The Spontaneously Adhesive Leukocyte Function-associated Antigen-1 (LFA-1) Integrin in Effector T Cells Mediates Rapid Actin- and Calmodulin-dependent Adhesion Strengthening to Ligand under Shear Flow*

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Integrins in effector T cells are highly expressed and important for trafficking of these cells and for their effector functions. However, how integrins are regulated in effector T cells remains poorly characterized. Here, we have investigated effector T cell leukocyte function-associated antigen-1 (LFA-1) regulation in primary murine effector T cells. These cells have high LFA-1 integrin expression and display high spontaneous binding to intercellular adhesion molecule-1 (ICAM-1) ligand under static conditions. In addition, these cells are able to migrate spontaneously on ICAM-1. Atomic force microscopy measurements showed that the force required for unbinding of integrin-ligand interactions increases over time (0.5–20-s contact time). The maximum unbinding force for this interaction was ~140 piconewtons at 0.5-s contact time, increasing to 580 piconewtons at 20-s contact time. Also, the total work required to disrupt the interaction increased over the 20-s contact time, indicating LFA-1-mediated adhesion strengthening in primary effector T cells over a very quick time frame. Effector T cells adhered spontaneously to ICAM-1 under conditions of shear flow, in the absence of chemokine stimulation, and this binding was independent of protein kinase B/Akt and protein kinase C kinase activity, but dependent on calcium/calmodulin signaling and an intact actin cytoskeleton. These results indicate that effector T cell integrins are highly expressed and spontaneously adhesive in the absence of inside-out integrin signaling but that LFA-1-mediated firm adhesion under conditions of shear flow requires downstream integrin signaling, which is dependent on calcium/calmodulin and the actin cytoskeleton.

Integrins (such as LFA-1, αLβ2, CD11a/CD18, and VLA-4, α4β1) are adhesion receptors that are of fundamental importance in immune responses. In lymphocytes, integrins are crucial for firm adhesion to endothelium under conditions of shear flow and therefore for lymphocyte migration into lymph nodes and inflamed tissue. Integrins are also important for lymphocyte activation and effector functions. Integrins mediate these events by binding to ligands (intercellular adherence molecules (ICAMs) and vascular adherence molecule) on endothelial cells surrounding blood vessels, on other leukocytes (dendritic cells, B cells) or on target cells (virus-infected cells, cancer cells). Integrins on the surface of naïve lymphocytes are not constitutively active; instead, their ligand-binding ability is regulated through “inside-out” signaling. In naïve lymphocytes, these signaling events are initiated for example through ligation of the T cell receptor, B cell receptor, or chemokine receptors, and inside-out signaling ultimately leads to integrin activation and increased binding to ligands (1). The exact sequence of signaling events leading to integrin activation is currently unclear, but the small GTPase Rap1 (2, 3) and integrin activators talin (4) and kindlin-3 (5) are important proximal elements in integrin regulation in lymphocytes (1). After ligand binding, integrins mediate “outside-in” signaling which results in actin reorganization, adhesion strengthening, and other downstream effects.

Integrins in effector T cells are highly expressed, and these molecules are important for trafficking of these cells (6) and for

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3 The abbreviations used are: LFA-1, leukocyte function-associated antigen-1; ANOVA, analysis of variance; ICAM-1, intercellular adhesion molecule-1; PDBu, phorbol 12,13-dibutyrate; SDF-1, stromal cell-derived factor-1; ADAP, adhesion and degranulating promoting adapter protein.
their effector functions, such as the killing of target cells (7). However, a detailed understanding of integrin regulation and integrin-ligand interaction forces in primary effector T cells is currently lacking. In addition, the LFA-1-mediated outside-in downstream signaling pathways involved in firm adhesion of effector T cells under shear flow conditions are poorly understood. We have now investigated LFA-1-mediated adhesion to ICAM-1 in primary murine effector T cells using static adhesion assays, shear flow adhesion assays, two-dimensional migration assays, and atomic force microscopy measurements.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The integrin ligand ICAM-1, E-selectin, and the chemokine SDF-1 were from R&D Systems. Akti-1/2 isoyme selective, Akt-VIII inhibitor, Syk inhibitor II, and PI3-kinase inhibitor LY294002 were from Calbiochem; MK2206 (Akt inhibitor) and GDC-0941 (PI3-kinase inhibitor) were from Selleck Chemicals. PKC inhibitor Ro-318220, calmodulin antagonist (calmodulin inhibitor) and Rap1 pulldown kit to isolate activated (GTP-loaded) Rap1 molecules (GST-RalGDS-RBD pulldown assay; Thermo Scientific). Prior to the pulldown assay, cells were suspended at a concentration of 2 × 10^5/3 ml, washed with PBS, resuspended in binding medium, and either treated with Akt-VIII inhibitor (10 mM) or left untreated for 30 min at 37 °C. The cells were then stimulated with 200 nM PDBu for 5 min. Cell lysates were prepared and used in the pulldown assay according to the manufacturer’s instructions. Total lysates and pulldown samples were analyzed for Rap1 content using standard SDS-PAGE and immunoblotting techniques.

