

# Statins inhibit T-acute lymphoblastic leukemia cell adhesion and migration through Rap1b

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## ABSTRACT

Statins are known to inhibit signaling of Ras superfamily GTPases and reduce T cell adhesion to ICAM-1. Here, we address the hypothesis that statins affect T cell adhesion and migration by modulating the function of specific GTPases. Statins inhibit the synthesis of mevalonic acid, which is required for farnesyl and geranylgeranyl isoprenoid synthesis. Ras superfamily GTPases are post-translationally isoprenylated to facilitate their anchorage to membranes, where they function to stimulate signal transduction processes. We demonstrate that 1  $\mu$ M statin inhibits the adhesion, migration, and chemotaxis of the T-ALL cell line CCRF-CEM and TEM of CCRF-CEM and PEER T-ALL cells, but higher statin concentrations are needed to inhibit adhesion of primary T cells. Similar effects are observed following treatment with GGTI-298 or RNA interference-mediated knockdown of Rap1b but not Rap1a, Rac1, Rac2, RhoA, or Cdc42. Statins also alter Rap1 activity and Rap1b localization. Rap1 levels are higher in primary T cells than T-ALL cells, which could explain their reduced sensitivity to statins. These results demonstrate for the first time that the closely related Rap1a and Rap1b isoforms have different functions and suggest that statins or Rap1b depletion could be used to reduce tissue invasion in T-ALL. *J. Leukoc. Biol.* 89: 577–586; 2011.

## Introduction

Statins are widely used to lower cholesterol levels in patients, thereby reducing risk of cardiovascular disease. In particular,

in patients with heart failure, statins are beneficial as a result of their effects on inflammation, oxidative stress, and vascular tone [1, 2]. These drugs have been reported to have anti-inflammatory properties [3, 4], and their possible role in cancer prevention has also attracted attention [5, 6].

Statins act by competitive inhibition of HMG-CoA reductase, resulting in a decrease in mevalonic acid synthesis. Mevalonic acid is an upstream substrate in the formation of cholesterol and also of the farnesyl and geranylgeranyl isoprenoids. These isoprenoids are post-translationally added to a carboxy terminal cysteine of CAAX motifs on proteins, such as small Ras-superfamily GTPases, and facilitate anchorage of these proteins in membranes, where they signal to downstream effectors [7]. By inhibiting synthesis of isoprenoids, statins, therefore, reduce the localization of Ras-superfamily proteins to membranes. A beneficial effect of statins has been reported in colorectal and melanoma cancer prevention, and the inhibition of Ras/Rho GTPase prenylation, leading to their mislocalization, could contribute to the cancer prevention property of statins [8].

Ras and Rho family GTPases are signal transduction proteins involved in cell proliferation, cell survival, cytoskeletal reorganization, membrane trafficking, and motility. These proteins are normally activated by GEFs that promote the switching between an inactive GDP to an active GTP state and inactivated by GAPs, which enhance the intrinsic GTPase activity of the proteins. Some Rho GTPases are also regulated by GDP dissociation inhibitors that bind to prenyl groups and thereby, inhibit membrane localization [9]. Statins have been shown to increase the level of RhoA-GTP yet inhibit its signaling to downstream targets such as Rho-associated kinases [10–12].

T-ALL is a hematological cancer of T lymphocytes [13], for which poor prognosis has often been shown to correlate with the migration and accumulation of leukemia cells in the tissues [14]. To transmigrate from the blood to tissues, leukocytes interact with and adhere to the endothelial cells lining

Abbreviations: CMFDA=5-chloromethylfluorescein diacetate, FFI=farnesyl transferase inhibitor, FN=fibronectin, GAP=GTPase-activating protein, GEF=guanine nucleotide exchange factor, GGTI=geranylgeranyl transferase inhibitor, HMG=3-hydroxy-3-methylglutaryl,  $Mn^{2+}$ =manganese ion,  $MnCl_2$ =manganese chloride, PAK-PBD=p21-activated kinase-p21 binding domain, RalGDS-RBD=Ral-guanine nucleotide dissociation stimulator-Ras-binding domain, Rap=Ras-related protein, SDF-1=stromal cell-derived factor-1, siRNA=small interfering RNA, T-ALL=T-acute lymphoblastic leukemia, TEM=transendothelial migration

The online version of this paper, found at [www.jleukbio.org](http://www.jleukbio.org), includes supplemental information.

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the blood vessels. This involves the activation of integrins such as LFA-1, which is expressed on the leukocyte surface and binds the endothelial cell adhesion molecule ICAM-1 [15]. Chemokines and cytokines can activate the Ras GTPase Rap1, which induces integrin activation through inside-out signaling [16].

Here, we demonstrate that low concentrations of statins inhibit LFA-1 activation on T-ALL cells and consequently, reduce adhesion to ICAM-1 and TEM of T-ALL cells but do not affect migration of primary T cells. Down-regulation of Rap1b but not Rap1a induces similar effects on T-ALL cells, implying that the response to statins is primarily a result of inhibition of Rap1b-mediated activation of LFA-1.

## MATERIALS AND METHODS

### Cell culture and drug treatment

CCRF-CEM (American Type Culture Collection, LGL Promochem, Middlesex, UK), PEER, SUPT-1 and Jurkat (kind gift of Prof. Asim Khwaja, University College London, UK) cells were maintained in RPMI 1640 containing 2 mM glutamine and supplemented with 10% FCS, 1 mM sodium pyruvate, 10 mM Hepes, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were used between Passages 1 and 10. T-lymphoblasts were derived from human PBMCs. Cells were cultured in RPMI 1640 supplemented with 10% human AB serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) and stimulated with 8 µg/ml PHA for 48 h. Cells were subsequently maintained in medium containing 10 U/ml IL-2 (Roche, Mannheim, Germany) and used between Passages 4 and 10. Where indicated, T-ALL cells and T-lymphoblasts were treated for 16 h with 1 µM simvastatin or lovastatin (Calbiochem, Nottingham, UK), 250 µM mevalonolactone (mevalonic acid; Sigma-Aldrich, Dorset, UK), 10 µM FFI (Enzo, Exeter, UK), or GGTI-298 (Calbiochem). Cell viability was determined with 0.4% Trypan blue solution.

HUVECs (Biowhittaker, Wokingham, UK) were cultured in flasks coated with 10 µg/ml FN (Sigma-Aldrich) in endothelial basal medium-2 supplemented with 2% FCS and endothelial cell growth supplements. Cells were used between Passages 1 and 4.

