting an asymmetric redistribution of CD44 to basal levels (Fig. 9, B and C). Collectively, these results indicate that Rap1 activation triggers lymphocyte polarization even in nonadherent lymphocytes. Thus, Rap1-mediated cell polarization triggered by chemokines does not depend on integrin-mediated adhesion.

## Discussion

Our report is the first to demonstrate the requisite involvement of Rap1 in chemokine-stimulated integrin activation and transendothelial migration. Our work reveals that Rap1 is associated with chemokine-mediated rapid integrin activation, enhancement of cell migration, and cell polarization in lymphocytes. These characteristics of Rap1, not observed for other Ras/Rho GTPases, likely contribute in the induction of lymphocyte extravasation and tissue migration in response to chemokines.

Integrin adhesiveness is regulated by ligand binding affinity and/or lateral mobility/clustering (avidity; van Kooyk and Figdor, 2000). Previous studies indicated that chemokines (SDF-1, SLC, and EBII-ligand chemokine) regulate LFA-1 adhesive activity by modulating avidity and affinity of LFA-1, and demonstrated the existence of a PI3K-independent pathway responsible for the attachment of lymphocytes to high density ICAM-1 molecules or high endothelial venules (Constantin et al., 2000). Rap1V12 up-regulates soluble ICAM-1-Fc binding and a conformational epitope associated with the high affinity state of LFA-1 (Katagiri et

\*,  $P < 0.002$  (1 min), 0.001 (5 min), and 0.001 (10 min), compared with unstimulated control adenovirus-infected lymphocytes. \*\*,  $P < 0.003$  (1 min), 0.001 (5 min), and 0.001 (10 min), compared with SLC-stimulated control lymphocytes. \*\*\*,  $P < 0.01$  (1 min), 0.001 (5 min), and 0.001 (10 min), compared with unstimulated control lymphocytes. (C) PTX had little effect on Rap1V12-induced adhesion and transmigration. Lymphocytes infected with the control or Rap1V12 adenovirus were cultured for 2 h with 50 ng/ml PTX, and were then incubated with or without 100 nM SLC, as indicated, for 10 min on the MBEC4 monolayers. Shear stress was then applied at 2 dyne/cm<sup>2</sup> for 20 min. \*,  $P < 0.005$ , compared with adhesion of SLC-stimulated lymphocytes without PTX pretreatment. \*\*,  $P < 0.002$ , compared with transmigration of SLC-stimulated lymphocytes without PTX pretreatment.



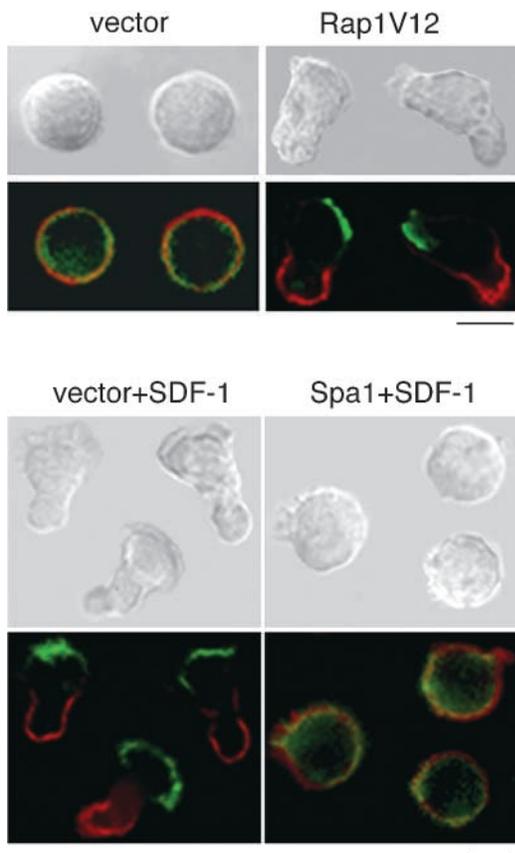
**Figure 7. Time-lapse images of transendothelial migration under flow.** The transmigration of SLC-stimulated (top) or PMA-stimulated (bottom) T cells infected with the control adenovirus and Rap1V12-expressing T cells (middle), was recorded at the indicated times after the induction of shear flow. Composite images of phase-contrast and GFP fluorescence are shown. Both control T cells stimulated with SLC and Rap1V12-expressing cells transmigrated through the endothelial monolayer (became phase-dark) within 10 min. Bars, 20  $\mu$ m.

