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Small molecule agonists of integrin CD11b/CD18 do not induce global conformational changes and are significantly better than activating antibodies in reducing vascular injury

Mohd Hafeez Faridi[†], Mehmet M. Altintas[†], Camilo Gomez[‡], Juan Camilo Duque[‡], Roberto I. Vazquez-Padron^{‡,*}, and Vineet Gupta^{‡,§,*}

[†]Peggy and Harold Katz Family Drug Discovery Center, Division of Nephrology and Hypertension, Department of Medicine, University of Miami, Miami, FL 33136

[‡]Department of Surgery, University of Miami, Miami, FL 33136

[§]Department of Internal Medicine, Rush University Medical Center, Chicago, IL 60612

Abstract

BACKGROUND—CD11b/CD18 is a key adhesion receptor that mediates leukocyte adhesion, migration and immune functions. We recently identified novel compounds, *leukadherins*, that allosterically enhance CD11b/CD18-dependent cell adhesion and reduce inflammation *in vivo*, suggesting integrin activation to be a novel mechanism of action for the development of anti-inflammatory therapeutics. Since a number of well-characterized anti-CD11b/CD18 activating antibodies are currently available, we wondered if such biological agonists could also become therapeutic leads following this mechanism of action.

METHODS—We compared the two types of agonists using *in vitro* cell adhesion and wound-healing assays and using animal model systems. We also studied effects of the two types of agonists on outside-in signaling in treated cells.

RESULTS—Both types of agonists similarly enhanced integrin-mediated cell adhesion and decreased cell migration. However, unlike leukadherins, the activating antibodies produced significant CD11b/CD18 macro clustering and induced phosphorylation of key proteins involved in outside-in signaling. Studies using conformation reporter antibodies showed that leukadherins did not induce global conformational changes in CD11b/CD18 explaining the reason behind their lack of ligand-mimetic outside-in signaling. *In vivo*, leukadherins reduced vascular injury in a

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*Corresponding Author Information: Roberto I. Vazquez-Padron, PhD; Tel: (305)-243-1154; rvazquez@med.miami.edu or Vineet Gupta, PhD; Tel: (312)-942-0102; Fax: (312)-942-0051; vineet_gupta@rush.edu.

Declarations of conflict of interests

V.G. is an inventor of pending patents related to the study, and V.G. and the University of Miami have the potential for financial benefit from their future commercialization. The pending patents have been licensed to Adhaere Pharmaceuticals Inc., a company cofounded by V.G. The authors have no additional financial interests.

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dose-dependent fashion, but, surprisingly, the anti-CD11b activating antibody ED7 was ineffective.

CONCLUSIONS—Our results suggest that small molecule allosteric agonists of CD11b/CD18 have clear advantages over the biologic activating antibodies and provide a mechanistic basis for the difference.

GENERAL SIGNIFICANCE—CD11b/CD18 activation represents a novel strategy for reducing inflammatory injury. Our study establishes small molecule leukadherins as preferred agonists over activating antibodies for future development as novel anti-inflammatory therapeutics.

Keywords

Cell adhesion; leukocytes; CD11b/CD18; drug discovery; anti-adhesion therapy; inflammation

1. Introduction

Integrins are a family of cell surface expressed α/β heterodimeric adhesion receptors that mediate diverse cellular functions [1]. The $\beta 2$ family of integrins consists of four members with distinct alpha-chains (CD11a, CD11b, CD11c or CD11d) and a common beta-chain ($\beta 2$, CD18). The $\beta 2$ integrin CD11b/CD18 (also known as $\alpha_M \beta 2$, CR3, Mac-1) is expressed primarily in leukocytes (including in neutrophils, macrophages and monocytes) and plays an important role in the development of the innate immune response [2–4]. CD11b/CD18 recognizes a number of physiologic ligands, including fibrinogen, the complement C3 fragment iC3b, blood-clotting factor X and CD54 (ICAM-1) [5], making it an important therapeutic target for reducing inflammatory responses [6]. To that end, a number of agents, biological and chemical, have been developed that inhibit the binding of CD11b/CD18 (and other $\beta 2$ integrins) to its various ligands [7–9]. However, such blocking agents have had limited success in humans [8, 10] and some have shown unexpected side effects [11]. Taking an alternative approach, we recently showed that small molecule mediated activation of CD11b/CD18, rather than its inhibition, also reduces leukocyte migration, tissue accumulation and inflammatory injury in various experimental models [12]. This suggests that integrin activation may represent a novel and effective approach for targeting CD11b/CD18 and for the development of therapeutic agents against inflammation.