### Transfection

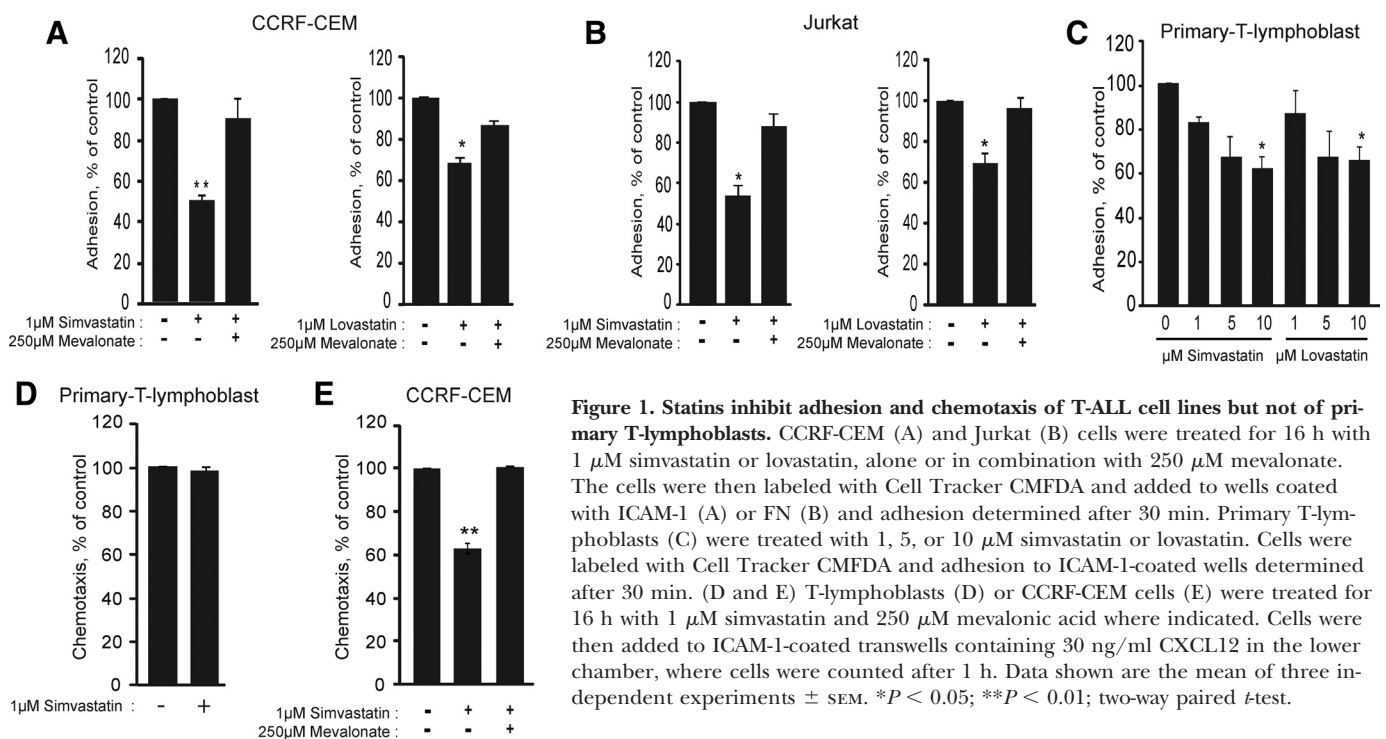
CCRF-CEM cells ( $5 \times 10^5$ ) were transfected by nucleofection (Amaxa Biosystems Nucleofection System, Lonza Biologics, Slough, UK) with 20 µg plasmid DNA or 1.2 µM siRNA (Dharmacon, Lafayette, CO, USA) in 100 µl Nucleofection reagent (Kit C, Lonza Biologics). Cells were used for experiments 48–72 h after siRNA transfection or 24 h after DNA plasmid transfection. pMT2-HA-Rap1b was a kind gift of Prof. Johannes Bos (University of Utrecht, Netherlands).

### Adhesion assays

Ninety-six-well plates were coated overnight with 5 µg/ml ICAM-1 (R&D Systems, Abingdon, UK) or 10 µg/ml FN and then blocked in 1% BSA in PBS for 30 min. Treated cells were centrifuged at 240 g for 4 min and resuspended in PBS at  $10^6$  cells/ml. Cells were then incubated at 37°C for 15 min with 2 µM Cell Tracker CMFDA (Invitrogen, Paisley, UK). Samples were resuspended in warm media, and  $10^5$  cells/well (each sample in triplicate) were incubated at 37°C for 30 min. After washing with PBS, the plate was read on a Fusion-α-fetoprotein plate reader (PerkinElmer, Cambridge, UK) at 485 nm excitation and 525–535 nm emission. Alternatively, images of CMFDA-labeled cells were acquired using Metamorph software (MDS Analytical Technologies, Wokingham, UK) on a Nikon Eclipse TE2000 microscope with a 4× objective. Cell numbers were determined using Volocity software (PerkinElmer).

### TEM

HUVECs were grown to confluency on FN-coated 5 µm transwell filters (Costar, Corning, NY, USA) and then stimulated with 10 ng/ml TNF-α (R&D Systems) for 16 h. After washing,  $2 \times 10^5$  T cells were added onto the HUVECs in the top chamber, and 600 µl medium containing 30 ng/ml



**Figure 1. Statins inhibit adhesion and chemotaxis of T-ALL cell lines but not of primary T-lymphoblasts.** CCRF-CEM (A) and Jurkat (B) cells were treated for 16 h with 1 µM simvastatin or lovastatin, alone or in combination with 250 µM mevalonate. The cells were then labeled with Cell Tracker CMFDA and added to wells coated with ICAM-1 (A) or FN (B) and adhesion determined after 30 min. Primary T-lymphoblasts (C) were treated with 1, 5, or 10 µM simvastatin or lovastatin. Cells were labeled with Cell Tracker CMFDA and adhesion to ICAM-1-coated wells determined after 30 min. (D and E) T-lymphoblasts (D) or CCRF-CEM cells (E) were treated for 16 h with 1 µM simvastatin and 250 µM mevalonic acid where indicated. Cells were then added to ICAM-1-coated transwells containing 30 ng/ml CXCL12 in the lower chamber, where cells were counted after 1 h. Data shown are the mean of three independent experiments  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$ ; two-way paired  $t$ -test.

CXCL12 (R&D Systems) was added to the bottom chamber. Cells in the lower chamber were counted after 1 or 2 h using a Casy Counter (Roche Innovatis, Bielefeld, Germany).

## Timelapse microscopy

HUVECs were grown to confluency on FN-coated coverslips and stimulated with TNF- $\alpha$  for 16 h. Cells were incubated for 8 min with 30 ng/ml CXCL12 and then washed and incubated for 1 h at 37°C. CCRF-CEM cells ( $0.75 \times 10^5$ ) in 500  $\mu$ l CEM media were added to the HUVECs, and time-lapse images were acquired every minute for 1 h using Metamorph software on a Nikon Eclipse TE2000 microscope with a 20 $\times$  objective. Cells were tracked and migration speed ( $\mu$ m/min) determined using ImageJ analysis software.

