

B-Raf Regulation of Integrin $\alpha4\beta1$ -mediated Resistance to Shear Stress through Changes in Cell Spreading and Cytoskeletal Association in T Cells*

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Background: Contribution of MAPK members to integrin adhesion is not established.

Results: Inhibition of B-Raf function or expression selectively regulates integrin $\alpha4\beta1$ in T cells.

Conclusion: B-Raf is a signaling component for integrin $\alpha4\beta1$ cytoskeletal association, cell spreading, and adhesion.

Significance: This novel association of B-Raf and integrin $\alpha4\beta1$ suggests new therapeutic targets in T cells and indicates potential off-target effects of sorafenib.

The regulation of integrin-mediated adhesion is of vital importance to adaptive and innate immunity. Integrins are versatile proteins and mediate T cell migration and trafficking by binding to extracellular matrix or other cells as well as initiating intracellular signaling cascades promoting survival or activation. The MAPK pathway is known to be downstream from integrins and to regulate survival, differentiation, and motility. However, secondary roles for canonical MAPK pathway members are being discovered. We show that chemical inhibition of RAF by sorafenib or shRNA-mediated knockdown of B-Raf reduces T cell resistance to shear stress to $\alpha4\beta1$ integrin ligands vascular cell adhesion molecule 1 (VCAM-1) and fibronectin, whereas inhibition of MEK/ERK by U0126 had no effect. Microscopy showed that RAF inhibition leads to significant inhibition of T cell spreading on VCAM-1. The association of $\alpha4\beta1$ integrin with the actin cytoskeleton was shown to be dependent on B-Raf activity or expression, whereas $\alpha4\beta1$ integrin affinity for soluble VCAM-1 was not. These effects were shown to be specific for $\alpha4\beta1$ integrin and not other integrins, such as $\alpha5\beta1$ or LFA-1, or a variety of membrane proteins. We demonstrate a novel role for B-Raf in the selective regulation of $\alpha4\beta1$ integrin-mediated adhesion.

The dynamic regulation of T cell adhesion is a critical component of the adaptive immune response (1). Events such as T cell trafficking into lymphoid organs or sites of inflammation are generally mediated by $\beta1$ integrins ($\alpha4\beta1$ integrin/VLA-4) binding to vascular cell adhesion molecule 1 (VCAM-1)² and

fibronectin (FN), or $\beta2$ integrins ($\alphaL\beta2$ integrin/LFA-1) binding to intercellular adhesion molecule 1 (ICAM-1) (2). Integrin-mediated adhesion is regulated by a process known as inside-out signaling and occurs either by a conformational change of the integrin determining the affinity of integrin for ligand or by avidity modulation due to receptor clustering (3). Integrin-mediated adhesion is also regulated by events following receptor occupancy that stabilize adhesion and coordinate cell spreading and migration, such as linkage of the integrin to the actin cytoskeleton and initiation of intracellular signaling (outside-in signaling) (4–6). This dynamic process is incompletely understood, more so when considering that each specific α/β integrin heterodimer or distinctly differentiated subset of activated lymphocytes may use a unique combination of interacting proteins to regulate each of these adhesive events (7).

The mitogen-activated protein kinase (MAPK) pathway is part of the outside-in signaling cascade initiated by integrin-mediated adhesion (8). The highly conserved Ras/Raf/MEK/ERK signaling module relays signals from the extracellular membrane to cellular effectors that regulate cell fate by influencing cellular proliferation, differentiation, apoptosis, and motility (9). Receptor stimulation activates Ras family small GTPases, subsequently recruiting RAF family kinases to the plasma membrane for activation. Activated RAF then binds and activates MEK to bind and activate ERK, which then translocates to the nucleus and activates transcription factor complexes. The RAF family of serine/threonine protein kinases consists of A-Raf, B-Raf, and C-Raf (Raf-1), which share a common modular structure and some binding partners but are unique in many aspects (10, 11).

There is accumulating evidence that members of the MAPK pathway have secondary roles outside their described signaling module regulating transcription (11, 12). For example, Raf-1 can regulate mitosis and apoptosis independent from MEK, and MEK can regulate autophagy independent from RAF and ERK (13–15). In adherent cell lines, MAPK pathway members were shown to regulate motility and cytoskeletal dynamics by interacting with paxillin, myosin light chain kinase, and the Rho family of small GTPases (16–19). However, our understanding

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² The abbreviations used are: VCAM-1, vascular cell adhesion molecule 1; FN, fibronectin; ICAM-1, intercellular adhesion molecule 1; KD, knockdown; GM1, monosialotetrahexosylganglioside.

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of the role of MAPK in the regulation of lymphocyte adhesion or the direct regulation of integrin activity is still limited.

We set out to study secondary roles of the MAPK pathway members as regulators of integrin function in T cells. Inhibition of RAF but not MEK/ERK reduces the adhesion of Jurkat T cells to VCAM-1 under shear stress, demonstrating the independence of this function from downstream signaling of the MAPK pathway. shRNA-mediated knockdown of B-Raf reproduced the effect of chemical RAF inhibition, confirming a role for B-Raf in lymphocyte adhesion.

EXPERIMENTAL PROCEDURES

Reagents and Cells—The human T cell line, Jurkat, was cultured in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, 100 $\mu\text{g}/\text{ml}$ penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin). Human fibronectin was purified from plasma (Gulf Coast Blood Center, Houston, TX) as described (20). Normal human T cells were negatively selected from healthy donors (Gulf Coast Blood Center, Houston, TX) using the RosetteSep Human T-cell enrichment mixture (Stemcell Technologies Inc., Vancouver, Canada) and then maintained in complete medium supplemented with human recombinant IL-2 (20 units/ml), anti-CD3 (1 $\mu\text{g}/\text{ml}$), and anti-CD28 (1 $\mu\text{g}/\text{ml}$). Human VCAM-1 was purified from the supernatant of CHO cells engineered to express soluble VCAM-1-Fc (21). AlexaFluor- and HRP-conjugated secondary antibodies, non-specific rabbit and mouse IgGs, and mouse anti-GM1 were purchased (Invitrogen). The rabbit anti-p44/42 MAPK (Erk1/2), anti-phospho-p44/42 MAPK, anti-phospho-B-Raf (pSER445), and anti- β -actin were purchased (Cell Signaling, Danvers, MA). Rabbit anti-B-Raf (clone EP152Y) (Epitomics, Burlingame, CA), mouse anti-CD59, and anti- $\alpha 5$ integrin (clone P1D6) (Santa Cruz Biotechnology, Inc.) were purchased. The mouse anti- $\alpha 4$ integrin (clone L25), anti-LFA-1 (clone MHM24), anti-CD3 (clone OKT3), anti-CD4 (clone OKT4), anti- $\beta 1$ integrin (clone 33B6), anti- $\alpha 4\beta 1$ integrin (clone 19H8), anti- αL integrin (clone 32E6), anti-CD43 (clone IB7), and anti-CD28 (clone 95F12) were purified from mouse ascites (22–25). Doxycycline, puromycin, saponin, U0126 (Sigma-Aldrich), recombinant human ICAM-1/Fc (R&D Systems, Minneapolis, MN), and sorafenib (LC Laboratories, Woburn, MA) were purchased.

Generation of Inducible B-Raf Knockdown Cells—Knockdown cells were generated using shRNA-pTRIPZ clones (Open Biosystems, Huntsville, AL). Lentivirus was generated by Lipofectamine 2000 transfection of the packaging cell line, 293T-METR, with packaging plasmids containing p Δ R8.91, CMV-pVSVG, and either scrambled non-silencing controls or a BRAF targeting sequence. Viral supernatants were collected at 48 h and concentrated by ultracentrifugation. Jurkat cells (1×10^6) were transduced and then selected in medium containing puromycin (1 $\mu\text{g}/\text{ml}$). After 1 week, cells were divided into a no treatment group and a doxycycline (1 $\mu\text{g}/\text{ml}$)-treated group. After 72 h, doxycycline-induced fluorescent red (TurboRFP) cells were purified with a FACS Aria IIu high-speed cell sorter (BD Biosciences) and cultured in complete medium with doxycycline.

