A Small Molecule Agonist of an Integrin, $\alpha_{L}\beta_{2}$

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The binding of integrin $\alpha_{L}\beta_{2}$ to its ligand intercellular adhesion molecule-1 is required for immune responses and leukocyte trafficking. Small molecule antagonists of $\alpha_{L}\beta_{2}$ are under intense investigation as potential anti-inflammatory drugs. We describe for the first time a small molecule integrin agonist. A previously described $\alpha/\beta$ I allosteric inhibitor, compound 4, functions as an agonist of $\alpha_{L}\beta_{2}$ in Ca$^{2+}$ and Mg$^{2+}$ and as an antagonist in Mn$^{2+}$. We have characterized the mechanism of activation and its competitive and noncompetitive inhibition by different compounds. Although it stimulates ligand binding, compound 4 nonetheless inhibits lymphocyte transendothelial migration. Agonism by compound 4 results in accumulation of $\alpha_{L}\beta_{2}$ in the uropod, extreme uropod elongation, and defective adhesion. Small molecule integrin agonists open up novel therapeutic possibilities.

Integrins are a large family of $\alpha/\beta$ heterodimeric cell surface receptors that mediate cell-cell and cell-extracellular matrix adhesion and transduce signals bidirectionally across the plasma membrane. Integrin $\alpha_{L}\beta_{2}$ (lymphocyte function associated antigen-1 (LFA-1)) Belongs to the $\beta_{2}$ integrin subfamily and is constitutively expressed on all leukocytes. $\alpha_{L}\beta_{2}$ remains in a low affinity state in resting lymphocytes and undergoes dramatic conformational change during lymphocyte activation, which greatly increases its binding affinity for its ligands intercellular adhesion molecule-1, -2, and -3 (ICAM-1, -2, and -3). Regulation of $\alpha_{L}\beta_{2}$ activation is pivotal for controlling leukocyte trafficking and immune responses in health and diseases (1–3).

$\alpha_{L}\beta_{2}$ is an important pharmaceutical target for treating autoimmune and inflammatory diseases (4–8). A humanized antibody to $\alpha_{L}\beta_{2}$ that blocks its binding to the ligand ICAM-1 has been approved by the FDA for treatment of psoriasis, a T cell-mediated autoimmune disease of the skin (9, 10). Furthermore, small molecule antagonists of $\alpha_{L}\beta_{2}$ have been discovered and are in development (11–17).

$\alpha_{L}\beta_{2}$ contains two von Willebrand factor-type A domains, the inserted (I) domains in the $\alpha_{I}$ and the $\beta_{2}$ subunits (18–20). Both $\alpha_{I}$ and $\beta_{2}$ domains have a Rossman fold (i.e. a central $\beta$-sheet surrounded by $\alpha$-helices) with a metal ion-dependent adhesion site (MIDAS) formed by $\beta$-$\alpha$ loops at the “top” face of the domain (20–23). In ligand binding the Mg$^{2+}$ ion in the MIDAS of the $\alpha_{I}$ I domain coordinates directly to a Glu residue that is in the center of the ligand binding sites in domain 1 of ICAM-1 and ICAM-3 (20, 24). The affinity of the $\alpha_{I}$ I domain for ICAMs is regulated by down;ward axial displacement of its C-terminal $\alpha$-helix, which is conformationally linked to reshaping of MIDAS loops and increases affinity for ligand by up to 10,000-fold (25, 26). During activation, the $\beta$ I domain undergoes similar $\alpha$-$\gamma$ helix downward axial movement, which is induced by the swing out of the hybrid domain (27–30). Previous data suggested that when activated, the $\beta_{2}$ I domain binds (through the Mg$^{2+}$ in its MIDAS) to the Glu residue (Glu-310) in the C-terminal linker of the $\alpha_{I}$ I domain, exerts a downward pull on its $\alpha$-$\gamma$ helix, and thereby activates the $\alpha_{I}$ I domain (Fig. 1A) (32, 33).