al., 2000; Reedquist et al., 2000) and clustering (Sebzda et al., 2002). These findings support the notion that Rap1 plays an essential role in chemokine-induced lymphocyte adhesion. Although both Rap1 and PI3K $\gamma$  are thought to function in chemokine-stimulated integrin activation, we did not find a significant effect of PI3K inhibitors on lymphocyte attachment and transmigration through endothelial layers in our experimental system. The relative contribution

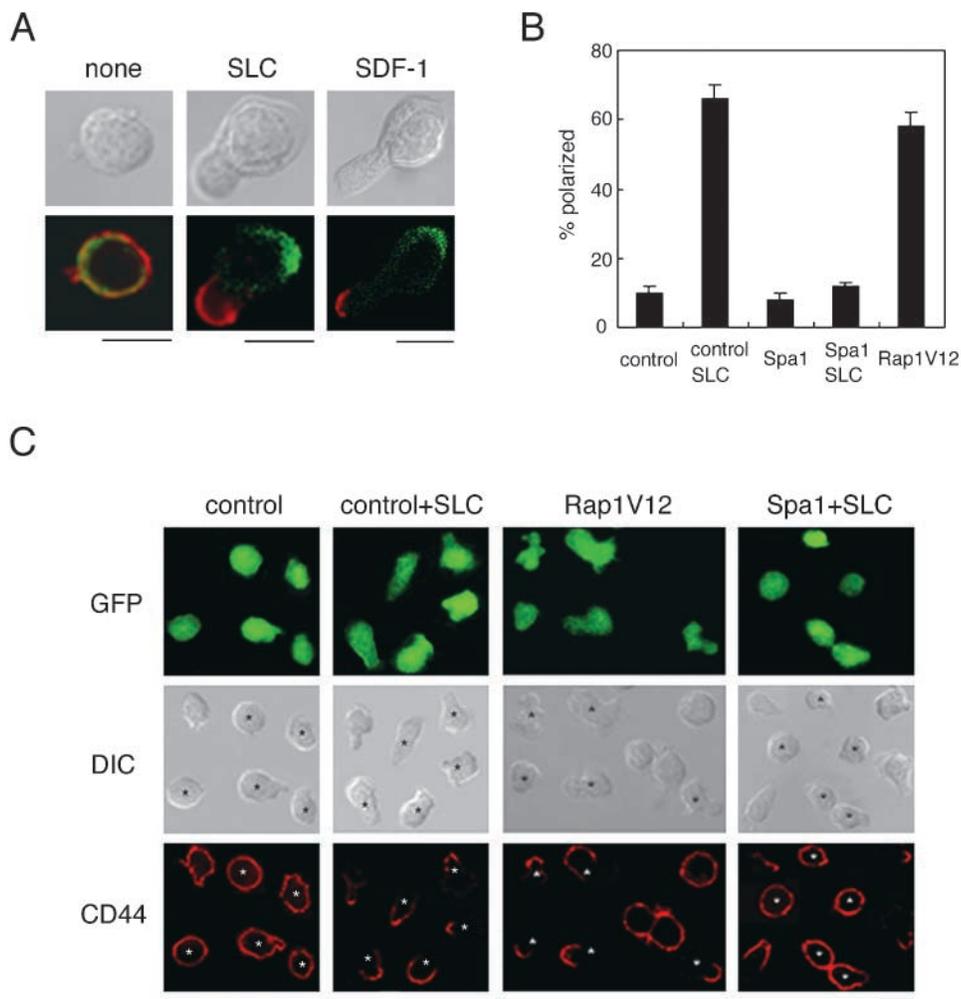
of these signaling pathways may vary according to the expression levels of Rap1 and PI3K $\gamma$  in leukocytes and the density of ICAM-1 on endothelial cells.