Parallel Plate Flow Detachment Assay—The detachment assay was performed as described (26). In brief, human FN (5 $\mu\text{g}/\text{ml}$), VCAM-1 (10 $\mu\text{g}/\text{ml}$), or mAbs (1 $\mu\text{g}/\text{ml}$) were immobilized to plastic slides, washed with PBS, blocked with 2% BSA in PBS, and assembled to a parallel plate flow chamber. Cells (4×10^6) in running buffer (10 mM Tris, 103 mM NaCl, 24 mM NaHCO_3 , 5.5 mM glucose, 5.4 mM KCl, and 0.2% BSA, pH 7.4) were injected into the flow chamber and allowed to settle on the slide for 10 min. A computer-controlled syringe pump (Harvard Apparatus) was used to apply an increasing linear gradient of fluid flow to the cells for 300 s and recorded by digital microscopy. Shear stress calculations were determined every 50 s, and the shear stress in dynes/cm² was defined as $(6\mu Q)/(wh^2)$, where μ is the viscosity of the medium (0.007), Q is the flow rate in cm³/s, w is the width of the chamber (0.3175 cm), and h is the height of the chamber (0.0254 cm).

Bright Field Microscopy—Human VCAM-1 was immobilized to 6-well non-tissue culture-treated plates (Falcon), washed with PBS, and blocked with 2% BSA in PBS. Cells (1×10^6) in complete medium were added, incubated at 37 °C for 10 min, and then fixed with 2% paraformaldehyde in PBS for 20 min at room temperature. Images were captured at $\times 20$ magnification using a Nikon Diaphot-TMD microscope, equipped with a VI-470 CCD video camera (Optronics Engineering). Images were analyzed using Slidebook software (version 5.0) to distinguish spread cells from non-spread cells by creating a mask of spread cells and counting all cells that were larger or smaller than the threshold.

Super-resolution Immunofluorescence—Human VCAM-1 (10 $\mu\text{g}/\text{ml}$) was immobilized to glass coverslips, washed with PBS, and blocked with 2% BSA in PBS. Cells (5×10^5) in complete medium were added and incubated at 37 °C for 10 min and then fixed with 2% paraformaldehyde in PBS for 20 min at room temperature. Cells were permeabilized by adding saponin to a concentration of 0.1% for 30 min at room temperature. Cells were washed three times with PBS, 2% BSA, 0.1% saponin, stained for total B-Raf (AlexaFluor 647) and $\beta 1$ integrin (AlexaFluor 488), and mounted to slides using Prolong Gold anti-fade reagent (Invitrogen). Images were acquired at room temperature using the OMX Blaze V4 structured illumination microscope (Applied Precision) with a $\times 100$ numerical aperture 1.40 objective lens, two EM-CCD Photometrics Evolve 512 cameras, and DeltaVision OMX acquisition software. The images were reconstructed and rotated in three dimensions by 90°, and the height of cells was measured using the softWoRx software (version 6.0 beta 19). The image stacks were then transferred to either Slidebook software (version 5.0) to measure the area of contact of the cell with the glass coverslip or Imaris Bitplane software (version 7.6.1) to measure the colocalization of $\beta 1$ integrin and B-Raf. The colocalization was quantified from the reconstructed three-dimensional image using the spot detection function for absolute fluorescence of both $\beta 1$ integrin and B-Raf channels. Spots were generated with a 200-nm maximum xy diameter and a 500-nm maximum z diameter, identifying between 2000 and 15,000 spots for each channel per reconstructed image. Then the spots-to-spots colocalization function was used to identify all spots within 300 nm of spots from the other channel.

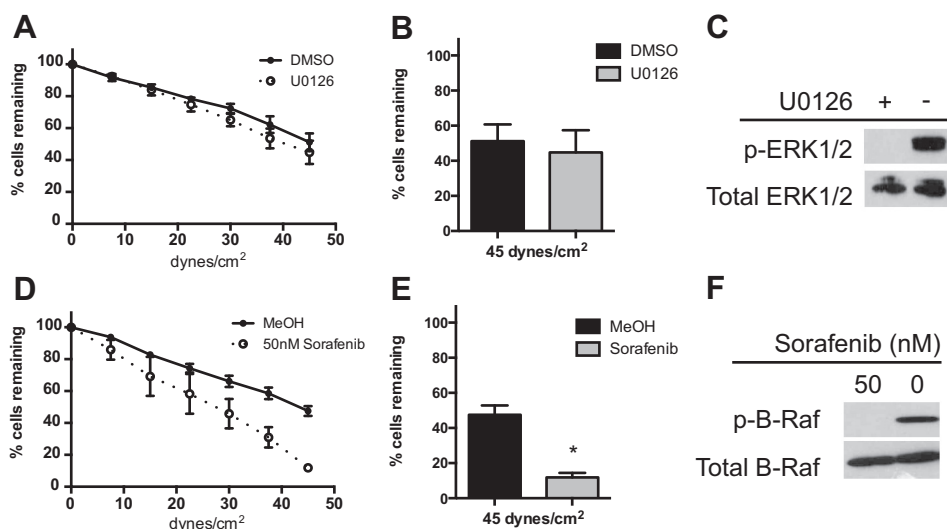


FIGURE 1. **Sorafenib reduces adhesion to fibronectin.** *A* and *D*, parallel plate flow detachment assay to slides coated with human FN (5 $\mu\text{g}/\text{ml}$) of Jurkat cells incubated for 1 h with U0126 (1 μM) and DMSO vehicle control (*A*) or sorafenib (50 nM) and methanol vehicle control (*D*). *B* and *E*, percentage of cells remaining at 45 dynes/cm² (maximum shear) from the parallel plate flow assay of cells incubated with U0126 (1 μM) and DMSO vehicle control (*B*) or sorafenib (50 nM) and methanol vehicle control (*E*). *C* and *F*, Western blots using SDS-PAGE (10% gel) of whole cell lysates (1×10^7 cells) after 10-min adhesion to FN and pretreated for 1 h with U0126 (1 μM) and DMSO vehicle control and probed for phosphorylated (*p*-) and total ERK (*C*) or sorafenib (50 nM) and methanol vehicle control and probed for phosphorylated and total B-Raf (*F*). Error bars, S.E. *, $p < 0.01$ using Student's *t* test.

Soluble VCAM-1 Binding Assay—The soluble VCAM-1 binding assay was modified from a previous procedure (27). In brief, cells (1×10^6) in 100 μl of serum-free medium were incubated with human VCAM-1-Fc (10 $\mu\text{g}/\text{ml}$) at 37 $^\circ\text{C}$ for 10 min. The cells were then diluted and fixed by adding 2 ml of RPMI 1640 with 2% paraformaldehyde for 20 min at room temperature. The cells were washed twice with 2% BSA in PBS and incubated with AlexaFluor 488-conjugated rabbit anti-mouse for 20 min at room temperature. The cells were then washed and analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

Cytoskeletal Stabilization Assay—The quantification of integrin-cytoskeleton attachment was modified from a previous procedure (26–28). Cells (2×10^6) in 100 μl of complete medium were incubated with mAb (1 $\mu\text{g}/\text{ml}$) at 4 $^\circ\text{C}$ for 30 min, and then either they were left untreated or AlexaFluor-conjugated rabbit anti-mouse was added at 4 $^\circ\text{C}$ for 30 min. The cells were incubated at either 4 or 37 $^\circ\text{C}$ for 10 min. The cells were then washed and resuspended in cytoskeletal stabilizing buffer (50 mM NaCl, 2 mM MgCl₂, 0.22 mM EGTA, 13 mM Tris, 1 mM PMSF, 10 mM iodacetamide, and 2% FBS, pH 8.0) with or without 0.1% Nonidet P-40. After 5 min at room temperature, 1 ml of cytoskeletal stabilizing buffer was added, and cells were immediately pelleted and fixed with 2% paraformaldehyde in PBS for 20 min at room temperature. The cells were then washed three times in PBS, and the amount of remaining bound mAb was determined by flow cytometry using a FACSCalibur (BD Biosciences).

RESULTS

RAF Inhibition, but Not ERK Inhibition, Leads to Decreased Adhesion of T Cells to Fibronectin—The parallel plate flow assay was used to measure adhesion and investigate the role of ERK signaling in integrin-mediated adhesion to human FN under conditions of shear stress. Two inhibitors of the ERK pathway

were used, U0126, a MEK inhibitor, and sorafenib, a RAF inhibitor. Jurkat cell adhesion to FN was unchanged by 1 h of 1 μM U0126 treatment (Fig. 1*A*). The U0126- and vehicle (DMSO)-treated cells show very similar rates of detachment, with ~49% of the initial cells remaining at 45 dynes/cm² (maximum shear) (Fig. 1*B*). The phosphorylation of ERK was inhibited in these cells (Fig. 1*C*). In contrast to MEK/ERK inhibition, adhesion to FN was significantly reduced by 50 nM sorafenib when compared with vehicle control (MeOH) (Fig. 1*D*). The cells pretreated with vehicle show 47.5% of cells remaining at maximum shear, whereas the cells pretreated with sorafenib show an increased rate of detachment with only 11.9% of cells remaining, a 75% inhibition (Fig. 1*E*). The phosphorylation of B-Raf was inhibited by 50 nM sorafenib (Fig. 1*F*). These results suggest that RAF, and not MEK/ERK, contributes to T cell resistance to shear stress after adhesion to FN.

