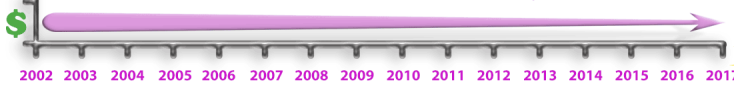




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Cyclin-Dependent Kinase Inhibitors Block Leukocyte Adhesion and Migration¹

Li Liu,^{2*} Barbara Schwartz,^{2*} Yoshiaki Tsubota,[‡] Elaine Raines,[‡] Hiroaki Kiyokawa,[§] Karyn Yonekawa,^{*} John M. Harlan,^{3,4*} and Lynn M. Schnapp^{3,4†}

Leukocyte trafficking is a tightly regulated process essential for an appropriate inflammatory response. We now report a new adhesion pathway that allows unstimulated leukocytes to adhere to and migrate through exposed endothelial matrix or high-density ligand, a process we have termed ligand-induced adhesion. This ligand-induced adhesion is integrin mediated, but in contrast to phorbol ester-stimulated adhesion, it is not dependent on the small GTPase Rap-1 activity. Instead, we show a critical role for cyclin-dependent kinase (Cdk) 4 in ligand-induced adhesion by three independent lines of evidence: inhibition by pharmacological inhibitors of Cdk, inhibition by dominant-negative construct of Cdk4, and inhibition by Cdk4 small interfering RNA. The major substrate of Cdk4, Rb, is not required for ligand-induced adhesion, suggesting the involvement of a novel Cdk4 substrate. We also demonstrate that Cdk4^{-/-} mice have impaired recruitment of lymphocytes to the lung following injury. The finding that Cdk inhibitors can block leukocyte adhesion and migration may expand the clinical indications for this emerging class of therapeutics. *The Journal of Immunology*, 2008, 180: 1808–1817.

Cyclin-dependent kinases (Cdks)⁵ are serine/threonine kinases that regulate progression through the cell cycle. Because of their critical role in cell proliferation and transcriptional regulation, Cdks are attractive therapeutic targets in different diseases and a number of pharmacological inhibitors have been developed to Cdks with varying degrees of specificity. All of the Cdk inhibitors to date act by competing with ATP for binding in the kinase ATP binding site (reviewed in Ref. 1). Cdk inhibitors are being evaluated for the treatment of malignancies, cardiovascular disease, and glomerulonephritis, based on the role of Cdks in cell proliferation (1, 2). However, it is increasingly clear that Cdks as well as cyclins and Cdk inhibitors are important for other functions, including cytoskeleton rearrangement (3), cell motility (4), regulation of apoptosis (5), and neurite outgrowth (6). Thus, there is increasing evidence that Cdks may have nontraditional roles in various cell behaviors, including those related to adhesion and migration.

Leukocyte trafficking from blood stream to tissue plays a key role in response to inflammation and infection. This process is a well-orchestrated series of adhesion, de-adhesion, signaling, and cytoskeletal changes that are tightly regulated. Leukocytes do not adhere to underlying endothelial cells (EC) when in a resting state. However, upon activation, that is, by cytokines or chemokines, leukocytes rapidly modulate changes in integrin conformation and/or clustering to alter integrin affinity and/or avidity that permit targeted integrin-mediated adhesion to the vascular EC and subsequent migration between EC (reviewed in Ref. 7). Following diapedesis, the leukocytes migrate through subendothelium and extravascular tissue via the interaction of integrin receptors with extracellular matrix components.

We previously demonstrated that phorbol ester-stimulated adhesion of Jurkat cells to fibronectin required activation of the small GTPase Rap1 (8). We also showed that leukocytes could adhere spontaneously to high-density fibronectin, a process we refer to as “ligand-induced adhesion.” We now further characterize the mechanism of ligand-induced adhesion in leukocytes and show that this pathway allows leukocyte adhesion to physiological relevant substrates such as the exposed endothelial matrix in the absence of exogenous stimulation. In contrast to phorbol ester-stimulated adhesion, this ligand-induced adhesion is not dependent on Rap1 but is dependent on Cdk4. Inhibition of ligand-induced adhesion and migration by Cdk inhibitors suggest that some of the in vivo effects of Cdk inhibitors may be due to blockade of leukocyte adhesion and migration, rather than, or in addition to, blockade of cell cycle.

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⁵ Abbreviations used in this paper: Cdk, cyclin-dependent kinase; BAEC, bovine aortic endothelial cell; EC, endothelial cell; siRNA, small interfering RNA; BALF, bronchoalveolar lavage fluid; Rb, retinoblastoma; DN, dominant negative; PDBu, phorbol dibutyrate.

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Materials and Methods

Cells

Jurkat T, Ramos B, and THP-1 cells were obtained from the American Type Culture Collection and were cultured in RPMI 1640 (Mediatech) supplemented with glutaMAX-1 (Invitrogen Life Technologies), 1 mM sodium pyruvate (BioWhittaker), nonessential amino acids (BioWhittaker), and 10% FBS (HyClone). Peripheral blood was obtained from healthy donors with informed consent according to protocols approved by the Human Subjects Review Committee of the University of Washington. PBMC were isolated by Ficoll-Hypaque (Pharmacia) gradient centrifugation and

