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Tetraspanin CD37 Regulates $\beta_2$ Integrin–Mediated Adhesion and Migration in Neutrophils

Janet L. Wee,‡,§ Keith E. Schulze, ‡ Eleanor L. Jones,*, Louisa Yeung,*,‡ Qiang Cheng, ‡ Candida F. Pereira, ‡ Adam Costin, ‡ Georg Ramm, ‡ Annemiek B. van Spriel, ‡ Michael J. Hickey,‡,† and Mark D. Wright*,‡,†

Deciphering the molecular basis of leukocyte recruitment is critical to the understanding of inflammation. In this study, we investigated the contribution of the tetraspanin CD37 to this key process. CD37-deficient mice showed impaired neutrophil recruitment in a peritonitis model. Intravital microscopy analysis indicated that the absence of CD37 impaired the capacity of leukocytes to follow a CXCL1 chemotactic gradient accurately in the interstitium. Moreover, analysis of CXCL1-induced leukocyte-endothelial cell interactions in postcapillary venules revealed that CXCL1-induced neutrophil adhesion and transmigration were reduced in the absence of CD37, consistent with a reduced capacity to undergo $\beta_2$ integrin–dependent adhesion. This result was supported by in vitro flow chamber experiments that demonstrated an impairment in adhesion of CD37-deficient neutrophils to the $\beta_2$ integrin ligand, ICAM-1, despite the normal display of high-affinity $\beta_2$ integrins. Superresolution microscopic assessment of localization of CD37 and CD18 in ICAM-1–adherent neutrophils demonstrated that these molecules do not significantly cocluster in the cell membrane, arguing against the possibility that CD37 regulates $\beta_2$ integrin function via a direct molecular interaction. Moreover, CD37 ablation did not affect $\beta_2$ integrin clustering. In contrast, the absence of CD37 in neutrophils impaired actin polymerization, cell spreading and polarization, dysregulated Rac-1 activation, and accelerated $\beta_2$ integrin internalization. Together, these data indicate that CD37 promotes neutrophil adhesion and recruitment via the promotion of cytoskeletal function downstream of integrin-mediated adhesion. *The Journal of Immunology, 2015, 195: 5770–5779.

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eukocyte recruitment from the bloodstream and migration throughout the body are of fundamental importance in the function of the immune system. It is well established that for circulating leukocytes to enter sites of inflammation, they must undergo a complex sequence of interactions with the endothelium lining the microvasculature of the inflamed site (1). This process is initiated by the leukocyte tethering to and rolling on the endothelial surface, interactions predominantly mediated by the selectin family of adhesion molecules. Rolling leukocytes are then able to undergo arrest and firmly adhere to the endothelial surface in response to chemotactic signals presented on the endothelium. Chemotactic signals cause leukocyte integrins to increase their affinity for their endothelial ligands, thereby facilitating arresting the rolling leukocyte. Adherent leukocytes then undergo integrin-dependent migration over the endothelial surface to encounter an optimal site for transmigration and exit the vasculature. Once in the interstitium, leukocytes migrate to the inflammatory focus via chemotaxis. Although the major adhesion molecules responsible for these interactions have been identified and studied in detail, a growing body of evidence indicates that another family of cell membrane molecules, the tetraspanins, has previously unrecognized functions in controlling adhesion molecule function during leukocyte recruitment (2–5).

Tetraspanins are a large family of proteins present in all multicellular eukaryotic organisms, and they are characterized by their prototypical four transmembrane domain structure. Tetraspanins have been shown to contribute to diverse cellular functions, including cell viability, proliferation, adhesion, and migration (6). Tetraspanins achieve these effects via organization of the cell membrane into microdomains consisting of tetraspanins and a diverse assortment of partner proteins. In immune cells, critical cell surface receptors including CD4, CD8, CD28, CD19, pattern recognition receptors, and MHC class I and II have all been reported to be components of these tetraspanin-enriched microdomains (7). Moreover, several of the proteins associated with these domains, including integrins and other adhesion molecules, signaling molecules such as protein kinase C, and proteins associated with cytoskeletal function, have important roles in leukocyte recruitment and migration. As such, the role of tetraspanins in
controlling the movement of immune cells is a growing area of interest. In B cells, the tetraspanin CD81 has been shown to associate with the α4 integrin and to promote interaction with VCAM-1 (8). Similarly, in endothelial cells, tetraspanins CD9, CD81, and CD151 assemble in docking structures around adherent leukocytes, where they associate with adhesion molecules ICAM-1 and VCAM-1 and promote transendothelial migration (2, 3). However, although the actions of tetraspanins have been examined in detail in lymphocytes, dendritic cells, and endothelial cells, little is known about their actions in neutrophils.

CD37 is a leukocyte-restricted tetraspanin with documented functions in many different leukocyte lineages. In B cells, the actions of CD37 contribute to development of humoral immunity (9–11). Moreover, CD37 is a target for novel immunotherapies to treat B cell malignancies (12–18). CD37 has also been shown to control functions in T cells and dendritic cells (19–22). Notably, we recently demonstrated that CD37 promotes migration of dendritic cells (23). CD37 is also expressed in granulocytes (24), but its role in these cells has not been examined. In this study, we analyzed leukocyte recruitment in CD37−/− mice and found compelling evidence that tetraspanins regulate β2 integrin function in neutrophils and thereby modulate their capacity to respond to inflammatory stimuli in vivo. Intravital microscopic examination of wild-type (WT) and CD37−/− neutrophils revealed that CD37 promotes β2 integrin–mediated neutrophil adhesion and transmigration, as well as extravascular chemotaxis. These responses were associated with a role for CD37 in modulating cytoskeletal-dependent parameters including actin polymerization, neutrophil spreading and polarization, and β2 integrin internalization.

