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DOCK2 is Required for Chemokine-Promoted Human T Lymphocyte Adhesion Under Shear Stress Mediated by the Integrin $\alpha_4\beta_1$

David García-Bernal,* Elena Sotillo-Mallo,* César Nombela-Arrieta,† Rafael Samaniego,‡ Yoshinori Fukui,§ Jens V. Stein,† and Joaquin Teixido ´2*

The $\alpha_4\beta_1$ integrin is an essential adhesion molecule for recruitment of circulating lymphocytes into lymphoid organs and peripheral sites of inflammation. Chemokines stimulate $\alpha_4\beta_1$ adhesive activity allowing lymphocyte arrest on endothelium and subsequent diapedesis. Activation of the GTPase Rac by the guanine-nucleotide exchange factor Vav1 promoted by CXCL12 controls T lymphocyte adhesion mediated by $\alpha_4\beta_1$. In this study, we investigated the role of DOCK2, a lymphocyte guanine-nucleotide exchange factor also involved in Rac activation, in CXCL12-stimulated human T lymphocyte adhesion mediated by $\alpha_4\beta_1$. Using T cells transfected with DOCK2 mutant forms defective in Rac activation or with DOCK2 small interfering RNA, we demonstrate that DOCK2 is needed for efficient chemokine-stimulated lymphocyte attachment to VCAM-1 under shear stress. Flow chamber, soluble binding, and cell spreading assays identified the strengthening of $\alpha_4\beta_1$-VCAM-1 interaction, involving high affinity $\alpha_4\beta_1$ conformations, as the adhesion step mainly controlled by DOCK2 activity. The comparison of DOCK2 and Vav1 involvement in CXCL12-promoted Rac activation and $\alpha_4\beta_1$-dependent human T cell adhesion indicated a more prominent role of Vav1 than DOCK2. These results suggest that DOCK2-mediated signaling regulates chemokine-stimulated human T lymphocyte $\alpha_4\beta_1$ adhesive activity, and that cooperation with Vav1 might be required to induce sufficient Rac activation for efficient adhesion. In contrast, flow chamber experiments using lymph node and spleen T cells from DOCK2$^{−/−}$ mice revealed no significant alterations in CXCL12-promoted adhesion mediated by $\alpha_4\beta_1$, indicating that DOCK2 activity is dispensable for triggering of this adhesion in mouse T cells, and suggesting that Rac activation plays minor roles in this process. The Journal of Immunology, 2006, 177: 5215–5225.

Lymphocyte recruitment into lymphoid organs and peripheral tissues during immune surveillance and response to inflammation depends on a tightly regulated multistep adhesion cascade. In this process, selectins mediate initial lymphocyte rolling on endothelium, and, following integrin activation, cells firmly attach to endothelial ligands and proceed to diapedesis (1–4). Firm attachment consists of at least two steps: 1) $\alpha_4\beta_1$ and $\alpha_4\beta_2$ integrins must be activated into high avidity states that are competent for ligand binding and 2) an adhesion strengthening process dependent on actin reorganization should proceed to generate a highly adherent cell phenotype that is resistant to shear stress (5).

Chemokines are main stimulators of integrin activation on T lymphocytes through inside-out signaling (6, 7). The chemokine CXCL12 stimulates integrin $\alpha_4\beta_1$-mediated T lymphocyte adhesion on its ligand VCAM-1 on endothelium, leading to T lymphocyte firm arrest (8). This response indicates that intracellular signaling originated upon binding of CXCL12 to its receptor CXCR4 finally impinges on $\alpha_4\beta_1$ molecules that switch from inactive to high avidity states capable of strong interactions with their ligands.

Investigation of intracellular signaling pathways involved in chemokine-promoted T cell attachment mediated by $\alpha_4\beta_1$ has led to the identification of several key molecules whose activation is required for efficient induction of adhesion. One of these pathways includes Ras family GTPase members such as Rap1, R-ras, and H-ras (9–11). Another key pathway involves activation of the guanine-nucleotide exchange factor (GEF) Vav1, which in turn, activates the GTPase Rac in human T lymphocytes (12). The activation of the Vav1-Rac pathway by CXCL12 controls subsequent strengthening of $\alpha_4\beta_1$-VCAM-1 interactions, such as interference with the expression of either Vav1 or Rac almost completely abrogates the up-regulation of this adhesion (12). Vav1 functions as a GEF predominantly for Rac, and its absence results in important defects in T cell development and activation (13–15). T cells from vav1$^{−/−}$ mice have decreased Rac activation (16), and constitutively active Rac rescues developmental defects in vav1$^{−/−}$ pre-T cells (17). In addition to its role as a GEF for Rac, Vav1 also functions as an adaptor molecule assembling different signaling pathways independently of its action on Rac activation (13, 14).

DOCK2 is a member of the Caenorhabditis elegans Cdc-5, mammalian DOCK180, and Drosophila melanogaster myoblast...
city family of scaffolding proteins that are involved in the regulation of actin dynamics by acting upstream of Rac (18–22). The Dock or DHR-2 C-terminal region on DOCK2 is capable of interacting with Rac promoting its activation in the presence of ELMO-1 (23), although it has also been reported (24) that DOCK2 can directly activate Rac in the absence of ELMO-1. DOCK2 is expressed in hemopoietic cells and its absence results in inhibition of both chemokine-induced Rac activation and T and B lymphocyte migration (21). Furthermore, DOCK2 is essential for Ag-induced TCR translocation, and for attenuation of allograft rejection (25, 26).