**Adhesion Assay under Shear Flow**—The assay was performed essentially as described (8, 9). Ibidi µ-slides VI were coated with 6 μg/ml (unless otherwise stated) ICAM-1 overnight at 4 °C. Where indicated, E-selectin at 3 μg/ml and ICAM-1 at 6 μg/ml were co-immobilized by overnight incubation at 4 °C. SDF-1 (5 μg/ml) was coated onto plates for a further 30 min where described. For adhesion assays to endothelial cells, bEnd.3 cells were seeded onto plates at a concentration of 0.5 × 10^6 cells/ml and grown under 15 dynes/cm^2 shear stress for 6 days using an Ibidi shear flow system or alternatively for 2 days under static conditions. TNF-α at a concentration of 10 ng/ml was added into the growth medium 18 h before the experiment to stimulate ICAM-1 expression. For effector T cell stimulation, cells were stimulated with 200 nM PDBu for 5 min. Where inhibitors were included, cells were pretreated for 30–60 min at 37 °C. Cells at a density of 1 × 10^6 cells/ml in binding medium or PBS were then injected into a flow system that used a silicone tubing loop of ~60 cm connected to a Multi-phase NE-1000 syringe pump (New Era Pump Systems), allowing the cells to flow at a continuous shear flow rate of 0.3 dyne/cm^2, unless otherwise stated. Cells were monitored by microscopy over a 10-min period, and the number of adhered cells in the field of view was determined at 1-min intervals by manual counting.

**Migration on ICAM-1**—8-well µ-slides (Ibidi) were coated with 1, 2, or 6 μg/ml ICAM-1 overnight at 4 °C. After washing with PBS, 70,000 cells were added to the plate in a volume of 400 μl of binding medium and left to adhere for 30 min at 37 °C. Time lapse microscopy was performed on a Nikon Eclipse Ti microscope with perfect focus and multipoint site visiting stage, using a CFI Plan Fluor ELWD 40× phase objective and a Photometrics Cascade II 1024 black illuminated EMCCD camera. Videos were subsequently analyzed using Imaris software (Bitplane AG).

**Atomic Force Microscopy**—Arrow TL2 cantilevers (Nano-Sensors) were functionalized with purified anti-CD43 (ebioscience; clone R2/60) at 0.5 μg/ml for 30 min at room temperature. Before the experiment, cantilevers were calibrated for sensitivity and spring constants. A small region of tissue culture plates (TPP) were coated with 3 μg/ml ICAM-1 overnight at 4 °C. The wells were blocked with 1% milk/PBS for 1 h 15 min at 37 °C. The cells were suspended in binding medium (RPMI 1640 medium with 0.1% BSA, 40 mM Hepes, 2 mM MgCl, 2 before being added to blocked wells, which were rinsed twice with PBS. Immediately prior to the addition to the plate, the cells were activated with 200 nM PDBu, 10 μg/ml anti-CD3 (clone 2C11) or left unstimulated. The cells were allowed to adhere for 30 min at 37 °C before unbound cells were removed by gentle washing in PBS containing 2 mM MgCl, 2 . The bound cells were lysed in 1% Triton X-100, 50 mM sodium acetate, pH 5, containing 3 mg/ml p-nitrophenyl phosphate (Calbiochem) and incubated for 1 h at 37 °C in the dark. The reaction was terminated with 1 mM NaOH, and the absorbance at 405 nm was measured.