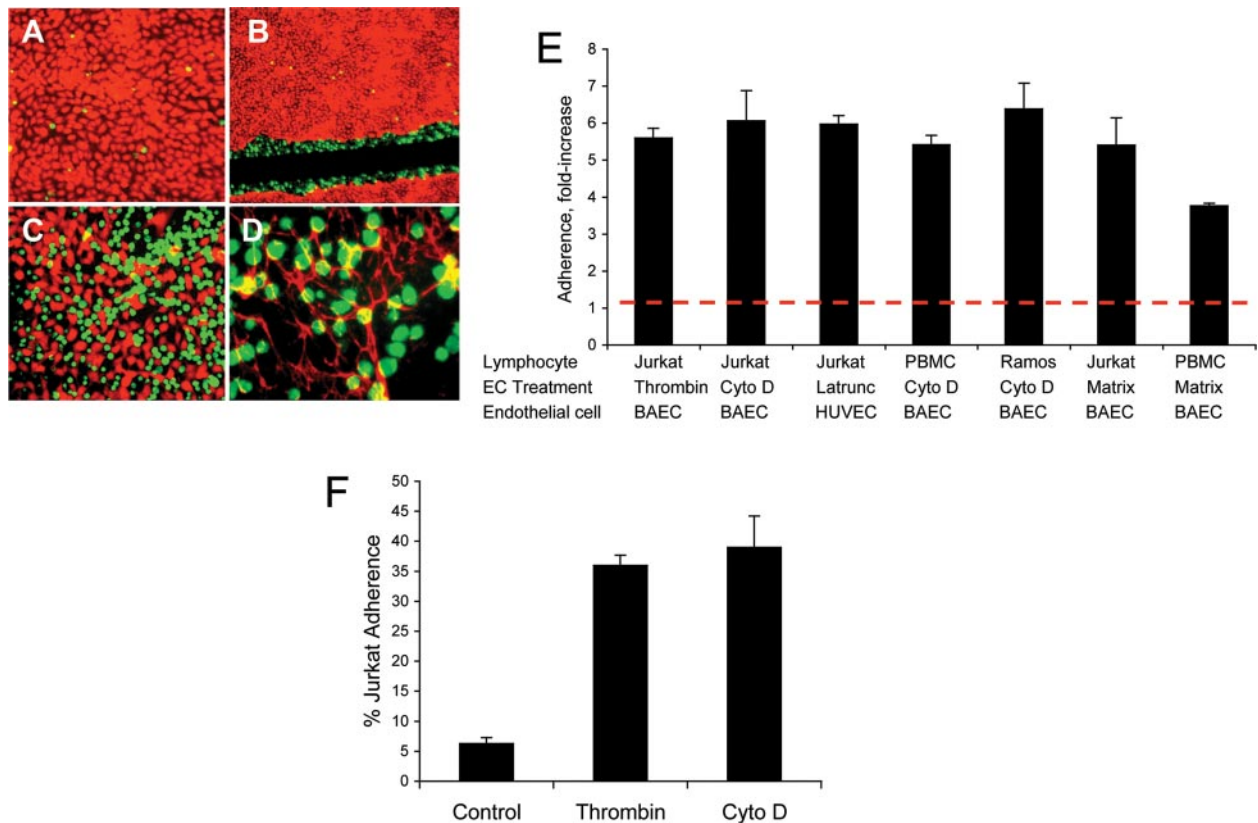


FIGURE 1. Unstimulated leukocytes adhere to disrupted EC monolayers and the endothelial matrix. *A*, Adhesion of unstimulated Jurkat cells (green) to a confluent monolayer of BAEC (red) after 30 min. *B*, Adhesion of unstimulated Jurkat cells to the edges of a wounded BAEC monolayer. A confluent endothelial monolayer was wounded by scratching and the intervening matrix was removed by scraping and aspirating. *C*, Adhesion of unstimulated Jurkat cells to cytochalasin-retracted BAEC monolayer. *D*, Adhesion of unstimulated Jurkat cells to the BAEC-derived matrix. Fibronectin fibrils are visualized in red with an Ab to bovine fibronectin. Original magnification: $\times 10$ (*A* and *C*); $\times 4$ (*B*); and $\times 20$ (*D*). *E*, PBMC, Jurkat cells, or Ramos cells were allowed to adhere to the BAEC-, HUVEC-, or EC-derived matrix for 20 min at 37°C. EC treatments were: human thrombin, 10 U/ml for 10 min; cytochalasin D (Cyto D), 1 μ M for 75 min; and latrunculin A, 1 μ M for 50 min. The EC matrix was prepared by treating confluent EC monolayers with 20 mM NH_4OH at 37°C for 5 min. *x*-Axis: fold-increase in adhesion compared with adhesion to the untreated endothelial monolayer of at least six independent experiments performed in triplicate wells. Dotted line represents adhesion to untreated endothelial monolayer. Values of $p < 0.01$ for all compared with controls. *F*, A representative experiment of Jurkat cell adhesion to thrombin- or cytochalasin D-retracted BAEC or untreated BAEC monolayer (control) is shown ($n = 6$ independent experiments). Data are the means of triplicate wells \pm SD.

washed with PBS. HUVEC were isolated and cultured as previously described (9) and were grown in RPMI 1640 supplemented with 2 mM glutamine, sodium pyruvate, nonessential amino acids, 10 mM HEPES, 100 U/ml penicillin, 100 U/ml streptomycin, 250 ng/ml Fungizone (BioWhittaker), 90 mg/ml heparin (Sigma-Aldrich), bovine hypothalamic extract, and 10% FBS (HyClone). HUVEC were cultured on surfaces coated with 2% gelatin (Sigma-Aldrich). BAEC were a gift from Helene Sage (Hope Heart Institute, Seattle WA) and were grown in DMEM (Mediatech) supplemented with glutaMAX-1, sodium pyruvate, and 8% FBS. The EC matrix was prepared by lysis of a confluent layer of EC with 20 mM NH_4OH at 37°C for 5 min (10) and blocked briefly with growth medium.

Adhesion assays

Lymphocytes were labeled with 2.5 mM calcein AM (Molecular Probes) at room temperature for 20–40 min, washed, and resuspended in HBSS with calcium and magnesium (Mediatech) supplemented with 0.1% BSA and 4 mM HEPES. Lymphocytes were incubated with inhibitors or function-blocking Abs at 37°C for 10–30 min and tested for ligand-induced adhesion or phorbol ester-stimulated adhesion to EC or to the EC matrix for 15–30 min at 37°C. Adhesion was measured by a Cytofluor Series 4000 fluorescence plate reader (PerSeptive Biosystems). Plates were scanned before and after washing for total and adherent cells, respectively. Statistical significance was determined by Student's *t* test. In some cases, EC were labeled with Cell Tracker Orange (Molecular Probes) per the manufacturer's instructions before adhesion. Cells were fixed after adhesion assays and adherent cells were directly visualized by fluorescent microscopy. Underlying fibronectin fibrils were visualized with polyclonal anti-bovine

fibronectin Ab (Accurate), followed by Alexa 568-conjugated secondary Ab (Molecular Probes).

Transfections

Cdk2 and Cdk4-dominant negative (DN) constructs were a generous gift from J. Roberts (Fred Hutchinson Cancer Research Center, Seattle, WA) (11). Cdk5 DN construct was a generous gift from P. Zelenka (National Eye Institute, National Institutes of Health, Bethesda, MD) (12). Generation of Rap 1 DN transfectants was previously described (8). A phosphorylation-deficient construct of pRb was a gift from Dr. J. Lukas (Institute of Cancer Biology for Center for Genotoxic Stress Research, Copenhagen, Denmark) (13, 14). Transfections were performed with TransIT-Jurkat Transfection Reagent (Mirus, Gene Transfer Specialist TM) according to the manufacturer's instructions. Controls consisted of cells transfected with empty vector pCMV alone. Stably transfected cell lines were selected in medium containing the neomycin analog G418 (1 mg/ml). Expression was verified by Western blot analysis. Cdk and retinoblastoma (Rb) siRNAs were purchased from Invitrogen Life Technologies and transient transfections were performed using a Nucleofactor Device (Amaxa Biosystems).

Western blot analysis

Equal numbers of Jurkat cells (1×10^6) were lysed in buffer containing 50 mM HEPES (pH 7.5), 10 mM NaCl, 5 mM MgCl_2 , 1 mM EGTA, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 1% Triton X-100, and 10% glycerol and Complete Mini Protease Inhibitor Cocktail (Roche). Equal amounts of protein were separated by SDS-PAGE, transferred to Immobilon, and blocked