Our work demonstrates that Rap1 is the major inside-out signal for LFA-1 and VLA-4 by chemokines, thus playing a critical role in lymphocyte attachment to immobilized ICAM-1, VCAM-1, and endothelial cells under flow. Rap1 activation by chemokines occurs in seconds, which is followed by the integrin-triggering effect of Rap1 in a minute. Thus, the integrin-triggering effect of Rap1 occurs within a time window of chemokine-induced conversion of rolling lymphocytes to firm arrest. It has recently been shown that endothelial chemokines also trigger earlier VLA-4-mediated capture by subsecond modulation of integrin avidity (Grabovsky et al., 2000). We showed the requisite involvement of Rap1 in lymphocyte arrest under the suboptimal shear stress (0.1 dyne/cm<sup>2</sup>). Our experimental system failed to support efficient lymphocyte rolling or tethering at a higher shear stress (1–5 dyne/cm<sup>2</sup>). This precludes us from examining whether Rap1 is also involved in extremely rapid integrin modulation. Therefore, it is still an open question of whether Rap1 could function as a subsecond integrin modulator in lymphocyte arrest. It is also possible that Rap1 activation by apical endothelial chemokines could convert transient lymphocyte attachment to shear-resistant adhesion necessary for the later progression to lymphocyte transendothelial migration.

The Rho family of small GTPases regulates cytoskeletal rearrangements underlying morphological transformations such as lamellipodia, filopodia, and focal adhesion (Hall, 1998). These signal transducers likely contribute to leukocyte adhesion and migration through such rearrangements, but little is known about their contributions to rapid integrin activation after chemokine stimulation. Rho was previously reported to be involved in IL-8-induced adhesion of leukocytes via VLA-4 and Mac1 (Laudanna et al., 1996). However, it remains to be determined whether Rho modulates either integrin affinity or clustering. Rac1 was reported to regulate integrin spreading in T cells, resulting in the enhancement of cell adhesion without affecting integrin affinity (D'Souza-Schorey et al., 1998). In BAF/LFA-1 cells, Rac1V12, Cdc42V12, and RhoV14 expression stimulates membrane ruffling, dendrite extension, or cell rounding, respectively (unpublished data). However, these GTP-



**Figure 8. Polarized morphology and membrane protein redistribution induced by SDF-1 and Rap1V12.** Rap1V12- or Spa1-expressing BAF/LFA-1 cells stimulated with or without 100 nM SDF-1 for 10 min, as indicated, were fixed in suspension and stained for CXCR4 (green) and CD44 (red), as described in Materials and methods. Bars, 10  $\mu$ m.



**Figure 9. Rap1 is involved in lymphocyte polarization.** (A) Asymmetrical morphology and distribution of CXCR4 (green) and CD44 (red) in chemokine-stimulated T cells. T cells were stimulated with 100 nM SLC or 100 nM SDF-1 for 10 min, fixed in suspension, and stained as in Fig. 8. (B) Quantitative analysis of lymphocyte polarization. T cells infected with adenoviruses encoding either GFP alone (control), Spa1, or Rap1V12 adenovirus were incubated without or with 100 nM SLC, as indicated, for 10 min and fixed in suspension. GFP-positive lymphocytes with CD44-marked uropods were quantified (100–150 cells), and are expressed as a percentage of the total GFP-positive cells. The mean and SE of triplicate experiments are shown. (C) Effects of Rap1V12 and Spa1 expression on lymphocyte polarization. T cells infected with the control, Spa1, or Rap1V12 adenovirus were incubated without or with 100 nM SLC as indicated, for 10 min, and fixed in suspension. Images detail GFP fluorescence (top), morphology by differential interference contrast (middle), and CD44 (bottom). Control adenovirus-infected lymphocytes exhibited a homogeneous distribution of CD44 without typical uropods. Polarized cell shape and CD44 redistribution were induced in control lymphocytes by SLC treatment and Rap1V12 expression. Spa1-expressing T cells failed to develop polarized morphology and redistribute CD44 when stimulated with SLC. Asterisks indicate adenovirus-infected cells. Bars, 10  $\mu$ m.