RAF Inhibition Decreases $\alpha 4\beta 1$ Integrin-mediated Adhesion to VCAM-1—Both $\alpha 5\beta 1$ and $\alpha 4\beta 1$ integrin bind FN, so to address the specificity of the adhesion, we tested adhesion to VCAM-1, which is a ligand for $\alpha 4\beta 1$ and not $\alpha 5\beta 1$. Adhesion to VCAM-1 was unchanged by 1 h of 1 μM U0126 treatment (Fig. 2*A*). The U0126- and vehicle (DMSO)-treated cells show very similar rates of detachment to VCAM-1, with ~40% of cells remaining at maximum shear (Fig. 2*B*). The phosphorylation of ERK was inhibited in these cells (Fig. 2*C*). However, adhesion to VCAM-1 was reduced by 50 nM sorafenib (Fig. 2*D*). The cells pretreated with either vehicle or 10 nM sorafenib show very similar rates of detachment to VCAM-1, with ~65% of the initial cells remaining at maximum shear, whereas the cells pretreated with 50 nM sorafenib show an increased rate of detachment with only 29.1% of the initial cells remaining, a 57% inhibition (Fig. 1*E*). The phosphorylation of B-Raf was inhibited at the 50 nM concentration of sorafenib (Fig. 2*F*). Sorafenib is most specific for Raf-1 (6 nM IC₅₀) and B-Raf (22 nM IC₅₀) (29), and

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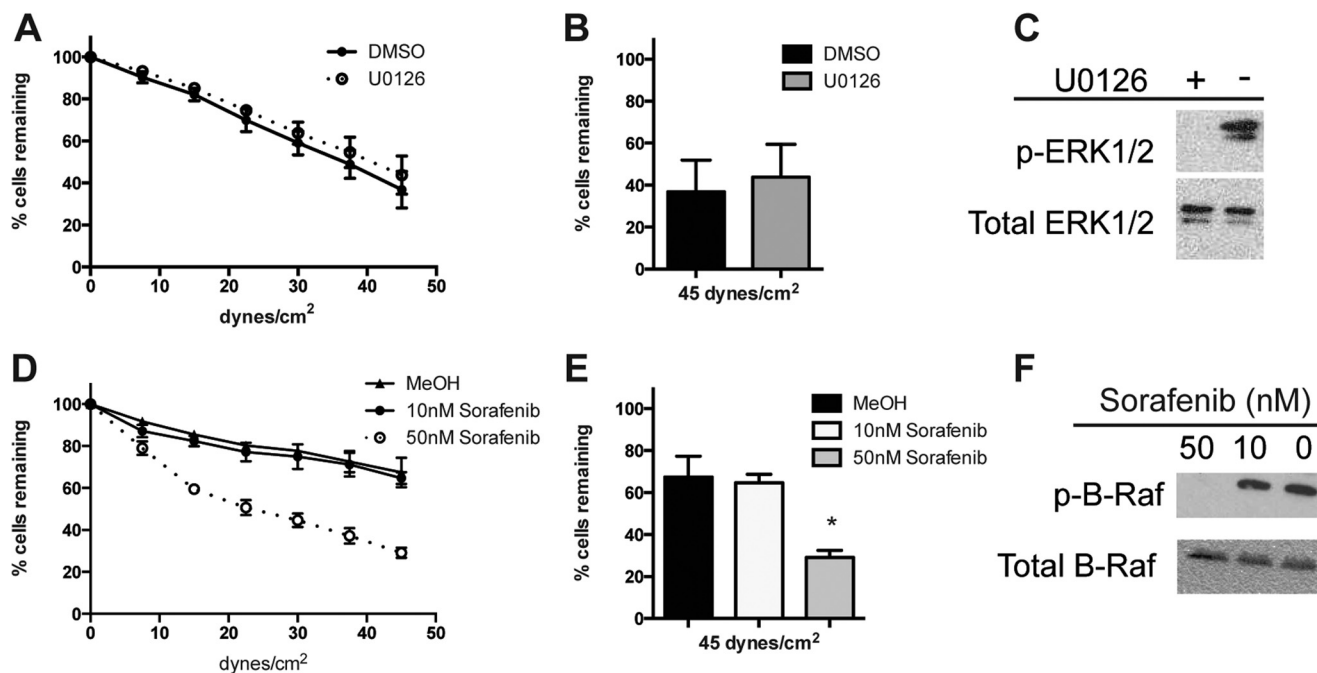


FIGURE 2. **Sorafenib reduces adhesion to VCAM-1.** A and D, parallel plate flow detachment assay to slides coated with human VCAM-1 (10 $\mu\text{g}/\text{ml}$) of Jurkat cells incubated for 1 h with U0126 (1 μM) and DMSO vehicle control (A) or sorafenib (10 or 50 nM) and methanol vehicle control (D). B and E, percentage of cells remaining at maximum shear from the parallel plate flow assay of cells incubated with U0126 (1 μM) and DMSO vehicle control (B) or sorafenib (10 or 50 nM) and methanol vehicle control (E). C and F, Western blot using SDS-PAGE (10% gel) of whole cell lysates (1×10^7 cells) after 10-min adhesion to VCAM-1 and pretreated for 1 h with U0126 (1 μM) and DMSO vehicle control and probed for phosphorylated (*p*-) and total ERK (C) or sorafenib (10 or 50 nM) and methanol vehicle control and probed for phosphorylated and total B-Raf (F). Error bars, S.E. *, $p < 0.01$ using Student's *t* test.

combined, these data suggest that B-Raf phosphorylation contributes to $\alpha 4\beta 1$ integrin-mediated adhesion to VCAM-1.

RAF Inhibition Leads to Decreased Adhesion of Normal T Cells to VCAM-1 and Fibronectin—Given the reported differences between Jurkat and non-transformed T cells, we investigated the response of normal human T cells to sorafenib under conditions of shear stress after adhesion to $\alpha 4\beta 1$ integrin ligands. Adhesion to both VCAM-1 (Fig. 3A) and FN (Fig. 3B) was significantly reduced by 50 nM sorafenib, compared with MeOH vehicle control. Three healthy donors were tested, and these results demonstrate that sorafenib reduces adhesion of normal human T cells to $\alpha 4\beta 1$ integrin ligands.

BRAF Knockdown Decreases Adhesion to VCAM-1 and Fibronectin—To confirm the role of B-Raf in $\alpha 4\beta 1$ integrin-mediated adhesion, Jurkat cells were stably transduced with doxycycline-inducible shRNA specific for BRAF or a scrambled shRNA vector control sequence. B-Raf protein expression was reduced in the B-Raf knockdown (KD) cells cultured with doxycycline (Fig. 4A). B-Raf knockdown cells (KD+D) compared with control cells (KD-NT, VC-NT, and VC+D) show decreased adhesion to both VCAM-1 (Fig. 4B) and FN (Fig. 4C). The control cells show very similar rates of detachment to VCAM-1, with ~55% of the initial cells remaining at maximum shear, whereas the KD+D cells show an increased rate of detachment with only 4.9% of the initial cells remaining (Fig. 4D). Similarly, with FN, the control cells show equal rates of detachment, with ~36% of the initial cells remaining at maximum shear, whereas the KD+D cells show an increased rate of detachment, with only 7.4% of the initial cells remaining (Fig. 4E). These results demonstrate that knockdown of B-Raf leads to decreased adhesion to $\alpha 4\beta 1$ integrin ligands.