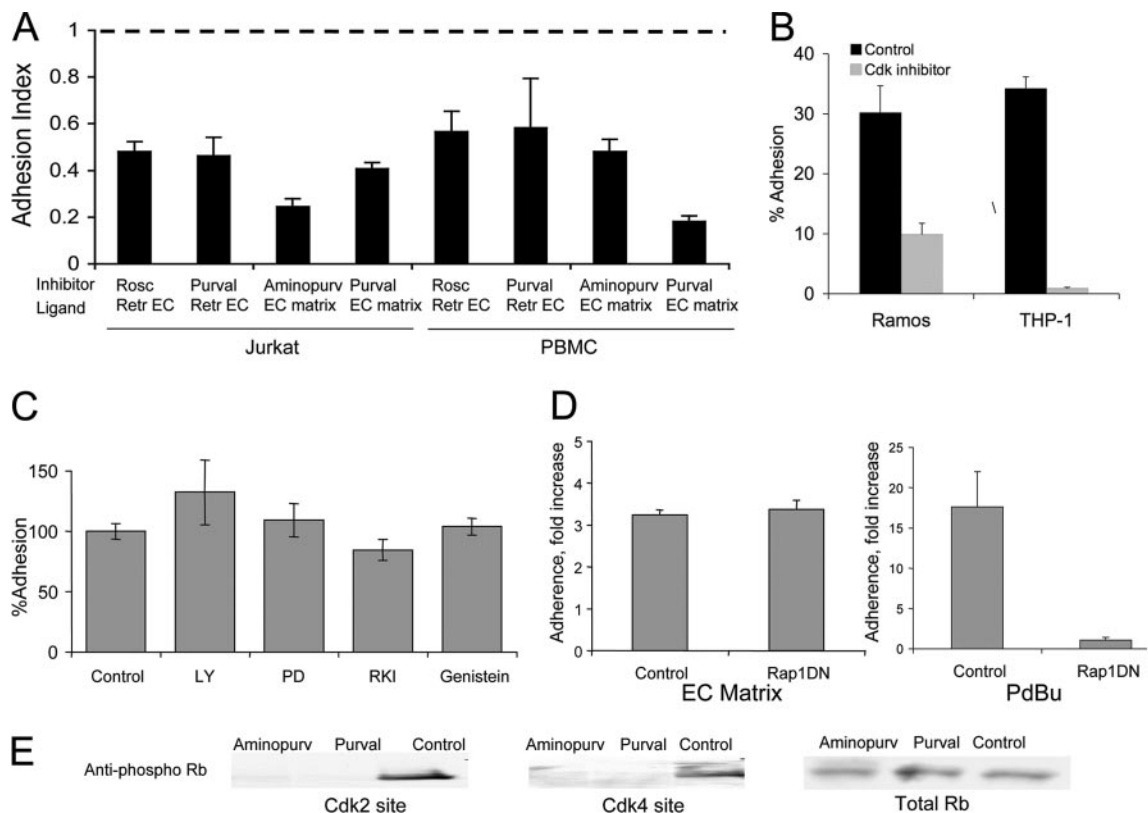


FIGURE 2. Cdk inhibitors block ligand-induced adhesion to the endothelial matrix and retracted EC, but Rap1 inhibition has no effect. *A*, PBMC or Jurkat cells were treated with 20 μ M roscovitine, purvalanol A, or aminopurvalanol A for 30 min, then allowed to adhere to cytochalasin D-retracted BAEC or BAEC-derived matrix for 30 min. Adhesion index is reported as the ratio of adhesion with inhibitors/adhesion without inhibitors of six replicate wells \pm SD. Dotted line represents adhesion to untreated EC monolayer. *B*, Ramos B cells or THP-1 cells with or without 10 μ M purvalanol A were allowed to adhere to the BAEC-derived matrix for 30 min. Average of three replicates \pm SD is shown. *C*, Jurkat cells were treated with PI3K inhibitor (50 μ M LY294002), MAPK inhibitor (50 μ M PD98059), RhoA kinase inhibitor (30 μ M Y7632), or tyrosine kinase inhibitor (10 μ M genistein) before adhesion to the EC matrix. Average of six replicates \pm SD is shown. *D*, Jurkat cells were transfected with Rap1DN construct or control vector and unstimulated (*left*) or stimulated with phorbol dibutyrate (PDBu; *right*) and allowed to adhere to the EC matrix for 30 min. Values represent means of three independent experiments performed in triplicate. *E*, Cdk inhibitors block Rb phosphorylation. Jurkat cells were treated with 20 μ M aminopurvalanol A or purvalanol A for 30 min and then lysed. Equal amounts of protein were separated by SDS-PAGE, then blotted with a phospho-specific Ab to the Cdk2-specific site on Rb (T821; *left*) or Cdk4-specific site on Rb (249/252; *middle*) or total Rb (*right*) as loading control.

for 1 h. Blots were incubated with primary Ab for 1 h, followed by peroxidase-conjugated secondary Ab for 1 h and then developed with ECL (Amersham Biosciences). Anti-phosphorylated Rb (T821) and anti-phosphorylated Rb (249/252) were obtained from BioSource International. Anti-Cdk2, anti-Cdk4, and anti-Cdk5 Abs were obtained from eBioscience.

Reagents and Abs

Cdk inhibitors aminopurvalanol A, purvalanol A, and roscovitine were obtained from Tocris Bioscience. Cytochalasin D and additional inhibitors were obtained from Calbiochem. Function-blocking Abs to α_4 and α_5 integrin subunits were obtained from Chemicon. The β_1 integrin-blocking Ab 5D1 was previously characterized (9). The β_1 integrin Ab 9EG7, which recognizes the activation epitope (15), was obtained from BD Biosciences. The β_1 integrin-activating Ab 8A2 was previously characterized (16). All Abs were used at saturating concentrations. Actin and tubulin Abs were obtained from Chemicon.

Flow cytometry

Integrin subunit expression levels were analyzed by a FACScan (BD Biosciences) instrument. Analyzed cells were incubated with integrin Abs for 30 min, followed by incubation with fluorescein-labeled secondary Abs for 30 min. In some cases, cells were pretreated with the β_1 -activating Ab 8A2 for 30 min at 37°C before analysis. PBMC cellular populations before and after adhesion were analyzed on the basis of forward and side scatter properties. Data were acquired and analyzed using CellQuest 3.3 (BD Biosciences).

Migration assays

Confluent HUVEC, grown on gelatin-coated Transwell filter (3 μ m; Corning), were treated with 20 mM NH_4OH for 20–30 min to remove the EC

and then the remaining EC matrix was washed with PBS. Calcein-labeled PBMC were allowed to adhere to the EC matrix for 30 min, followed by washing with Percoll to remove nonadherent cells. Cells were then treated with 20 μ M purvalanol or vehicle (DMSO). PBMC were allowed to migrate for 4.5 h at 37°C and the number of migrated cells in the bottom chamber was counted.

Bleomycin-induced lung injury

This animal experiment was approved by the Institutional Animal Care and Use Committee of the University of Washington. Generation of Cdk4^{-/-} mice was previously described (17). Cdk4^{-/-}, Cdk4^{+/-}, or Cdk4^{+/+} littermates underwent intratracheal instillation with 0.033 U of bleomycin in 50 μ l of saline (SICOR Pharmaceuticals) as previously described (18). Seventy-two hours later, mice were sacrificed, the right main stem bronchus was tied off, and the left lung was isolated and lavaged with 1 ml of PBS containing 0.6 mM EDTA warmed to 37°C. Bronchoalveolar lavage fluid (BALF) total cell count was determined by trypan blue exclusion and cell differential was determined on DiffQuik (Dade Behring)-stained cytopins.

Results

Leukocytes adhere spontaneously to the EC matrix

Confluent bovine aortic EC (BAEC) or HUVEC were treated with low-dose cytochalasin D to cause them to retract. Untreated Jurkat cells were allowed to adhere to cytochalasin-treated EC. We found minimal Jurkat cell adhesion to intact endothelial monolayers (Fig. 1A), but large numbers of Jurkat cells attached adjacent to the retracted EC (Fig. 1C). In addition, wounding an endothelial monolayer by scratching also

Table I. Cell differential following ligand-induced adhesion^a

	Lymphocytes (%)	Monocytes (%)
Total PBMC	67	31
Adherent cells	25	73

^a Freshly isolated PBMC and cells adherent to the EC matrix after 20 min at 37°C were evaluated by forward and side scatter to assess cell distributions. The data are the means of two separate experiments, and similar data were obtained in an additional experiment in which cell differentials were evaluated from cytopsins of the two populations.

caused Jurkat cells to adhere spontaneously to the margin of the retracted monolayer (Fig. 1B). When EC were removed by NH₄OH treatment, the remaining underlying matrix, which contains fibronectin, also supported spontaneous Jurkat cell adhesion (Fig. 1D). The underlying EC matrix is a complex substrate that includes matrix proteins, such as fibronectin, and proteoglycans (19). High-concentration fibronectin also supported unstimulated adhesion of Jurkat cells.