ases stimulated little or only mild adhesion to ICAM-1, which was previously shown to be dependent on PI3K activity (Katagiri et al., 2000). Recent work by McLeod and colleagues showed both Rap1 and Rap2 activation after SDF-1 stimulation of B cell lines and the expression of RapGAPII inhibited chemotaxis toward SDF-1 in Transwell assays using bare membranes (McLeod et al., 2002). It was not clear from this paper whether Rap1 and Rap2 are critically involved in integrin-mediated adhesion and migration induced by chemokines. Although this work could identify a stimulatory effect of Rap2 on chemotaxis, our work could not identify an effect of constitutively active Rap2 on LFA-1 activation and migration on immobilized ICAM-1. Rap2 likely acts in a manner unrelated to integrin-mediated adhesion and migration. Thus, our work indicates the unique

ability of Rap1 to rapidly trigger LFA-1 activation on chemokine stimulation.

Leukocyte emigration from the bloodstream across the microvessel wall into tissues is an essential step in the inflammatory response and lymphocyte homing. This process is based on robust leukocyte cell motility. In addition to an effect on integrin triggering, our paper demonstrates a promigratory effect of Rap1 on lymphocytes. Rap1V12 expression stimulated lymphocyte motility on ICAM-1 and VCAM-1 at similar levels observed for chemokine stimulation. Cell migration requires the coordination of front adhesion and rear de-adhesion. Detachment of the cell rear of Rap1V12- or SDF-1-stimulated migratory cells was impaired by mutation of the tyrosine-based endocytosis motif of the  $\beta$ 2 integrin subunit (Tohyama et al., 2003). This

mutation did not affect LFA-1 adhesive activity, suggesting that detachment is regulated by endocytosis of LFA-1 rather than a switch between on and off states of integrin activity during migration. Therefore, firm attachment does not necessarily hamper cell migration. Our paper demonstrates that the activation of Rap1 induces both integrin-mediated cell adhesion as well as migration.

Chemokines critical for T cell homing such as SLC are distributed on the apical side of high endothelial venule (Stein et al., 2000; Warnock et al., 2000). Thus, it is unlikely that a chemokine gradient across the endothelium stimulates transmigration of T cells *in vivo*. Retention of chemokines on the apical side is instrumental both to prevent dilution of chemokines by the flowing blood and to restrict the site of lymphocyte emigration. Our work indicated that both soluble chemokines and Rap1V12 expression were able to trigger initial arrest and promote subsequent diapedesis. Thus, a chemokine gradient is not necessarily required for transendothelial migration, which is in agreement with other works (Cinamon et al., 2001; Cuvelier and Patel, 2001).

Our paper suggests that haptokinesis, rather than chemotaxis, is important for transmigration. We showed in this paper that Rap1V12 expression in lymphocytes induced robust cell motility and transmigration without exogenous chemokines. PTX treatment did not inhibit transmigration of Rap1V12-expressing lymphocytes, excluding the possibility that Rap1 enhances transmigration by promoting endogenous PTX-sensitive Gi machineries. The transmigration stimulated by soluble chemokines and Rap1V12 still required the application of shear stress, consistent with results observed for lymphocyte transmigration through chemokine-immobilized HUVEC layers (Cinamon et al., 2001). This result suggests that chemokine activation of Rap1 and shear activation of transmigration are two separable and probably sequential processes. Shear stress dependency of transmigration implies that shear-induced mechanotransduction in lymphocytes and/or endothelial cells is required in addition to Rap1 signaling. Alternatively, shear stress may induce cell deformation, helping to redirect free membrane protrusions at the leading edge to junctions in the endothelium.