Materials and Methods

Mice

CD37−/− mice were generated by homologous recombination (9) and backcrossed 10 times to a C57BL/6 background. WT and CD37−/− mice were bred and housed under specific pathogen-free conditions in the Alfred Medical Research and Education Precinct (AMREP) or Monash Medical Centre Animal facilities and were used between 6 and 12 wk of age. The animal ethics committees at AMREP or Monash Medical Centre approved all animal experiments.

Abs

Anti-ly6G conjugated to allophtocyanin and anti-CD11a (α4 integrin, clone M17/4) conjugated to Alexa 488 were purchased from eBioscience (San Diego, CA). Nonspecific anti-LFA-1 (αβ2 integrin, clone H155-78) conjugated to PE was obtained from BioLegend (San Diego, CA). Rabbit polyclonal Abs to human CD18 (β2 integrin) and human transferrin receptor were sourced from Abcam (Cambridge, U.K.). Hamster anti-mouse CD18 (β2 integrin, clone 2E6), mouse anti-human CD37 (clone WR17), and rat anti-mouse CD11b (αM integrin, clone M1/70) were purified in-house.

Thioglycollate-induced peritonitis

Mice were inoculated i.p. with 500 μl of 3% thioglycollate (Sigma-Aldrich); 24 h later, mice were euthanized and cells were harvested by peritoneal lavage. Total cell counts were obtained, and neutrophil numbers were derived from the percentage of Ly6G cells determined by analysis of stained cells by flow cytometry (LSR-Fortessa Cell Analyzer, BD Biosciences).

Intravital microscopy

CXCL1-induced leukocyte recruitment was examined in the mouse cremaster muscle by intravital microscopy, as described previously (25). Mice were anesthetized (ketamine hydrochloride, 150 mg/kg; xylazine hydrochloride, 10 mg/kg, i.p.); and a heat pad was used to maintain body temperature. The jugular vein was cannulated for administration of additional anesthetic. The cremaster muscle was dissected free of surrounding tissues and exteriorized onto an optically clear viewing pedestal. The muscle was cut longitudinally with a cautery and held flat against the pedestal by attaching silk sutures to the edges of the tissue. The muscle was then superfused with bicarbonate-buffered saline (pH 7.4, 37°C) and placed under a coverslip.

Intravital microscopy (Axiovert 2 Imaging; Zeiss) with an ×20 objective lens was used to examine the cremasteric microcirculation. A video camera (Sony SSC-DCS50AP) was used to project the images onto a monitor (Sony PVM-20N5E), and the images were recorded using a videocassette recorder. Unbranched postcapillary venules (25–40 μm in diameter, two per experiment) were selected for observation in each experiment. To minimize variability, the same section of venule was recorded at each time interval. Leukocyte rolling flux, rolling velocity, adhesion, and transmigration were quantitated offline using playback analysis. Leukocyte rolling flux was defined as the number of leukocytes moving at a velocity less than that of erythrocytes in the same vessel per minute. Leukocyte rolling velocity was determined by measuring the time taken for leukocytes to roll over a 100-μm length of the venule, and was determined for 20 cells per time point. Adherent leukocytes were defined as cells that remained stationary for 30 s or longer. Leukocyte transmigration was defined as the number of extravascular leukocytes per field of view adjacent to the selected venule.

CXCL1-induced leukocyte recruitment

Two models of CXCL1-induced leukocyte recruitment were examined (25). The first model was CXCL1-induced leukocyte adhesion and transmigration. After an initial baseline recording, the cremaster muscle was superfused with 5 μM CXCL1 (R&D Systems, Minneapolis, MN) dissolved in bicarbonate superfusion buffer, and leukocyte recruitment parameters were examined after 30 and 60 min. The second model was CXCL1-induced directed extravascular leukocyte migration. Directed leukocyte recruitment was induced using CXCL1 incorporated in an agarose bead directly applied to the exteriorized cremaster muscle, as described previously (25). The gel bead (~1 mm3), which also incorporated a small amount of charcoal to aid visualization, was placed ~550 μm from a postcapillary venule and held in place with a coverslip. The cremaster muscle was superfused with bicarbonate buffer at a minimum rate (<0.2 mL/min) to avoid disrupting the chemotactic gradient and viewed with transillumination microscopy. Experiments ran for ~2 h. During the first hour, leukocytes underwent adhesion and migrated into the interstitial tissue adjacent to the selected venule. In the second hour, interstitial migration of leukocytes toward the gel bead was recorded using time-lapse microscopy (UltraView; PerkinElmer) and videocassette image acquisition software (PerkinElmer). Leukocyte migration parameters (velocity, track length, displacement and meandering index [displacement/track length]) were determined using Image J (National Institutes of Health).