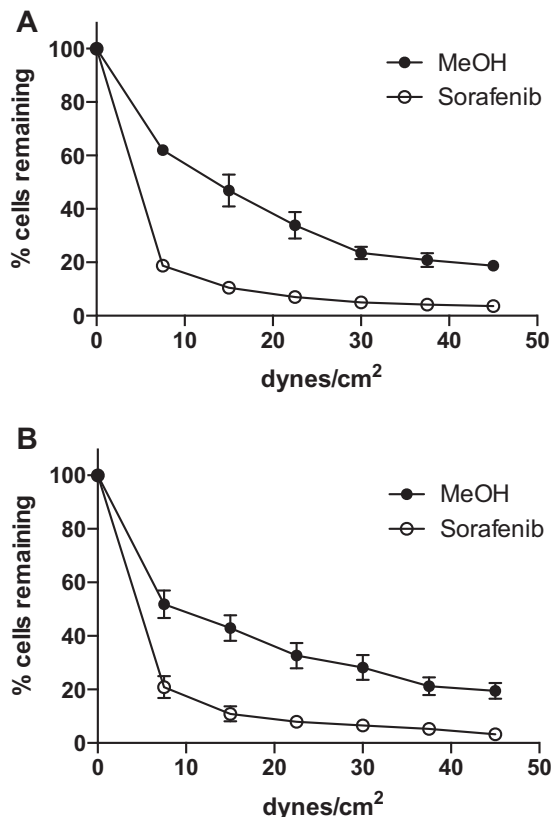


FIGURE 3. **Sorafenib reduces adhesion of activated normal human T cells to VCAM-1 and fibronectin.** A and B, parallel plate flow detachment assay of activated normal human T cells pretreated for 1 h with methanol vehicle control or sorafenib (50 nM) to slides coated with human VCAM-1 (10 $\mu\text{g}/\text{ml}$) (A) or human FN (5 $\mu\text{g}/\text{ml}$) (B). Data are shown for an experiment performed in triplicate from one healthy donor. Error bars, S.E.

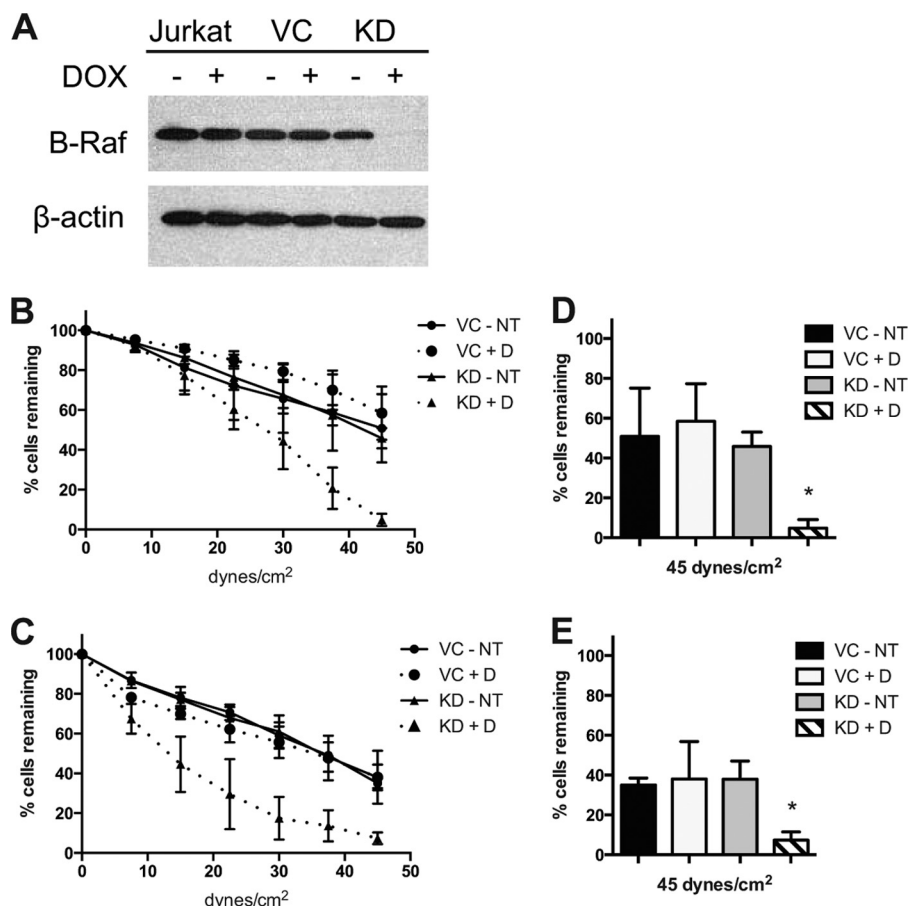


FIGURE 4. **B-Raf knockdown reduces adhesion to VCAM-1 and fibronectin.** *A*, Western blot using SDS-PAGE (10% gel) of whole cell lysates (1×10^7 cells) of Jurkat, vector control (VC), and B-Raf KD cells cultured with and without doxycycline ($\pm D$), and probed for total B-Raf or β -actin. *B* and *C*, parallel plate flow detachment assay of vector control and KD cells cultured with doxycycline (+D) or without doxycycline (NT) to slides coated with human VCAM-1 (10 μ g/ml) (*B*) or human FN (5 μ g/ml) (*C*). *D* and *E*, percentage of cells remaining at maximum shear from the parallel plate flow assay of vector control and KD cells to slides coated with VCAM-1 (*D*) or FN (*E*). Error bars, S.E. *, $p < 0.01$ using Student's *t* test.

B-Raf Is Unessential for T Cell Proliferation or $\alpha 4$ and $\beta 1$ Integrin Expression—A reduction in viability or $\alpha 4\beta 1$ integrin expression of B-Raf knockdown cells could account for decreased adhesion. To confirm that B-Raf knockdown did not produce a defect in cell viability, the proliferation of the transduced or control cells cultured with or without doxycycline was measured for 10 days and found to remain unchanged (Fig. 5A). To confirm that B-Raf knockdown does not lead to reduced surface expression of integrin subunits, $\alpha 4$ or $\beta 1$ integrin subunits were measured by flow cytometry and were unchanged (Fig. 5B). Thus, the decreased adhesion observed under conditions of B-Raf knockdown is not due to reduced viability or $\alpha 4\beta 1$ integrin expression.

$\alpha 4\beta 1$ Integrin Affinity Is Not Affected by B-Raf Knockdown or Sorafenib—Another mechanism of regulating the adhesion strength of integrins is the modulation of binding affinity. To test the role of B-Raf in the regulation of $\alpha 4\beta 1$ integrin affinity, the binding affinity for soluble VCAM-1 was measured and found to be unchanged by pretreatment with 50 nM sorafenib (Fig. 5C) or by B-Raf knockdown (Fig. 5D). Cells were incubated with Mn^{2+} as a positive control for maximal integrin affinity, and the binding affinity for VCAM-1 of cells incubated with 1 mM Mn^{2+} was increased over the cells that did not receive Mn^{2+} but unchanged by B-Raf knockdown or sorafenib, indi-

cating that the ability of the integrin to achieve a maximal affinity conformation is unaffected. These results indicate that B-Raf is unessential to $\alpha 4\beta 1$ integrin affinity for soluble VCAM-1, and the reduced adhesion is not due to affinity regulation of $\alpha 4\beta 1$.

Sorafenib or B-Raf Knockdown Specifically Reduces Adhesion Mediated by $\alpha 4\beta 1$ Integrin—The fact that $\alpha 4\beta 1$ affinity was shown to be unaffected suggested that we could bypass physiologic ligand coupling by using adhesion to immobilized mAbs to address the specificity of B-Raf inhibition on resistance to shear stress. Jurkat cell adhesion to an anti- $\alpha 4\beta 1$ integrin mAb (19H8) was significantly reduced by 50 nM sorafenib when compared with vehicle control, whereas adhesion to an anti-CD28 mAb was unchanged by sorafenib (Fig. 6A). Adhesion was measured to mAbs to $\alpha 4\beta 1$ integrin (19H8), $\beta 1$ integrin (33B6), $\alpha 5$ integrin, LFA-1 (32E6 and MHM24), CD28, GM1, CD43, CD59, CD3 (OKT3), and CD4 (OKT4) (Fig. 6B). Adhesion was only reduced by sorafenib to $\alpha 4\beta 1$ or $\beta 1$ integrin mAbs. Adhesion was also measured to the LFA-1 natural ligand ICAM-1 and found to be unaffected by sorafenib (Fig. 6B). The shRNA vector control cells (VC+D) and B-Raf knockdown cells (KD+D) show equal detachment when bound to immobilized anti-LFA-1 (32E6), anti-CD3 (OKT3), and ICAM-1 (Fig. 6C). However, the adhesion of B-Raf knockdown cells to the anti-