**Rap1 Assays**—Rap1 activity was determined using a Rap1 pulldown assay kit to isolate activated (GTP-loaded) Rap1 molecules (GST-RalGDS-RBD pulldown assay; Thermo Scientific). Prior to the pulldown assay, cells were suspended at a concentration of 2 × 10^5/3 ml, washed with PBS, resuspended in binding medium, and either treated with Akt-VIII inhibitor (10 mM) or left untreated for 30 min at 37 °C. The cells were then stimulated with 200 nM PDBu for 5 min. Cell lysates were prepared and used in the pulldown assay according to the manufacturer’s instructions. Total lysates and pulldown samples were analyzed for Rap1 content using standard SDS-PAGE and immunoblotting techniques.

**Flow Cytometry**—For flow cytometry, CD11a-PE (clone 2D7) and CD18-FITC (β2 integrin, clone C71/16) antibodies were from BD Bioscience. Live cells were gated according to their forward scatter and side scatter profiles. Data were acquired on an LSR Fortessa flow cytometer (BD Biosciences) and analyzed using Flowjo software (Treestar).

**Static Adhesion Assay**—ICAM-1 (6 μg/ml, unless otherwise stated) was coated in duplicate/triplicate onto flat-bottom 96-well Maxisorp plates (Nunc) by overnight incubation at 4 °C. The wells were blocked with 1% milk/PBS for 1 h 15 min at 37 °C. The cells were suspended in binding medium (RPMI 1640 medium with 0.1% BSA, 40 mM Hepes, 2 mM MgCl, 2 before being added to blocked wells, which were rinsed twice with PBS. Immediately prior to the addition to the plate, the cells were activated with 200 nM PDBu, 10 μg/ml anti-CD3 (clone 2C11) or left unstimulated. The cells were allowed to adhere for 30 min at 37 °C before unbound cells were removed.
Integrin Regulation in Effector T Cells

A

CD18

CD11a

B cells

CD4+ T cells

CD8+ T cells

Effector T cells

B

% cell adhesion

Unstimulated
PdBu
anti-CD3

C

pAkt
pErk
Akt

- + PdBu

D

active Rap1

total Rap1

- - + PdBu
Akt VIII (10μM)

E

F

G

H

Track Displacement
Track length

µm

Track speed max.
Track speed mean
Track speed min.

µm/s

Track straightness

arb. units

1ug/ml ICAM-1
2ug/ml ICAM-1
8ug/ml ICAM-1

1ug/ml ICAM-1
2ug/ml ICAM-1
8ug/ml ICAM-1
RESULTS

LFA-1 Is Highly Expressed and Spontaneously Adhesive in Primary Murine Effector T Cells—LFA-1-mediated effector T lymphocyte adhesion is important for immunological synapse formation with target cells and effective cytolytic activity of effector CD8\(^+\) T cells (7) as well as for adhesion and transmigration of both CD4\(^+\) and CD8\(^+\) effector T cells under flow conditions (10). However, a detailed understanding of adhesion forces and signaling processes involved in effector T cell integrin-mediated adhesion is currently lacking. We therefore set out to investigate integrin-mediated adhesion in effector T cells. Cultivation of murine T cells with anti-CD3 followed by IL-2 results in an effector T cell phenotype of ~90% CD8\(^+\) and 10% CD4\(^+\) (11) which are CD62L\(^{low}\) and CD44\(^{high}\) (data not shown). Effector T cells have high integrin LFA-1 expression compared with naïve murine B cells, CD4\(^+\) or CD8\(^+\) T cells (Fig. 1A). Effector T cells also display high spontaneous LFA-1-mediated adhesion to ICAM-1 under static conditions, which cannot be much further up-regulated with agents that stimulate inside-out signaling to integrins, such as phorbol ester or cross-linking of the T cell receptor (Fig. 1B). Effector T cell adhesion to ICAM-1 is completely dependent on β2 integrins (Fig. 1B). Therefore, effector T cell integrins are highly expressed and adhesive (Fig. 1, A and B). Effector T cells display high PI3-kinase/Akt activity, as this pathway is activated by IL-2, but basal MAPK activity (Fig. 1C). In addition, they have high basal Rap1 activity, which cannot be much further up-regulated with phorbol ester stimulation and is not affected by inhibition of Akt (Fig. 1D).