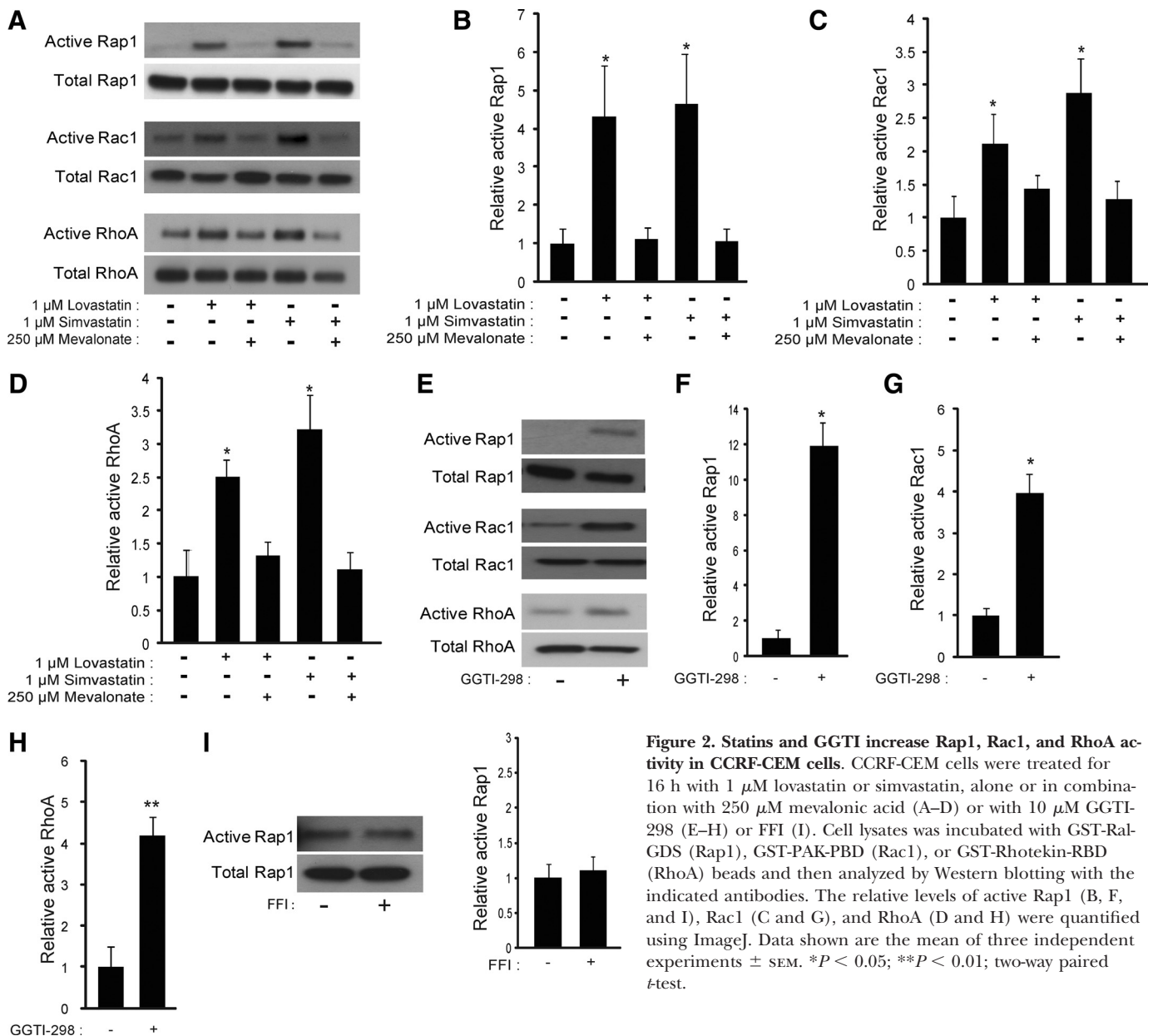
## Flow cytometry

To measure total surface LFA-1, CCRF-CEM cells ( $2 \times 10^5$ ) were incubated for 30 min at 4°C with 1:100 CD11a antibody (BD Biosciences, Oxford,

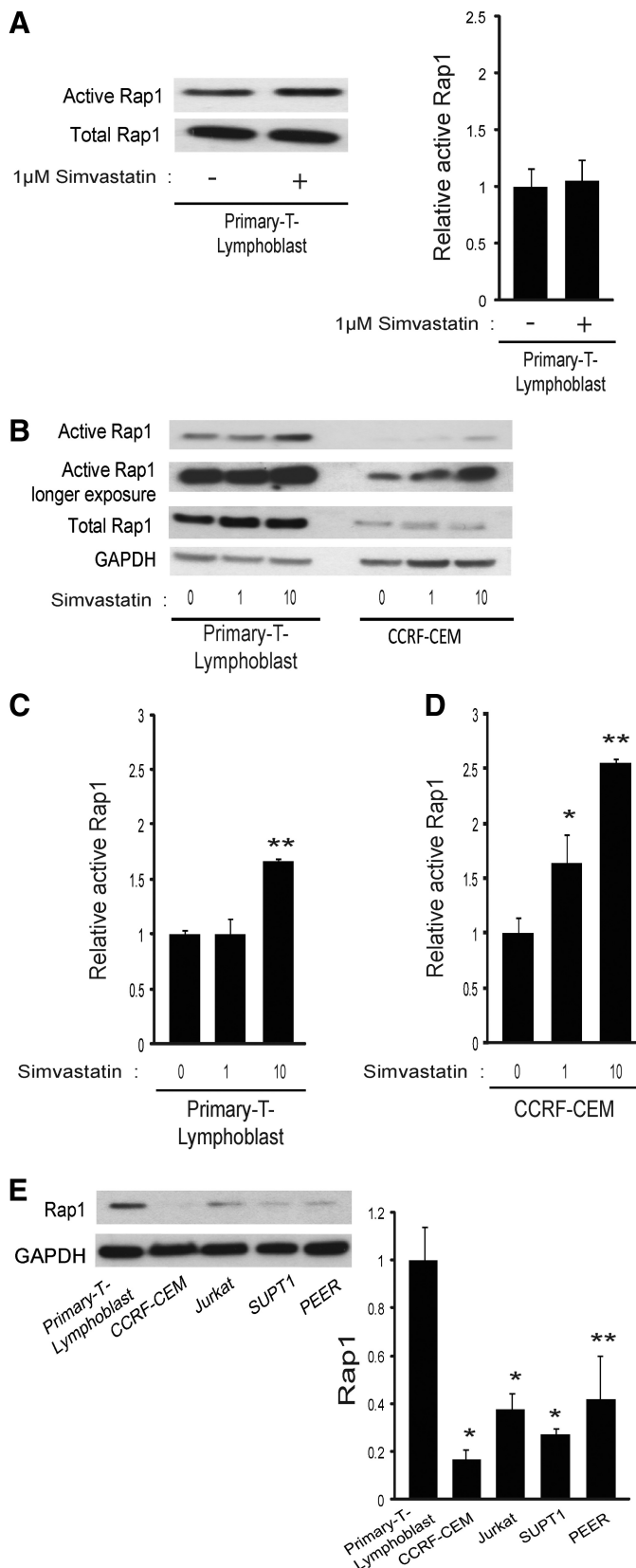
UK) or 1:50 IgG2a as an isotype control (Serotec, Kidlington, UK). To measure surface levels of active LFA-1, cells were incubated for 15 min at 37°C with 200  $\mu$ M MnCl<sub>2</sub> and then for 30 min at 37°C with 1:100 mAb24 (kind gift of Prof. Nancy Hogg, Cancer Research UK London Research Institute, UK). Cells were incubated with Alexa Fluor 488-labeled anti-mouse antibody (Invitrogen) and then analyzed on a Becton Dickinson FACSCalibur machine using the FlowJo program.