The above data indicate that Vav1 and DOCK2 are key regulators of chemokine-triggered Rac activation on T cells. Therefore, similarly to Vav1, DOCK2 could potentially mediate chemokine-promoted T cell adhesion mediated by αβ1 through Rac activation. In the present study, we have investigated the role of DOCK2 in chemokine-promoted T lymphocyte adhesion mediated by αβ2, both under static and flow conditions, and compared DOCK2 implication to that of Vav1. Furthermore, we have dissected DOCK2 involvement in the initial αβ1-VCAM-1 interactions, as well as in the subsequent adhesion-strengthening step of the adhesion cascade. The results from this study should shed light into the mechanisms controlling Rac activation that are required for efficient T cell extravasation in response to chemokines.

Materials and Methods

Cells, Abs, and reagents

The human T cell line Molt-4, human peripheral blood T lymphocytes, and CD4+ T cells were cultured and prepared as described previously (12). Purity of T lymphocytes and CD4+ cells populations was assessed by flow cytometry using anti-CD3 T6b and anti-CD T4 mAbs. Human CD45RA and CD45RO mAbs, purified from mouse ascites using CDs45RA and CD45RO microbeads (Miltenyi Biotec). Purity was >95% as analyzed by flow cytometry using anti-CD45RA and CD45RO Abs (BD Pharmingen). For the isolation of control and DOCK2-deficient T cells, single-cell suspensions were obtained from lymph nodes and spleens of control and DOCK2−/− mice, and negative selection was conducted using magnetic beads coated with anti-B220 and anti-Mac1 (Dynal Biotech), as described (27). Purity was >95% for CD4+ CD8 cells. Anti-CXCR4 mAb was purchased from R&D Systems, anti-Vav1 from Santa Cruz Biotechnology, anti-Rac from BD Pharmingen, and anti-GFP from Abcam. Abs (BD Pharmingen). For the isolation of control and DOCK2-deficient T cells, single-cell suspensions were obtained from lymph nodes and spleens of control and DOCK2−/− mice, and negative selection was conducted using magnetic beads coated with anti-B220 and anti-Mac1 (Dynal Biotech), as described (27). Purity was >95% for CD4+ CD8 cells. Anti-CXCR4 mAb was purchased from R&D Systems, anti-Vav1 from Santa Cruz Biotechnology, anti-Rac from BD Pharmingen, and anti-GFP from Abcam.

Transfections, RNA interference, and RT-PCR

Vectors pB-L-KIAA0209 and pCXN2-FLAG-KIAA (DOCK2)-dCS, codons for wild-type (WT) and dominant negative DOCK2, respectively, were obtained from Dr. M. Matsuda (Osaka University, Osaka, Japan). WT and mutant DOCK2 were amplified by PCR and subcloned into Xhol/SalI sites of pEGFP-C1 (Clontech Laboratories). The entire coding sequence was verified by DNA sequencing of the constructs. Three small interfering RNAs (siRNA) duplexes were designed corresponding to human DOCK2 (28) (DOCK2-A, bases 745–765; DOCK2-B, bases 2233–2253; and DOCK2-C, bases 4489–4509 from gene sequence). Sense strands were GCAAACGCUCAUAUGAGTTdT for DOCK2-A, GUCUAUUGUGGCGUGCGG GdTdT for DOCK2-B, and UGCCAUAGAAACCAUGUCCdTdT for DOCK2-C. For control and Vav1 siRNA, we used the sequences already described (Vav1.1 and Vav1.3; Ref.12). Control, DOCK2, and Vav1 siRNA duplexes were purchased from Dharmacon and Ambion, and verified to be specific for their targets by Blast search against the human genome. Vectors and siRNA were nucleofected (Amaxa Nucleofector device; Amaxa) following the described procedure (12). Molt-4 transfectants were assayed 16 h posttransfection, whereas PBL T lymphocytes (PBL-T), CD4+, CD45RA−, and CD45RO− transfectants were used after 6 h. Transfection of the different siRNA did not affect cell viability as assessed in cell cycle analysis by flow cytometry and by trypan blue exclusion (data not shown). For RT-PCR, cells were lysed in TriReagent (Sigma-Aldrich), and RNA was extracted and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega). Amplification of DOCK2 was performed using primers 5′-GGGAAAGCATCTGGAAACAA-3′ and 5′-ATGGAAACAGCTGCTAGAC-3′ and TaqDNA polymerase (Invitrogen Life Technologies). The PCR profile consisted of a 5-min initial denaturation at 94°C followed by 40 cycles of 30-s denaturation at 94°C, 30-s annealing at 58.5°C, and 1-min polymerization at 72°C, and finally by a 10-min extension at 72°C. Aliquots of each sample were amplified using the same conditions with human GAPDH primers (12).