To further investigate integrin regulation in effector T cells, we assessed the motility and morphology of effector T cells on the LFA-1 ligand, ICAM-1. Murine effector T cells placed on an ICAM-1-coated surface are polarized (Fig. 1E). The cells migrate randomly and spontaneously on 6 µg/ml ICAM-1. Lower ICAM-1 concentrations do not significantly affect cell motility under static conditions (Fig. 1, F–H).

FIGURE 1. Effector T cells display high levels of LFA-1 integrin expression and high basal adhesion to ICAM-1. A, expression levels of CD18 and CD11a subunits of LFA-1 in naïve B cells (top panel), CD4\(^+\) T cells (second panel), CD8\(^+\) T cells (third panel), and effector T cells (bottom panel) were assessed by flow cytometry. B, adhesion of untreated, PDBu-, and anti-CD3-treated wild type or β2 integrin\(^{−/−}\) effector T cells to ICAM-1 was assessed in a static adhesion assay. Black bars represent wild type, and white bars represent β2 integrin\(^{−/−}\) (n = 2). C, detection of pAkt, total Akt, and pERK levels in effector T cells, with or without stimulation with 200 nM PDBu, by Western blotting. Data are representative of n = 3. D, detection of active Rap1 from untreated, Akt-VIII-treated, and PDBu-treated effector T cells. Data are representative of n = 3. E, untreated effector T cells viewed by microscopy at 40×, using phase contrast after 60 min on ICAM-1. F–H, effector T cell tracking of track displacement, track length, track speed, and track straightness on 1, 2, or 6 µg/ml ICAM-1 by time lapse microscopy. For C–H, data are from three or four independent experiments. Error bars represent S.D.

FIGURE 2. Atomic force microscopy measurements reveal that LFA-1 is spontaneously adhesive in effector T cells and that adhesion strengthening occurs rapidly after ligand binding in these cells. A, phase contrast image of an effector T cell attached to anti-CD43 functionalized cantilever (top) and schematic representation of distinct stages (I–IV) in a single cell adhesion measurement (bottom). B, example of force-distance curve acquired during approach (i and ii) and retraction (iii and iv) steps. C, maximum unbinding force measurements of untreated effector T cells. D, total amount of work required to detach untreated effector T cells. In C and D, error bars represent S.E., with n = 20 individual cells for each condition.
These results indicate that LFA-1 on murine effector T cells is highly expressed and can spontaneously form adhesive contacts to ICAM-1. These contacts are sufficient to mediate integrin outside-in signaling, resulting in successful cell spreading, polarization, and migration on ICAM-1.

Atomic Force Microscopy Reveals That LFA-1 on Effector T Cells Mediates Rapid Adhesion Strengthening after Initial Spontaneous ICAM-1 Binding—Cell adhesion to integrin ligands is a complex process regulated both by inside-out signaling (activation) of integrins, including conformational changes of the integrin extracellular domain and, thereafter, integrin-induced adhesion strengthening and actin reorganization events (outside-in signaling). To investigate the mechanism of integrin-mediated adhesion in effector T cells, we used force spectroscopy to measure the forces required to disrupt integrin-ligand interactions. The setup is described in Fig. 2A. The effector T cell was attached to the tip of the cantilever and allowed to make very short (0.5 s) and longer (up to 20 s) contacts with integrin ligand (ICAM-1) coated on a plate. To measure the force acting on the cantilever, cantilever deflection was determined using a laser beam reflected from the back of the cantilever onto a quadrant photodiode. The maximum unbinding force and total work required for cell detachment from the ligand (i.e. area under the curve) were measured for each interaction, as described in the example force curve displayed in Fig. 2B. As controls, we measured effector T cell adhesion to plastic; here, both the maximum unbinding force and the total work required to disrupt the binding of cells to plastic were comparatively low, although it increased somewhat over time (Fig. 2, C and D). We thereafter investigated effector T cell binding to ICAM-1; the maximum unbinding force for this interaction was approximately 140 piconewtons at 0.5-s contact time, increasing to 580 piconewtons at 20-s contact time (Fig. 2C). The total work required to disrupt integrin-ligand interactions followed a similar pattern, increasing significantly over the 20-s time frame, from 4 joules at the 0.5-s contact time up to 30 joules at the 20-s contact time (Fig. 2D). The integrin-ligand interaction was abolished by EDTA, which chelates the divalent cations that are essential for integrin binding to ligand (Fig. 2, C and D). These data show that the interaction of effector T cell LFA-1 with its ligand, ICAM-1, is strong and undergoes spontaneous maturation and adhesion strengthening over a short time frame of just 20 s.