## Immunofluorescence

CCRF-CEM cells ( $5 \times 10^5$ ) were transfected with 20  $\mu$ g pMT2-HA-Rap1b. After 24 h, cells were treated with 1  $\mu$ M simvastatin for 16 h and cytospun onto coverslips by centrifugation at 300 g for 5 min. HUVECs ( $1.5 \times 10^5$ ) were seeded onto FN-coated coverslips and treated for 16 h with 1  $\mu$ M simvastatin. Samples were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and then incubated for 1 h with 1:200 mouse anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 1:200 anti-mouse vascular endothelial-cadherin antibody (BD Biosci-



**Figure 2. Statins and GGTI increase Rap1, Rac1, and RhoA activity in CCRF-CEM cells.** CCRF-CEM cells were treated for 16 h with 1  $\mu$ M lovastatin or simvastatin, alone or in combination with 250  $\mu$ M mevalonic acid (A–D) or with 10  $\mu$ M GGTI-298 (E–H) or FFI (I). Cell lysates were incubated with GST-Ral-GDS (Rap1), GST-PAK-PBD (Rac1), or GST-Rhotekin-RBD (RhoA) beads and then analyzed by Western blotting with the indicated antibodies. The relative levels of active Rap1 (B, F, and I), Rac1 (C and G), and RhoA (D and H) were quantified using ImageJ. Data shown are the mean of three independent experiments  $\pm$  SEM. \* $P$  < 0.05; \*\* $P$  < 0.01; two-way paired  $t$ -test.



ences), followed by 1:500 Alexa Fluor 488 anti-mouse antibody (Invitrogen). Coverslips were mounted onto slides using anti-fade mounting medium (Dako, Ely, UK).

### GTPase activity assays

GST-RalGDS-RBD, GST-PAK-PBD, and GST-Rhotekin-RBD were purified from *Escherichia coli* as described previously [17]. CCRF-CEM cells ( $10^6$ /pull-down) were lysed in pull-down buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM  $MgCl_2$ , 1 mM EDTA, 25 mM NaF, 1 mM  $Na_3VO_4$ , 10 µg/ml aprotinin, 100 µM PMSF, and 10% glycerol). Some lysate (40 µl) was retained to determine total GTPase levels. The remaining lysate was incubated with the protein-bound beads on a rotor for 1 h at 4°C. The beads were washed three times in pull-down buffer, boiled in Laemmli sample buffer, and used for Western blotting.

### Western blot analysis

Cells were lysed in 1% SDS, followed by addition of 1% Triton X-100, 25 mM NaF, 1 mM  $Na_3VO_4$ , and 10 mg/ml aprotinin, then incubated for 10 min on ice, and clarified by centrifugation. Lysates were separated on 4–12% Bis-Tris polyacrylamide gels (Invitrogen) or on a 12.5% polyacrylamide gel and then transferred to a nitrocellulose membrane (Millipore, Watford, UK). Membranes were incubated in blocking buffer (5% nonfat dried milk in TBS containing 0.1% Tween-20) for 1 h at room temperature and then incubated for 1 h with 1:1000 dilutions of the following antibodies diluted in blocking buffer: Rap1b (36E1), Rap1a/Rap1b (26B4), RhoA (67B9), or Cdc42 (11A11; Cell Signaling Technology, Beverly, MA, USA); Rap1a (C-17; Santa Cruz Biotechnology); and Rac1 (23A8) or Rac2 (07-604, Millipore). Membranes were then incubated with HRP-conjugated anti-mouse, anti-rabbit, or anti-goat antibodies (GE Healthcare, Chalfont St Giles, UK). Antibodies were visualized using the ECL detection system (GE Healthcare).

## RESULTS

### Statins inhibit adhesion and chemotaxis of T-ALL cell lines but not of primary T-lymphoblasts

To investigate the effect of statin treatment on T-ALL cell adhesion, we first performed an adhesion assay on ICAM-1, which is expressed on the endothelial cell surface and is fundamental in mediating adhesion between the endothelium and leukocytes [18]. We observed a 50% reduction in the binding of CCRF-CEM cells to ICAM-1 following treatment with 1 µM simvastatin or lovastatin (Fig. 1A). Addition of 250 µM mevalonic acid rescued the statin-induced inhibition of adhesion (Fig. 1A). This indicates that statins are acting by inhibiting HMG-CoA reductase, rather than binding directly to the  $\beta_2$  integrin (LFA-1) L-site and thereby, inhibiting adhesion to ICAM-1 [19, 20]. We also observed a

**Figure 3. Primary T-lymphoblasts are less sensitive to statins and have higher Rap1 levels than T-ALL cells.** Primary T-lymphoblasts (A–C) and CCRF-CEM cells (B and C) were treated for 16 h with the indicated concentrations of simvastatin. Cell lysates were incubated with GST-RalGDS beads and then analyzed by Western blotting with Rap1 antibody. Relative levels of active Rap1 were quantified using ImageJ (A, C, and D). (E) Total Rap1 expression in primary T-lymphoblasts and T-ALL cell lines CCRF-CEM, Jurkat, SUPT-1, and PEER was analyzed by Western blotting and relative levels quantified using ImageJ. Data shown are the mean of three independent experiments  $\pm$  SEM. \* $P$  < 0.05; \*\* $P$  < 0.01; two-way paired  $t$ -test.



significant decrease in Jurkat cell adhesion to FN that was rescued by mevalonic acid (Fig. 1B). As T cells bind FN mostly through the  $\beta 1$  integrin [21], this effect cannot be attributed to statin binding to  $\beta 2$  integrin. The effect of statins on adhesion was not a result of changes in T-ALL cell viability (Supplemental Fig. 1A and B).