Cell adhesion, soluble binding, and cell spreading assays

Recombinant human sVCAM-1 (rVCAM-1) consisting of domains 1–4 fused to the Fc portion of IgG1 (28) and the recombinant FN-H89 fragment of fibronectin, which contains the CS-1 site (29), were coated on 96-well plates (High Binding; Costar). For static adhesions, nontransfected cells, or siRNA, transfectants were labeled before addition to wells with 2′,7′-bis(carboxymethyl)-5(6′)-carboxyfluorescein-acetoxyethyl ester (BCECF-AM; Molecular Probes). Cells resuspended in adhesion medium (RPMI 1640/0.5% BSA) were added to wells (7.5 × 104 for Molt-4 or 1.25 × 105 for PBL-T cells in 100 μl) containing sVCAM-1 or FN-H89, alone or coimmobilized withCXCL12 (R&D Systems). After a short centrifugation, plates were incubated for 2 min at 37°C, and washing and quantification of adhesions was performed as earlier reported (30). For GFP transfectants, after the 2-min adhesion step and washes, bound cells were detached, counted, and analyzed by flow cytometry to determine GFP expression. For some adhesions of control and DOCK2−/− T cells, we followed the method already described (31). Briefly, slides were coated with sVCAM-1 and, after blocking, 1 × 105 cells were added to each well and allowed to settle for 15 min at 37°C. CXCL12 (final concentration, 1 μM) was added to the 12 o’clock position of each well except the 0-min time point. Under these conditions, cells attach adjacent to the site of chemokine addition. Following washing and fixation, adherent cells were counted using NIH Image software version 1.62. Flow chamber adhesion assays with human T cell transfectants followed the procedure described elsewhere (31, 32) with some modifications. Briefly, sVCAM-1 and CXCL12 were coimmobilized on petri dishes that were incorporated as lower walls of a parallel flow chamber. Transfectants were infused at a flow rate of 1 dyne/cm2 and cell attachment was visualized on at least two fields of view (0.58 mm2) using a 10× objective of an inverted phase-contrast microscope. Events were recorded for 4–5 min for each cell. Total cell migration from the flow chamber was calculated as the percentage of cells that became firmly adherent for at least 20 s (adhesion strengthening) were expressed as stable arrest, whereas cells that attached for a maximum of 5 s but resumed rolling were expressed as transient arrest (the percentage of rollers becoming adherent related to total number of interacting cells). Otherwise, tethering cells that did not arrest at any moment were expressed as rolling cells (the percentage of rollers related to total number of interacting cells). Tethering cells, cells that were rolling, cells that were attached for >5 s, then flow was increased at 1 dyne/cm2 every 30 s. The number of cells remaining bound was determined as the percentage of total adhered cells after a 4-min adhesion stage. For experiments of soluble binding, cells were exposed to CXCL12 or MnCl2 before adding sVCAM-1-Fc at saturating concentrations (20 μg/ml) as described previously (12). Bound sVCAM-1-Fc was detected by flow cytometry using PE-conjugated AffiniPure Gt (Jackson Immunoresearch Laboratories). To analyze cellular spreading, GFP transfectants were allowed to adhere for different times for VCAM-1-coated coverslips in the absence or presence of CXCL12. Subsequently, cells were fixed with paraformaldehyde 4% in PBS, mounted with mountol (Sigma-Aldrich), and images were captured by confocal microscopy (Leica TCS-SP2-AOBS-UV; Leica) with a 63× oil immersion objective. Areas and perimeters of adhered cells were measured using Leica confocal software (version 2.6.1, build 1537; Leica) and roundness was calculated using the formula 100 π(area/perimeter2) as earlier described (33).

Western blotting and GTPase assays

Following lysis (34), supernatants were resolved by SDS-PAGE and proteins were transferred to polyvinylidene fluoride membranes (Amersham Biosciences). Membranes were incubated with Abs, followed by washing and incubation with HRP-conjugated secondary Abs. Proteins were visualized using SuperSignal West Pico or Femto (Pierce) chemiluminescent substrate as described elsewhere (35, 36). Following washing and blocking, blots were reprobed with control Abs to test for total protein content. For GTPase assays, we followed essentially the method described previously (35). Briefly, cells exposed to CXCL12 were lysed, aliquots of extracts were kept for total lysate controls, and the remaining volume was mixed with GST-PAK-CD fusion protein (36) in the presence of [γ-35S]GTPγS, GTPγS, Diagn-somes injected into electrophoresis buffer, separated by SDS-PAGE, and transferred to polyvinylidene fluoride membranes that were incubated with Abs against Rac. Protein detection was performed as above.
Statistical analyses

Data were analyzed by one-way ANOVA, followed by Tukey-Kramer multiple comparisons. In both analyses, the minimum acceptable level of significance was \( p < 0.05 \).

Results

Role of DOCK2 on CXCL12-promoted human T lymphocyte adhesion mediated by \( \alpha_\beta_1 \) integrin

Molt-4 T cells, as well as PBL-T and the CD4\(^{+}\) subpopulation of PBL-T express notable amounts of DOCK2 (Fig. 1A). To investigate the potential involvement of DOCK2 in CXCL12-promoted adhesion of T lymphocytes mediated by \( \alpha_\beta_1 \) integrin, we first expressed in Molt-4 and PBL-T cells GFP-fused WT DOCK2 and dCS-DOCK2 (Fig. 1B, top), a mutant form comprising aa 1–515 that is unable to activate Rac (37), and subsequently compared transfectant attachment to sVCAM-1 under static conditions. Expression of GFP-fused WT and mutant DOCK2 forms was monitored by Western blotting using anti-GFP Abs (Fig. 1B, bottom). Mock and DOCK2 WT transfectants displayed similar levels of CXCL12-promoted up-regulation of adhesion to VCAM-1 (Fig. 1C). Enhancement of attachment to VCAM-1 of dCS-DOCK2 transfectants in response to the chemokine was of lesser magnitude compared with DOCK2 WT transfectants, but still statistically significant (\( p < 0.05 \)) with respect to basal (unstimulated) adhesion.