Our newly described small molecule agonists (termed “leukadherins”) activate CD11b/CD18 by binding to the integrin’s ligand-binding αA domain (also known as the CD11bA-domain and the αI -domain [13]), an approximately 200 amino acid von Willebrand factor type A (VWFA) domain in the CD11b chain. Modeling studies showed that leukadherins bind to an allosteric pocket in the αA domain, shifting the equilibrium to its more active conformation, thereby promoting ligand engagement by CD11b/CD18 [12]. Leukadherin-mediated integrin activation increases CD11b/CD18-dependent adhesion of leukocytes, which leads to a significant reduction in their migration *in vitro* and *in vivo* and results in a significant decrease in inflammatory injury.

A number of monoclonal antibodies (mAbs) that activate CD11b/CD18 and other $\beta 2$ integrins or that bind in an activation-sensitive manner (together referred to as “activating mAbs”) have also been previously described in the literature [14–23]. KIM127 is an

activation-dependent antibody that also activates human CD11b/CD18 by recognizing sites in the CD18 EGF2 domain that are buried in the inactive integrin conformation [15, 19, 24]. Antibody 24 (mAb 24) detects and stabilizes the ligand-bound active conformation of human $\beta 2$ integrins and recognizes an activation-sensitive epitope in the CD18 A-domain (βA domain) [17]. Similarly, activating antibodies against murine and rat $\beta 2$ integrins have also been described in the literature. M18/2 recognizes the murine CD18 chain and simulates CD11b/CD18-dependent cell adhesion and rosetting [25–27]. The anti-rat CD11b antibodies ED7 and ED8 enhance CD11b/CD18-dependent granulocyte adhesion and homotypic aggregation, suggesting that they activate CD11b/CD18 [28].

As a therapeutic agent, the small molecule compounds and the antibody-based biologics each have distinct advantages and disadvantages. While small molecules are easily delivered (typically orally), they are rapidly cleared and require frequent dosing, although the oral route of administration makes it an easy process. The route of administration of antibody-based biological agents is less than desirable, as they are typically injected intravenously into the circulation, although their long *in vivo* half-life means that they need to be typically administered weekly or every other week. However, this delayed clearance of antibody-based biologics is also a liability, in case they lead to serious side effects, as the side effects take a much longer time to subside. Additionally, biologics have the potential to develop an immune response against them, generating new complications in the treated patients. Having established that CD11b/CD18 activation is a novel and pharmacologically useful mechanism for the development of anti-inflammatory therapeutics, we wondered if both types of integrin agonists – small molecule based chemical compounds and the antibody based biologics – would be equally effective and reasonable to use *in vivo* to treat inflammation via this mechanism of action (MOA). To address this question, we decided to perform a head-to-head testing of the two types of agents using our newly developed leukadherins compounds and a number of anti-CD11b/CD18 activating antibodies that are widely available.

Here, we report our findings that indeed CD11b/CD18 activation via both types of reagents (the chemical leukadherins and the biologic activating mAbs) increases integrin-mediated cell adhesion and decreases cell migration and wound healing *in vitro*, suggesting that both types of agents have a similar mechanism of action. However, we also show that while leukadherins do not induce CD11b/CD18 clustering on cell surface or intracellular signaling pathways in the treated cells, the activating antibodies produced significant CD11b/CD18 clustering and increased phosphorylation of key intracellular signaling proteins. This suggests that unlike the binding with leukadherins, engagement of CD11b/CD18 with activating mAbs mimics a ligand-bound state and induces significant outside-in signaling. Further mechanistic investigations using conformation-specific probes showed that leukadherin binding did not induce large global conformational changes in CD11b/CD18 that are typically associated with binding of ligands or activating antibodies [29–31]. This explains the lack of ligand-mimetic outside-in signaling by leukadherins. Finally, in a head-to-head comparison in a vascular injury model *in vivo*, while both LA1 and ED7 similarly and significantly reduced the influx of macrophages into the injured arteries, only leukadherin LA1 dose-dependently reduced vascular injury and showed significantly higher

efficacy than the anti-CD11b activating antibody ED7. The results suggest that this small molecule agonists of CD11b/CD18 has a clear therapeutic advantage over the biologic activating antibody. Together, the data presented here show that small molecule agonists of CD11b/CD18 have significant advantages over biological agonists, which may require significant optimization before biologic agonists can be used *in vivo* to take advantage of this new mechanism of action for the development of novel anti-inflammatory therapeutics. Thus, leukadherins represent a preferred class of agents for development into future anti-inflammatory therapeutics.