To quantify adhesion, we performed cell adhesion assays as previously described (8). PBMC adhesion to cytochalasin-treated EC and the underlying EC matrix demonstrated a 5- to 6-fold increase in adhesion relative to an untreated EC monolayer (Fig. 1E). Similar results were also found with Jurkat T cells and Ramos B cells (Fig. 1, E and F). To determine which

cells in PBMC were adherent, we performed flow cytometry analysis of total PBMC and adherent PBMC. By flow cytometric analysis of forward and side scatter, we found that both monocytes and lymphocytes adhered to the EC matrix (Table I). To examine a more physiological agent to provoke EC retraction, we treated BAEC with thrombin. Thrombin, like cytochalasin D, caused EC retraction and increased Jurkat cell adhesion by 4- to 5-fold (Fig. 1F). Additional reagents that caused EC retraction, including staurosporine, latrunculin, EDTA, and PBS without calcium and magnesium, showed similar enhancement of adhesion (Fig. 1E and data not shown). Of note, treatment of EC with agents that stimulated apoptosis but failed to induce retraction, such as colchicine, vinblastine, nocadazole, and paclitaxel, also failed to produce comparable leukocyte adhesion (data not shown). Similar results were found whether BAEC or HUVEC were used.

Pharmacological inhibitors of Cdk, but not other kinases, inhibit spontaneous adhesion to the matrix

To determine the pathway(s) involved in ligand-induced adhesion, we tested the effects of pharmacological inhibitors of various pathways. Only Cdk inhibitors blocked ligand-induced adhesion to retracted EC or the EC matrix (Fig. 2A). When PBMC or Jurkat were treated with the Cdk inhibitors roscovitine, purvalanol A, or aminopurvalanol A, their adhesion to retracted BAEC or the EC matrix

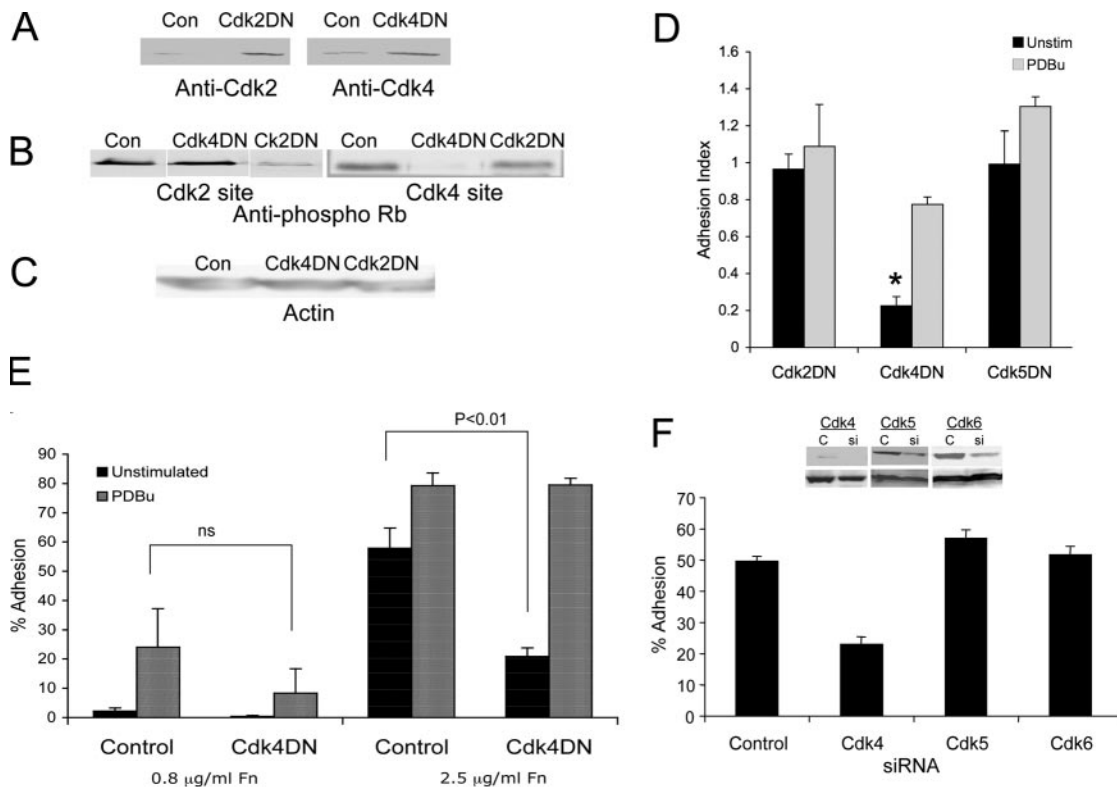


FIGURE 3. Cdk4 DN construct and Cdk4 siRNA inhibit ligand-induced adhesion. *A*, Jurkat cells stably transfected with Cdk2 DN (*left*) or Cdk4 DN (*right*) or control vector were lysed and equal amounts of protein were separated by SDS-PAGE and then blotted with an Ab to Cdk2 or Cdk4. *B*, Cdk2 and Cdk4 DN constructs specifically inhibit phosphorylation of Rb protein. Jurkat cell transfectants were lysed, and equal amounts of protein were separated by SDS-PAGE and then blotted with a phospho-specific Ab to a Cdk2-specific phosphorylation site on Rb (T821; *left*) or Cdk4-specific site on Rb (249/252; *right*). *C*, Blots were probed with Ab to actin as loading control. *D*, Cdk4 DN, but not Cdk2 DN or Cdk5DN, inhibits ligand-induced adhesion to the EC matrix. Control, Cdk2 DN, Cdk4 DN, or Cdk5DN Jurkat cells were tested for unstimulated and phorbol ester-stimulated adhesion to the BAEC matrix. Adhesion index is the adhesion of DN transfectants compared with adhesion of controls. *, $p < 0.0001$ compared with control. *E*, Unstimulated Jurkat cell adhesion to high-density fibronectin is inhibited by Cdk4 DN. Data are means of at least six independent experiments of five replicate wells each \pm SEM. *F*, Jurkat cells were transiently transfected with Cdk4, Cdk5, or Cdk6 siRNA or control vector. After 48 h, cells were lysed, and equal amounts of protein were separated by SDS-PAGE and then blotted with Ab to the indicated Cdk (*top*) or total Rb as loading control (*bottom*). Concurrent transfectants were allowed to adhere to the endothelial matrix for 30 min.