Flow chamber assay

In vitro leukocyte adhesion was examined using a whole blood flow chamber assay, as described previously (23, 26), with some modifications. Blood was harvested from anesthetized mice by cardiac puncture, diluted 1:10 in HBSS, and perfused at a physiological shear rate of 2 dynes/cm2 through a parallel flow chamber apparatus (Glycotech, Rockville, MD) assembled over 35-mm Petri dishes precoated with P-selectin (1 μg/ml), CXCL1 (5.2 nM), and either mouse ICAM-1-Fc or Fc (9 nM; all reagents were from R&D Systems). In some experiments, blood samples were incubated with blocking anti-β2 mAb 2E6 for 15 min at 37°C prior to perfusion. Leukocyte interactions were visualized using an inverted microscope (Axiovert 200; Zeiss) with an ×10 objective. After a 6-min equilibration period, the chamber was flushed with HBSS and recordings were made along the length of the flow chamber using a camera (Sony SSC-DCS50AP) and video recorder. The numbers of interacting (rolling or adherent) cells were determined in 100 fields of view for each experiment, and rolling velocity was determined by measuring the time taken for cells (20 per experiment) to roll a defined distance in the chamber.

Neutrophil enrichment from bone marrow

Neutrophils were enriched from bone marrow as described previously (27). Within these isolates, neutrophils were identified with flow cytometry as Ly6G+ cells.

Flow cytometric analysis of neutrophil integrins

Expression of α4 and αM integrins on neutrophils (identified via Ly6G expression) was determined using flow cytometry, by staining with M17/4 and M1/70.
respectively. To assess β2 integrin ligand binding function, a soluble ICAM-1 binding assay was used (28). Bone marrow–derived neutrophils (1 × 10⁶) were incubated with mICAM-1 Fc or Fc alone (100 µg/ml for both), in the presence of Mg²⁺ (10 mM) and EGTA (1 mM) for 30 min at 37°C. Cells were then fixed with paraformaldehyde, incubated with Fc block (1:100) for 5 min, and then stained with FITC-conjugated anti-human Fc (1:50) and allophycocyanin-conjugated anti-Ly6G (1:200) for 30 min. ICAM-1 binding was examined on Ly6G⁺ cells.

**Integrin internalization assay**

Integrin internalization was examined as described previously (29), with some modifications. Bone marrow neutrophils were chilled on ice for 20 min prior to labeling with anti-LFA-1-PE (clone H155-78, 1:400). The mean fluorescence intensity (MFI) of total anti-CD11a-PE staining on neutrophils (MFIₚ) was determined at this time point, identifying neutrophils on the basis of anti-Ly6G (1:200) staining. Additional aliquots of these labeled cells were then resuspended in ice-cold RPMI 1640 containing 1% goat serum as a control experiment, which revealed that this technique removed the vast majority of the surface-bound Ab. Subsequently, adherent cells were detached from the coverslips with trypsin-EDTA, fixed with 2% paraformaldehyde, and cytocentrifuged with anti-Ly6G Ab prior to FACS analysis. The MFI of internalized anti-LFA-1-PE in cells (MFIᵢ) was established from cell populations gated for Ly6G⁺ expression. The internalization index (II) was calculated from normalizing MFIᵢ values to MFIₚ. In parallel experiments to examine the time course of LFA-1 internalization, the formyl peptide receptor agonist peptide, WKYMVm (5 nM; Phoenix Pharmaceuticals, Burlingame, CA), was used as an activating stimulus (30). Cells were removed from the assay after 5, 15, 30, and 60 min, and assessed as described above.

**Confocal microscopy**

Bone marrow–derived neutrophils (in RPMI 1640/1% sheep serum) were allowed to adhere to coverslips coated with ICAM-1 Fc (9 nM), in the presence of WKYMVm or sheep serum, for 20 min at room temperature, and then transferred to 37°C for 15 min. After washing, adherent cells were fixed with 2% paraformaldehyde and labeled with allophycocyanin-conjugated anti-Ly6G. Cells were then permeabilized with 0.5% Triton-X prior to staining with phallolidin–FITC (0.5 µg/ml). Cells were examined via confocal microscopy (Nikon A1R). The average fluorescence intensity of phallolidin–FITC staining and cell surface area of Ly6G⁺ cells were determined in captured images using Image J. Thirty to thirty-five cells per experiment were examined. Cell polarization was assessed utilizing the Coclustering plugin in Image J. Circularity is defined as 4π × ([Area] / [Perimeter]²), where values range from 0 (elongated polygon, maximally polarized) to 1 (perfect circle, no polarization). Cells with areas of 40 µm² or less were excluded from the analysis.