LFA-1-mediated Adhesion of Effector T Cells under Flow Conditions Is Independent of Chemokines but Dependent on the Actin Cytoskeleton, Intracellular Calcium, and Calmodulin Signaling Pathways—Integrins are essential for firm adhesion of effector T cells under shear flow conditions (10), but signaling pathways required for this process are currently poorly understood. We therefore further focused on LFA-1–ICAM-1 interactions under shear flow conditions and the requirements for inside-out and outside-in signals for this process. To do this, we used a shear flow assay we have previously described and characterized, where lymphocytes are allowed to adhere to ICAM-1 under relatively low shear flow in the presence or absence of integrin inside-out stimulation (8, 9). This allows for the analysis of the integrin component of cell adhesion under shear flow conditions, without interference from other adhesion molecules such as selectins (which are required for cell rolling/capture at higher shear forces). It is also important to note that such low or disturbed shear forces are physiologically relevant in pathophysiological conditions such as atherosclerosis (12).

There was significant binding of effector T cells to ICAM-1 under conditions of shear flow of up to 0.5 dyne/cm² without inclusion of any activation stimulus (Fig. 3A). The cells could also roll on the ICAM-1 substrate under these conditions, presumably allowing their capture (Fig. 3B). Effector T cells also bound spontaneously to endothelial cells (bEnd.3 cells) under similar shear flow conditions; in addition, endothelial cells also supported cell adhesion at higher shear rates of up to 1.2 dynes/cm², presumably because they express molecules such as selectins which aid the capture of cells under higher shear flow conditions (Fig. 3C). This hypothesis was supported by co-immobilization of ICAM-1 with E-selectin; as has previously been shown for neutrophils (13), these conditions indeed supported much higher effector T cell adhesion under high shear flow conditions than ICAM-1 alone (Fig. 3D).

Lower ICAM-1 concentrations decreased adhesion under flow conditions (Fig. 3A), and the adhesion was abolished in β2 integrin−/− effector T cells (Fig. 3E), demonstrating that adhesion to immobilized ICAM-1 is mediated by specific β2 integrin-ICAM-1 interactions. In addition, adhesion of β2 integrin−/− effector T cells to endothelial cells under shear flow was also significantly reduced compared with wild-type effector T cells (Fig. 3F). Phorbol ester stimulation of cells did not markedly increase adhesion of effector T cells to ICAM-1 under conditions of shear flow (Fig. 3G). In contrast, naïve B cells (and CD4+ T cells; data not shown) required chemokine stimulation (or B cell receptor stimulation; data not shown) to be adhesive to the ICAM-1 substrate under similar conditions (Fig. 3H), confirming a requirement for chemokine-induced inside-out signaling to integrins in these cells. Therefore, integrins in effector T cells and naïve B cells are regulated differently; B cells require integrin inside-out signaling through chemokines (or other stimuli) to mediate firm adhesion under flow, whereas effector T cells do not.