Two distinct classes of small molecule antagonists of $\alpha_{L}\beta_{2}$ have been developed as anti-inflammatory agents. One group of antagonists binds the hydrophobic pocket underneath the $\alpha$-$\gamma$ helix of the $\alpha_{I}$ I domain (e.g. LFA703 or BIRT377), blocks the downward axial movement of the $\alpha$-$\gamma$ helix, and inhibits ligand binding of $\alpha_{L}\beta_{2}$ allosterically by stabilizing the $\alpha_{I}$ I domain in the low affinity conformation (11–14, 34). These antagonists are called $\alpha$ I allosteric inhibitors. The other group of antagonists appears to bind to the $\beta_{2}$ I domain MIDAS near a key regulatory interface with the $\alpha_{I}$ I domain, blocking communication of conformational change to the $\alpha_{I}$ I domain while at the same time activating conformational rearrangements elsewhere in integrins (35–37). These antagonists, such as compounds 3 and 4 from Genentech and XVA143 from Hoffmann-La Roche, are called $\alpha/\beta$ I allosteric inhibitors (Fig. 1B).

In this report, however, we describe that compound 4, previ-
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Binding of Soluble ICAM-1—Binding of soluble ICAM-1-IgA Fc fusion protein complexed with affinity-purified, fluorescein isothiocyanate-conjugated anti-human IgA was measured by flow cytometry (37).

Cell Adhesion to Immobilized ICAM-1—Binding of fluorescently labeled transfectants to immobilized ICAM-1 was as described (40). Briefly, ICAM-1-IgG Fc fusion protein at 10 μg/ml was immobilized on microtiter plates previously coated with 20 μg/ml protein A and blocked with 2% human serum albumin. Binding of transfectants to immobilized ICAM-1 was determined in HEPES, NaCl, glucose, bovine serum albumin (BSA; 20 mM HEPES, pH 7.5, 140 mM NaCl, 2 mg/ml glucose, 1% BSA) supplemented with divalent cations and compounds as indicated. After incubation at 37 °C for 30 min, unbound cells were washed off, and bound cells were quantitated (40).

Flow Chamber Assay—Binding and detachment in shear flow of αLβ2 transfectants on immobilized ICAM-1 substrates was done in a parallel plate flow chamber as described (42).

Fluorescence Resonance Energy Transfer (FRET) Assay—FRET assay using αL-monomeric cyan fluorescent protein (mCFP)/β2-monomeric yellow fluorescent protein (mYFP) K562 stable transfectants was as described (43).

Cell Migration Assays—Lymphocyte transendothelial migration assays were as described (41). Briefly, before each experiment confluent HUVEC monolayers were activated for 12 h with TNF-α (100 ng/ml). HUVECs were then washed 3 times in buffer A (Hanks’ balanced salt solution supplemented with 20 mM HEPES, pH 7.2, and 1% human serum albumin). Interleukin 2-cultured primary human lymphocytes were pelleted, resuspended at 100,000 cells/ml in 500 μl of buffer A containing compound 4 (1 μM), compound 5 (1 μM), BIRT377 (20 μM), or CBR LFA-1/2 Fab (20 μg/ml) and then added to HUVECs and incubated at 37 °C for 10 or 60 min. Samples were fixed in 3.7% formaldehyde in phosphate-buffered saline for 5 min and stained for leukocyte αL integrin (TS2/4 mAb conjugated to Cy3), endothelial cell ICAM-1 (IC1/11 mAb conjugated to Alexa488), and F-actin (phallolidin-Alexa647; Molecular Probes) as described (41). Imaging was conducted using Bio-Rad Radiance 2000 Laser-scanning confocal microscope system. For each condition complete Z-stacks (0.5 μm thickness) were obtained in each of ten randomly selected fields. Using LaserSharp 2000 software (Bio-Rad) Z-stacks were analyzed (based on previously described criteria (41)) to determine the

EXPERIMENTAL PROCEDURES

Antibodies and Small Molecule Inhibitors—mAbs to human αL and β2 are as described (34). m24 (38) and KIM127 (39) were kind gifts of N. Hogg (London Research Institute) and M. Robinson (Celltech, Slough, UK), respectively. Compound 5 (XVA143) was synthesized according to example 345 of the patent (35) and was also obtained from P. Gillespie (Hoffmann-La Roche). Compounds 3 and 4 were obtained from Genentech (South San Francisco, CA) through the research reagents program. LFA703 (11, 12) was provided by Novartis Pharma AG (Basel, Switzerland), and BIRT377 was from T. Kelly (Boehringer Ingelheim Pharmaceuticals Inc, Ridgecy, CT).