**Labeling of samples for N-STORM imaging**

Anti-mouse IgG Alexa 405-Alexa 647 and anti-rabbit IgG Cy3-Alexa 647 activator-reporter-labeled secondary Abs were prepared by dissolving dyes Alexa 405 and Alexa 647 in PBS at a molar ratio of activator dye Ab:reporter dye of 1:1.2 molar in PBS at a concentration of 1.2 mg/ml in PBS. Activator and reporter dye conjugates were purified by gel filtration using Nap-5 columns (GE Healthcare). Two-dimensional STORM images were acquired with an N-STORM microscope (Nikon). Each sample was illuminated repetitively with each frame of a Rookie laser (405 or 561 nm) followed by three frames of the reporter laser (647 nm) with an alternating sequence of the two activation lasers used for two-color imaging. Emission was recorded onto a 256 × 256-pixel region of an electron multiplying charge coupled device camera (iXon DU-897; Andor Technology, Belfast, U.K.), giving a pixel size of 157.17 nm. The N-STORM images were reconstructed from a series of 8000 images using NIS-Elements AR software (version 4.1; Nikon). Background was determined by measuring fluorescence intensity in intracellular areas where no blinking was visible. Minimum peak size was determined by measuring the fluorescence intensity of the dimmest spots above background while maximum peak size was set at 20,000.

Localization coordinates from each image were exported from NIS-Elements AR as a text file and imported into the statistical analysis environment R (31). These coordinates were used to generate Planar Point Pattern objects to be analyzed further using the spatstat package (32). Coclustering of CD37, β2 integrin, CD11b and the transferrin receptor (TR) were assessed using univariate and bivariate Getis and Franklin’s local Ripley’s K-function analysis (33, 34). Under this method, coclustering is quantified by correlating the degree to which each localization clusters with localizations of its own species (i.e., L(rij)) and the degree to which they cluster with localizations of the opposite protein species (i.e., L(rl)), at a specified spatial scale r. Getis and Franklin’s local version of Ripley’s K-function (33) was used to calculate L(rij) and bivariate/cross-type L(rl)cross values for each point as follows:

\[
L(rij) = \frac{\sum_{i,j} e(x_i,r) e(x_j,r) \delta_{ij}}{\pi} \text{ where } \delta_{ij} = \begin{cases} 1 & \text{if } d_{ij} \leq r \\ 0 & \text{else} \end{cases}
\]

where r is the spatial scale radius, A is the area of three analysis windows distributed across and contained within the cell (each 3 × 3 µm), d_ij is the distance between two points i and j, n is the number of points, and e(x_i,r) and e(x_j,r) are the edge-correction weighting. Ripley’s isotropic edge correction weighting, was implemented in the spatstat package (32, 33), was used for all analyses considered in this study. In our analyses, a spatial scale of r = 50 nm was used, which corresponds approximately to the maxima observed for the overall Ripley’s K analysis of CD37, β2 integrin, CD11b, and the TR (data not shown).

**Electron microscopy analysis of the surface distribution of β2 integrin**

For whole-mount transmission electron microscopy, bone marrow–derived neutrophils from WT and CD37−/− mice were allowed to adhere for 20 min at room temperature on ICAM-1-coated 100-mesh nickel grids pre- covered with Formvar and then transferred to 37°C in the presence of the chemotactic peptide WKYMVm (Peprotech, Rocky Hill, NJ) and Ca²⁺ and Mg²⁺ for 20 min for full activation. After washing with PBS, the cells were fixed in 2% paraformaldehyde for 15 min, washed, blocked with 1-buffer (PBS, 1% BSA, 0.25% gelatin, 20 mM glycine), and incubated with anti-α₅ integrin (clone M171/4) Ab or isotype control Ab in 1-buffer for 30 min at 4°C. After washing, cells were fixed in 1% paraformaldehyde containing 0.1% glutaraldehyde to prevent capping induced by secondary Abs. After washing and blocking in 1-buffer, samples were incubated with anti-rat IgG (Cappel Laboratories) with 15 nm gold particles coupled to Protein A, and were fixed in 1% glutaraldehyde. Subsequently, samples were washed, dried, dehydrated, and analyzed in a Hitachi H-7500 transmission electron microscope operating at 80 kV. Digital images of electron micrographs were collected on a Gatan Multiscan camera and were processed with custom-written software based on MATLAB (MathWorks). The distribution pattern of α₅β₁ integrin and repartition distance analysis were described as previously (36).

**Assessment of Rho GTPase activation**

Activation of RhoA, Rac-1, and Cdc42 were assessed in WT and CD37−/− neutrophils using G-LISA activation assay kits (Cytoskeleton, Denver, CO).
CD37 ablation impairs neutrophil recruitment and endothelial interactions

We first addressed whether the absence of CD37 affects the ability of neutrophils to undergo recruitment to sites of inflammation. In the thioglycollate peritonitis model, CD37−/− mice showed a significant reduction in the number of recruited neutrophils (Fig. 1A), despite having normal numbers of circulating neutrophils (WT 9.1 ± 1.8 × 106/ml versus CD37−/− 7.7 ± 1.0 × 106/ml; n = 11 per group). This provides evidence of a role for CD37 in neutrophil recruitment. To examine this contribution of CD37 further, we used intravital microscopy to examine neutrophil-endothelial interactions in the microvasculature of WT and CD37−/− mice. In the absence of an inflammatory stimulus, leukocyte rolling flux and velocity did not differ between WT and CD37−/− mice (Fig. 1B–E basal time point and data not shown; see also Supplemental Videos 1, 2), indicating that in contrast to the tetraspanin CD63 (4), CD37 does not regulate P-selectin–dependent leukocyte rolling interactions. We next examined the response during acute exposure to CXCL1, a chemokine previously shown to induce neutrophil adhesion and transmigration (25, 37, 38). In WT mice, CXCL1 induced robust increases in leukocyte adhesion and transmigration after 30 and 60 min, without markedly affecting leukocyte rolling (Fig. 1B–E, Supplemental Video 3). At these same time points, CD37−/− mice displayed significant reductions in adhesion and transmigration (Fig. 1D, 1E; Supplemental Video 4). In addition, leukocyte rolling velocity was significantly faster in CD37−/− mice after 30 and 60 min of exposure to CXCL1 (Fig. 1C), possibly reflecting the reduction in leukocyte adhesion in these mice. Transmigration efficiency, which represents the ratio of leukocyte adhesion to transmigration, did not differ between the two strains of mice (Fig. 1F). This result indicated that the defect in leukocyte transmigration observed in CD37−/− mice stemmed primarily from an impairment in adhesion. These impaired responses were observed despite normal expression of L-selectin, CXC2R2, and the α1, α3M, and α4 integrins on CD37−/− neutrophils (Fig. 2).