To more directly analyze the DOCK2 role in CXCL12-triggered, \( \alpha_\beta_1 \)-mediated adhesion, we transfected Molt-4 cells with three siRNA covering different regions on DOCK2. Only DOCK2-C siRNA exerted a substantial reduction in DOCK2 expression, whereas transfection of DOCK2-A and -B siRNA resulted in no or minor alterations in DOCK2 expression (Fig. 2A, top left). Molt-4, PBL-T, and CD4\(^{+}\) cells transfected with DOCK2-C siRNA displayed enhanced adhesion to VCAM-1 in response to CXCL12 but to a lower extent compared with control or DOCK2-A siRNA transfectants (Fig. 2B). Instead, T cells transfected with siRNA for Vav1 (Vav 1.3; Fig. 2A, top right and bottom left) exhibited a large impairment in CXCL12-promoted up-regulation of adhesion to VCAM-1 (Fig. 2B), as we also previously described (12). Control experiments indicated that transfection with DOCK2 or Vav1 siRNA specifically reduced the expression of their own targets (Fig. 2A, right gels). In addition, \( \alpha_4\beta_1 \) and CXCRII expression did not change by transfection of WT or mutant DOCK2, or siRNA for DOCK2 or Vav1, as compared with WT DOCK2 or control siRNA transfectants (data not shown).

After migrating across endothelium, T lymphocytes travel through subendothelial basement membranes and interstitial tissues rich in extracellular matrix proteins following chemotactic cues. We used FN-H89, a fibronectin fragment containing the CS-1 region which constitutes an interacting site for \( \alpha_\beta_1 \), to test the effect of interfering with DOCK2 expression in CXCL12-triggered T cell attachment to extracellular matrix proteins. DOCK2 siRNA transfectants displayed a significant up-regulation of cell attachment to FN-H89 in response to CXCL12 to levels similar to those of control siRNA transfectants, whereas enhanced adhesion was abolished in cells transfected with Vav1.3 siRNA (Fig. 2C) as previously reported.

To compare the extent of Rac activation in cells transfected with DOCK2 or Vav1 siRNA, we subjected Molt-4 transfectants to GTPase assays using times of incubation with CXCL12 close to those used in the adhesion assays. Notably, DOCK2 and Vav1 siRNA Molt-4 transfectants exhibited a drastic inhibition of Rac activation in response to CXCL12 as compared with activation obtained with control siRNA transfectants, although a residual level of Rac activation (10–20% of control) was detected in DOCK2 siRNA transfectants (Fig. 2D).

Since chemokines activate integrins on T lymphocytes under shear stress during tethering to endothelium, we next examined the behavior of DOCK2 siRNA transfectants during CXCL12-promoted adhesion to VCAM-1 under flow conditions. Molt-4 and PBL-T transfectants were perfused in flow chambers at an initial shear rate of 1 dyne/cm\(^2\) and their rolling, arrest, and detachment at increasing shear rates was analyzed. In the absence of CXCL12, transfectants predominantly rolled on VCAM-1 (data not shown), whereas the presence of the chemokine triggered a very rapid (\(<1\) s) development of firm adhesion (stable arrest for \(>20\) s) in a cell population close to 90% in control siRNA Molt-4 transfectants and 75% in PBL-T transfectant counterparts (Fig. 3, A and B, left panels). A minor cell population (5–15%) showed transient arrest (\(<5\) s) but resumed rolling. In addition, DOCK2-A siRNA Molt-4 transfectants, as well as cells transfected with a siRNA for Vav1 (Vav1.1) that only minimally affects Vav1 expression (12), displayed stable arrest in a cell population \(>75\% \). Firm adhesion induced by the chemokine was abolished by cell pretreatment with...

![FIGURE 1. CXCL12-promoted adhesion to VCAM-1 of WT and mutant DOCK2 T cell transfectants.](http://www.jimmunol.org/)
FIGURE 2. CXCL12-promoted adhesion and Rac activation of siRNA DOCK2 and Vav1 human T cell transfectants. A. Molt-4 cells were transfected with control or the indicated DOCK2 or Vav1 siRNA and, following solubilization, lysates were analyzed by RT-PCR (top gels) or Western blotting (bottom gels), using DOCK2-, Vav1-, or Rac1-specific reagents. Results were compared with loading controls GAPDH and paxillin. Also shown are results from nontransfected (−) Molt-4 cells. B and C. Cells were transfected with control, DOCK2, or Vav1 siRNA, and BCECF-AM-labeled transfectants were subjected to adhesion assays under static conditions to sVCAM-1 (B) or FN-H89 (C) coinmobilized with or without CXCL12. Adhesion data represent the mean ± SD of triplicate samples from representative results of at least two independent experiments for each panel. Adhesion was significantly up-regulated. ***, p < 0.001; **, p < 0.01; or *, p < 0.05 according to one-way ANOVA test. D. Molt-4 cells transfected with control or the indicated Vav1 or DOCK2 siRNA were incubated for the indicated times with CXCL12 and subjected to GTPase assays to detect active Rac (left). A representative result of three independent experiments is shown, whereas the right panel displays densitometer analyses in arbitrary units showing fold induction of Rac activation (mean ± SD) from the three GTPase experiments. Activation was significantly inhibited. ***, p < 0.001 or ***, p < 0.01 according to one-way ANOVA test.

DOCK2 IS NEEDED FOR T LYMPHOCYTE ADHESION MEDIATED BY α4β1

pertussis toxin or with anti-α4 mAb (data not shown). Instead, Molt-4 and PBL-T cells transfected with DOCK2-C siRNA mainly rolled, with only 30–35% of cells displaying stable arrest after some rolling (p < 0.01). Furthermore, both transfectants exhibited a significant increase in transient arrest compared with control siRNA transfectants. Stable arrest of Vav1.3 siRNA Molt-4 and PBL-T transfectants was even lower, with 15–20% of cells firmly sticking, and displayed also higher levels of transient arrest than control counterparts (Fig. 3, A and B, left panels).