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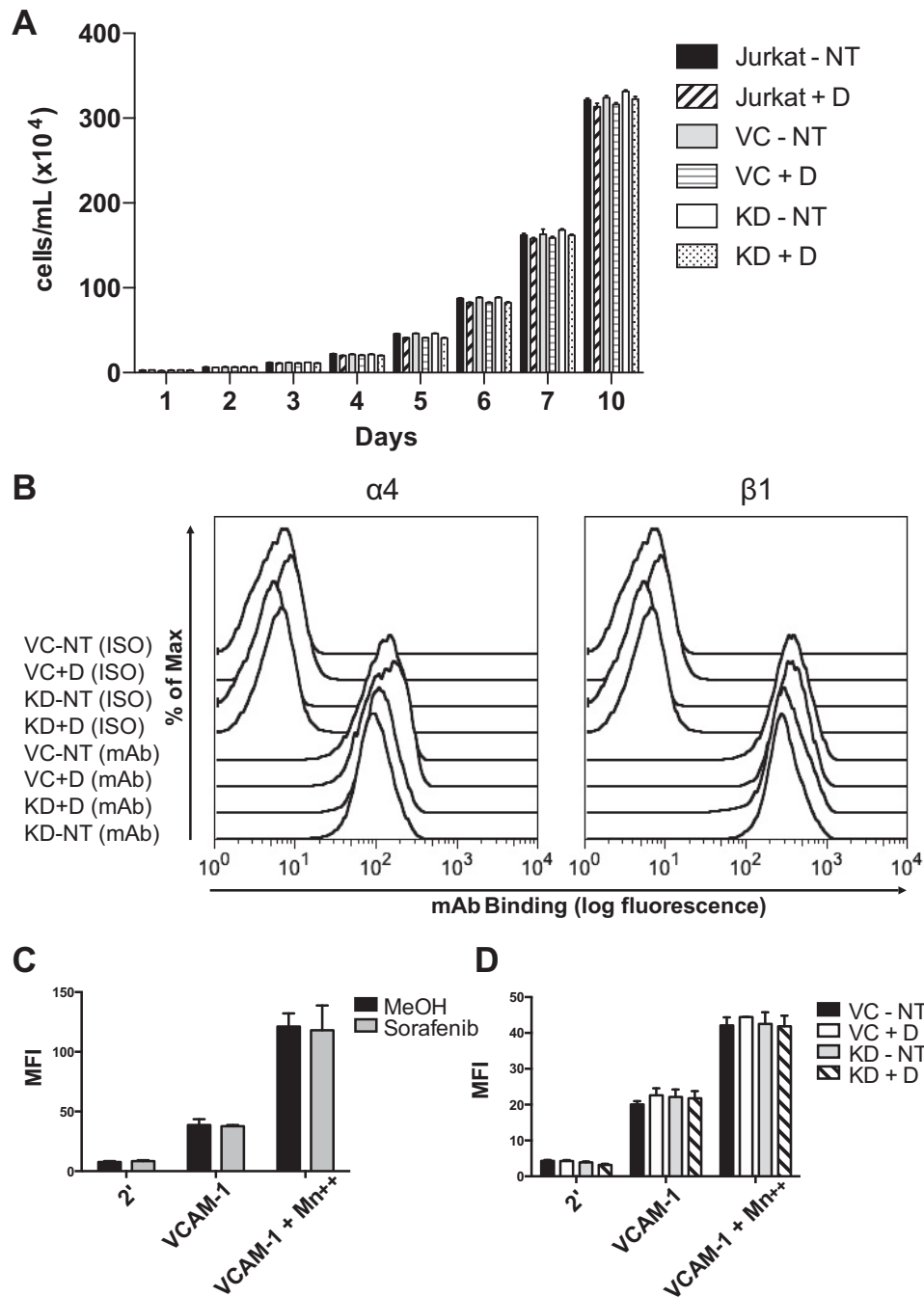


FIGURE 5. Cell proliferation, integrin expression, and affinity for VCAM-1 are not affected by B-Raf knockdown or sorafenib. *A*, proliferation measured by trypan blue exclusion of Jurkat, shRNA vector control (VC), and B-Raf KD cells cultured with and without doxycycline ($\pm D$) for 10 days. *B*, flow cytometry using a FACSCalibur (BD Bioscience) of isotype control (ISO) (nonspecific mouse IgG) and $\alpha 4$ (mAb L25) or $\beta 1$ (mAb 33B6) integrin subunits of vector control (VC) and B-Raf KD cells cultured with (+D) and without (NT) doxycycline stained with Alexa Fluor 488 goat anti-mouse secondary antibody. *C* and *D*, soluble VCAM-1 (10 $\mu\text{g}/\text{ml}$) binding assay of Jurkat cells incubated for 1 h with sorafenib (50 nM) or methanol vehicle control (*C*) and vector control (VC) and B-Raf KD cells cultured with doxycycline (+D) or without doxycycline (NT) (*D*). For *C* and *D*, cells were incubated with secondary (2') AlexaFluor 488 rabbit anti-mouse IgG and no soluble VCAM-1 to show background fluorescence. Error bars, S.E. MFI, mean fluorescence intensity.

$\alpha 4\beta 1$ integrin mAb (19H8) was significantly reduced when compared with shRNA vector control cells (Fig. 6C). These results indicate that B-Raf specifically regulates $\alpha 4\beta 1$ integrin and not other integrins or a variety of membrane components.

Sorafenib or B-Raf Knockdown Inhibits Cell Spreading after Adhesion to VCAM-1—Bright field microscopy was used to investigate the effects of RAF inhibition on cell morphology after adhesion to VCAM-1. Images were captured and quanti-

fied after 10 min of adhesion to VCAM-1 (Fig. 7A). An average of 84.4% of MeOH vehicle-treated cells were spread, compared with 9% of cells treated with 50 nM sorafenib (Fig. 7B). Similarly, an average of 77.3% of shRNA vector control cells (VC+D) were spread, compared with 7.9% of B-Raf knockdown cells (KD+D) (Fig. 7C). These results demonstrate that B-Raf activity or expression is required for efficient $\alpha 4\beta 1$ integrin-driven cell spreading.

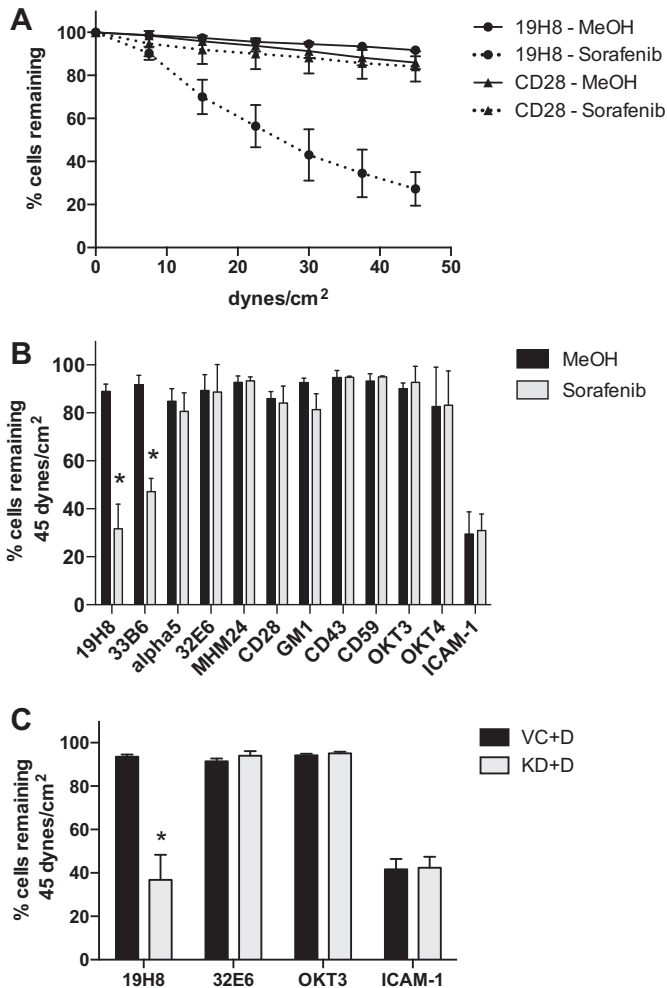


FIGURE 6. Sorafenib or B-Raf knockdown specifically reduces adhesion mediated by $\alpha 4\beta 1$ integrin. *A*, parallel plate flow detachment assay of Jurkat cells incubated for 1 h with sorafenib (50 nM) and methanol vehicle control to slides coated with anti- $\alpha 4\beta 1$ integrin mAb (19H8) or anti-CD28 mAb (1 μ g/ml). *B*, percentage of cells incubated for 1 h with sorafenib (50 nM) and methanol vehicle control remaining at maximum shear to slides coated with anti- $\alpha 4\beta 1$ integrin (19H8) ($n = 8$), anti- $\beta 1$ integrin (33B6) ($n = 6$), anti- $\alpha 5$ integrin ($n = 4$), anti-LFA-1 (32E6) ($n = 4$), anti-LFA-1 (MHM24) ($n = 3$), anti-CD3 (OKT3) ($n = 3$), anti-CD4 (OKT4) ($n = 3$), anti-CD28 ($n = 6$), anti-CD43 ($n = 3$), anti-CD59 ($n = 3$), anti-GM1 ($n = 3$), or human ICAM-1 (5 μ g/ml) ($n = 6$). *C*, percentage of B-Raf KD or vector control (VC) cells cultured with doxycycline (+D) remaining at maximum shear to slides coated with anti- $\alpha 4\beta 1$ integrin (19H8) ($n = 5$), anti-LFA-1 (32E6) ($n = 4$), anti-CD3 (OKT3) ($n = 4$), or human ICAM-1 (5 μ g/ml) ($n = 6$). Error bars, S.E. *, $p < 0.01$ using Student's *t* test.