To confirm the role of CD37 in promoting leukocyte adhesion, we next compared the ability of WT and CD37−/− neutrophils to adhere to adhesion molecule-coated coverslips in an in vitro flow chamber assay (Fig. 3). Previous studies have demonstrated that neutrophil adhesion induced via CXC2R2 ligands is primarily dependent on L-selectin (39). Therefore, in these experiments, we assessed β2 integrin–dependent interactions via incorporation of the β2 integrin ligand ICAM-1 in the adhesive substrate, along with P-selectin and CXC2R1 to promote rolling and arrest respectively. Similar to the in vivo findings, in the in vitro flow chamber assay neither the number of rolling leukocytes (Fig. 3A) nor leukocyte rolling velocity (WT, 15.7 ± 0.9 μm/s; CD37−/−, 13.6 ± 0.5 μm/s; n = 5 mice/group) differed between WT and CD37−/− leukocytes. In contrast, adhesion of CD37−/− leukocytes was significantly impaired relative to WT leukocytes (Fig. 3B). In these experiments, adhesion could be inhibited using a function-blocking anti-β2 integrin mAb, confirming that it was β2 integrin–dependent (Fig. 3B). Integrins can be displayed at the cell surface in both low- and high-affinity conformations. Therefore, one possible explanation for the reduced capacity of CD37−/− leukocytes to adhere to ICAM-1 is that CD37 regulates the expression of high-affinity β2 integrin on the cell surface. To determine whether CD37 affected integrin inside out signaling, we examined the display of high-affinity β2 integrins by measuring the binding of soluble ICAM-1–Fc to activated neutrophils. Binding of ICAM-1–Fc to CD37−/− neutrophils did not differ from that of WT neutrophils under the conditions examined (Fig. 3C), indicating that CD37 has no role in regulating the conformational state of the β2 integrins. Together with the in vivo findings, these data demonstrate that the absence of CD37 results in a reduced capacity of leukocytes to undergo β2 integrin–dependent adhesion under flow conditions, but has no effect on the conformational state of the β2 integrins.

Absence of CD37 impairs the ability of neutrophils to respond to chemotactic cues in the interstitium

Effective neutrophil recruitment to sites of inflammation requires leukocytes to respond appropriately to chemotactic stimuli in the interstitium. Therefore, we next assessed the effect of CD37 deficiency on interstitial leukocyte migration. This was achieved using an in vivo chemotaxis assay in which extravascular leukocytes

**Results**

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Absence of CD37 impairs the ability of neutrophils to respond to chemotactic cues in the interstitium

Effective neutrophil recruitment to sites of inflammation requires leukocytes to respond appropriately to chemotactic stimuli in the interstitium. Therefore, we next assessed the effect of CD37 deficiency on interstitial leukocyte migration. This was achieved using an in vivo chemotaxis assay in which extravascular leukocytes

**FIGURE 1.** Neutrophil recruitment is impaired in CD37−/− mice. (A) Comparison of thioglycollate-induced peritoneal neutrophil recruitment in WT and CD37−/− mice. Neutrophils were enumerated by flow cytometry 24 h after thioglycollate injection. Data points represent individual mice. Mean ± SEM are also shown. (B–F) Leukocyte-endothelial cell interactions in cremasteric postcapillary venules of WT and CD37−/− mice, prior to (“Basal”) and after 30 and 60 min of CXCL1 superfusion (n = 5 mice/group). Data are shown for (B) leukocyte rolling flux, (C) leukocyte rolling velocity, (D) leukocyte adhesion, and (E) transmigration. (F) Transmigration efficiency (i.e., ratio of leukocyte adhesion to transmigration after 30 and 60 min CXCL1 superfusion). Data are shown as mean ± SEM. See also Supplemental Videos 1–4. *p < 0.05, **p < 0.01, ***p < 0.001 versus WT basal conditions in same genotype; †p < 0.05 versus WT.
neutrophils, we analyzed their distribution using two-color superresolution microscopy (N-STORM). As an appropriate Ab against mouse CD37 is not available, for these experiments we used the human HL-60 cell line. Cells were imaged following adhesion to ICAM-1 and activation with fMLF. Coclustering of CD37 and the β2 integrin was assessed using univariate and bivariate Getis and Franklin’s local Ripley’s K-function analysis (34) (Fig. 5). As a positive control, coclustering of CD18 with CD11b was assessed, and coclustering of CD37 with the TR receptor was assessed as a negative control, as previous work has shown that these two molecules do not coassociate (40). As expected, CD18 and CD11b achieved a high level of coclustering (Fig. 5A–C, Supplemental Fig. 1A). In contrast, the level of coclustering of CD37 and CD18 was significantly lower than that of CD18 with CD11b (Fig. 5C–E, Supplemental Fig. 1B) and not significantly different from that of the CD37/TR negative control (Fig. 5F–H, Supplemental Fig. 1C). Thus, using this approach, we were unable to detect significant interactions between CD37 and β2 integrins.