Cell Isolation and Culture—K562 transfectants expressing wild-type and mutant αLβ2 were described (40). Preparation of human peripheral blood mononuclear cells (PBMCs) and interleukin-2 cultured primary lymphocytes was previously described (41). Primary human umbilical vein endothelial cells (HUVECs) were from Cambrex (Walkersville, MD) and cultured as confluent monolayers on fibronectin (10 μg/ml) coated on glass coverslips or ΔT live-cell imaging chambers (Biotechs, Butler, PA) in EGM-2 complete media (Cambrex, Walkersville, MD).

Binding of Soluble ICAM-1—Binding of soluble ICAM-1-IgA Fc fusion protein complexed with affinity-purified, fluorescein isothiocyanate-conjugated anti-human IgA was measured by flow cytometry (37).
number of cells in the process of, or having completed diapedesis.

Morphological analysis of the apically adherent lymphocyte population was based both on the overall cell shape and the distribution of actin and $\alpha_L\beta_2$. Cells exhibiting generally even actin and $\alpha_L\beta_2$ distributions and either spherical or symmetrically spread shapes were designated as "round" or "spread", respectively. Cells exhibiting polarized shapes with an actin-enriched leading edge and roughly even distribution of LFA-1 were designated "polarized". Cells that exhibited both extended uropods and sequestration of the majority of the cellular LFA-1 to the uropod were designated as "X-polarized" (i.e. extremely polarized).

For live-cell experiments confluent TNF-$\alpha$-activated HUVEC monolayers were prepared on Bioptechs $\Delta T$ imaging chambers, rinsed three times with buffer A, and maintained at 37 °C. Lymphocytes (100,000) were added to the chambers, and differential interference contrast images were acquired (using a Zeiss Axiovert S200 epifluorescence microscope (Germany) equipped with a 63× oil objective coupled to a Hamamatsu Orca CCD (Japan)) at 5-s intervals over a course of 30 min. Cell migration was analyzed by manually tracing the outline of each cell in selected frames (i.e. at 180-s intervals) for each time course. Lines connecting the centroid of each cell outline (automatically calculated by OpenLab software) were generated to represent the migration path or “track” followed by each lymphocyte. The total length of the cell tracks was divided by the total time interval during which the track was recorded to calculate average migration velocity. The linear distance between the beginning and endpoint of each track was measured to determine the overall displacement of each cell. Measurement of cell lateral migration parameters was restricted to lymphocytes during their migration over the apical surface of the endothelium and discontinued upon diapedesis across the endothelial monolayer to the subendothelial space. The percentage of diapedesis was obtained by dividing the number of cells that initiated diapedesis by the total number of migrating cells.

To analyze the qualitative details of migration behavior, representative cells were traced at 50-s intervals. The distance separating the centroid of the cell in the initial frame and the centroid of the cell at each subsequent interval was plotted against the cumulative time elapsed.

**Online Supplemental Material**—Supplemental Videos 1 and 2 are representative videos of lymphocyte migration in the absence (Video 1) and presence (Video 2) of compound 4 as described in Figs. 7, C and D, respectively.

**RESULTS**

**Compound 4 Activates $\alpha_L\beta_2$ in Physiologic Cations ($Ca^{2+}$/Mg$^{2+}$) but Inhibits in Mn$^{2+}$**

K562 cells expressing $\alpha_L\beta_2$ showed little binding to soluble multimeric ICAM-1 in $Ca^{2+}$/Mg$^{2+}$ (Fig. 2A), whereas binding was greatly increased by Mn$^{2+}$ (Fig. 2B) or the activating mAb CBR LFA-1/2 (Fig. 2C). In Mn$^{2+}$, compounds 3–5 potently inhibited soluble, multimeric ICAM-1 binding by $\alpha_L\beta_2$ (Fig. 2B), consistent with previous observations (17, 37). However, in physiologic cations (i.e. 1 mM $Ca^{2+}$ and 1 mM Mg$^{2+}$) we found, unexpectedly, that compound 4 greatly increased ligand binding, whereas compounds 3 and 5 had no effect (Fig. 2A). Furthermore, activation of $\alpha_L\beta_2$ binding to ICAM-1 in $Ca^{2+}$/Mg$^{2+}$ by CBR LFA-1/2 mAb was further increased by compound 4 but inhibited by compounds 3 and 5 (Fig. 2C).