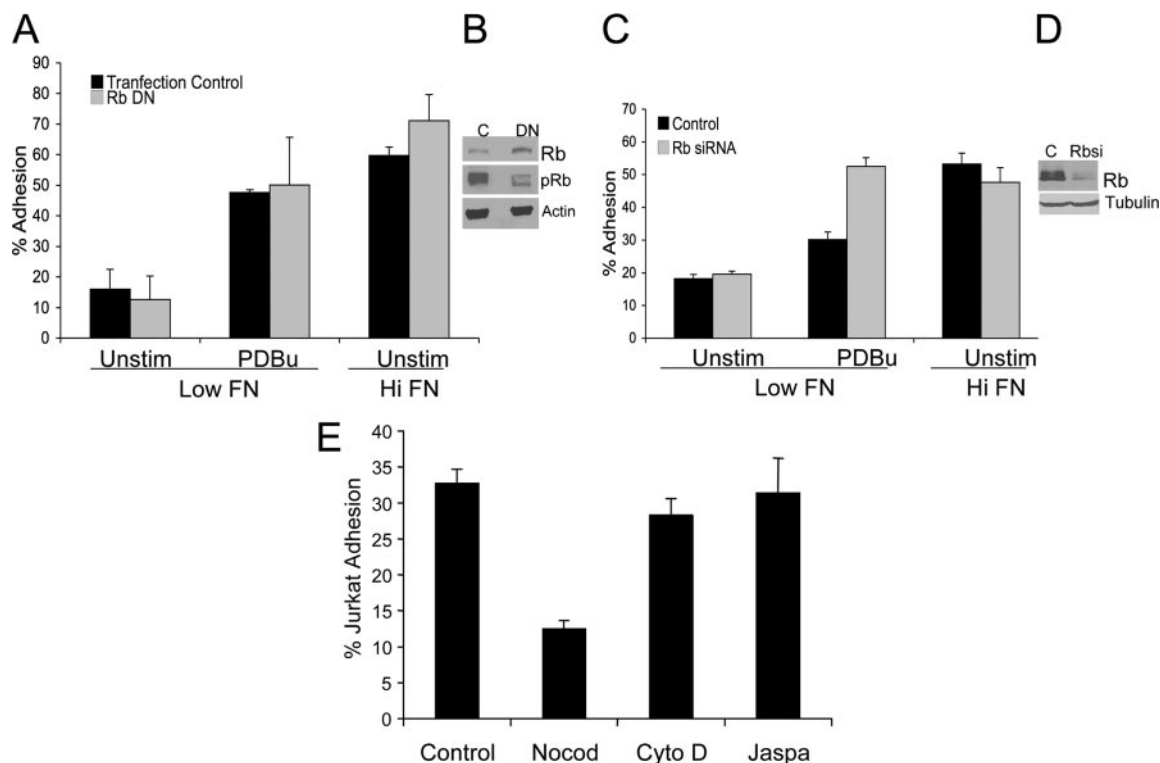


FIGURE 4. A, Rb is not required for ligand-induced adhesion of Jurkat cells to high-density fibronectin (FN). Jurkat cells stably transfected with DN Rb or control vector were allowed to adhere to low- or high-density fibronectin with or without PDBu stimulation. B, Lysates of Jurkat cells transfected with DN Rb or control were blotted with Ab to total Rb (*top*), phospho-specific Rb (*middle*), or actin as a loading control (*bottom*). C, Jurkat cells were transiently transfected with Rb siRNA or control construct. After 48 h, cells were allowed to adhere to low- or high-density fibronectin with or without PDBu stimulation. D, Concurrent Jurkat cell transfectants were lysed, and equal amounts of protein were separated by SDS-PAGE and then blotted with Ab to Rb (*top*) or tubulin as a loading control (*bottom*). E, Microtubules but not actin cytoskeleton are involved in ligand-induced adhesion. Jurkat cells were pretreated with nocodazole (20 μ M), jasplakinolide (50 ng/ml), or cytochalasin D (1 μ M) for 20 min, then were allowed to adhere to the EC matrix. Data are the means of triplicate wells \pm SD.

was significantly reduced (Fig. 2A). In contrast, the Cdk inhibitors had no effect on phorbol ester-stimulated adhesion (data not shown). Cell viability was similar in Cdk inhibitor- and vehicle-treated cells by calcein fluorescence and trypan blue exclusion (>95%). In addition to the T lymphocytic cell line (Jurkat), we showed that ligand-induced adhesion in the B lymphocytic cell line (Ramos) and monocytic cell line (THP-1) was inhibited by Cdk inhibitors (Fig. 2B). Inhibitors of other pathways, including RhoA kinase (Y-7632), MAPK (PD98059), PI3K (LY294002), and tyrosine kinases (genistein) did not inhibit ligand-induced adhesion (Fig. 2C). We previously showed that phorbol ester-stimulated Jurkat adhesion to low-density fibronectin was dependent on Rap1A activity (8). In contrast, overexpression of the DN Rap1 construct N17Rap1 had no effect on ligand-induced adhesion of Jurkat cells to the BAEC-derived matrix, whereas phorbol ester-stimulated Jurkat adhesion was almost completely inhibited (Fig. 2D).

To verify the efficacy and specificity of the Cdk inhibitors, we examined phosphorylation of Rb protein, the major substrate of Cdk. Roscovitine and aminopurvalanol A reportedly inhibit Cdk2 but not Cdk4 (1). Cdk2 and Cdk4 phosphorylate Rb at different sites: Cdk2 phosphorylation sites include threonine 821 and Cdk4 phosphorylation sites include serine 249 and threonine 252 (20). Although treatment with the Cdk inhibitors did block phosphorylation of Rb, we found that the inhibitors blocked phosphorylation of both Cdk sites (Fig. 2E). Thus, at the doses tested in leukocytes, the Cdk inhibitors function as broad-spectrum Cdk inhibitors, rather than subtype-specific in-

hibitors. Nevertheless, these data suggested that Cdks might be involved in ligand-induced adhesion.

Cdk4 is involved in ligand-induced adhesion

Recent studies have shown that Cdk inhibitors can target additional pathways, including MAPK (21). Therefore, to confirm our inhibitor studies and to determine which Cdk was involved in ligand-induced adhesion, we tested the ability of Cdk DN constructs to inhibit ligand-induced adhesion. We stably transfected Jurkat cells with a Cdk4 DN or Cdk2 DN expression vector that contained a mutation D145N that prevents their activity (11) or Cdk5 DN with inactivating mutation at T33 (12). Construct expression and inhibition of Rb phosphorylation was confirmed (Fig. 3, A–C, and data not shown). Only Cdk4 DN reduced the adhesion; Cdk2 DN and Cdk5 DN did not inhibit ligand-induced adhesion (Fig. 3D). We previously showed that lymphocytes were able to adhere spontaneously to high-density fibronectin in a Rap-1-independent manner. We now show that spontaneous adhesion to high-density fibronectin is inhibited by Cdk4 DN (Fig. 3E). Importantly, the Cdk DN did not affect phorbol ester-stimulated adhesion (Fig. 3, D and E).

To further confirm the role of Cdk4, we examined the effect of Cdk4 siRNA on ligand-induced adhesion of Jurkat and Ramos cells. Maximal reduction in Cdk4 protein was achieved at 48 h after initial transfection, as indicated by Western blot analysis (Fig. 3F). At that time, ligand-induced adhesion was almost completely abolished, while control vector had no effect (Fig. 3F). In addition, Cdk5 and Cdk6 siRNA did not inhibit ligand-induced adhesion.