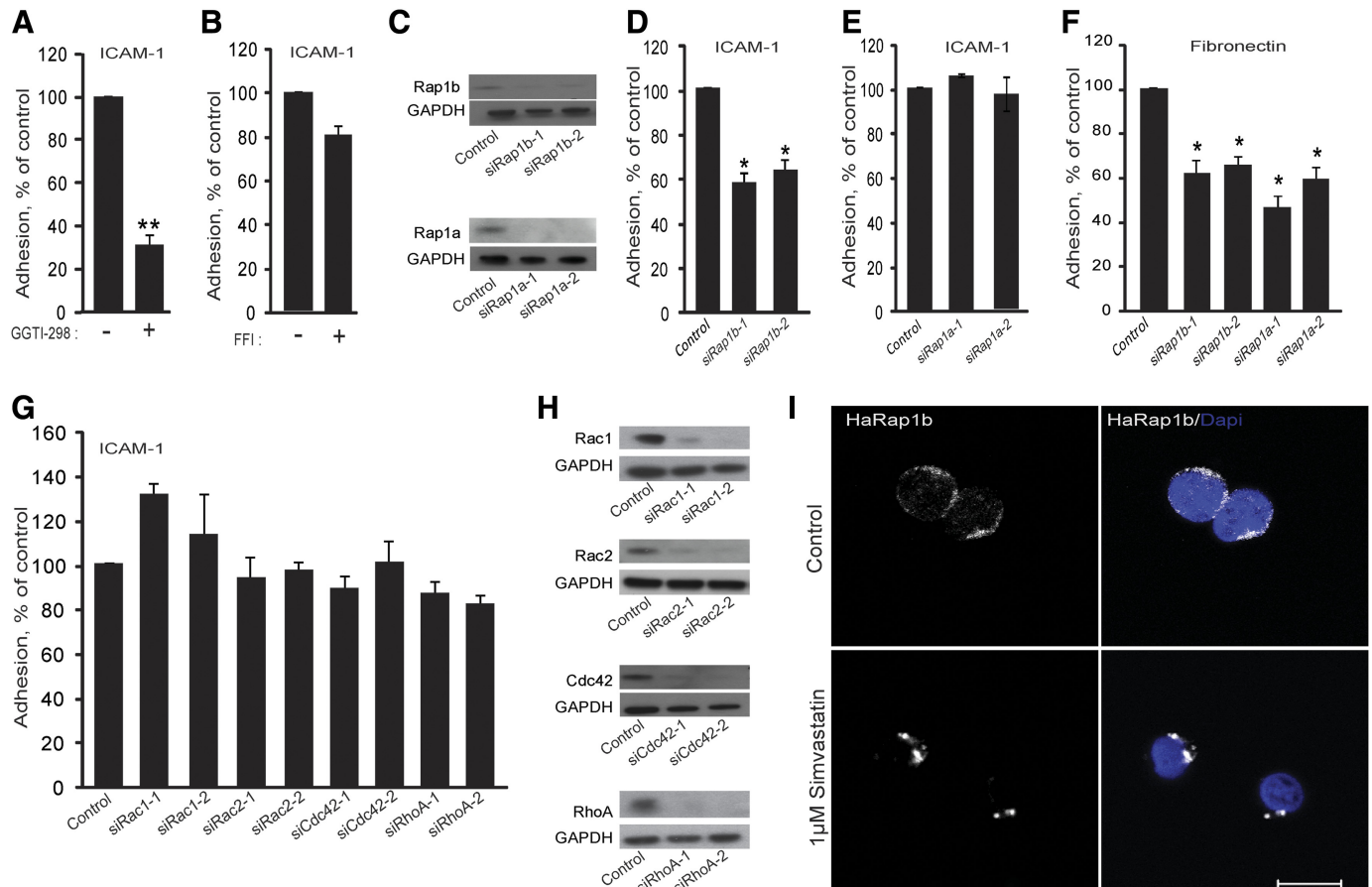
Surprisingly, 1  $\mu$ M simvastatin or lovastatin did not reduce the adhesion of primary T-lymphoblasts, but some inhibition was observed at 10  $\mu$ M statins (Fig. 1C). This was not a result of a difference in adhesion to ICAM-1 between primary T-lymphoblasts and CCRF-CEM cells (Supplemental Fig. 1C). Similarly, 1  $\mu$ M statin treatment did not affect T-lymphoblast chemotaxis toward CXCL12 (SDF-1), whereas it inhibited chemotaxis of CCRF-CEM cells (Fig. 1D and E). The reduction in CCRF-CEM cell chemotaxis was also rescued by mevalonic acid (Fig. 1E), and mevalonic acid by itself had no effect on chemotaxis (Supplemental Fig. 1D). Together, these results indicate that statins inhibit adhesion

and migration of T cells and that T-lymphoblasts are less sensitive to statins than CCRF-CEM or Jurkat T-ALL cells.

### Statins dysregulate Rap1, Rac1, and RhoA activity in CCRF-CEM cells

Given the effect of statin treatment on CCRF-CEM and Jurkat cell adhesion and the known effect of statins in inhibiting protein prenylation, we investigated whether they altered the activity of GTPases. In particular, we focused on Rap1, Rac1, and RhoA, as these GTPases have been implicated in integrin-mediated adhesion [22–24].

Incubation of CCRF-CEM cells for 16 h with simvastatin increased the level of GTP-bound Rap1 (Fig. 2A and B). This was prevented by cotreatment with mevalonic acid (Fig. 2A). Statin treatment induced a shift in the mobility of Rap1 on SDS-PAGE, reflecting the reduced prenylation (Supplemental Fig. 2A). Statins have been reported to increase levels of RhoA-GTP and Rac1-GTP in THP-1 cells



**Figure 4. Rap1b depletion leads to reduced adhesion of CCRF-CEM cells.** CCRF-CEM cells were treated for 16 h with 10  $\mu$ M GGTI-298 (A) or FFI (B). Cells were then labeled with Cell Tracker CMFDA and plated on ICAM-1 and adhesion measured after 30 min. CCRF-CEM cells were transfected with siRNAs targeting Rap1b or Rap1a (C–F) and Rac1, Rac2, Cdc42, or RhoA (G and H). (C and H) Levels of each protein were analyzed by Western blotting. GAPDH was used as a loading control. Blots shown are representative of three independent experiments. (D–G) Adhesion assays were performed on ICAM-1 or FN as indicated. \* $P$  < 0.05; \*\* $P$  < 0.01; two-way paired  $t$ -test. (I) CCRF-CEM cells were transfected with a plasmid encoding pMT2-HA-Rap1b. After 24 h, cells were treated for 16 h with 1  $\mu$ M simvastatin and then cytospun for 5 min at 300 g onto coverslips. After fixation and permeabilization, samples were stained with an anti-HA antibody and with DAPI to stain nuclei and imaged by confocal microscopy. Scale bar, 10  $\mu$ m.