Next we assessed the effects of these compounds on physiologic leukocytes (i.e. primary human PBMCs). The PBMCs showed weak binding to soluble multimeric ICAM-1 in $Ca^{2+}$/Mg$^{2+}$ alone and significant binding in Mn$^{2+}$ alone (Fig. 2D). Consistent with our observations with K562 transfectants (Fig. 2, A–C), compound 4 strongly increased binding of soluble ICAM-1 to PBMCs in $Ca^{2+}$/Mg$^{2+}$ but inhibited Mn$^{2+}$-in-
duced binding (Fig. 2D). Both compound 4- and Mn²⁺-induced ICAM-1 binding was α₄β₇-dependent, as such binding was completely inhibited by the α₄I domain-specific blocking antibody TS2/14 (Fig. 2D).

The activating effect of compound 4 was confirmed and further analyzed using static cell adhesion and flow chamber assays. In the static cell adhesion assay, K562 cells expressing α₄β₇ were allowed to adhere to immobilized ICAM-1, and the unbound cells were removed with an automatic plate washer. In the presence of Ca²⁺/Mg²⁺ alone very little cell adhesion was observed, whereas in the presence of Mn²⁺ alone adhesion was greatly enhanced (Fig. 2E). The addition of either compound 4 or 5 abolished Mn²⁺-induced adhesion. In contrast, in Ca²⁺/Mg²⁺-compound 4, but not compound 5, greatly increased cell adhesion (Fig. 2E). In a flow chamber assay, K562 cells expressing α₄β₇ showed week rolling and firm adhesion to immobilized ICAM-1 in Ca²⁺/Mg²⁺ (Fig. 2F). As demonstrated previously, the addition of compound 5 in Ca²⁺/Mg²⁺ significantly increased rolling adhesion, and Mn²⁺ increased firm adhesion (42). At a shear stress of 2 dyn/cm², compound 4 in Ca²⁺/Mg²⁺ induced firm adhesion to a similar extent as observed with Mn²⁺ alone. Under a high shear regime of 32 dyn/cm² compound 4 still promoted significant adhesion (Fig. 2F). However, the total number of rolling and firmly adherent cells was reduced by about half, whereas the amount of adhesion in Mn²⁺ alone remained essentially unchanged. Thus, α₄β₇ adhesiveness induced by compound 4 is less shear-resistant than adhesiveness induced by Mn²⁺.

The Activating Effect of Compound 4 Is Inhibited by Compound 5 Competitively—Compound 4 and compound 5 have homologous structures, and our previous findings suggested that both compounds bind to the MIDAS of the β₂ I domain (37). However, in Ca²⁺/Mg²⁺, compound 4 was activating, whereas compound 5 was inhibitory to wild type α₄β₇ (Fig. 2). Therefore, we studied whether ICAM-1 binding to α₄β₇ in Ca²⁺/Mg²⁺ stimulated by compound 4 could be competitively inhibited by compound 5. We found that α₄β₇ activation by 50 nM compound 4 was reversed by compound 5 in a dose-dependent manner (Fig. 3A). Importantly, the inhibitory dose-response curve of compound 5 was shifted significantly to the right in the presence of a higher concentration (1 μM) of compound 4 (Fig. 3A). Such concentration dependence demonstrates a competitive mode of inhibition. Binding to ICAM-1 stimulated by compound 4 was also inhibited by an α₁ allostERIC inhibitor, LFA703, that binds the hydrophobic pocket underneath the α₁I helix of the α₁ I domain (38). However, the inhibitory dose-response curve of LFA703 was identical with 50 and 1000 nM compound 4, demonstrating non-competitive inhibition.