2. Material and Methods

2.1. Reagents and antibodies

The anti-CD11b monoclonal antibody (mAb) 44a (an immunoglobulin G (IgG) 2a (IgG2a) isotype) [3], the heterodimer-specific mAb IB4 (IgG2a) [32, 33], the activating anti-CD18 mAb KIM127 (IgG1) [19] and the anti-CD11b mAb ED8 (IgG1) [34] were from ATCC. The activating anti-CD18 mAb 24 (IgG1) [17] was obtained from Abcam, the activating anti-CD11b mAb ED7 (IgG1) [34] was from Sigma-Aldrich, the activating anti-CD18 mAb M18/2 (IgG2a) [25] was from ebiosciences, the blocking anti-CD11b mAb OX42 (IgG2a) [35] was obtained from Millipore and the isotype control antibodies clone X40 (IgG1) and clone X39 (IgG2a), fluorescein isothiocyanate (FITC)-conjugated mAb A85-1 (rat anti-mouse IgG1), FITC-conjugated R19-15 (rat anti-mouse IgG2a), FITC-conjugated goat antibody against mouse immunoglobulin, rat antibody against mouse GR-1 (GR1-FITC), and phycoerythrin (PE)-conjugated rat antibody against mouse CD11b were obtained from BD Pharmingen. M1/70, a rat mAb against mouse CD11b (IgG2b) [36] was from the monoclonal antibody core at University of California, San Francisco (UCSF). Human fibrinogen (depleted of plasminogen, von Willebrand factor, and fibronectin) was from Enzyme Research Laboratories, bovine serum albumin (BSA) was from Sigma, LPS (O111:B4) was from Invivogen, and phorbol-12-myristate-13-acetate (PMA) was from Cell Signaling. Maxisorp and Highbind 384-well plates were obtained from Nalgene and Corning, respectively. Non-fat milk was obtained from BioRad. All cell culture reagents were from Invitrogen Corp. and Mediatech. Fetal bovine serum (FBS) was purchased from Atlanta Biologicals, Inc. The antibiotic G418 was purchased from Invivogen.

2.2. Animals

The wild type Sprague-Dawley (SD) rats were purchased from Harlan Laboratories. Animal care and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and were performed in accordance with institutional guidelines.

2.3. Cells and cell lines

K562 cells stably transfected with plasmid encoding wild-type integrin CD11b/CD18 (K562 WT cells) have been described previously [37, 38] and were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS and G418 (0.5 mg/ml). The murine macrophage cell line (RAW 264.7 cells) was obtained from ATCC and the cells were maintained in DMEM supplemented with 10% heat-inactivated FBS according to the manufacturer's instructions.

The primary rat peritoneal macrophages were isolated from the wild type Fisher 344 rats that had been previously injected with 5mL of Brewer's thioglycolate broth (Sigma-Aldrich). Macrophage purity was directly analyzed by single channel flow cytometry using rat macrophage specific monoclonal antibodies ED1 (AbD Serotec, Raleigh, NC), WT.5 (BD Biosciences) and His36 (BD Biosciences). Purity was typically > 85%. Macrophages were used in assays immediately upon isolation.

2.4. Cell adhesion assays

Cell adhesion assays using K562 WT, murine RAW 264.7 cells and the primary rat macrophages were performed as previously described and used immobilized fibrinogen as the ligand of CD11b/CD18 [37]. A stock solution of the *leukadherin* family of small-molecule agonists LA1, LA2 and L15 was prepared by dissolving each compound in DMSO at a concentration of 10 mM. The final concentration of DMSO in the assay was approximately 1%. Cells were suspended in serum-free DMEM and incubated in the presence of various agents in ligand-coated wells in a 384-well plate for 10 min at 37°C. LA1, LA2 and LA15 were used at a final concentration of 15µM each and the mAbs were used at a final concentration of 20 g/mL each in the assays. The assay plates were then gently inverted and kept in the inverted position for 30 min at room temperature to dislodge the nonadherent cells. The remaining adherent cells were fixed using 4% formaldehyde and quantified by imaging microscopy as previously described (22, 63). Assays were performed in triplicate wells. Data reported are from one of at least three independent experiments.