Sorafenib Inhibits Cell Spreading and $\beta 1$ Integrin Colocalization with B-Raf after Adhesion to VCAM-1—Super-resolution microscopy was used to investigate RAF inhibition on cell morphology and localization of $\beta 1$ integrin and B-Raf after 10-min adhesion to VCAM-1. Cell spreading after pretreatment with 50 nM sorafenib was measured using the height of cells (Fig. 8A) and the area of cellular contact with the glass coverslip (Fig. 8B) as indicators of spreading. The average height of cells pretreated with vehicle control (MeOH) was 5.5 μ m, whereas the average height of cells pretreated with sorafenib was 10.2 μ m (Fig. 8C). The average area of cellular contact with VCAM-1 of control cells was 512 μ m², whereas the average with sorafenib was 110 μ m², a 78.4% reduction (Fig. 8D). The colocalization of $\beta 1$ integrin and B-Raf was quantified from reconstructed three-dimensional images using the spots generated from absolute

fluorescence (Fig. 8E). The control cells had 31.6% of $\beta 1$ integrin colocalize with B-Raf, and the cells pretreated with sorafenib had only 13.3% of $\beta 1$ integrin colocalize with B-Raf, a 58% reduction (Fig. 8F). A trend was observed in the decreased amount of B-Raf to colocalize with $\beta 1$ integrin in sorafenib-treated cells, but it failed to reach significance (Fig. 8G). These results demonstrate that sorafenib inhibits cell spreading on VCAM-1 and reduces $\beta 1$ integrin colocalization with B-Raf after adhesion to VCAM-1.

Sorafenib Prevents $\beta 1$ Integrin Association with the Cytoskeleton—Integrin association with the actin cytoskeleton increases post-ligand binding and is necessary for focal adhesion formation and cellular resistance to shear stress. It has been shown that antibody cross-linking of $\alpha 4$ integrin on Jurkat cells leads to a significant increase in the amount of anti- $\alpha 4$ mAb able to resist solubilization by a non-ionic detergent, and this is interpreted to result from an increase in $\alpha 4$ integrin association with the cytoskeleton (30). The association of $\beta 1$ integrin with the cytoskeleton after activation by cross-linking of an anti- $\beta 1$ mAb was measured. Cells pretreated for 1 h with 50 nM sorafenib show significantly reduced $\beta 1$ integrin association with the cytoskeleton after activation at 37 °C (5.2%) compared with control (23.6%), a 78% reduction (Fig. 9A). These results show that RAF inhibition reduces $\beta 1$ integrin association with the cytoskeleton and indicate a role for B-Raf phosphorylation in this pathway.

B-Raf Is Essential for $\beta 1$ Integrin Association with the Cytoskeleton—The association of $\beta 1$ integrin with the cytoskeleton was measured in B-Raf knockdown or shRNA vector control cells pretreated with 50 nM sorafenib or vehicle control. Consistent with results in Fig. 9A, shRNA vector control cells pretreated for 1 h with sorafenib show significantly reduced $\beta 1$ integrin association with the cytoskeleton after activation at 37 °C (4.2%) compared with vehicle control (20.5%), a 79.6% reduction (Fig. 9B). In comparison with vector control cells (20.5%), B-Raf knockdown cells show significantly reduced $\beta 1$ integrin association with the cytoskeleton (6%) after activation at 37 °C, a 71.8% reduction. These results indicate that B-Raf is essential for $\beta 1$ integrin association with the cytoskeleton.

Sorafenib Specifically Prevents $\alpha 4\beta 1$ Integrin Association with the Cytoskeleton—The resistance to Nonidet P-40 solubilization was measured for mAbs to $\alpha 4\beta 1$ integrin, LFA-1, CD28, and CD3. Compared with control cells at 4 °C, cross-linking at 37 °C significantly increased the amount of detergent-resistant $\alpha 4\beta 1$ (from 27.6 to 59%), LFA-1 (from 31.3 to 60.8%), and CD3 (from 39.2 to 65.3%), but there was very little CD28 association with the cytoskeleton (~4%) (Fig. 9C). Treatment with 50 nM sorafenib had little effect on the amount of detergent-resistant LFA-1 (65.3%), CD28 (~2%), or CD3 (65.7%), whereas the amount of detergent-resistant $\alpha 4\beta 1$ mAb was significantly reduced (from 59 to 26.2%). These results show that sorafenib inhibits $\alpha 4\beta 1$ integrin association with the cytoskeleton and indicate that phosphorylation of B-Raf is specifically mediating the $\alpha 4\beta 1$ integrin association with the cytoskeleton while having no effect on LFA-1, CD28, or CD3 association with the cytoskeleton.

Integrin $\alpha 4\beta 1$ Regulation of T Cell Adhesion by B-Raf

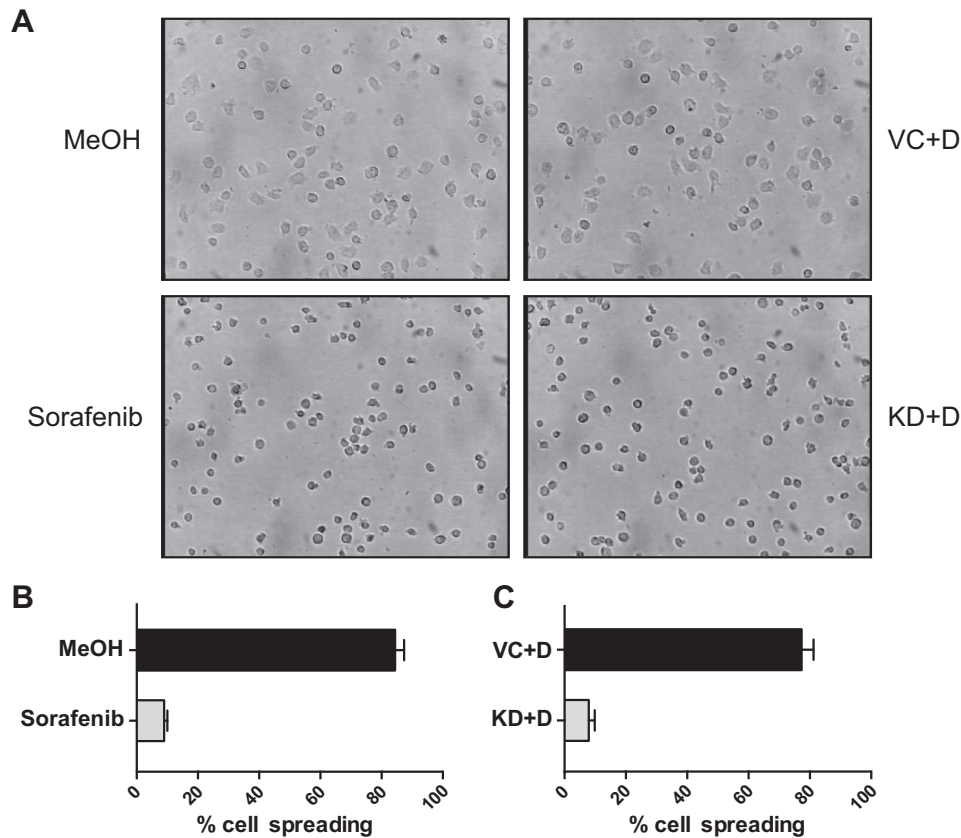


FIGURE 7. Sorafenib or B-Raf knockdown inhibits cell spreading on VCAM-1. *A*, bright field microscopy after 10-min adhesion to VCAM-1 (10 $\mu\text{g}/\text{ml}$) of Jurkat cells pretreated for 1 h with methanol vehicle control or sorafenib (50 nM) (left panels) and shRNA vector control (VC) or B-Raf knockdown (KD) cells cultured with doxycycline (+D) (right panels). Images were quantified, and results are shown as the mean percentage of cell spreading from two independent experiments performed in triplicate for vehicle- and sorafenib-treated cells (*B*) and control and B-Raf KD cells (*C*). Error bars, S.E. *, $p < 0.001$ using Student's *t* test.