Compound 4 and Mn²⁺ Activate α₄β₇ by Different Mechanisms—The interaction between the β₂ MIDAS and an acidic residue in the C-terminal linker of α I domains, e.g. Glu-310 in α₄I, is indispensable for Mn²⁺-induced activation of β₂ integrins (32, 33, 44). Mutation of either the metal-coordinating MIDAS residue Ser-114 in the β₂I domain or Glu-310 in the α₄I domain C-terminal linker totally abolished Mn²⁺-induced ICAM-1 binding (Fig. 4). Mutation of another nearby acidic residue in the C-terminal linker of the α₄ I domain, α₄-E316, only partially reduced Mn²⁺-induced ligand binding and served as a control (Fig. 4). Consistent with our previous conclusion that compound 4 binds to the MIDAS of the β₂ I domain (37), the β₂ Ser-114 mutation completely abolished both inhibition of ICAM-1 binding in Mn²⁺ by compound 4 and stimulation of ICAM-1 binding in Ca²⁺/Mg²⁺ by compound 4 (Fig. 4). Despite the absolute requirement for α₄I-Glu-310 in Mn²⁺-induced ICAM-1 binding by α₄β₂, compound 4 was able to activate binding to ICAM-1 by the α₄-E310A mutant, demonstrating that compound 4 activates α₄β₂ by a mechanism that is distinct from that of Mn²⁺.

Susceptibility to α₄β₂ Inhibitory Antibodies—mAbs exist that inhibit α₄β₂ function by distinct mechanisms. Whereas some mAbs bind to the α₁ I domain and competitively block ICAM-1 binding, other α₄I domain and β₂I domain mAbs block ICAM-1 binding indirectly through allosteric mechanisms (34, 45, 46). We compared inhibition by a panel of these mAbs of CBR LFA-1/2-activated α₄β₂ (wild type + mAb); α₄-Glu-310C/β₂-A210C (CC), an α₄β₂ mutant that is constitutively activated by introducing an intersubunit disulfide bond between residue 210 in a β₂ I domain MIDAS loop and the α₄-Glu-310 residue (33); α₄β₂ activated by compound 4 in Ca²⁺/Mg²⁺ (wild type + #4); and α₄β₂ activated by a disulfide bond mutationally introduced into the α₁I domain (HA) (Table 1). The α₄-E310C/β₂-A210C mutant and
TABLE 1
Inhibition by αL and β2 I domain antibodies of multimeric ICAM-1 binding to αLβ2 mutants

<table>
<thead>
<tr>
<th>mAb</th>
<th>Domain</th>
<th>Epitope</th>
<th>WT + mAb</th>
<th>CC</th>
<th>WT + #4</th>
<th>HA</th>
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<td>154–183</td>
<td>97</td>
<td>96</td>
<td>99</td>
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<td>α1</td>
<td>Lys-197, His-201</td>
<td>98</td>
<td>98</td>
<td>99</td>
<td>97</td>
</tr>
<tr>
<td>MHM24</td>
<td>α1</td>
<td>Lys-197</td>
<td>96</td>
<td>97</td>
<td>98</td>
<td>96</td>
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<td>TSI/22</td>
<td>α1</td>
<td>Gln-266, Ser-270</td>
<td>96</td>
<td>97</td>
<td>96</td>
<td>92</td>
</tr>
<tr>
<td>TS2/14</td>
<td>α1</td>
<td>Ser-270, Glu-272</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>14</td>
</tr>
<tr>
<td>May.017</td>
<td>β1</td>
<td>Glu-175, ?</td>
<td>98</td>
<td>70</td>
<td>82</td>
<td>3</td>
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<td>β1</td>
<td>Glu-175</td>
<td>97</td>
<td>40</td>
<td>12</td>
<td>2</td>
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<tr>
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<td>4</td>
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<td>YF651</td>
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<td>His-332, Asn-339</td>
<td>97</td>
<td>2</td>
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wild-type αLβ2 activated by compound 4 showed almost identical susceptibility, i.e. they were inhibited by both the competitive αL1 domain mAbs and the allosteric TS2/14 α1 I domain mAb, were partially inhibited by mAb to Glu-175 in the specificity-determining loop of the β2 I domain, and were resistant to mAbs to residues in the α1 helix (133) and α7 helix (332 and 339) of the β2 I domain.