To evaluate shear resistance, after transfectants were allowed to adhere, then flow was increased from 1 to 10 dyne/cm², measuring cells remaining bound. Control, DOCK2-A, and Vav1.1 Molt-4 siRNA transfectants developed a substantially higher resistance to detachment than DOCK2-C and Vav1.3 transfectants (Fig. 3A, right panel). At a shear rate of 3 dyne/cm², between 65 and 80% of control, DOCK2-A and Vav1.1 siRNA transfectants still remained bound, whereas only 25% and 10–15% of DOCK2-C and Vav1.3 transfectants, respectively, displayed shear resistance. Notably, there were between three and four times more DOCK2-C siRNA transfectants bound than the Vav1.3 counterparts at shear rates between 5 and 10 dyne/cm² (Fig. 3A, right panel).

In addition, DOCK2-C siRNA PBL-T transfectants showed significantly higher detachment rates at increasing shear stress compared with control siRNA transfectants, but differences were of lesser magnitude compared with Molt-4 transfectants (Fig. 3, B, right panel, and D). Again, DOCK2 PBL-T siRNA transfectants revealed a slightly higher resistance to detachment than Vav1.3 counterparts. Together, these results indicate that DOCK2 is needed for an efficient induction by CXCL12 of human T lymphocyte attachment to VCAM-1 under shear stress, mainly controlling the adhesion strengthening step and that interference with Vav1 expression affects cell adhesion strengthening to a higher degree compared with DOCK2 knockdown.

To explore the possibility that the defect in stable arrest on VCAM-1 promoted by CXCL12 shown by DOCK2 siRNA PBL-T transfectants was based on specific impairments in either CD45RA⁺ and CD45RO⁺ T lymphocyte attachment, we purified these cell populations and upon transfection with DOCK2, as well as with Vav1 or control siRNA, transfectants were tested in flow chambers. The results indicated that knocking-down DOCK2 or Vav1 affected arrest on VCAM-1 at similar levels on CD45RA⁺ and CD45RO⁺ transfectants, and closely reflected the reduced attachment observed in whole PBL-T transfectants (Fig. 3C, left panel). Moreover, cell detachment at increasing shear was similarly affected in both transfectants (Fig. 3C, right panels), indicating that defects in adhesion strengthening due to inhibition in DOCK2 or Vav1 expression detected in PBL-T transfectants similarly targeted both CD45RA⁺ and CD45RO⁺ T lymphocytes.
Role of DOCK2 on CXCL12-promoted soluble binding of sVCAM-1-Fc

To gain further insights into the steps of the adhesion cascade that are regulated by DOCK2, we first tested soluble binding of sVCAM-1-Fc to αvβ1 on T lymphocytes transfected with DOCK2 siRNA. Soluble binding assays allow characterization of αvβ1-VCAM-1 interactions in the absence of spreading processes, mimicking the initial steps in the sequence of adhesion events. DOCK2 siRNA transfectants exhibited a reduced capacity to bind sVCAM-1-Fc in response to CXCL12 in comparison with the binding detected on control siRNA transfectants (Fig. 4A). The binding of sVCAM-1-Fc to Vav1 siRNA transfectants was impaired to an
even greater extent, confirming the important role of Vav1 activity for efficient CXCL12-stimulated \(\alpha_\delta\beta_1\) interactions (12). Control experiments revealed that binding was blocked by anti-\(\alpha_\delta\) mAb and that DOCK2, Vav1, or control siRNA transfectants retained similar levels of sVCAM-1-Fc binding upon exposure to \(\text{Mn}^{2+}\), a commonly used potent positive control for affinity regulation (Fig. 4A). No binding was observed without \(\text{Mn}^{2+}\) or sVCAM-1-Fc, or when this cation was present but no soluble ligand was added (data not shown).

To analyze whether alterations in \(\alpha_\delta\beta_1\) high-affinity conformations represented a mechanism whereby DOCK2 siRNA transfectants could bind VCAM-1-Fc less efficiently than control siRNA counterparts, we used the 15/7 Ab (38), an anti-\(\beta_1\) mAb that recognizes an activation epitope on \(\beta_1\) integrins and that constitutes a useful tool for detection of \(\alpha_\delta\beta_1\) integrin high-affinity conformations. Preliminary experiments indicated that the \(\beta_1\) epitope recognized by 15/7 mAb on Molt-4 cells was already induced upon 30-s exposure to CXCL12, induction peaked at 1 min and gradually decreased after prolonged incubation times (data not shown). Flow cytometry analyses on transfectants revealed a significant reduction in the binding of 15/7 mAb to both DOCK2 and Vav1 siRNA transfectants, as compared with control siRNA counterparts (Fig. 4B). Control experiments displayed similar levels of 15/7 mAb staining on control, and DOCK2 and Vav1 siRNA transfectants upon incubation with \(\text{Mn}^{2+}\). These results suggest that impaired development of \(\alpha_\delta\beta_1\) high-affinity conformations on DOCK2 and Vav1 siRNA transfectants represents a mechanism that could contribute to their reduced \(\alpha_\delta\beta_1\)-dependent adhesion stimulated by CXCL12.