2.5. Wound healing assay

Murine RAW 264.7 macrophage cells were grown in DMEM 10% FBS medium in T75 flasks to 70% confluence. The cells were detached by trypsin treatment for 10 min at 37°C and evenly reseeded in 24 well plates and allowed to attain >70% confluency. A scratch was introduced horizontally across the well using 200 µL tip applying even force throughout the etching and holding the tip at approximately 80degree angle. The wells were subsequently washed and fresh medium containing various treatment groups was introduced. The wound was imaged using 10x objective. The cells were allowed to grow for 48 hrs and the wound was reimaged at the same field after 48h. The average extent of wound closure was evaluated by measuring the width of the wound. The area healed was analyzed using ImageJ software and is expressed as percentage healing after 48 hrs.

2.6. Immunofluorescence microscopy

To examine localization and clustering of CD11b/CD18 on the cell surface, 1×10^4 K562 CD11b/CD18 cells were suspended in serum-free IMDM and incubated in the presence of control DMSO (1%), leukadherins LA1, LA2 or LA15 (15 µM each), mAb KIM127 (1:100 dilution of ascites), mAb 24 (20 µg/mL) or Mn^{2+} (1 mM) for 1h at 37°C, as described previously [12, 30]. To visualize changes upon ligand binding, fibrinogen (50 µg/mL) was also added to the cell suspension media in a second set of incubations. The cells were fixed in suspension and incubated with mAb IB4, which is specific for CD11b/CD18, followed by Alexa Fluor 488-conjugated goat antibody against mouse Ig (Sigma). Fluorescence images were recorded with a Leica DMI16000 deconvolution microscope using an HCX APO 40×/

0.75 DRY objective with a DCF360FX camera and with Leica LAS-AF software. A 3-dimensional representation of CD11b/CD18 fluorescence intensity and the analysis of the number of CD11b/CD18 clusters per cell was performed using ImageJ software as described [39]. Clustering determination was carried out by counting individual fluorescent peaks that were projected from the basal to the apical side of the cell and were at least 50% above the base line levels. The images presented are representative of at least 20 cells analyzed for each condition from at least three independent experiments.

2.7. Western blotting analysis

K562 WT cells were incubated with LA1, LA2, or LA15 (15 μ M), or fibrinogen (200 μ g) in serum-free medium for 1 hour at 37°C. Cell lysates were resolved on 4-12% NuPAGE Bis-Tris SDS-PAGE gels using MES running buffer and transferred to a polyvinylidene difluoride (PVDF) membrane (ThermoScientific) using established protocols. Membranes were incubated with a 1:1,000 dilution of the anti-phospho-protein antibodies (anti phospho-p44/42 MAPK (pERK1/2) antibody (Thr²⁰²/Tyr²⁰⁴, Cell Signaling) or anti-phospho-SAPK/JNK (pJNK) antibody (Thr¹⁸³/Tyr¹⁸⁵, Cell Signaling #81E11)), or with an antibody against either total ERK1/2 (p44/42 MAPK (Erk1/2), Cell Signaling #137F5) or total JNK (SAPK/JNK, Cell Signaling #56G8) and developed according to the manufacturer's instructions (ThermoScientific). Proteins were quantified by densitometry using ImageJ software. Data presented are representative of two to three independent experiments.

2.8. Flow Cytometry

Flow cytometric analyses of K562 WT cells were performed according to published protocols [12, 15, 40]. Briefly, cells were suspended in the assay buffer (Tris-buffered saline (TBS) containing 1mM of ions Ca²⁺ and/or Mg²⁺ and 0.1% BSA). Cells (5 X 10⁵) were incubated with primary mAb (1:100 dilution of IB4 ascites or the isotype controls, 1:50 dilution of KIM127 ascites or 15 μ g/mL of mAb 24) in 100 μ l buffer in the absence or presence of 20 μ M agonist LA1 on ice (except for mAbs KIM127 and 24 and the relevant isotype controls, where incubations were performed at 37°C) for 30 minutes. Subsequently, the cells were washed three times with the assay buffer and incubated with goat anti-mouse-APC (1 μ g/ml, Invitrogen) for 20min at 4°C. Cells were washed twice with the assay buffer and analyzed using FACSCaliber flow cytometer (BD Biosciences, CA), counting at least 10,000 events. Data was analyzed using the CellQuest software (BD Biosciences). Data shown is from one of at least three independent experiments.