To determine whether CD37 ablation affected β2 integrin clustering, WT and CD37−/− mouse neutrophils were adhered to ICAM-1, and LFA-1 was labeled with 15-nm gold particles. Samples were analyzed by whole-mount electron microscopy (Supplemental Fig. 2A) where cluster formation was determined by calculating interparticulate distances with a near-neighbor variate Getis and Franklin’s local Ripley’s K-function analysis, and cluster size was rated according to the number of beads per cluster (Supplemental Fig. 2B, 2C). Although we are visualized while responding to a chemotactic gradient, established via release of CXCL1 from an agarose bead immobilized on the cremaster muscle (25). In this assay, neither the velocity nor the mean displacement of migrating leukocytes differed between WT and CD37−/− mice (Fig. 4A, B). In contrast, the track length of migrating leukocytes was significantly greater in CD37−/− versus WT mice (Fig. 4C). Similarly, the meandering index, indicative of the capacity of the leukocyte to follow the directional cues provided by the CXCL1 gradient effectively, was significantly reduced in CD37−/− mice (Fig. 4D). The latter finding is further illustrated by comparison of the paths of migration of WT and CD37−/− leukocytes, with the WT leukocytes migrating more directly toward the source of the chemotactic stimulus than the CD37−/− leukocytes (Fig. 4E, 4F). These data indicate that CD37 is not required for detection of a CXCL1-derived chemotactic gradient by leukocytes, but is important in enabling the leukocyte to follow the gradient accurately.

**CD37 and β2 integrin do not cocluster significantly in human neutrophils**

The previous data show that CD37 ablation impairs β2 integrin-dependent processes of neutrophil adhesion to the vascular endothelium (Fig. 1) and to ICAM-1 (Fig. 3). To understand the molecular relationship between CD37 and the β2 integrin in

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**FIGURE 2.** Absence of CD37 does not alter neutrophil expression of homing molecules. Expression of (A) LFA-1 (αL), (B) Mac-1 (αM), (C) L-selectin (CD62L), (D) CXCR2, and (E) α4 integrin were compared on circulating neutrophils (Ly6G+ cells) from WT and CD37−/− mice using flow cytometry. Histograms shown are representative of n = 3–4 independent experiments.

**FIGURE 3.** CD37−/− leukocytes adhere less efficiently to ICAM-1 under flow conditions, but display normal levels of high-affinity β2 integrin. (A) and (B) An in vitro flow chamber adhesion assay was used to assess interactions of WT and CD37−/− leukocytes. Blood from WT or CD37−/− mice was diluted in HBSS and perfused over a substrate consisting of P-selectin, CXCL1, and ICAM-1 Fc fusion protein. (A) Rolling of WT and CD37−/− leukocytes on complete substrate. (B) Adhesion of WT and CD37−/− leukocytes on substrates containing either ICAM-1-Fc or Fc alone. In some experiments, inhibitory anti–β2 integrin Ab was included to confirm that adhesion was β2 integrin–dependent. For ICAM-1-Fc/Fc–alone experiments, n = 5 mice per genotype. For anti–β2 integrin experiments, n = 3. Data are shown as mean ± SEM. (C) Assessment of high-affinity β2 integrin expression on WT and CD37−/− neutrophils. Neutrophil binding of soluble ICAM-1 Fc, or Fc alone (both at 100 μg/ml), under static conditions, was determined using flow cytometry. Data are shown as mean ± SEM of n = 4 independent experiments. *p < 0.05, **p < 0.01 versus WT ICAM-1 Fc.
CD37 restrains further that CD37 is not required for CD18 clustering. We conclude that CD37 does not cluster significantly with CD18, and absence of CD37 did not affect their size and frequency. We expression upon ligand engagement. To address this issue, we the absence of CD37 and consequent reduced stability of integrin might be explained by dysregulation in integrin internalization in

readily detected β2 integrin clusters on mouse neutrophils, the absence of CD37 did not affect their size and frequency. We conclude that CD37 does not cluster significantly with CD18, and further that CD37 is not required for CD18 clustering.