Effect of Compounds on αLβ2 Conformation—mAbs m24 and KIM127 represent reporters for αLβ2 active conformations. Whereas m24 recognizes the active conformation of the β2 I domain, KIM127 binds to an epitope in the β2 EGF2 domain that is buried in the bent (i.e. latent) integrin conformation and exposed in the extended (i.e. active) conformation. Compounds 3–5 induced exposure of the m24 and KIM127 epitopes on cell surface αLβ2 with similar dose responses (Fig. 5A and B), in agreement with previous measurements on purified αLβ2 with compounds 4 and 5 (37).

We previously developed a FRET method to monitor the spatial proximity of αL and β2 cytoplasmic domains in living cells by fusing mCFP and mYFP to the C termini of αL and β2, respectively (43). Efficient FRET can only be observed when the cytoplasmic tails of αL and β2 (and, therefore, the fused mCFP and mYFP) are in close proximity. Consistent with our previous observations (43), we found here that stable K562 cell transfec-
tants expressing αLβ2 with similar dose responses (Fig. 5A and B), in agreement with previous measurements on purified αLβ2 with compounds 4 and 5 (37).

Compounds 4 and 5 Inhibit Lymphocyte Transendothelial Migration by Distinct Mechanisms—To assess the effects of compounds on transendothelial migration, i.e. diapedesis, we monitored migration of interleukin-2–cultured primary human lymphocytes through TNF-α-activated HUVEC monolayers in medium with Ca2+/Mg2+ by confocal microscopy. Under control conditions, efficient lymphocyte transendothelial migration was observed (~45% by 10 min and ~70% by 60 min). Compared with control, compound 4, compound 5, and BIRT377, an αL allosteric antagonist (Fig. 6A), all inhibited transendothelial migration by greater than 2-fold. Interestingly, Fab fragments of the αLβ2–activating antibody, CBR LFA-1/2, also produced a comparable inhibition of diapedesis (Fig. 6A).

Despite similarity in overall extent of inhibition of diapedesis, morphological analysis (as described under “Experimental Procedures”) revealed dramatic differences among these antagonists (Fig. 6, B–D). Under control conditions (Me2SO), the majority of the cells were polarized, whereas the remaining cells were equally divided into round and spread populations. In the presence of either compound 5 or BIRT377, the polarized cell population was reduced by greater than 2-fold, and the round cell population was dominant (Fig. 6, C and D). In stark contrast, for both compound 4 and CBR LFA-1/2 Fab treatments,
the major cell population was in an unphysiologic “extremely polarized” (X-polarized) state in which the uropod was extended in length and dramatically enriched in the major cell population in an unphysiologic “extremely polarized” (X-polarized) state in which the uropod was observed among the apically retracted back toward the uropod.

The major cell population was in an unphysiologic “extremely polarized” (X-polarized) state in which the uropod was extended in length and dramatically enriched in $\alpha_i\beta_2$, concomitant with depletion of $\alpha_i\beta_2$ from other regions of the cell (Fig. 6, B–D).

The findings that compound 4 and CBR-LFA1/2 activate adhesiveness and induce extreme polarization and localization of LFA-1 to the uropod suggest that they may suppress lymphocyte migration by preventing de-adhesion of the uropod. To test the hypothesis that compound 4 inhibits migration, we performed live-cell imaging of lymphocytes migrating on endothelial monolayers (Fig. 7 and supplemental Videos 1 and 2). Quantitative analysis of more than 50 lymphocytes revealed a greater than 2-fold reduction by compound 4 in both average lateral migration velocity and in the mean displacement of the lymphocytes and a nearly 3-fold reduction in the frequency of diapedesis (Fig. 7, A and B). Analysis of the live-cell imaging demonstrates that, in contrast to the relatively steady and smooth migration observed under control conditions (Fig. 7, C and E, and Video 1), compound 4 promotes “jerky” or “frustrated” migration in which the leading edge and cell body repeatedly advance, then become partially retracted back toward the uropod (Fig. 7, D–E, and Video 2).