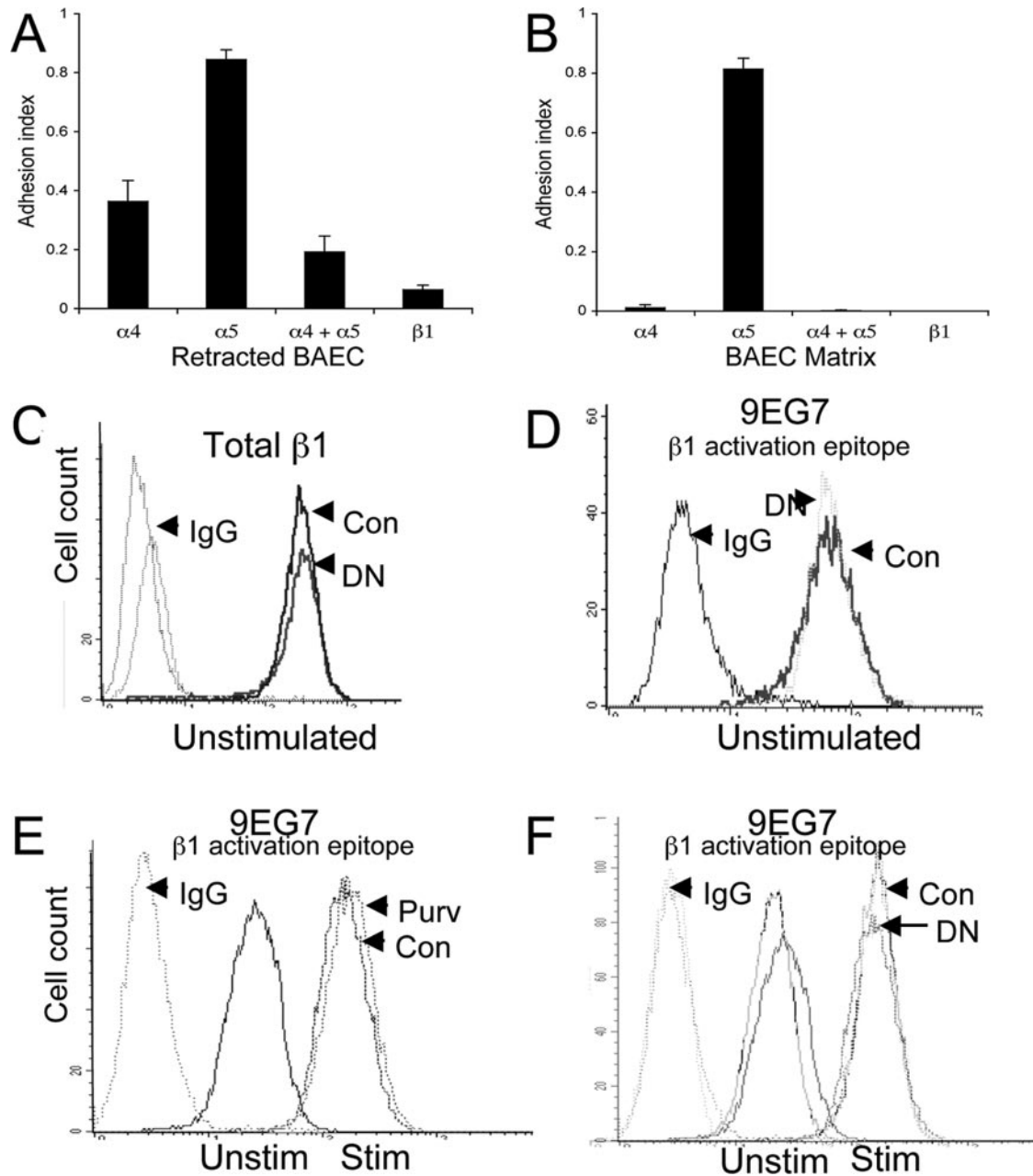


FIGURE 5. Ligand-induced adhesion is dependent on β_1 integrins. *A*, Jurkat cells were pretreated with the indicated function-blocking Ab for 10 min, then allowed to adhere to cytochalasin D-retracted BAEC for 20 min. *B*, Jurkat cells were pretreated with the indicated function-blocking Ab for 10 min, then allowed to adhere to the BAEC-derived matrix for 20 min. Adhesion index is reported as the ratio of adhesion with Abs/adhesion without Abs of six replicate wells \pm SD. *C–F*, No change in integrin expression or activation following Cdk4 blockade. Wild-type (Con) or Cdk4 DN Jurkat cells were incubated with an Ab to β_1 (*C*) or with 9EG7, a mAb that recognizes an activation epitope of β_1 integrins (*D*) and analyzed by flow cytometry. *E*, Jurkat cells with or without purvalanol or (*F*) Cdk4 DN Jurkat cells were stimulated (Stim) for 15 min with the β_1 integrin-activating mAb 8A2, then analyzed for cell surface expression of the activation epitope of β_1 by incubation with 9EG7 Ab. *x*-Axis represents log fluorescent units; *y*-axis is cell count. Each experiment was independently replicated at least three times. Unstim, Unstimulated.

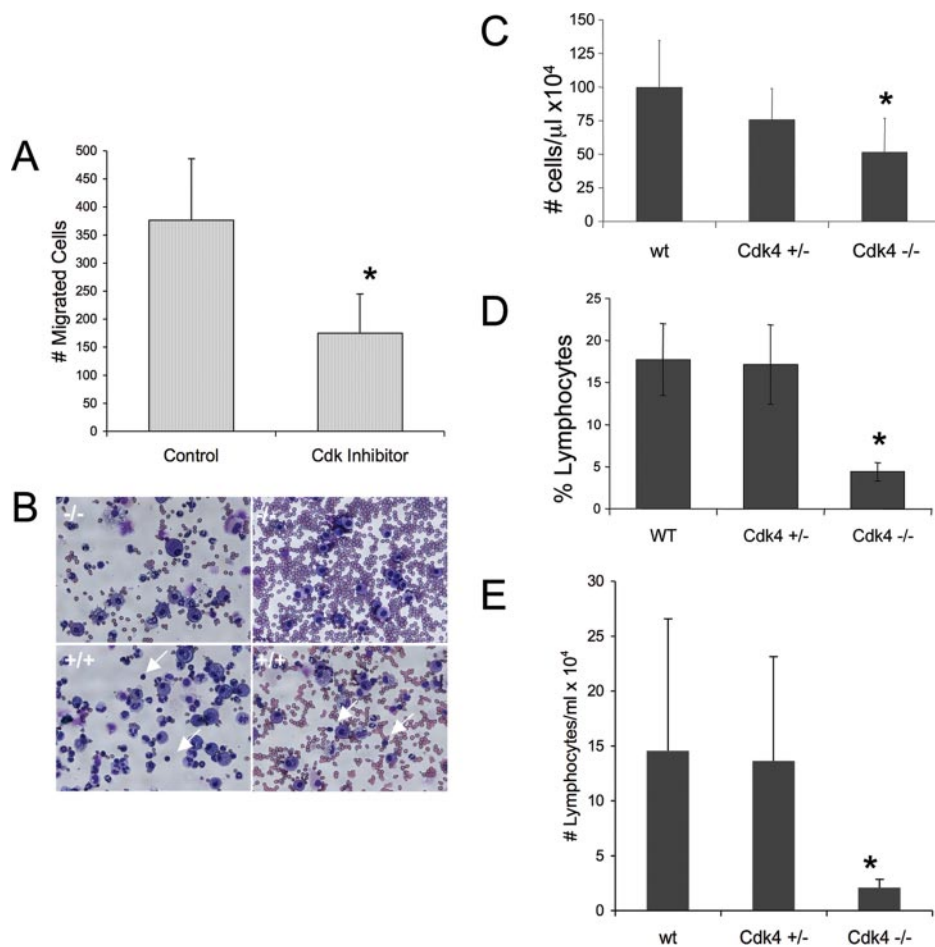
Taken together, these results show that Cdks, specifically Cdk4, are involved in ligand-induced adhesion.