Given the presence of chemokines on the apical side of the endothelium, it is feasible that attached lymphocytes are stimulated by chemokines to migrate over endothelial cells under the control of the Rap1 promigratory signal, as seen in our work. When migrating lymphocytes reach intercellular junctions, they begin diapedesis between tightly apposed endothelial cells. Although the detailed process of diapedesis is still unclear, several adhesion molecules of the immunoglobulin superfamily, including CD31, CD99 (Schenkel et al., 2002), and JAM-1 (Martin-Padura et al., 1998; Ostermann et al., 2002) that are concentrated at endothelial junctions, have been reported to play a critical role in leukocyte transmigration. Antibody cross-linking of CD31 and CD99 on leukocytes also activates LFA-1 and VLA-4, acting through Rap1 signaling in the case of CD31 (Hahn et al., 1997; Reedquist et al., 2000). Thus, it is conceivable that chemokines and junctional adhesion molecules sequentially activate Rap1, stimulating haptotactic migration through endothelial cell layers.

The acquisition of front-rear polarity is critical for cell migration. Chemokines induce lymphocyte polarization, associated with the development of both a leading edge and uropod. The polarized characteristics induced by chemokines and Rap1V12 are indistinguishable in terms of both morphology and cell surface receptor distribution, and appear to be inherent to lymphocytes because it occurs without the spatial cues such as adhesion or chemokine gradients. Many lines of evidence indicate that the Rho family of GTPases is involved in leukocyte chemotaxis through cytoskeletal remodeling and cell polarity (Sanchez-Madrid and del Pozo, 1999; Worthylake and Burridge, 2001). Constitutively activated Rac1, Cdc42, or Rho failed to stimulate cell polarization and migration in our system. This result is consistent with previous reports demonstrating their inhibitory effects on both CSF-1-stimulated macrophage chemotactic migration (Allen et al., 1998) and lymphocyte polarization (del Pozo et al., 1999). As yet, no constitutively active Ras/Rho family GTPases, with the exception of Rap1, have been reported to stimulate lymphocyte polarization and migration, suggesting a unique function for Rap1 in lymphocyte polarization and migration. The relationship of Rap1 with cell polarity determination and migration was previously indicated from studies in yeast (Chant and Stowers, 1995) and flies (Asha et al., 1999). In budding yeast, Bud1 (closest homologue to Rap1) determines the bud site by recruiting polarity-determining factors, such as Cdc42 and Cdc24 (a guanine exchange factor for Cdc42; Gulli and Peter, 2001). However, Rap1 does not directly activate Cdc42 or Rac in lymphoid cells (unpublished data). There is little evidence indicating a pathway triggered by chemokines inducing the hierarchical activation of GTPases. Chemokines may activate Rap1 and Rho family GTPases in parallel. It is also conceivable that Rap1 may control spatial regulation of Rho family GTPases and regulatory proteins, as implied from studies in budding yeast.

Our paper demonstrates the critical contributions of Rap1 to integrin activation, enhancement of integrin-mediated migration, and cell polarization, resulting in chemokine-triggered lymphocyte transmigration. The unexpected relationship between integrin triggering and cell polarization and migration suggests that Rap1 governs not only integrins, but also lymphocyte migration machinery. The mechanism by which Rap1 accomplishes this task awaits further study. In particular, the identification of Rap1 effector molecules is crucial to clarify whether Rap1 controls these biological effects through single or multiple biochemical events, which will provide important information facilitating a better overall understanding of Rap1 function.