**DISCUSSION**

The interaction between $\alpha_i\beta_2$ and ICAM-1 plays a critical role in the formation of the immunological synapse in immune responses and in leukocyte adhesion and extravasation through endothelium. $\alpha_i\beta_2$ is a clinically validated target for the treatment of autoimmune disease, and small molecule antagonists of $\alpha_i\beta_2$ are under intense investigation. Here, we show that a class of compounds previously classified as $\alpha/\beta$ I allosteric antagonists includes among its members a compound that is an agonist of $\alpha_i\beta_2$ in the presence of physiologic divalent cations, i.e. $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$. In contrast, compound 4 is an antagonist in $\text{Mn}^{2+}$, as previously reported (17, 37). Agonism in $\text{Ca}^{2+}/\text{Mg}^{2+}$ and antagonism in $\text{Mn}^{2+}$ was consistently observed in soluble multimeric ICAM-1 binding assays, static cell adhesion, and flow chamber assays and with both K562 transfectants expressing $\alpha_i\beta_2$ and physiologic leukocytes, i.e. PBMCs. In parallel assays the structurally homologous compounds 3 and 5 (XVA143) exhibit only antagonistic properties. The finding that compound 4 can act as both an agonist and antagonist support our previous conclusion that it is an allosteric effector (37) and does not mimic and directly compete binding of ICAM-1 (17, 47).

Compounds 3–5 (XVA143) have very similar structures and appear to have overlapping binding sites. The ability of all three
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FIGURE 7. Dynamics of lymphocyte lateral migration and diapedesis across endothelium. Live-cell imaging and analysis of lymphocytes migrating on TNF-α-activated HUVEC monolayers was as described under "Experimental Procedures." For each condition, greater than 50 cells, taken from four separate imaging experiments (see representative experiments in supplemental Videos 1 and 2) were analyzed. A and B, two-dimensional tracks of lymphocytes migrating over a 30-min period under control conditions (A) and in the presence of 1 μM compound 4 (B). Tracks of cells that initiated diapedesis during the imaging time course are terminated at the point of initiation of diapedesis and are depicted in red. C–E, kinetics of migration of representative lymphocytes. C–D, left panels are selected frames from representative live-cell imaging experiments under control condition (C, see Video 1) and in the presence of compound 4 (D, see Video 2). Representative cells (boxed region in left panels) were tracked at 50-s intervals. The outline (red) of the cell position at relative time 0 is shown in all panels. Note that in control condition (C) the migrating cell steadily increases its distance from its origin over time, whereas in the presence of compound 4 (D) the cell repeatedly moves away from and then contracts back toward the origin. E, the distances from the origin of the centroids of the two migrating cells shown in C and D are plotted against time for control (black) and compound 4 (red) conditions. The control cell is only tracked for 7 min because after this it left the boxed region in Fig. 7C.

compounds to stabilize non-covalent association of the αL and β2 subunits in SDS-PAGE is not dependent on the αI domain and is absolutely dependent on divalent cations and the β2 I domain MIDAS residue Ser-114. Mn2+ and Ca2+/Mg2+ each support stabilization of αLβ2 and αIβ2 noncovalent complexes in SDS-PAGE. All three compounds inhibit ligand binding by αMβ2 as well as αIβ2 (37). Antagonism and agonism by compound 4 appear to occur at the same binding site, since the closely related compound 5 competitively antagonizes agonism by compound 4, and agonism requires β2 I domain residue Ser-114.

The mechanism of αI domain activation by compound 4 differs somewhat from mechanisms previously described for other αI domain activators. For αIβ2 stimulated with either Mn2+ or CBR LFA-1/2, mutation of Glu-310 to Ala at the C-terminal αI domain linker results in loss of ligand binding by abolishing the interdomain communication between the αI and β I domains (33). The lack of dependence on Glu-310 in compound 4-induced αIβ2 activation suggests that compound 4 makes distinct contacts with the αI domain or its linker that cause activation. However, at the same time, compound 4 (like other α/β I allosteric antagonists) apparently blocks the Glu-310–β2 MIDAS interaction through competition for the binding to the MIDAS (37). Wild-type αIβ2 activated by compound 4 showed almost identical susceptibility to inhibitory antibodies as αI–E310C/β2–A210C, which is consistent with the notion that compound 4 induces interaction between the β2 I domain MIDAS and the C-terminal αI I domain linker similarly to the engineered disulfide bond in αI–E310C/β2–A210C. The similarity between these activation mechanisms is further supported by our previous finding that αI–E310C/β2–A210C exhibits less binding to soluble multimeric ICAM-1 in Mn2+ than in Ca2+/Mg2+ (33).