We then further investigated the requirements for this chemokine-independent adhesion of effector T cells under shear flow. Integrin attachment to the actin cytoskeleton has been previously suggested to be important for the ability of

![Figure 3](https://example.com/figure3.png)
integrins to load forces and undergo spontaneous activation when binding to ligands under shear flow (14). To investigate the role of the actin cytoskeleton in effector T cell adhesion to ICAM-1 under shear flow conditions, we pretreated cells with cytochalasin D, which disrupts actin filaments. Indeed, cytochalasin D pretreatment of cells completely abolished effector T cell integrin-mediated adhesion to ICAM-1 under conditions of shear flow (Fig. 4A), indicating that actin-integrin interactions are essential for effector T cell firm adhesion under these conditions. In contrast, the ADAP module, which has previously been shown to be important in Rap1-mediated adhesion of LFA-1 (15), is not important for effector T cell adhesion under flow, as ADAP−/− effector T cells and WT effector T cells adhered equally to ICAM-1 under shear flow conditions (Fig. 4B). Zap-70 tyrosine kinase, which is upstream of Slp-76/ADAP, is also not important for adhesion, as inhibition of this kinase with a specific Syk/Zap-70 inhibitor (Syk inhibitor II) had no significant effect on adhesion to ICAM-1 under shear flow (Fig. 4C).

What signaling processes downstream of LFA-1 regulate firm adhesion of effector T cells under shear flow? Phospholipase C activity has previously been implicated in LFA-1 regulation in human effector T cells (10), and this enzyme is upstream of protein kinase C (PKC) activation in cells. PKC isoforms have been implicated in LFA-1 adhesion under flow in T cells and neutrophils (16, 17). We therefore used the general PKC inhibitor, Ro-318220, to investigate the dependence of effector T cell adhesion under shear flow on PKC activity. Effector T cell adhesion to ICAM-1 was not affected by Ro-318220, indicating that PKC is not important for this process (Fig. 4D).

Phospholipase C activity also regulates calcium release in cells. Interestingly, chelation of intracellular calcium with BAPTA-AM reduced effector T cell adhesion under flow conditions (Fig. 4E), although the reduction was not statistically significant, implicating intracellular calcium in regulation of firm adhesion in these cells. Calmodulin is a major mediator of calcium responses in cells and has been previously implicated in integrin-mediated adhesion and spreading responses of leukocytes (18, 19). Here, we show that calmodulin is important for adhesion of effector T cells to ICAM-1 under shear flow conditions, as W-7 (Fig. 4F) or calmidazolium (Fig. 4G) pretreatment of cells significantly reduced adhesion. Importantly, adhesion to ICAM-1 co-immobilized with E-selectin, or endothelial cells under higher shear flow conditions were also inhibited by the calmodulin antagonist (Fig. 4, H and I). Therefore, firm adhesion of effector T cells to ICAM-1 under shear flow conditions requires an intact actin cytoskeleton and Ca2+/calmodulin signaling downstream of LFA-1–ICAM-1 interactions.

LFA-1 outside-in signaling can also induce PI3-kinase/Akt activation in T cells (20), and we observed high PI3-kinase/Akt activity in effector T cells under basal conditions (Fig. 1C). Additionally, specific Akt inhibitors (MK2206 and Akt-VIII) partially reduced effector T cell adhesion to ICAM-1 under static conditions (data not shown). We therefore investigated the involvement of the PI3-kinase/Akt pathway in the regulation of effector T cell adhesion under conditions of shear flow. The Akt inhibitors Akt-VIII and MK2206 did not inhibit effector T cell adhesion under flow conditions (Fig. 5A, C, and D, and data not shown), although these inhibitors inhibited the high Akt activity seen in these cells (Fig. 5B). These results show that the Akt pathway does not play a role in LFA-1-mediated effector T cell adhesion under shear flow conditions. Also, SDF-1-induced naïve CD4+ and CD8+ T cell adhesion was unaffected by Akt inhibition (Fig. 5, E and F). In contrast, SDF-1-induced adhesion of naïve B cells to ICAM-1 under similar conditions was much reduced by PI3-kinase and Akt inhibition (Fig. 5, G–I), indicating a role for this signaling pathway in LFA-1-mediated adhesion in naïve B cells.

Together, these data confirm that integrin-mediated firm adhesion of effector T cells and naïve B cells under conditions of shear flow is fundamentally differently regulated. In B cells, this process requires chemokine-induced inside-out signaling and the PI3K/Akt pathway. In contrast, effector T cells chemokine-induced inside-out signaling to integrins is not required, but calmodulin-dependent outside-in signals and the actin cytoskeleton are necessary for firm adhesion to occur.