Rb phosphorylation is not required for ligand-induced adhesion

The ability of inhibitors to rapidly (within 20 min) down-regulate ligand-induced adhesion suggests that Cdk activity is required for sustained ligand-induced adhesion and is independent of transcription. In addition, transcription and translation inhibitors actinomycin and cycloheximide had no effect on ligand-induced adhesion (data not shown). Rb family members are the major substrate for Cdks (22). To determine whether Rb is involved, we transfected

Jurkat cells with a phosphorylation-deficient construct of pRb (pRb^{cdk}) lacking 10 CDK consensus sites (13). Because Rb is unable to be phosphorylated, it remains constitutively active (continues to bind transcription factors and prevents progression through the cell cycle G₁) (14). If Rb phosphorylation is required for ligand-induced adhesion, overexpression of this construct should inhibit it. However, overexpression of phosphorylation-deficient Rb did not inhibit ligand-induced adhesion (Fig. 4, *A* and *B*), suggesting that Rb is not required for ligand-induced adhesion. As further confirmation, knockdown of Rb by siRNA had no effect on ligand-induced adhesion (Fig. 4, *C* and *D*).

FIGURE 6. A, Leukocyte migration across the EC matrix is inhibited by Cdk inhibitors. PBMC were allowed to adhere to the endothelial matrix for 30 min, nonadherent cells were removed, then control diluent (DMSO) or purvalanol (20 μ M) was added. Cells that migrated through the Transwell filters after 4.5 h were counted. Data are presented as the mean of two independent experiments performed in triplicate \pm SD and expressed as a migration index (cell number/average of control DMSO). The number of migrated cells for the control ranged from 285 to 467 in the two experiments. *, $p < 0.001$ compared with control. B–D, Decreased lymphocyte recruitment in Cdk4^{-/-} mice. Wild-type (wt, WT) ($n = 9$), Cdk4^{+/-} ($n = 4$), and Cdk4^{-/-} ($n = 5$) mice were treated with bleomycin administered intratracheally. Bronchoalveolar lavage was performed 72 h later B, Cytospin demonstrating relative paucity of lymphocytes (arrows) in Cdk4^{-/-} mice compared with wild-type mice (two mice per condition). Original magnification, $\times 40$. C, Total BALF cell count. D and E, Percent and absolute number of lymphocytes in BALF. *, $p < 0.04$ -/- compared with wild type.



We previously reported that a moderate dose of cytochalasin D (1 μ M), which targets actin microfilaments, inhibited phorbol ester-stimulated adhesion (8). However, the same dose of cytochalasin, or jasplakinolide, a stabilizer of actin filaments, had no effect on ligand-induced adhesion (Fig. 4E). In contrast, treatment with nocodazole, an inhibitor of microtubules, significantly inhibited ligand-induced adhesion, but had no effect on phorbol ester-stimulated adhesion (Fig. 4E). Taken together, the data suggest a non-traditional role of the cytoskeleton in ligand-induced adhesion.

β_1 integrins are involved in ligand-induced adhesion

Integrins mediate binding to many components of the endothelial matrix. We previously reported that increased Jurkat adhesion to EC treated with staurosporine was β_1 integrin dependent (9). To determine the cell adhesion receptors involved in ligand-induced adhesion, we pretreated Jurkat cells with function-blocking mAbs to β_1 , α_4 , and α_5 integrin subunits. Pretreatment with β_1 -blocking mAb almost completely abrogated adhesion to retracted EC or the EC matrix (Fig. 5, A and B). Pretreatment with α_4 -blocking mAb also significantly decreased cell adhesion. In contrast, blockade of α_5 had no significant effect on adhesion to retracted EC or the EC matrix, although blockade of both α_4 and α_5 further reduced cell adhesion to retracted EC. The results suggest that $\alpha_4\beta_1$ is the primary integrin involved in ligand-induced adhesion of leukocytes to the EC matrix. However, $\alpha_5\beta_1$ may also contribute to ligand-induced adhesion.

No change in β_1 integrin expression or conformation following Cdk blockade

Because ligand-induced adhesion is spontaneous and Cdk inhibitors rapidly (within 20 min) inhibit the adhesion, it is unlikely to

involve changes in surface expression of β_1 integrin. As confirmation, we found no difference in total surface expression of α_4 or β_1 in Jurkat cells with or without Cdk4 DN or Jurkat cells with or without Cdk inhibitors (Fig. 5C and data not shown). However, integrins can assume different conformational states and many integrins at rest exhibit a low affinity state. Thus, a change in the conformation state of β_1 could contribute to ligand-induced adhesion. Therefore, we determined the effect of Cdk blockade on the expression of the β_1 activation epitope defined by mAb 9EG7 (15). We found no difference in baseline expression of the activation epitope by flow cytometry in Jurkat with or without Cdk inhibitors or Jurkat cells with or without Cdk4 DN (Fig. 5, D and E). Furthermore, there was no difference in the induction of β_1 activation epitope by the β_1 -activating Ab 8A2 (16) in the presence of Cdk4 blockade (Fig. 5, E and F). Therefore, inhibition of ligand-induced adhesion by Cdk blockade is not due to changes in integrin expression or activation.

Leukocyte migration across an EC matrix is inhibited by Cdk inhibitors

We hypothesized that ligand-induced adhesion could occur in vivo with localized retraction or loss of endothelium and during migration through the subendothelial matrix once transmigration has occurred. To test potential biological relevance of this Cdk-mediated pathway, we examined the effect of Cdk inhibitors on PBMC migration across an EC-derived matrix. Equal numbers of PBMC were allowed to adhere before treatment with Cdk inhibitor to ensure that differences in migration were not simply due to differences in the number of adherent cells. Migration in the presence of purvalanol A was inhibited by 50% (Fig. 6A). Thus, Cdk-mediated

pathway plays a role in unstimulated adhesion and migration of PBMC across the exposed EC matrix.

Impaired cell recruitment in *Cdk4* knockout mice following lung injury

The response of *Cdk4*^{-/-} mouse to injury and inflammation has not been examined. Based on our data showing a novel role of Cdk4 in leukocyte adhesion and migration, we asked whether the absence of Cdk4 would affect the recruitment of leukocytes following injury. To test this, we examined the response of *Cdk4*^{-/-} mice to intratracheal instillation of bleomycin. Bleomycin lung injury response involves an initial inflammatory influx of leukocytes, followed by fibroblast proliferation and fibrosis (23). We examined BALF cell count and cell differential 72 h after bleomycin-induced lung injury. We found that *Cdk4*^{-/-} mice had significantly decreased total cell count compared with wild-type mice ($p < 0.04$; Fig. 6C). Although *Cdk4*^{+/-} mice appeared to have an intermediate phenotype, *Cdk4*^{+/-} cell counts were not statistically significantly different from wild-type or *Cdk4*^{-/-} mice. When we analyzed the cell populations in BALF, we found a selective decrease in lymphocytes in *Cdk4*^{-/-} mice, while the absolute number of BALF neutrophils was similar in all groups (Fig. 6, B, D, and E). This suggests that the decrease in total BALF cell number is due to a selective blockade of lymphocyte recruitment. There were no differences in peripheral blood counts of *Cdk4*^{-/-} mice compared with *Cdk4*^{+/-} and wild-type littermates at baseline or following bleomycin injury (data not shown), suggesting that differences in BALF were not simply reflecting alterations in peripheral cell counts. These results suggest that Cdk4 plays a role in lymphocyte trafficking in vivo.