Our working model for agonism by compound 4 is as follows. Ca2+ and Mn2+ compete for binding to the Adjacent to MIDAS (ADMIDAS) metal ion binding site and by binding to this site inhibit and stimulate ligand binding, respectively, and coordinate with alternative ADMIDAS residues (48). In both Ca2+/Mg2+ and Mn2+, compounds 3–5 (XVA143) bind to the β2 MIDAS and block its interaction with αI–Glu-310. In Ca2+/Mg2+, the complex between compound 4 and the β2 I domain is slightly altered compared with its conformation in Mn2+ so
that it is complementary to and can bind to the $\alpha_L$ 1 domain or its linker and induce the open conformation of the $\alpha_L$, 1 domain through interactions that do not involve, but functionally substitute for, the $\alpha_C$-Glu-310: $\beta_2$-MIDAS interaction.

Despite agonistic stimulation of ligand binding, compound 4 can still block physiologic functions of $\alpha_L\beta_2$, that require cycles of adhesion and detachment. It has been proposed that integrins are active at the leading edge, whereas they are inactive at the trailing edge of migrating leukocytes (49, 50). Inactivation of integrins at the trailing edge is thought to be important for detaching the uropod (51). Indeed, sustained activation of $\beta_2$ or $\beta_2$ via activating antibodies (52, 53) or blockade of Rho signaling (54) suppressed eosinophil and monocyte transmigration by preventing the trailing edge from being detached.

We found that although compounds 4 and 5, BIRT377, and CBR LFA-1/2 all inhibit lymphocyte transmigration across the endothelial cell layer, they do so by different mechanisms. Compound 5 and BIRT377 distinctly promoted a predominant round cell population, with greatly reduced spreading and polarization consistent with a reduction in overall adhesiveness. In contrast, compound 4 and CBR LFA-1/2 Fab induced the migrating lymphocytes to display unusually long uropods that were highly enriched in $\alpha_L\beta_2$, consistent with increased adhesion and decreased de-adhesion in the trailing edge. This was confirmed by live-cell imaging analysis that demonstrated frustrated lateral migration induced by compound 4, in which failure of the uropod to detach limited lymphocyte migration. Thus, compound 5 (XVA143) blocks transendothelial migration by reducing adhesion, whereas compound 4 and CBR LFA-1/2 Fab block transendothelial migration by activating $\alpha_L\beta_2$ and interfering with uropod detachment. In a related finding, mutant mice expressing constitutively active $\alpha_L\beta_2$ were impaired in T cell migration, T cell proliferation stimulated by antigen presenting cells, cytotoxic T cell activity, T-dependent humoral immune responses, and neutrophil recruitment during aseptic peritonitis, although signaling through $\alpha_L\beta_2$ was not affected (31). The above observations are consistent with the previous report that compound 4 is a potent inhibitor of the mixed lymphocyte reaction (17). Our study demonstrates for the first time a small molecule integrin allosteric agonist that functions as an anti-inflammatory drug through a novel mechanism of action, perturbation of integrin de-adhesion.

Compound 4 is the first small molecule agonist reported for any integrin. Integrin agonists open up novel opportunities for therapeutics that increase rather than decrease integrin-dependent adhesion. For example, immune recognition of tumor cells is LFA-1-dependent, and agonists might enhance immune responses, including cytotoxic killing of tumor cells. Although we have found that agonism of $\alpha_L\beta_2$ decreases cell migration, and mice with permanently up-regulated $\alpha_L\beta_2$ are functionally impaired, appropriate dosing could allow cycles of agonism at peak drug levels to be alternated with cell migration during intervening toughts. There is extensive precedent with G-protein-coupled receptors for closely related compounds to act as agonists and antagonists (inverse agonists), and both types of compounds have important therapeutic applications.

REFERENCES

Small Molecule Agonist of an Integrin, $\alpha_L\beta_2$