DISCUSSION

This work has demonstrated a novel role for B-Raf in the direct regulation of the $\alpha 4\beta 1$ integrin in lymphocytes. Both the chemical inhibition and knockdown of B-Raf lead to decreased resistance to shear stress of T cells after adhesion to $\alpha 4\beta 1$ ligands and decreased $\alpha 4\beta 1$ association with the cytoskeleton after mAb cross-linking. These effects were both specific for $\alpha 4\beta 1$ integrin and independent from affinity regulation or downstream MEK/ERK activity. We have also shown that sorafenib inhibits $\alpha 4\beta 1$ integrin-driven cell spreading and $\beta 1$ integrin colocalization with B-Raf. The increased height and reduced area of cellular contact with VCAM-1 of cells treated with sorafenib suggest that these cells are experiencing greater shear stress distributed over a smaller area of anchorage, probably contributing to decreased adhesion in our laminar flow system. Cell adhesion has been shown to be dependent on the dynamics of the actin cytoskeleton and cell spreading, promoting adhesion strengthening and migration, in part by providing a more streamlined shape for the cell to reduce the shear stress imposed by laminar flow (6, 31, 32).

At present, it is not clear on the molecular level how B-Raf regulates $\alpha 4\beta 1$ integrin function. Chemokine binding to G protein-coupled receptors induces the activation of phospholipase C and calcium signaling, leading to the rapid up-regulation of $\alpha 4\beta 1$ and LFA-1 integrin affinity (33, 34). Downstream of calcium signaling, Rap1 from the Ras family of small GTPases

plays an important role in $\beta 2$ integrin affinity regulation and adhesion, but this has not been established for $\alpha 4\beta 1$ integrin (4, 35). It has been shown that Rap1 mediates phorbol-ester (phorbol 12-myristate 13-acetate)-stimulated adhesion to FN, but Rap1 does not mediate SDF-1 α -stimulated affinity up-regulation of $\alpha 4\beta 1$ integrin for VCAM-1 or adhesion to VCAM-1 after SDF-1 α or phorbol 12-myristate 13-acetate stimulation (36, 37). However, Rap1 specifically activates B-Raf, not Raf-1, and precedence for RAF family members in adhesion regulation was provided by studies demonstrating that H-Ras activation of Raf-1 suppressed integrin activation in CHO cells, but there were conflicting results concerning whether the suppression was independent of ERK (38–42). Therefore, a secondary role for B-Raf in the direct regulation of integrin-mediated adhesion was feasible.

Identification of the specific B-Raf-containing complex and binding partners of this mechanism will be important to the understanding of lymphocyte adhesion. Our results indicate that B-Raf is specifically regulating the $\alpha 4\beta 1$ integrin and not LFA-1 or a variety of other membrane proteins. Integrin cytoplasmic tails binding to integrin-associated proteins mediate the events following receptor occupancy, such as adhesion strengthening and outside-in signaling. It has been shown that talin regulates cytoskeletal association, resistance to shear stress, and affinity of both $\alpha 4\beta 1$ and LFA-1 (43, 44). However, it is established that different integrin heterodimers expressed by

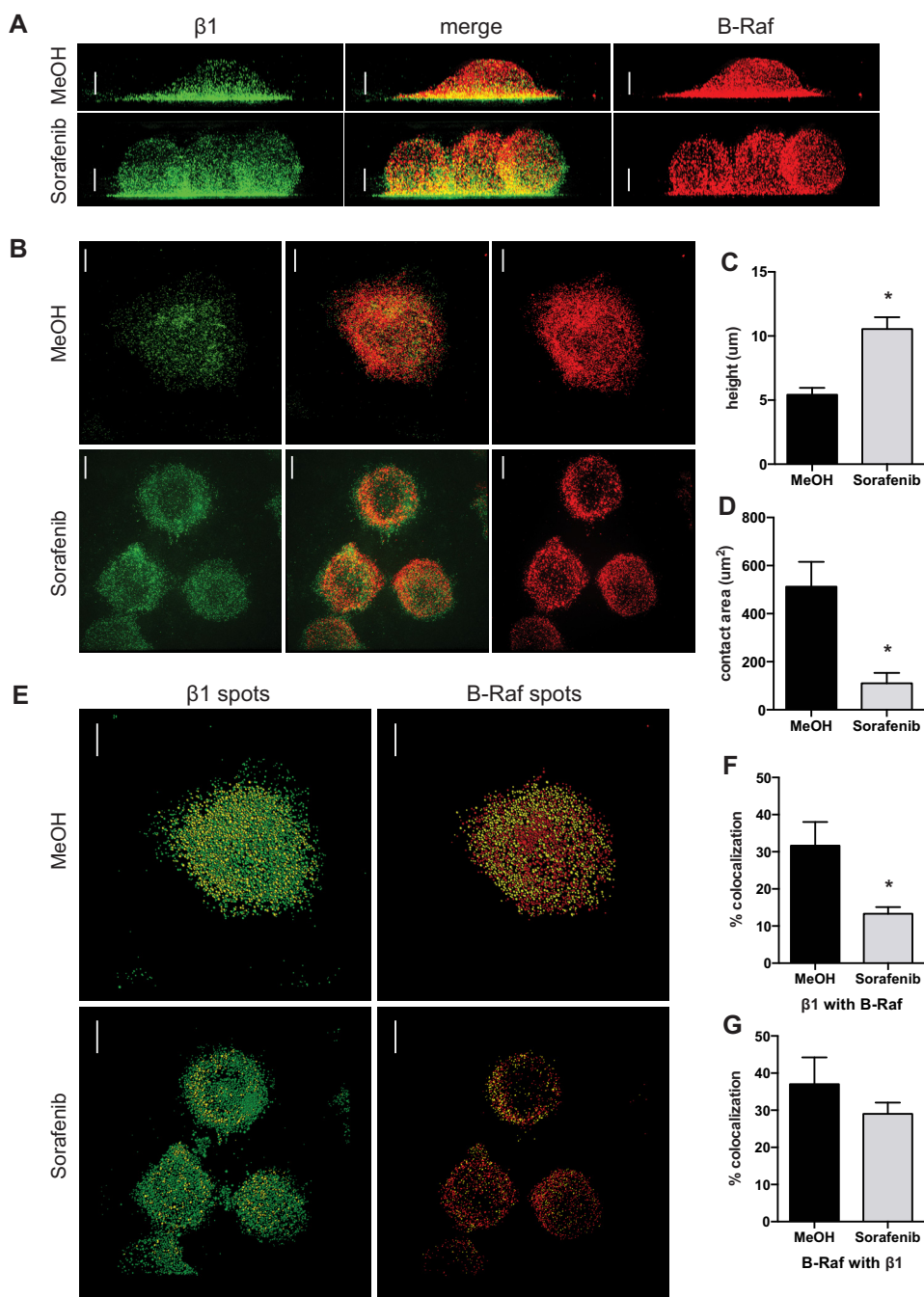


FIGURE 8. Sorafenib inhibits cell spreading and $\beta 1$ integrin colocalization with B-Raf after adhesion to VCAM-1. *A* and *B*, super-resolution immunofluorescence of $\beta 1$ integrin (green) and B-Raf (red) of Jurkat cells incubated for 1 h with methanol vehicle control or sorafenib (50 nM) and then fixed after 10 min of adhesion to glass coverslips coated with VCAM-1 (10 $\mu\text{g}/\text{ml}$). *A*, representative maximum intensity projection of three-dimensional reconstructed images rotated by 90° to illustrate the height of cells; *B*, representative maximum intensity projection of the five z-sections closest to the interface with the coverslip to illustrate the area of contact with VCAM-1. *C*, images were analyzed for the height of the cells when rotated by 90° in three dimensions; *D*, the area of contact at the interface of the glass coverslip. *E*, colocalization of $\beta 1$ integrin and B-Raf using the spot detection function of absolute fluorescence for total $\beta 1$ integrin (green) and $\beta 1$ integrin colocalized with B-Raf (yellow) (left) and total B-Raf (red) and B-Raf colocalized with $\beta 1$ integrin (yellow) (right). The percentage of colocalization was determined by dividing the number of colocalized spots by the total number of spots for $\beta 1$ integrin (*F*) and B-Raf (*G*). Data are shown from one experiment containing individual cells pretreated with methanol ($n = 10$) or sorafenib ($n = 14$), and the experiment was repeated with methanol ($n = 10$) and sorafenib ($n = 12$). Scale bars, 4 μm ; error bars, S.E. *, $p < 0.01$ using Student's *t* test.

the same cells can utilize distinct signaling components and downstream effectors. For example, paxillin binding to $\alpha 4$ integrin cytoplasmic domains is known to regulate cytoskeletal association and resistance to shear stress but not affinity of $\alpha 4\beta 1$, whereas paxillin does not bind to αL of LFA-1 (5, 30, 45).