## Materials and methods

### Antibodies

FD441.8 (anti-mouse LFA-1) and YN1/1 (anti-mouse ICAM-1) was obtained from the American Type Culture Collection. PS/2 (anti-mouse VLA-4; Miyake et al., 1992) was a gift from K. Miyake. MVCAM-A,429 (anti-mouse VCAM-1) and anti-mouse CD44 were purchased from BD Biosciences. Alexa<sup>®</sup> 546-conjugated goat anti-rat IgG was purchased from Molecular Probes, Inc. FITC-conjugated goat anti-human IgG was purchased from ICN Biomedicals. Anti-Rap1A was purchased from Transduction Laboratories.

### Pull-down assays

LN cells from Balb/c mice suspended at  $5 \times 10^7$ /ml in RPMI 1640 were stimulated with 100 nM mouse SLC or SDF-1 (R&D Systems) at 37°C for the indicated times, and were stopped by resuspending the cells in 1% Triton X-100 containing lysis buffer (Katagiri et al., 2002). Active GTP-bound Rap1 was measured using a GST-RalGDS-RBD fusion protein as described previously (Franke et al., 1997).

### Spa1 or Rap1V12 expression in lymphocytes via adenovirus

Spa1 and Rap1V12 were expressed in lymphocytes using adenoviruses produced according to the manufacturer's instruction (AdEasy™ adenoviral vector system; Stratagene). LN cells from transgenic mice carrying the adenovirus receptor expressed in T cells (Wan et al., 2000) were infected with recombinant adenoviruses, cultured in RPMI 1640 containing 10% FCS and 50  $\mu$ M 2-ME with 20  $\mu$ g/ml 2C11 for 2 h, and then with 5 U IL-2 for 36 h. Infection efficiencies were estimated by lymphocyte GFP expression levels with FACSCalibur™ (Becton Dickinson). Greater than 80% of the lymphocytes constantly expressed GFP.

### Plasmids and transfection

BAF/LFA-1 cells were transfected by electroporation with pcDNA3 (Invitrogen) containing FLAG-Spa1, or T7-Rap1AV12 (Katagiri et al., 2000), Cdc42V12 (a gift from Dr. S. Hattori, University of Tokyo, Tokyo, Japan), and Rap2AV12 (a gift from Drs. H. Kitamura and M. Noda, Kyoto University, Kyoto, Japan). H-Ras, Rac, and Rho were described previously (Katagiri et al., 2000).

### Adhesion to immobilized ICAM-1 and VCAM-1

SLC-induced adhesion to ICAM-1 and VCAM-1 was measured at 37°C in a parallel plate flow chamber (FCS2 system; Biopetech). 0.1  $\mu$ g/ml recombinant mouse ICAM-1 (Katagiri et al., 2002) and 0.05  $\mu$ g/ml VCAM-1 human IgG1-Fc was coated on polystyrene disks that were then blocked with 1% BSA. For production of recombinant mouse VCAM-1, the first three immunoglobulin domains (aa 1–309) of mouse VCAM-1 was obtained from lung mRNA by RT-PCR, and a fusion protein of this fragment and human IgG1-Fc was generated essentially as described previously (Katagiri et al., 2002). The flow chamber was mounted on the stage of an inverted confocal laser microscope (model LSM510, Carl Zeiss MicroImaging, Inc.). Shear stress was generated with an automated syringe pump (Harvard Apparatus).  $2 \times 10^6$  cells suspended in 500  $\mu$ l Lefkovitz L15 medium (GIBCO BRL) containing 0.5% BSA were loaded with or without 100 nM SLC (R&D systems) and were incubated for the indicated time before applying shear stress at 2 dyne/cm<sup>2</sup>. Phase-contrast and GFP fluorescence images were recorded and processed to count cells using ImagePro® Plus software (Media Cybernetics).