**CD37 restraints β2 integrin internalization**

Integrin-mediated cellular adhesion is a dynamic process, particularly in migrating cells, where endocytosis of integrins plays a critical role in detaching and removing integrins from the trailing edge, and redistributing these to the leading edge (41). As such, the defective adhesive and migratory behavior of CD37−/− leukocytes might be explained by dysregulation in integrin internalization in the absence of CD37 and consequent reduced stability of integrin expression upon ligand engagement. To address this issue, we compared internalization of LFA-1 in WT and CD37−/− neutrophils quantitatively using flow cytometry and confocal microscopy. WT neutrophils underwent substantial LFA-1 internalization within 15 min of adhesion to ICAM-1 (Fig. 6A, 6B, 6D). However, LFA-1 internalization was significantly greater in CD37−/− neutrophils (Fig. 6A), as indicated by an increase in the internalization index (Fig. 6B). Analysis of the kinetics of LFA-1 internalization revealed that internalization progressively increased over 60 min in both WT and CD37−/− cells, but was greater in CD37−/− neutrophils at every time point examined (Fig. 6C). These findings were confirmed with confocal microscopy (Fig. 6D). These data are consistent with a role for CD37 in stabilizing expression of LFA-1 on the surface under ligand-binding conditions.

**Neutrophil CD37 promotes cytoskeletal rearrangement, cell spreading, and polarization and dysregulates Rac-1 activation**

β2 integrin-mediated outside-in signaling is critical to stabilization of leukocyte adhesion via leukocyte spreading and cytoskeletal rearrangement, as well as subsequent polarization and migration (42). As such, alterations in these behaviors may represent a mechanism whereby the absence of CD37 modulates leukocyte adhesive function. Therefore, we next compared cytoskeletal function of WT and CD37−/− neutrophils by assessing actin polymerization and cell spreading on ICAM-1. FITC-phalloidin was used to label polymerized actin, and staining was quantified with flow cytometry and confocal microscopy (Fig. 7A, 7B). Both approaches revealed that actin polymerization was significantly impaired in CD37−/− neutrophils relative to WT cells. These changes were associated with a significant reduction in cell spreading of CD37−/− neutrophils (Fig. 7C). Indeed, although >40% of WT neutrophils achieved a cell area >120 μm², only ~20% of CD37−/− neutrophils reached this size (p < 0.05 versus WT; Fig. 7D). These findings were supported by a reduction in the capacity of adherent CD37−/− neutrophils to undergo polarization (Fig. 7E), as indicated by a circularity index

![Figure 4](http://www.jimmunol.org/)

Directed migration of leukocytes in the interstitium is altered in the absence of CD37. (A–D) Directed extravascular leukocyte migration was induced in cremaster muscles of WT and CD37−/− mice via controlled local release of CXCL1, and was examined by intravital microscopy. Migration was quantitatively assessed with analysis of the following parameters: (A) migration velocity, (B) displacement, (C) track length, and (D) meandering index (displacement/track length). Data were derived from 7–8 mice per group, 8–10 cells per mouse. Data are shown as mean ± SEM. (E and F) Representative migration paths of neutrophils in the CXCL1-induced in vivo chemotaxis assay in (E) WT and (F) CD37−/− mice. Cell migration tracks in XY plane are displayed as arising from a common point of origin. The position of the source of CXCL1 relative to the paths of migration is also depicted. *p < 0.05, **p < 0.01.

![Figure 5](http://www.jimmunol.org/)

CD37 and the β2 integrin do not cocluster significantly in human neutrophils. (A, D, and G) Reconstructed dual-color single molecule N-STORM images, and enlarged views (B, E, and H) showing the distributions of CD11b and CD18 (β2 integrin) (A and B), CD37 and β2 integrin (D and E) and CD37 and the transferrin receptor (TR) (G and H) in HL-60 cells following adhesion to ICAM-1 and activation with FMLP. White boxes indicate representative windows (3 × 3 μm) used for subsequent second-order Ripley’s K analysis. Yellow denotes coclustering. Scale bars represent 5 μm (A, D, and G) or 0.5 μm (B, E, and H). (C) Proportion of CD18 localizations coclustered with CD37 or CD11b (n = 7). (F) Proportion of CD18 localizations coclustered with CD18 (n = 5) or TR (n = 5). Data in (C) and (F) were derived from Quadrant 4 in Supplemental Fig. 1A–C.
We also compared activation of the Rho family of GTPases in WT and CD37$^{−/−}$ neutrophils via quantitation of the GTP-bound forms of RhoA, Rac-1, and Cdc42 in activated cells. After PMA stimulation, CD37$^{−/−}$ cells displayed significantly elevated active Rac-1 relative to that seen in WT cells (Fig. 7G), whereas Cdc42 activation was comparable between the genotypes and RhoA activation was undetectable under these conditions (data not shown). Together, these data indicate that CD37 plays an important role in the cytoskeletal rearrangements that occur after neutrophil binding to the $\beta_2$ integrin ligand ICAM-1, and that Rac-1 activation is dysregulated in the absence of CD37.