### Role of DOCK2 in human T cell spreading during CXCL12-promoted adhesion to VCAM-1

To test whether differences in adhesion strengthening shown by DOCK2 and Vav1 siRNA transfectants in flow chamber experiments could reflect morphological differences during the adhesion process, we subjected Molt-4 cells transfected with GFP-fused WT and mutant forms of DOCK2 and Vav1 to attachment to VCAM-1.

**FIGURE 4.** Soluble binding of sVCAM-1-Fc to DOCK2 siRNA T cell transfectants. A, Molt-4 cells transfected with control, DOCK2-C, or Vav1.3 siRNA were preincubated with control or anti-\(\alpha_\delta\) mAb, and subsequently incubated in the absence or presence of CXCL12 or \(\text{Mn}^{2+}\), before addition of sVCAM-1-Fc. Cell-bound ligand was detected by flow cytometry using PE-conjugated goat anti-human IgG. Insert numbers represent mean fluorescence intensity units. Note the three-decade log scale starting at 0 and ending at \(10^3\). A representative result of three independent experiments is shown. B, Control, DOCK2, or Vav1 siRNA transfectants were incubated for 1 min at 37°C with or without CXCL12 or \(\text{Mn}^{2+}\), samples were immediately placed in ice, and subsequently cells were incubated with 15/7 or control mAb, followed by the addition of FITC-conjugated secondary Ab and analysis by flow cytometry. A representative result of three independent experiments is shown.
and captured transfectant morphology at different times by confocal microscopy. We used dCS-DOCK2 for mutant DOCK2, whereas for mutant Vav1 we used Src homology (SH)3-SH2-SH3-Vav1, a truncated form of Vav1 that only contains the C-terminal SH3-SH2-SH3 region (39). This mutant should have interfered with the activation of endogenous Vav1 by sequestering tyrosine kinases important for its phosphorylation. We previously showed that expression of this mutant Vav1 in Molt-4 cells interfered with up-regulation of αβ1-mediated adhesion in response to CXCL12 (12). Confocal images revealed that in the absence of CXCL12, cells displayed a round morphology on VCAM-1 upon 5-min incubation, whereas the presence of the chemokine switched cell morphology to a clearly spread phenotype in WT DOCK2 and Vav1, as well as in mock transfectants (Fig. 5A). Instead, transfectants expressing mutant DOCK2 and Vav1 forms displayed a minimal spreading on VCAM-1 at a 5-min incubation in response to CXCL12. Analyses of longer incubation times revealed a considerable delay in the acquisition of a spread phenotype in SH3-SH2-SH3-Vav1 transfectants in comparison to dCS-DOCK2 counterparts (Fig. 5A), suggesting a more severe alteration in spreading of transfectants expressing mutant Vav1. Quantification of the areas and roundness of the different transfectants confirmed an early

**FIGURE 5.** Role of DOCK2 and Vav1 on T cell spreading during CXCL12-promoted adhesion to VCAM-1. A, Molt-4 cells were transfected with vectors coding for GFP-fused WT or mutant DOCK2 and Vav1, or GFP alone (mock). Transfectants resuspended in adhesion medium with or without CXCL12 (150 ng/ml) were placed on coverslips coated with sVCAM-1 (10 μg/ml) that were incubated at 37°C for the indicated times. Bound cells were fixed and analyzed by confocal microscopy. Only GFP transfectants are shown. B, Data indicate mean cell area ± SD (left panel) and roundness ± SD (right panel) corresponding to at least 30 cells per condition for the different GFP transfectants, measured as described in Materials and Methods. ***p < 0.001; **p < 0.01; and *p < 0.05, significant changes in cell area and roundness.
DOCK2 was determined by NIH Image software. Data represent the mean to adhesion assays to the indicated concentrations of sVCAM-1 in the presence or absence of CXCL12. After washing and fixation, the extent of adhesion was significantly up-regulated.

Adhesion was assessed by fluorescence in T cells from DOCK2+/+ and DOCK2−/− mice were labeled with BCECF-AM, added to wells coated with sVCAM-1 (2.5 μg/ml) alone (medium) or coimmobilized with CXCL12 and subjected to adhesions for 2 min at 37°C under static conditions. The extent of adhesion was determined in a fluorescence analyzer. Data represent the mean ± SD from five independent experiments, each performed with triplicate samples. Adhesion was significantly up-regulated. **, p < 0.01; ***, p < 0.001 according to one-way ANOVA test. A, T cells from control and DOCK2−/− mice were perfused at 0.5 dynes/cm² in a flow chamber coated with sVCAM-1 (10 μg/ml), coimmobilized with CXCL12 (1 μg/ml), and analyzed for rolling and firmly adherent cells (left panel), or for cell detachment after increasing shear rates (right panel). Data are presented as the mean ± SD of cell percentages from total cell population either rolling, or rolling and subsequently sticking (stable arrest, left panel), or as percentages from the initial number of bound cells that remain attached at the indicated increases of shear stress (right panel; n = 4).

Discussion

The α4β1 integrin is an essential adhesion molecule for lymphocyte migration from blood into tissues during immune responses (4). The activation of Rac is a key event that is required for efficient cell adhesion and migration, and we previously demonstrated that Rac activation by Vav1 promoted by CXCL12 controls subsequent T lymphocyte adhesion mediated by α4β1 (12). Because the C. elegans Ced-5, mammalian DOCK180, and D. melanogaster myoblast city family protein DOCK2 is also involved in Rac activation and migration of T lymphocytes in response to chemokines (21), we investigated the role of DOCK2 during...
CXCL12-promoted, αβ4-dependent, human T lymphocyte adhesion. In this study, we provide evidence supporting the involvement of DOCK2 for efficient CXCL12-triggered human peripheral blood T lymphocyte attachment to VCAM-1 under shear stress. Impairment in CXCL12-stimulated attachment to VCAM-1 under shear due to DOCK2 knocking-down similarly targeted both CD45RA⁺ and CD45RO⁺ cell populations from PBL-T lymphocytes. Instead, the participation of DOCK2-mediated signaling in static T cell adhesion to αβ4 ligands was rather modest. When we compared DOCK2 and Vav1 in these adhesions, a major role for Vav1 was observed, clearly under static but also under flow conditions.