Discussion

We report a new pathway that regulates leukocyte adhesion to the endothelial matrix and migration of leukocytes through the matrix in the absence of exogenous cytokine or chemokine stimulation. Many of the features of ligand-induced adhesion described in this report do not fit into classic integrin-mediated adhesion paradigms, including lack of dependence on Rap1, lack of inhibition by cytochalasin D, and a role for Cdks in leukocyte adhesion (Table II). Only Cdk inhibitors were able to block ligand-induced adhesion, demonstrating a new mechanism of action of Cdk inhibitors. Inhibitors of other signaling pathways (e.g., RhoA kinase, MAPK, PI3K) failed to block ligand-induced adhesion. The role of Cdk4, specifically in ligand-induced adhesion, is supported by two independent lines of evidence: inhibition by a DN construct of Cdk4, but not Cdk2, or Cdk5 and inhibition by Cdk4 siRNA but not Cdk5 or Cdk6 siRNA.

Although the rationale for use of Cdk inhibitors in many diseases is blockade of cell cycle progression and limitation of cell proliferation, our study and others suggest that Cdks have additional functions independent of cell cycle regulation. Cdks 4 and 6 were reported to regulate neuronal cell death (5). Cdk5 plays an important role in neuronal cell function and contributes to neurite outgrowth and axonal regeneration (6, 24). In non-neuronal cells, Cdk5 also regulates cytoskeletal remodeling, cell motility, and adhesion (4, 12, 25). Cdk1 (*cdc2*) localized to membrane ruffles of migrating cells and was noted to be a downstream effector of the integrin $\alpha_v\beta_3$, leading to cell migration (26). In murine models of glomerular disease (27, 28) and polycystic kidney disease (29), treatment with Cdk inhibitors improved outcomes. Furthermore, blockade of Cdks during injury resulted in increased neutrophil apoptosis and enhanced resolution of inflammation (30).

Our study shows that a Cdk inhibitor blocks leukocyte adhesion and migration in vitro and knockout Cdk4 activity blocks lympho-

Table II. Comparison of two pathways of leukocyte adhesion

Stimulated Adhesion ^a	Ligand-Induced Adhesion
Cdk-independent	Cdk dependent
Rap1 dependent	Rap1 independent
RhoA kinase dependent	RhoA kinase independent
Integrin dependent	Integrin dependent
Actin filaments involved (inhibited by cytochalasin D)	Actin filaments not involved
Microtubules not involved	Microtubules involved (inhibited by nocodazole)

^a By soluble agonists.

cyte recruitment to lung following injury in vivo. This may expand potential therapeutic indications for Cdk inhibitors to include diseases characterized by abnormal lymphocyte infiltration, such as psoriasis, or multiple sclerosis. The effect of Cdks on migration could also lead to unexpected outcomes. For example, the presence of tumor-infiltrating lymphocytes in several malignancies is associated with a better outcome (31–33). Cdk inhibitors could potentially decrease lymphocyte homing to tumors, which might lead to poorer outcomes unrelated to its effect on cell cycle.

Circulating leukocytes constitutively express $\alpha_4\beta_1$ in a low-affinity conformation. We now show that these cells are adhesion-competent without stimulation by chemokines or cytokines when presented with an appropriate substrate. In addition to our studies demonstrating that ligand (i.e., high-density fibronectin or EC matrix) regulates spontaneous adhesion of lymphocytes (8, 34), other examples of regulation of adhesion by ligand have been noted. For example, eosinophil spreading and migration is inhibited by high density, but not low-density fibronectin (35). In addition, initial adhesive strength increases with increasing ligand density (36, 37). Changes induced by ligand binding can be detected by Abs that recognize conformational changes on integrins (38). Adhesion of unstimulated leukocytes to the subendothelial matrix suggests an alternate pathway to initiate “outside-in”-mediated inflammatory responses. Posttrafficking interactions of leukocytes with subendothelial matrix result in “outside-in” signaling that contribute to the inflammatory response. For example, ligand binding of $\alpha_4\beta_1$ enhanced production of inflammatory mediators such as TNF- α , IL-1, and tissue factor (39–41), and $\alpha_4\beta_1$ binding to VCAM-1 increased β_2 -mediated cell adhesion (42). $\alpha_1\beta_1$ -mediated outside-in signaling on leukocytes also regulates cell proliferation, adhesion, migration, and activation, which contributes to the inflammatory response (reviewed in Ref. 43). Blockade of $\alpha_1\beta_1$ in animal models of immune-mediated disorders including graft-versus-host disease (44), arthritis (45), colitis (46), and glomerulonephritis (27) markedly decreased the inflammatory response. In addition, α_1 ^{-/-} mice demonstrate a decreased inflammatory response in several models of immune-mediated inflammation (43). Thus, posttrafficking leukocyte signaling is an important contributor to the inflammatory response, and ligand-induced adhesion may play an important role in this process.

We previously reported that leukocyte adhesion to staurosporine-treated EC was mainly $\alpha_4\beta_1$ integrin-dependent (9). Since staurosporine-induced EC retraction is an early stage of EC apoptosis, it now appears likely that the induced adhesion was due to exposure of the EC matrix rather than to apoptotic EC per se. Two lines of evidence support this: treatment of EC with agents that induced retraction but did not induce apoptosis increased adhesion of unstimulated cells and agents that induced apoptosis without causing EC retraction did not enhance adhesion.

The major defined substrate for Cdk4 is Rb. However, our data suggest that Rb phosphorylation is not required for ligand-induced

adhesion. Thus, it is likely that a novel substrate for Cdk is involved. We also show that integrin expression and conformation is not affected by Cdk blockade. Although our data support a role for Cdk4 in ligand-induced adhesion in leukocytes, additional Cdk family members may participate in other cell types.

Interestingly, Cdk4^{-/-} mice are viable, although the mice are infertile and small. Some of the mice have abnormalities in the hypothalamic-pituitary axis (47, 48) and develop diabetes due to abnormal pancreatic islet cell formation (17). Heterozygote mice do not display any overt phenotype. Cdk4^{-/-} mouse embryo fibroblasts proliferate normally under conditions that promote continuous growth, but have impaired proliferative capacity following induction of a short quiescent period (17). In addition, keratinocytes from Cdk4^{-/-} mice display normal proliferative capabilities in response to wounding (49). We now show that Cdk4^{-/-} mice have a selective block in recruitment of lymphocytes to the lung following injury, complementing our *in vitro* data demonstrating that Cdk4 blockade inhibits leukocyte migration across the subendothelial matrix.

In summary, we report a new pathway that allows leukocytes to adhere to and migrate through the exposed endothelial matrix in the absence of exogenous cytokine or chemokine stimulation, termed ligand-induced adhesion. Furthermore, we provide evidence for a novel role of Cdk4 in regulating this adhesion. Primary monocytes and lymphocytes are capable of ligand-induced adhesion using several ligands including fibronectin and EC-derived matrices. We demonstrate a role of Cdk4-mediated ligand-induced adhesion in leukocyte trafficking by regulating migration. Furthermore, we show that the absence of Cdk4 in mice decreases lymphocyte recruitment in the BALF following lung injury. Thus, Cdk4 may be a novel therapeutic target for regulating lymphocyte recruitment during injury and inflammation. Further characterization of this novel pathway should provide new insights into post-trafficking events in leukocyte emigration.

Disclosures

The authors have no financial conflict of interest.

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