[10], and we also observed an increase in active Rac1 and RhoA in CCRF-CEM cells (Fig. 2A–C). This increase in GTP-loading could be a result of mislocalization of unprenylated proteins, for example, leading to reduced interaction with GAPs. This hypothesis is supported by the observation that treatment with GGTI-298, which prevents prenylation of Rap1, Rac1, and RhoA [25, 26], also resulted in higher levels of GTP-bound Rap1, Rac1, and RhoA (Fig. 2E–H). Rap1, Rac1, and RhoA are post-translationally geranylgeranylated at the C terminus, whereas some other Rho and Ras family proteins are farnesylated [25, 26]. Consistent with this, no changes in GTP-loading of Rap1 were observed following incubation with 10  $\mu$ M FFI, which inhibits farnesyltransferases (Fig. 2I).

### Low concentrations of statins do not dysregulate Rap1 activity in primary T-lymphoblasts

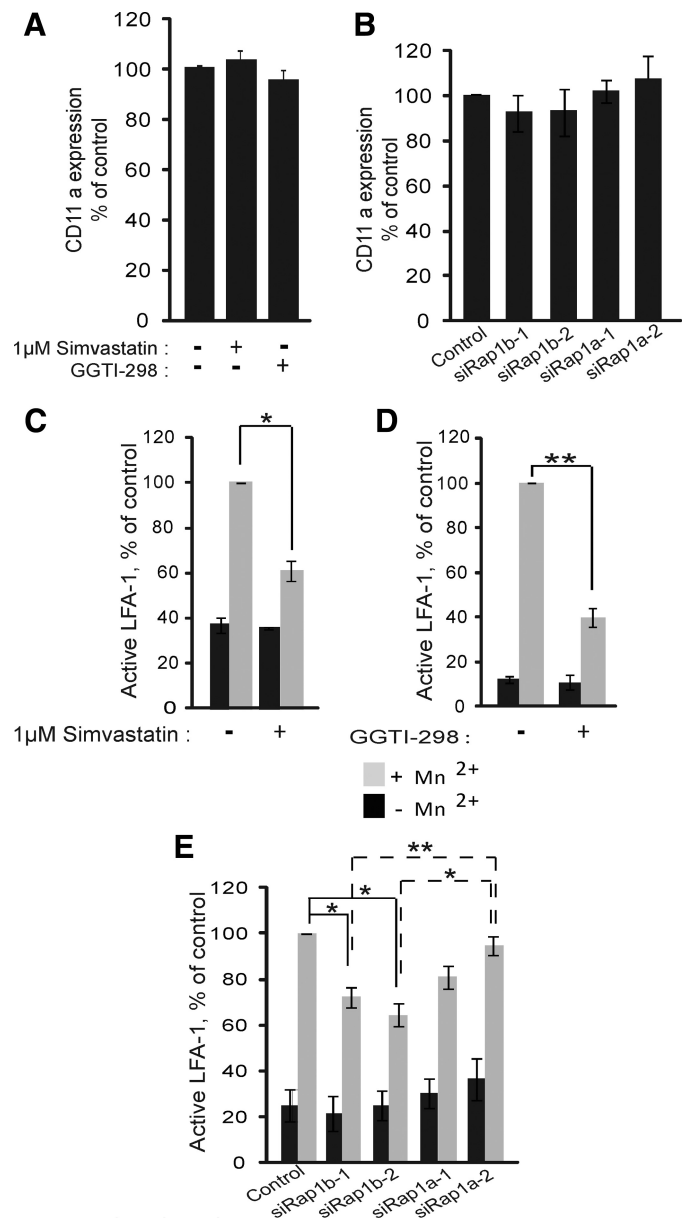
As we had observed that statins at 1  $\mu$ M did not affect the adhesion or chemotaxis of primary T-lymphoblasts (Fig. 1C and D), we next investigated the effect of statins on Rap1 activity in these cells. Treatment of T-lymphoblasts with 1  $\mu$ M simvastatin did not affect Rap1 activity (Fig. 3A). However, 10  $\mu$ M simvastatin did increase Rap1 activity significantly in T-lymphoblasts and induced a further increase in Rap1 activity in CCRF-CEM cells above that induced by 1  $\mu$ M simvastatin (Fig. 3B–D). This analysis also showed that the total level of Rap1 was much lower in CCRF-CEM cells than in T-lymphoblasts. Similarly, Rap1 levels were lower in a panel of T-ALL cell lines compared with primary T-lymphoblasts (Fig. 3E). These results suggest that the higher expression level of Rap1 protein in T-lymphoblasts could be responsible for their reduced sensitivity to statins.

### Rap1b is required for T-ALL cell adhesion and migration

To investigate the importance of prenylation for adhesion, we treated CCRF-CEM cells with 10  $\mu$ M GGTI-298 or FFI. There was a significant reduction of adhesion to ICAM-1 when cells are treated with GGTI-298 compared with untreated or FFI-treated samples (Fig. 4A and B). To determine whether GTPases implicated in integrin-mediated adhesion affected T-ALL cell adhesion to ICAM-1, we used siRNA to knockdown the expression of the two Rap1 isoforms (Rap1a and Rap1b; Fig. 4C) and RhoA, Rac1, Rac2, and Cdc42 in CCRF-CEM cells (Fig. 4H). Rap1b but not Rap1a depletion strongly reduced adhesion to ICAM-1 (Fig. 4D and E). Interestingly, knockdown of Rap1a led to reduced adhesion to FN, suggesting that this isoform is involved in  $\beta$ 1 but not  $\beta$ 2/LFA-1 activation (Fig. 4F). Knockdown of Rac1, Rac2, Cdc42, and RhoA did not affect adhesion (Fig. 4G). Interestingly, Rap1a and Rap1b levels were higher in T-lymphoblasts than in CCRF-CEM and Jurkat cells (Supplemental Fig. 1C), indicating that both Rap1a and Rap1b contribute to the increase in total Rap1 observed (Fig. 3E).

As statins can affect the localization of GTPases through inhibiting prenylation, we examined the effect of simvastatin on

Rap1b localization in CCRF-CEM cells. Rap1 has been shown previously to be activated by the chemokine CXCL12 [16]. Simvastatin treatment altered the localization of Rap1b and induced its accumulation on multiple punctate structures in the cytoplasm in CXCL12-stimulated cells (Fig. 4I). These might be endosomes, similar to the localization of Rap1a, prior to translocation and activation at the plasma membrane [27, 28].