In addition to possible Rap1 interaction, novel binding partners of B-Raf in T cells have been found (46). Of interest, B-Raf

was shown to interact with both Dock2 (dedicator of cytokinesis protein 2), a guanine nucleotide exchange factor known to specifically activate Rac, and IQGAP1 (Ras GTPase-activating-like protein 1), a scaffolding protein known to interact with actin, Rac1, calmodulin, and Src (46–48). Alternatively, B-Raf regulates cytoskeletal dynamics in melanoma cells by mediating cross-talk with the Rho/ROCK pathway through Rnd3 and

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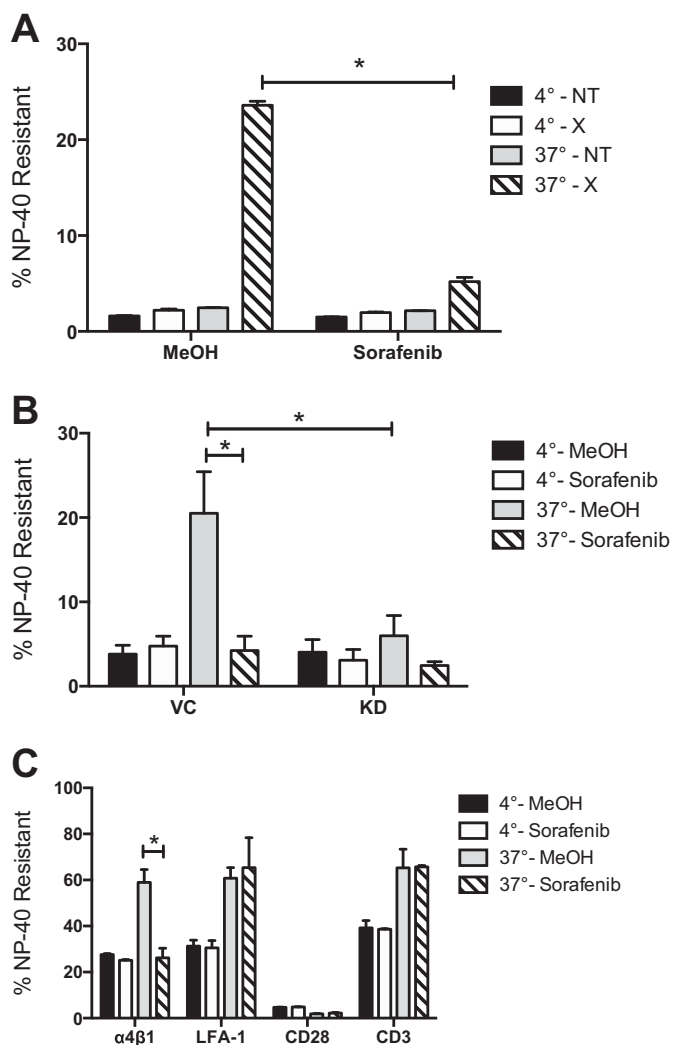


FIGURE 9. Sorafenib or B-Raf knockdown specifically reduces $\beta 1$ and $\alpha 4\beta 1$ integrin association with the cytoskeleton. *A*, cytoskeletal stabilization assay of Jurkat cells incubated for 1 h with sorafenib (50 nM) and methanol vehicle control and then incubated with AF-488-conjugated anti- $\beta 1$ integrin mAb and either cross-linked with an anti-mouse secondary mAb (X) or left untreated (NT) at 4 or 37 °C. *B*, cytoskeletal stabilization assay of vector control (VC) or B-Raf knockdown (KD) cells incubated for 1 h with sorafenib (50 nM) and methanol vehicle control and then incubated with AF-488-conjugated anti- $\beta 1$ integrin mAb and anti-mouse secondary mAb at 4 or 37 °C. *C*, Cytoskeletal stabilization assay of Jurkat cells incubated for 1 h with sorafenib (50 nM) and methanol vehicle control and then incubated with AF-647-conjugated anti-mouse secondary mAb and either anti- $\alpha 4\beta 1$ (19H8), anti-LFA-1 (32E6), anti-CD3, or anti-CD28 mAb at 4 or 37 °C. The percentage of Nonidet P-40-resistant mAb was determined by dividing the mean fluorescence intensity of Nonidet P-40-treated cells by the MFI of cells that received no Nonidet P-40. Error bars, S.E. *, $p < 0.001$ using Student's *t* test.

in fibroblasts through the ROCKII/LIMK/cofilin pathway (19, 49). Also, whereas an ERK-paxillin complex has since been identified in human adenocarcinoma cells, it was previously shown that Ras-induced serine phosphorylation of paxillin was mediated by induced expression of an activated B-Raf construct in a variety of cell types (17, 50). However, we found that sorafenib or B-Raf knockdown did not affect the induced phosphorylation of paxillin (Tyr-118) after adhesion to VCAM-1 (data not shown), a residue essential for actin cytoskeleton-dependent cell spreading and motility in lymphocytes (51–53), whereas other studies using adherent cell lines have identified

Raf-1 association with vimentin, myosin phosphatase, and the Rho-effector Rok- α , raising the possibility of similar interactions for B-Raf (54–56). Altogether, and given the inherent differences between how adherent cell types and lymphocytes regulate adhesion, more studies are required to explore the molecular details of how B-Raf and $\alpha 4\beta 1$ integrin interact in T cells.

The activities of all Raf isoforms are subject to complex regulation but have been shown to be dependent on Ras activity for the initiation of Raf activation. The activation of both A-Raf and Raf-1 requires phosphorylation on the B-Raf corresponding residue Ser-455, but this residue has been shown to be constitutively phosphorylated on B-Raf in many cell types (57, 58). Therefore, B-Raf activation is solely dependent on Ras activity and phosphorylation of Thr-598/Ser-601 (11). Constitutive activation of MAPK signaling by mutant forms of B-Raf (*i.e.* V600E) is observed broadly in solid cancers of multiple primary sites (59, 60). Leukemias of lymphoid origin express B-Raf, and other than the recent discovery of hairy cell leukemia, mutations of the *BRAF* gene are very rare, suggesting a fundamental and conserved role in T cell leukemia and possibly normal T cell function (61–67). Although there are few studies that focus on the importance of B-Raf to T cell physiology, it has been shown that MAPK signaling during T cell development progression beyond the CD4-CD8 double positive stage requires B-Raf, and rescue experiments with B-Raf can restore proliferation through MAPK signaling in anergic T cells (40, 67).

Specific inhibitors of mutant B-Raf were approved for treatment of melanoma in 2011, whereas general RAF inhibitors have been clinically used since 2007 (68–70). Chemical inhibition of MEK or RAF results in the inhibition of ERK and is sufficient to stop proliferation of many cancer cells (71). Sorafenib is a multikinase inhibitor once higher concentrations have been reached, but it is most specific for C-Raf (6 nM IC_{50}) and B-Raf (22 nM IC_{50}) and is still used in the clinic for the treatment of advanced renal cell carcinoma or hepatocellular carcinoma (29). The findings presented in this work should cause a reevaluation of clinical use of sorafenib having potential off-target effects on T cells (72–74). The effects of sorafenib on $\alpha 4\beta 1$ integrin could impact T cell migration and homing to sites of inflammation or potentially effector functions (75–77). This is significant because the selectivity of this interaction may provide a therapeutic target for $\alpha 4$ integrin-related diseases, such as asthma, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and certain leukemias and lymphomas (78). Given that we have focused on sorafenib inhibition of B-Raf Ser-445 and the rarity of B-Raf mutations in T cells, this work also suggests that specific V600E B-Raf inhibitors may have fewer off-target effects on T cells (79).

We propose a novel secondary role for B-Raf in the up-regulation of cytoskeletal association of $\alpha 4\beta 1$ and cell spreading mediated by the $\alpha 4\beta 1$ integrin to regulate resistance to shear stress. Interestingly, this effect is independent from downstream MEK/ERK signaling, and within the scope of our studies, this association is unique to $\alpha 4\beta 1$ integrin and not to other integrins or unrelated surface proteins. Both $\alpha 4\beta 1$ integrin and B-Raf play important roles in human diseases, and understanding the mechanisms of their functions will provide important

insights into the adaptive immune response and design of therapeutic strategies.

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