Flow chamber and soluble binding assays revealed that decreased efficiency of DOCK2 siRNA human T cell transfectants to attach to VCAM-1 upon stimulation with CXCL12 was mainly the result of an impairment in αβ4-VCAM-1 adhesion strengthening. Thus, main differences with respect to control siRNA transfectants were that DOCK2 siRNA counterparts displayed significant decreased stable arrest and increased rolling, as well as substantially reduced resistance to detachment upon increased shear stress. This fact might be also partially contributed by an early defect in developing efficient initial cell arrest, as transient arrests (<5 s) were higher in these transfectants than in control siRNA ones. In contrast, the experimental conditions required to obtain sufficient levels of VCAM-1-Fc binding promoted by CXCL12 in the soluble binding assays (45-s preincubation with the chemokine followed by 75-s incubation with VCAM-1-Fc) allows detection of bound VCAM-1 in the absence of spreading, but mainly reflects the adhesion strengthening step rather than the initial receptor-ligand interactions. The results from these assays using DOCK2 siRNA human T cell transfectants confirm that DOCK2 function is important for strengthening of αβ4-VCAM-1 interactions triggered by CXCL12.

Compared with DOCK2, knocking-down Vav1 not only affected, to a greater extent, the αβ4-VCAM-1 adhesion strengthening in response to CXCL12 of human T cell transfectants, but αβ4-VCAM-1 interactions at initial arrest were also affected, based on the notable increase in the levels of transient arrests with respect to control siRNA transfectants. Furthermore, Vav1 siRNA transfectants displayed lower resistance to detachment to increased shear stress and a large reduction (>$75\%$) in binding to VCAM-1 promoted by CXCL12, indicating major impairments in developing efficient adhesion strength. Finally, spreading of DOCK2 dominant negative mutant T cell transfectants on VCAM-1 stimulated by CXCL12 displayed a transient impairment that was faster and more efficiently recovered than that of Vav1 dominant negative counterparts, again pointing to a more prominent role of Vav1 on the reinforcement of adhesion.

To gain insights into mechanisms involved in increased strengthening of αβ4-VCAM-1 interactions by CXCL12 that is controlled by DOCK2 and Vav1, we used the 15/7 anti-β1 mAb, a useful reagent for detection of αβ4 high-affinity conformations (38). Binding of 15/7 mAb to CXCL12-stimulated DOCK2 and Vav1 siRNA transfectants was notably reduced compared with control transfectants and correlated with decreased VCAM-1-Fc binding observed with the same transfectants. These results suggest that DOCK2- and Vav1-dependent signaling contribute to the development of αβ4 high-affinity conformations in response to CXCL12, representing a mechanism that likely favors strengthening of αβ4-VCAM-1 interactions. Since talin directly interacts with the β1 subunit modulating β1 integrin affinity conformations (40, 41), it then constitutes a candidate for further studies aimed to characterize Vav1-DOCK2-Rac downstream effectors linking to talin regulation of αβ4-dependent human T cell adhesion stimulated by chemokines. Another potential mechanism involved in adhesion strength is integrin clustering. We have not been able to detect αβ4 macroclusters promoted by CXCL12 on human T cells using confocal laser microscopy, though we cannot exclude that the αβ4 microclusters, which were not detected by confocal microscopy, could form and could also contribute to the strengthening of adhesion in response to CXCL12.

Importantly, CXCL12-triggered activation of Rac was impaired to a larger extent on Vav1 than on DOCK2 siRNA human T cell transfectants, as detected at times corresponding to those used in the adhesion assays. Along with the more pronounced defects in the adhesion of Vav1 than DOCK2 siRNA transfectants, the data strongly suggest that Rac activation by Vav1 stimulated by CXCL12 exerts a predominant control on αβ4-dependent human PBL-T lymphocyte attachment under flow conditions, as compared with DOCK2-mediated Rac activation. Furthermore, our results provide potential hints on the mechanisms by which Rac could control this adhesion. A critical level of chemokine-promoted Rac activation might be required to activate downstream effectors to subsequently induce both αβ4 high-affinity conformations and actin reorganization leading to a spread phenotype resistant to detachment from shear stress during T cell extravasation. Upon DOCK2 or Vav1 knocking-down, Rac activation by chemokines might not reach a sufficient threshold level, resulting in weak cell adhesion and detachment. After extravasation, when the influence of shear stress is minimal or absent, Vav1-dependent Rac activation could be sufficient to promote T cell attachment to αβ4 ligands on subendothelial basement membranes and interstitial tissues, whereas DOCK2 involvement would be of lesser importance.

Although the data indicate that activation of the Vav-Rac pathway by CXCL12 stimulated αβ4-mediated human PBL-T cell adhesion, we cannot rule out that Vav-dependent, Rac-independent signaling could contribute to this adhesion. Thus, Vav1 can also function as an adaptor molecule in T cells that transduces inside-out signals independently of Rac activation (13–15). Some of these signals, such as tyrosine kinase activity, might modulate the pathways leading to integrin activation.