Discussion

Tetraspanins regulate molecular function by organizing their partner proteins into signal transducing microdomains. The best characterized of the tetraspanin molecular partners are integrins. Indeed, in nonimmune cells the evidence that tetraspanins can regulate integrin-mediated functions is overwhelming, incorporating roles in wound healing (43), angiogenesis (44), hemostasis (45), epidermal integrity (46), glomerular filtration (47, 48), and cancer cell metastasis (49–51). By comparison, the evidence that tetraspanins regulate adhesion and migration of leukocytes is limited. Whereas CD81 and CD37 have been found to modulate the function of $\alpha_4\beta_1$ in B cells (8, 11), there is little strong evidence that tetraspanins regulate $\beta_2$ integrin function in leukocytes. $\beta_2$ integrins have been reported to be components of tetraspanin-enriched microdomains (52, 53). However, the functional relevance of these observations is unclear. Currently the most compelling evidence that tetraspanins regulate $\beta_2$ integrins comes from in vitro studies by Quast et al. (54), who showed in migrating dendritic cells that the tetraspanin CD81 and $\alpha_L\beta_2$ colocalized at the leading edge, and that silencing CD81 reduced dendritic cell adhesion to ICAM-1. Whether tetraspanins regulate $\beta_2$ integrins in neutrophils or in vivo immune responses has not been examined.

In this study, we examined the role of the tetraspanin CD37 in neutrophil function by analyzing the classical sequence of leukocyte-endothelial cell interactions in WT and CD37$^{−/−}$ mice, revealing compelling evidence that CD37 regulates $\beta_2$ integrin function in an in vivo inflammatory response. CD37$^{−/−}$ leukocytes activated by the neutrophil-attracting chemokine CXCL1 demonstrated impaired adhesion to the vascular endothelium (Fig. 1C–F), a response previously shown to be LFA-1–dependent (37). This resulted in diminished leukocyte extravasation. This impairment in migration and adhesion was reflected in the reduced neutrophil inflammation induced by thioglycollate in the peritonitis model (Fig. 1A). In vitro assessment of CD37-deficient neutrophils under flow conditions provided further evidence that CD37 modulates $\beta_2$ integrin function, in that they showed reduced
adhesion to the $\beta_2$ integrin ligand, ICAM-1 (Fig. 3A, 3B). Together, these data provide clear evidence that CD37 has an important role in supporting $\beta_2$ integrin function.

These experiments also showed that CD37 ablation impairs outside-in dependent parameters, including actin polymerization and cell spreading and polarization (Fig. 7), but does not affect the display of high-affinity $\beta_2$ integrins on the neutrophil surface (Fig. 3C). These findings are in line with previous reports that have shown that tetraspanin-mediated effects on integrin function occur at the level of outside-in signaling, as evidenced by the effects on cell spreading (45, 55) and adhesion strengthening under flow (8, 56), observed for $\beta_1$ and $\beta_3$ integrins. In addition, we observed a role for CD37 in restraining the endocytosis of $\beta_3$ integrins following adhesion to ICAM-1 (Fig. 6), a function that is also dependent on the cytoskeleton (57). Together with the effects on $\beta_2$ integrin–dependent adhesion, these observations indicate that the leukocyte-restricted tetraspanin CD37 has a key role in regulating the function of the leukocyte-restricted $\beta_2$ integrin family. However, the underlying mechanism is likely to be different to the well-characterized regulation of $\beta_1$ integrins by tetraspanins, where several robust and direct molecular partner interactions have been demonstrated (58). In contrast, in the current study, high-stringency N-STORM analyses of CD37 and $\beta_2$ integrin distribution in human neutrophils adherent to ICAM-1 failed to detect coclustering that was significantly different from negative controls (Fig. 5). Moreover, the absence of CD37 from mouse neutrophils did not affect the size and frequency of CD18 clusters (Supplemental Fig. 2). Although we cannot rule out a functionally relevant transient or weak interaction between CD37 and $\beta_2$ integrin, we argue that the most likely model is that CD37 facilitates communication between $\beta_2$ integrins and the cytoskeleton. This model is consistent with several reports that tetraspanins modulate the activity of the cytoskeleton-regulating Rho GTPases (44, 51, 59–62), and interact with and regulate the activity of Ezrin-radixin-moesin proteins that can control cytoskeletal rearrangement through the Rho pathway (63, 64). Thus, the impairment in the ability of CD37$^{-/-}$ neutrophils to bind stably to the vascular endothelium can be explained first by a defect in cytoskeletal rearrangement (Fig. 7), which leads to an impairment of neutrophil spreading on the endothelium. Second, an elevated rate of microtubule-dependent $\beta_2$ integrin endocytosis in the absence of CD37 (Fig. 6) would result in weakening of the adhesive interaction of the neutrophil with the endothelium.

Impairments in spreading, polarity, and integrin recycling are also likely to contribute to the lack of directionality we observed in CD37$^{-/-}$ neutrophil migration in the interstitium (Fig. 4). In this study, we note that extracellular neutrophil migration is dependent on integrins other than the $\beta_2$ family, with studies indicating that $\alpha_4\beta_1$ is of particular importance to this function (65, 66). This raises the possibility that in addition to its effect on the function of $\beta_2$ integrins, CD37 also modulates the actions of other integrins that contribute to neutrophil extravascular migration. This would be consistent with our previous observations on the actions of CD37 in regulating $\alpha_4\beta_1$ integrin function in B cells (11).

In conclusion, these data demonstrate that the tetraspanin CD37 is a key player in neutrophil recruitment and, therefore, acute inflammation. These observations raise the possibility that immunotherapeutic reagents that target CD37 to treat B cell malignancies might have unforeseen consequences in impairing neutrophil recruitment, and potentially the capacity to mount an effective acute inflammatory response.

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Disclosures
The authors have no financial conflicts of interest.

References

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