### Under-flow adhesion and transmigration assays with endothelial cells

Mouse endothelial cells, BC1 and MBEC4 (Tatsuta et al., 1992), were cultured on fibronectin-coated disks for 2 d. The BC1 and MBEC4 monolayers were pretreated with TNF $\alpha$  for the last 6 and 24 h, respectively. The cultured disk was set in the flow chamber as described above. For lymphocyte attachment under flow, the BC1 monolayer was preincubated with SDF-1 for 10 min and then washed. Lymphocytes were resuspended at  $2 \times 10^6$ /ml in Lefkovitz L15 medium containing 5% FCS, and then loaded into the chamber under shear stress at 0.1 dyne/cm<sup>2</sup> for 10 min. For transmigration, an MBEC4 endothelial cell line was used because MBEC4 efficiently supports transmigration, when compared with BC1 and other mouse endothelial cells. Because lymphocyte rolling on the MBEC4 monolayer treated with TNF $\alpha$  for 24 h was insufficient for lymphocyte accumulation, lymphocytes were loaded into the flow chamber with or without SLC, and incubated on the endothelial monolayer for 1, 5, and 10 min. Then, shear stress was applied at 2 dyne/cm<sup>2</sup> for 20 min. Phase-contrast and GFP fluorescence images were recorded every 10 s. The GFP-positive cells that remained firmly adherent were scored for attachment. The GFP-positive cells that became phase-dark from phase-light during migration over the endothelial monolayer were scored for transmigration.

### Cell migration assays

Random cell migration was recorded at 37°C with a culture dish system for live-cell microscopy ( $\Delta$ T culture dish system; Biopetech). 0.1  $\mu$ g/ml mouse and human ICAM-1-Fc, and 0.05 mg/ml mouse VCAM-1-Fc were coated on thermoglass-based dishes (Biopetech). Cells were loaded in the ICAM-1-Fc- or VCAM-1-Fc-coated dish and mounted on an inverted confocal laser microscope (model LSM510, Carl Zeiss MicroImaging, Inc.). Phase-

contrast and GFP fluorescence images were taken every 15 s for 20 min. GFP-positive cells were traced and calculated for velocity using ImagePro® Plus software (Media Cybernetics).

### Immunofluorescent staining

BAF/LFA-1 or adenovirus-infected T cells were fixed with 3.3% PFA for 15 min at RT. Fixed cells were mounted on poly-L-lysine-coated slides. For double staining, fixed cells were first stained with 1  $\mu$ g/ml SDF-1-Fc, followed by FITC-conjugated goat anti-human IgG (1:100 dilution). Then, CD44 was detected by rat anti-mouse CD44 mAb (1:50 dilution; BD Biosciences), followed by Alexa® 546 conjugate goat anti-rat IgG (1:400 dilution; Molecular Probes, Inc.). Cells were incubated with each antibody for 1 h, and unbound antibodies were removed by washing with PBS/0.1% BSA five times. Stained cells were observed with an inverted confocal laser microscope (model LSM510, Carl Zeiss MicroImaging, Inc.).

### Online supplemental material

Time-lapse images of control and Rap1V12 adenovirus-infected lymphocytes on ICAM-1 or VCAM-1 were collected every 15 s, and videos were created at 6 frames/s using QuickTime Pro (Apple Computer, Inc.). Video 1 shows random migration of GFP-positive lymphocytes (green) infected with control adenovirus on ICAM-1. Video 2 shows random migration of GFP-positive lymphocytes (green) infected with Rap1V12 adenovirus on ICAM-1. Video 3 shows random migration of GFP-positive lymphocytes (green) infected with control adenovirus on VCAM-1. Video 4 shows random migration of GFP-positive lymphocytes (green) infected with Rap1V12 adenovirus on VCAM-1. Online supplemental material available at <http://www.jcb.org/cgi/content/full/jcb.200301133/DC1>.

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