In addition to the important roles that DOCK2 and Vav1 play during chemokine-stimulated human T cell attachment mediated by αβ4 under shear stress, our results from flow chamber and soluble binding assays also indicate that additional activated molecules are required for efficient induction of attachment. A likely candidate is Rap1, a target for chemokine activation involved in inside-out signaling leading to αβ4 and αβ2 activation through regulator of adhesion and polarization enriched in lymphoid tissues (RAPL) (10, 11), that could work along with Vav1 and DOCK2 to induce a switch to αβ4 high-affinity conformations and promote increased strengthening of αβ4-VCAM-1 interactions. Subsequently, Vav1-Rac- and DOCK2-Rac-dependent reorganization of the actin cytoskeleton in response to CXCL12 may play a more prominent role in a further reinforcement of the adhesion.

Similarly to human PBL-T lymphocytes, up-regulation by CXCL12 of attachment to VCAM-1 displayed by lymph node and splenic T cells from DOCK2⁻/⁻ mice was not, or only modestly, affected in comparison to T cells from control mice when assays were performed under static conditions. However, we encountered a different scenario when we analyzed CXCL12-promoted mouse T cell attachment to VCAM-1 under flow conditions. We found that DOCK2⁻/⁻ and control T cells achieved similar levels of stable arrest on VCAM-1 triggered by CXCL12 at low shear (0.5 dynes/cm²). Moreover, cell detachment at shear stresses up to 5 dynes/cm² was also similar between DOCK2⁻/⁻ and control T cells, indicating that DOCK2 absence did not affect cell-adhesion
strengthening. Since Rac activation by CXCL12 in splenic T cells from DOCK2−/− mice is strongly inhibited in comparison to control T cells (21), the data suggest that enhanced adhesion is not significantly dependent on chemokine-promoted Rac activation, similarly to CCL21-induced T cell adhesion to VCAM-1 (27). Therefore, Rac-independent mechanisms including Rap1, Vav1 adaptor functions, and other Rho GTPases similar to Rac, such as RhoG, could play key roles for efficient induction of αβ2-dependent mouse T cell adhesion in response to CXCL12, either under normal conditions, or as compensatory mechanisms in the absence of DOCK2 expression. Several explanations could account for the distinct DOCK2 role in adhesion under flow conditions from human and mouse samples. Most likely it depends on the intrinsic differences between human and mouse T cells and that their source was also different, e.g., T cells from peripheral blood in human samples, and spleen and lymph node T cells in mice. It is also possible that distinct proportions of naive vs memory T cells might be present in the human and mouse samples, which may use Rac-dependent and -independent pathways differentially. Indeed, PBL-T human samples displayed low CD69 and high L-selectin (CD62L) expression, whereas T cells from DOCK2−/− mice showed a decrease in CD62L expression on CD4+ cells compared with WT counterparts (27), small increases in CD69 expression on CD8+ cells, and in CD44 expression on CD4+ and CD8+ cells (data not shown). Therefore, whereas human samples displayed a resting phenotype, DOCK2−/− T cells exhibited a slightly more activated pattern than WT cells, based on the expression of these activation markers.

Furthermore, lymph nodes and spleen from DOCK2−/− mice display a remarkable decrease in T cellularity (21), which could have provoked a selection of T cells with enhanced, DOCK2-independent, adhesive, and migratory capacity. Thus, we have reported that spleen and lymph node T cells from DOCK2−/− mice have increased αα expression that frequently translates into higher levels of adhesion to VCAM-1 (this work and Ref. 27). Increased αα expression has been correlated with T cell memory phenotype and enhancement in adhesion to αβ2 ligands (42, 43). Therefore, if DOCK2 absence affected at some extent the stimulation by CXCL12 of αβ2-dependent mouse T cell adhesion, this fact might be somewhat obscured by increased basal adhesion due to increased αβ2 expression. Altogether, the data indicate that although DOCK2-dependent signaling is clearly involved in CXCL12-triggered human PBL-T lymphocyte adhesion to VCAM-1 under shear stress, its role in spleen and lymph node mouse T cells, if any, might not be as obvious.

Along with data from mice deficient for DOCK2 (21), our results suggest that efficient Rac activation by CXCL12 in T lymphocytes requires the presence of both DOCK2 and Vav1. Therefore, it is possible that DOCK2 and Vav1 could cooperate in the activation of Rac in response to chemokines for subsequent stimulation of αβ2-dependent cell adhesion. This cooperation could take place in macromolecular complexes in which both DOCK2 and Vav1 are present. Whether DOCK2 and Vav1 interact in chemokine-exposed T cells has not been addressed, although this interaction has been earlier reported (37) in T cell lines expressing them. It could be then hypothesized that DOCK2 might have a scaffold role by helping Vav1 localize close to Rac for efficient activation, as also previously suggested (25). Alternatively, cooperation between DOCK2 and Vav1 for Rac activation could happen in different complexes at distinct subcellular locations. Finally, it could also be hypothesized that Vav1 is an essential GEF for Rac activation whose downstream signaling mediates feedback activation of other essential GEFs, including DOCK2.

Given the key roles of αβ2-dependent adhesion in proper immune T cell functions and the implication of chemokines to stimulate this adhesion, characterization of intracellular signaling pathways activated by chemokines that are relevant for efficient induction of cell adhesion should widen our knowledge on the inside-out mechanisms governing integrin-mediated lymphocyte extravasation.

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References