DISCUSSION

Recently, it has been shown that integrins in human effector T cells can mediate adhesion to endothelial ICAM-1 under conditions of shear flow in the absence of chemokine-induced activation (10). Surprisingly, the integrins on the effector T cells were not in an active conformation (i.e. did not bind mAb24), but were expressed at high levels on the surface of the cells (10). In this report, we confirm the high expression of integrins in murine effector T cells. We reveal the spontaneous binding of effector T cell LFA-1 to its ligand ICAM-1 in several different assays (static adhesion assay without phorbol ester- or anti-CD3-mediated inside-out signaling, spontaneous two-dimensional migration on ICAM-1, and chemokine-independent adhesion under shear flow conditions). We report that these cells can roll on ICAM-1, confirming that the integrin on effector T cells is not in a high affinity state (21). In addition, we have investigated the very early interactions (0.5–20 s) between LFA-1 on the effector T cells and its ligand, ICAM-1, using atomic force microscopy. We show that adhesion of effector T

FIGURE 4. Effector T cell adhesion to ICAM-1 under shear flow conditions requires an intact actin cytoskeleton and calcium/calmodulin signaling. A, adhesion of untreated and cytochalasin D-treated (10 μg/ml) effector T cells to ICAM-1 under shear flow conditions (n = 4, p < 0.05 by ANOVA). B, adhesion of wild type and ADAP−/− effector T cells to ICAM-1 under shear flow conditions (n = 3, p > 0.05 by ANOVA). C, adhesion of untreated and Syk-ll inhibitor-treated (5 μM) effector T cells to ICAM-1 under shear flow conditions (n = 2, p > 0.05 by ANOVA). D, adhesion assay under shear flow conditions of untreated and Ro-318220-treated (5 μM) effector T cells to ICAM-1 (n = 3, p > 0.05 by ANOVA). E, adhesion under shear flow of untreated and BAPTA-AM-treated (30 μM) effector T cells to ICAM-1 (n = 4, p > 0.05 by ANOVA). F, adhesion of untreated and W-7-treated (50 μM) effector T cells to ICAM-1 (n = 5, p < 0.05 by ANOVA). G, adhesion of untreated and calmidazolium-treated (5 μM) effector T cells to ICAM-1 under shear flow conditions (n = 3, p < 0.05 by ANOVA). H, adhesion of untreated and calmidazolium-treated (5 μM) effector T cells to ICAM-1 under shear flow conditions (n = 3, p < 0.05 by ANOVA). I, adhesion of untreated and calmidazolium-treated (5 μM) effector T cells to E-selectin under high shear stress of 2 dynes/cm2 (n = 3, p < 0.05 by ANOVA). In all cases, solid bars represent untreated cells and white bars represent inhibitor-treated cells. Error bars represent S.D., except in E, which represent S.E. n, numbers refer to sets of cells generated from one to three mice; multiple experimental runs were performed for each set of cells.
Integrin Regulation in Effector T Cells

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cells to ICAM-1 undergoes spontaneous maturation over a short time frame (5–20 s). This is, to our knowledge, the first time integrin binding strength has been assessed in primary effector T cells. Most studies on the strength of immune cell integrin-ligand bonds have been performed using cell lines, and the relevance of these studies to integrin regulation in primary cells is unknown. Our data therefore provide great insight of the nature of integrin-ligand bonds in primary immune cells and how integrin-mediated adhesion matures during early interactions between the integrin and its ligand.