**Figure 5. Statins, GGTI, and Rap1b regulate LFA-1 integrin activity.** CCRF-CEM cells were treated for 16 h with 1  $\mu$ M simvastatin (A and C) or 10  $\mu$ M GGTI-298 (A and D) or transfected with siRNAs targeting Rap1b or Rap1a (B and E). Cells were then stained for  $\alpha$ L integrin (CD11a; A and B) or were incubated for 15 min with 200  $\mu$ M MnCl<sub>2</sub> (Mn<sup>2+</sup>) and then stained for active LFA-1 with mab24 (C–E). Cells were analyzed by flow cytometry. Data show the mean of three experiments  $\pm$  SEM. \* $P$  < 0.05; \*\* $P$  < 0.01; two-way paired  $t$ -test.

### Statins and Rap1b regulate integrin activity

We next investigated if statins, GGTI, or Rap1b depletion affected adhesion by inducing changes in the surface expression and/or activation of integrins. LFA-1 is normally expressed on the leukocyte surface in an inactive form, but it can be activated by addition of  $Mn^{2+}$  [29]. No significant changes were observed in the cell surface expression of LFA-1 subunit CD11a ( $\alpha L$ ) following statin or GGTI treatment (Fig. 5A) or knockdown of Rap1a or Rap1b (Fig. 5B). To investigate LFA-1 activation, we used the mAb24 antibody, which recognizes the active form of LFA-1 [30, 31]. Statins and GGTI-298 but not FFI inhibited LFA-1 activation (Fig. 5C and D). Similarly, knockdown of Rap1b but not Rap1a reduced LFA-1 activation (Fig. 5E). These results indicate that the statin-induced inhibition of adhesion to ICAM-1 is at least in part a result of reduced Rap1b prenylation and hence, decreased activation of the integrin LFA-1.

### Statin treatment and Rap1b down-regulation inhibit TEM of T-ALL cells

TEM of leukocytes is a crucial step during inflammation and immune responses and requires LFA-1 adhesion to endothelial ICAM-1 [32]. We found that statin treatment significantly decreased the TEM of CCRF-CEM and PEER T-ALL cells but not primary T-lymphoblasts (Fig. 6). Consistent with a specific involvement of Rap1b in LFA-1 activation, Rap1b but not Rap1a depletion reduced TEM of CCRF-CEM cells (Fig. 6B and C). Treatment of endothelial cells for 16 h with 1  $\mu M$  simvastatin did not disrupt endothelial cell–cell junctions or the endothelial monolayer (Supplemental Fig. 2E), indicating that, at this concentration, statins are unlikely to alter endothelial integrity.

Time-lapse movies were performed to visualize the effect of statins on T-ALL cell morphology and migration on endothelial cells. Most control CCRF-CEM cells had a polarized migratory morphology, with a lamellipodium at the front and uropod at the back [33], and migrated across the endothelial monolayer (Fig. 6D, top panels). Cells treated with 1  $\mu M$  simvastatin had a more rounded phenotype and were not polarized (Fig. 6D, middle panels). Some statin-treated cells transiently extended protrusions in different directions but without forming a stable lamellipodium or uropod (Fig. 6D and E). These effects were reversed by coincubation with mevalonic acid (Fig. 6D, bottom panels). Tracking of statin-treated cells showed that they had a reduced migration speed on the endothelial surface compared with controls (Fig. 6F). These data indicate that statins reduce TEM by reducing adhesion of T-ALL cells to ICAM-1 and inhibiting their migration on endothelial cells.

## DISCUSSION

A critical step in T-ALL progression is the accumulation of leukemic cells in the tissues, which requires adhesion to and transmigration through the endothelium. Here, we demonstrate that statins and a GGTI reduce T-ALL cell adhesion, LFA-1 integrin activation, migration, and TEM, indicating that

the effects of statins are a result of reduced protein prenylation rather than cholesterol depletion. Our results suggest that these responses are predominantly a result of inhibition of Rap1b, as depletion of Rap1b but not its closely related isoform Rap1a has similar effects to statins on T-ALL cell adhesion and migration.

The relative contributions of the two Rap1 isoforms, Rap1a and Rap1b, to integrin activation have not been investigated previously. Studies with Rap1a and Rap1b null mice indicate that each isoform affects integrin-mediated leukocyte adhesion, although the two isoforms have not been compared directly. For example, primary hematopoietic cells isolated from Rap1a null mice have reduced adhesion to FN but just a slight decrease of adhesion to ICAM-1 [34], and B cells from Rap1b null mice have reduced ICAM-1 adhesion and chemotaxis [35].

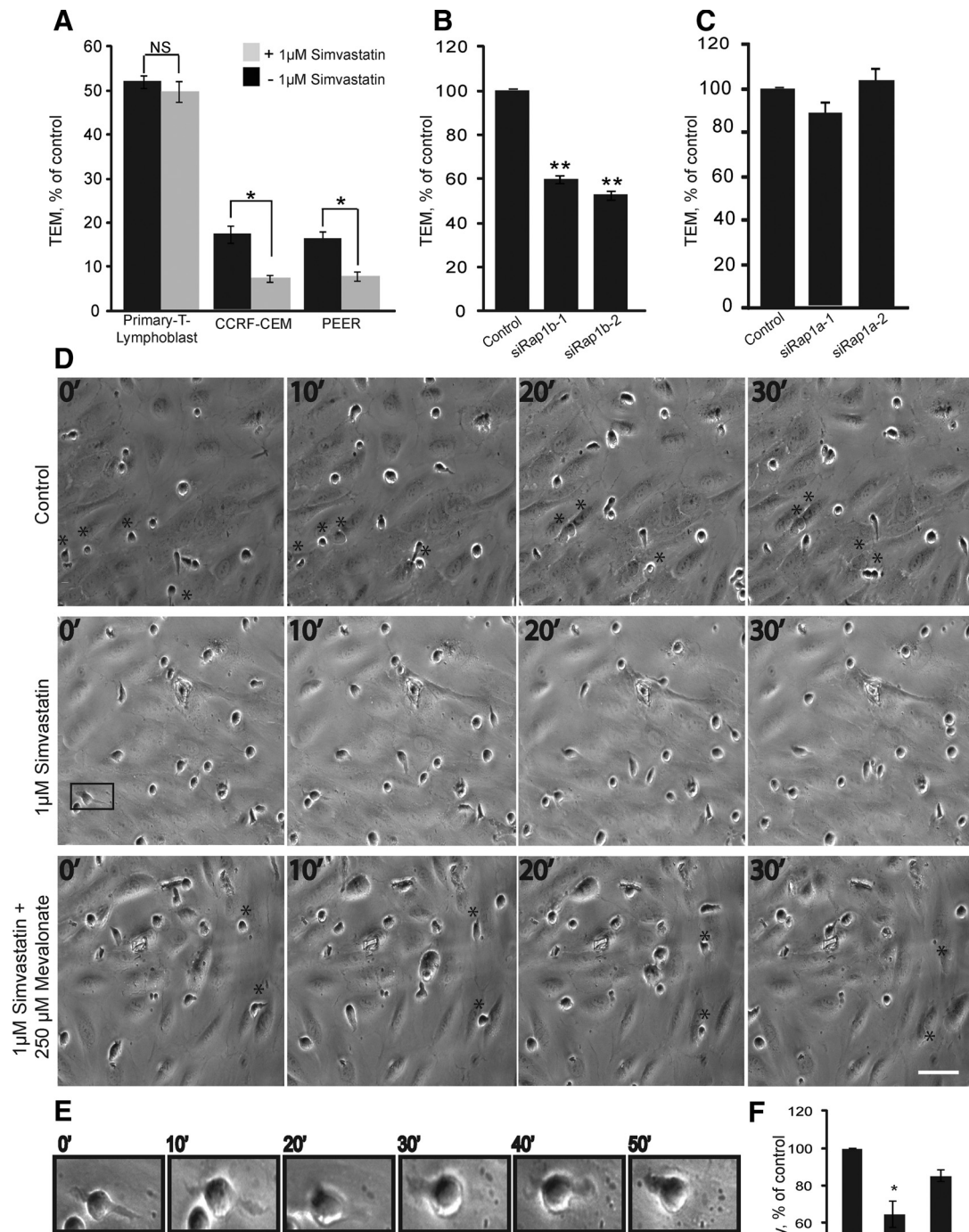
Rap1 stimulates integrin activation through its target Rap1-GTP-interacting adapter molecule, which in turn, regulates talin interaction with integrins [36, 37]. In addition, in T cells, the Rap1 target RapL interacts with and regulates the spatial distribution of LFA-1 [38, 39]. Rap1a and Rap1b differ by only 8 aa, most of which are close to the C terminus [40, 41]. Our results indicate that Rap1b is required specifically for T-ALL cell adhesion to ICAM-1, whereas both isoforms contribute to adhesion to FN. This suggests that the interacting partners and/or localization of Rap1a and Rap1b could be different.