The signaling pathways regulating integrin-mediated spontaneous adhesion of effector T cells under flow conditions were previously unknown. Integrin-mediated adhesion activates many intracellular signaling pathways, such as Src/Syk, Pyk2, phospholipase C, and PI3-kinase pathways. Phospholipase C and Src family kinases have been reported to be important for integrin-mediated firm adhesion and spreading on endothelial ICAM-1 in effector T cells (10). In addition, the kinase downstream of Src family kinases in T cells, Zap-70, has been reported to be important for adhesion strengthening under flow conditions in human T lymphoblasts (22). However, using a Syk/Zap-70 inhibitor, we could not confirm these results in murine effector T cells, and the Slp76/ADAP module that is downstream of Zap-70 does not appear to be important for effector T cell adhesion under shear flow. PI3-kinase is important for adhesion strengthening under flow conditions in neutrophils (23) and Akt has been previously reported to be important in integrin-dependent platelet adhesion under shear flow conditions downstream of PI3-kinase (24). In addition, LFA-1 can induce PI3-kinase/Akt activation in T cells (20). However, in this report we show that although effector T cells display high PI3-kinase/Akt activity, they do not require Akt kinase activity in the outside-in signaling pathway for adhesion to ICAM-1 under flow conditions. These data are in agreement with previous data reporting effector T cells treated with Akt inhibitors display increased rather than decreased homing to lymphoid tissue, which was shown to be due to a defect in the regulation of homing receptors in these cells (11). In contrast, we now show that SDF-1-induced naïve B cell adhesion to ICAM-1 under conditions of shear flow is dependent on the PI3-kinase/Akt-pathway. Our data are in agreement with a previous report, showing PI3-kinase to be important for homing of B cells to lymph nodes in vivo (25). Together, these data implicate that the PI3-kinase/Akt pathway is important for chemokine-induced adhesion under shear flow conditions in certain lymphocyte (and leukocyte) subtypes such as naïve B cells but not others, i.e. naïve or effector T cells.

What mediates effector T cell rolling adhesion, adhesion strengthening, and the ability of these cells to withstand shear flow forces downstream of integrin-ligand bonds? Here, we show that this process is absolutely dependent on an intact actin cytoskeleton. The actin cytoskeleton may aid in ICAM-1-induced integrin activation by shear flow forces by linking the integrin receptors to a solid support inside the cell, as suggested previously (26). As has previously been reported in certain other cellular environments (27), we have shown that LFA-1 in effector T cells can mediate rolling adhesion to ICAM-1 under low physiological shear flow. Under higher shear flow conditions, selectins are required to tether the cells. It is possible that the mechanical linkage to the cytoskeleton allows further activation of the integrin by shear flow forces after initial ICAM-1 contacts have formed, thereby allowing firm adhesion. In addition, effector T cells which have bound to ICAM-1 reorganize their actin cytoskeleton and spread out on the ligand under conditions of shear flow (data not shown), and this integrin-actin-induced shape change likely aids their resistance to shear flow forces. What mediates these integrin-mediated downstream cell spreading events in effector T cells? Phospholipase C signaling downstream of LFA-1 has been reported to be important for firm adhesion of effector T cells (10). PKC/protein kinase D (PKD) signaling are downstream of phospholipase C in cells, and PKC isoforms have recently been implicated in neutrophil adhesion strengthening under conditions of shear flow (17). However, effector T cell adhesion to ICAM-1 under shear flow conditions is independent of PKC (this report), and we have previously reported that it is also independent of PKD (9). In contrast, we now show the process to be dependent on calcium/calmodulin signaling downstream of the active integrin. Interestingly, calcium/calmodulin signaling has previously been implicated in LFA-1 integrin regulation in T cells through regulation of the actin cytoskeleton (18, 28), and calcium/calmodulin has also been reported to be important for cR481 integrin-mediated adhesion to vascular adhesion molecule under conditions of shear flow (19). Calmodulin regulates many target proteins within cells, such as calcineurin, myosin light chain kinase, and calmodulin kinase II and interestingly has also been reported to be important for Rap1 activation in cells (29). How calmodulin regulates effector T cell adhesion under conditions of shear flow will be important to study in the future.

Taken together, the results from this study indicate that effector T cell integrins are highly expressed and spontaneously active, mediating cellular adhesion under shear flow conditions independently of chemokine-induced inside-out signaling. However, integrin-mediated outside-in signaling events that could be involved in integrin strengthening downstream of shear flow forces could not be confirmed.
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modulate the actin cytoskeleton, potentially via calcium/calmodulin pathways, are essential for firm adhesion under conditions of shear flow. Therefore, integrins in effector T cells are fundamentally differently regulated than in, for example, naïve B cells.

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