It is interesting that Rac1, Rac2, and RhoA were not required for T-ALL adhesion to ICAM-1, as there is evidence that they and/or specific RhoGEFs regulate ICAM-1 adhesion in response to chemokines or BCR/TCR engagement [42]. The signal transduction pathways leading from Rac and Rho to integrin activation are not clear, in contrast to the well-characterized mechanisms linking Rap1 to integrins. It is thus possible that Rho GTPase involvement in adhesion is indirect and as a result of the effects on cytoskeletal dynamics. However, depletion of Rac and Rho proteins clearly affects T cell behavior: we have recently shown that RhoA and Rac2 are required in CCRF-CEM cells for TEM and that RhoA depletion dramatically alters T cell morphology and reduces migration on endothelial cells [43].

The increase in GTP-bound Rap1 and Rho GTPases in statin- and GGTI-treated cells is presumably a result of altered localization of the unprenylated proteins. As GTP levels are higher than GDP levels in cells, we predict that GTPase proteins are initially loaded with GTP when they are synthesized. If GTPases are unable to be prenylated and hence, are mislocalized, then it is unlikely they will encounter GAPs, and thus, they will remain GTP-loaded [44]. However, they will also not interact efficiently with their downstream targets when unprenylated, and thus, their downstream signaling will be inhibited, despite being GTP-loaded [11, 45].

Simvastatin and lovastatin are often used at 10  $\mu M$  or higher concentrations in experiments in vitro [46, 47], although they can increase RhoA-GTP levels at 1  $\mu M$  [10]. Plasma levels of lovastatin were reported to be between 200 and 250 nM, 6 h after administration of 160 or 200 mg lovastatin to human AML patients [48]. Thus, statin levels of 1  $\mu M$  could be pres-





**Figure 6. Statin treatment and Rap1b down-regulation reduce TEM of T-ALL cells.** Primary T-lymphoblasts, CCRF-CEM cells, and PEER cells were treated for 16 h with 1 μM simvastatin (A) or transfected with siRNAs targeting Rap1a or Rap1b (B and C) and then added to confluent HUVECs on transwell filters. After 1 h (primary T-lymphoblasts) or 2 h (CCRF-CEM and PEER), cells that had transmigrated through the HUVECs toward CXCL12 in the lower chamber were counted. Data shown are mean of three independent experiments ± SEM. \* $P < 0.05$ ; \*\* $P < 0.01$ ; two-way paired  $t$ -test. (D) CCRF-CEM cells treated with 1 μM simvastatin, with or without 250 μM mevalonic acid for 16 h, were added to HUVECs and imaged by time-lapse microscopy. Images show cells at 0, 10, 20, and 30 min; asterisks (top panels) indicate examples of CCRF-CEM cells undergoing TEM. Scale bar, 50 μm. (E) Zoom of a representative cell treated with 1 μM simvastatin (boxed region in D), showing extension of protrusions in different directions. (F) Relative velocity of CCRF-CEM cells on HUVECs compared with control. Data are from three independent experiments ± SEM. \* $P < 0.05$ ; two-way paired  $t$ -test.



ent at earlier time-points, but 10  $\mu$ M is likely to be much higher than most leukemia cells in the blood would be exposed to for any significant time in vivo. Statins at 10  $\mu$ M have been reported to reduce AML cell viability in vitro [49], but in our experiments, 1  $\mu$ M statin did not affect the viability of primary T-lymphoblasts or T-ALL cells. Furthermore, 1  $\mu$ M statin did not affect T-lymphoblast adhesion to ICAM-1 or migration but did inhibit T-ALL responses, suggesting that it may be possible to inhibit T-ALL recruitment to tissues selectively without affecting primary T cells. Interestingly, we found that the active form of Rap1 and the levels of Rap1a and Rap1b protein are higher in primary T-lymphoblasts than CCRF-CEM and Jurkat cells and that Rap1 was only activated by 10  $\mu$ M statin but not 1  $\mu$ M statin in T-lymphoblasts. This might explain why statins are less effective at inhibiting primary T-lymphoblast adhesion to ICAM-1.

In conclusion, our data indicate that Rap1b is the main Rap1 isoform required for adhesion to ICAM-1, chemotaxis, and TEM of T-ALL cell lines. Our results suggest that statins, GGTIs, or other inhibitors of Rap1b signaling could be potential therapies to reduce invasion and metastasis in T-ALL.

## AUTHORSHIP

E.I. designed and carried out experiments and wrote the manuscript; S.J.H. designed experiments and established conditions for studying CCRF-CEM, T-lymphoblast adhesion, and TEM; and A.J.R. planned experiments and wrote the manuscript.

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## KEY WORDS:

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