2.9. Balloon-induced arterial injury in rats

All surgeries were performed under anesthesia by isoflurane (Baxter). Activating mAb ED7 (4mg/kg/d), the isotype control mIgG1 (4mg/kg/d, Rockland) and the leukadherin LA1 (1mg/kg/d) were each administered intra peritoneally (i.p.) in saline daily until the end of the experiment. Balloon injury in the right iliac artery was inflicted with a 2F Fogarty catheter (Baxter) adapted to a custom angiographic kit (Boston Scientific, Scimed) [41]. An aortotomy in the abdominal aorta was made to insert a catheter to the level of the right iliac artery. The balloon was inflated to 1.5 to 1.6 atmospheres and retracted to the arteriotomy site three times. The aortic excision was repaired with eight sutures. The abdominal cavity was closed by planes with an interrupted suture pattern. Arterial specimens were collected

21 days after injury and fixed in 4% formalin-PBS (Sigma-Aldrich) for 5 min and analyzed by histology and immunostaining. Neointima were measured in H&E stained slides using ImagePro.

2.10. Flow cytometric analyses for quantitation of arterial macrophages in injured arteries

Agonist treated and control balloon-injured Fisher 344 rats were sacrificed 7 days post surgery at the onset of inflammation. The injured and non-injured iliac arteries were microdissected and digested with Collagenase/Elastase mix (Worthington Biochemical, NY) in DMEM containing 2% FBS for 2h at 37°C. The resulting single cell suspension was washed with cold PBS containing 2% FBS and filtered through 70 μ M sieve. Cells were re-suspended cold PBS containing 2% FBS at a concentration of 10^6 cells per 100 μ l and stained with biotinylated anti-rat CD11b antibody (clone WT.5, BD Biosciences) for 1h at 4°C. Subsequently, cells were washed and incubated with APC-Streptavidin for 30 min at 4°C. Stained cells were fixed with formalin 4% in PBS for 10 min. Cells were washed twice with the assay buffer and analyzed using FACS II Canto cytometer and the data were analyzed using FlowJo (Tree Star Inc.) software. Data presented is from 4–6 independent samples/group.

2.11. Statistical analysis

Data were analyzed with GraphPad Prism and compared with the Student's *t* test or by one-way analysis of variance (ANOVA) with posthoc analysis, where appropriate. *P* < 0.05 was considered statistically significant.

3. Results

3.1. Chemical and biological agonists of integrin CD11b/CD18 enhance cell adhesion

We have proposed that integrin activation could be a novel mechanism for the development of next-generation anti-inflammatory therapeutics that reduce inflammatory cell migration and recruitment by enhancing, rather than reducing, cell adhesion [12, 37]. To that end, we recently described novel small molecule agonists of CD11b/CD18, which we termed *leukadherins* [12], that we identified using a cell-based high-throughput screening assay [37, 42]. Leukadherin-1 (LA1) (Figure 1A) showed high-affinity for CD11b/CD18 and increased cell adhesion by binding to an allosteric pocket of the ligand-binding α A-domain (also known as α I-domain) of CD11b (Figure 1B) and stabilizing it in an active form [12]. We also identified additional analogs of LA1 that showed similar activity (including LA2 and LA15, Figure S1). Similarly, a number of biological agonists of CD11b/CD18 (and other β 2 integrin), in the form of activating antibodies, have been developed over the years by others, including anti-CD18 monoclonal antibody (mAb) KIM127 [43] and anti-CD18 mAb 24 [17]. To determine how the two types of agonists – chemical (LA1) and biological (mAbs KIM127 and 24) – affect ligand binding by CD11b/CD18, we performed a head-to-head comparison of LA1, mAb KIM127 and mAb 24 for their relative abilities to increase adhesion of CD11b/CD18 to immobilized physiologic ligand fibrinogen using K562 cells stably expressing wild type CD11b/CD18 (K562 WT). K562 WT cells constitutively express wild type CD11b/CD18 in a low affinity state (as in normal leukocytes) [24, 38], thus, providing an excellent system to examine the level of integrin activation afforded by

various agents [12, 24]. K562 WT showed minimal adhesion to immobilized fibrinogen in the presence of physiologic divalent cations (Ca^{2+} plus Mg^{2+} , each at 1mM) (Figure 2). The known non-selective integrin activator Mn^{2+} [44, 45] significantly enhanced cell adhesion, increasing it to a maximal level, which was significantly blocked to basal levels by a blocking anti-CD11b antibody 44a that binds to the α A-domain [3]. Similarly, agonists LA1 and activating mAbs KIM127 and 24 significantly increased K562 WT cell adhesion to immobilized fibrinogen, as compared to the basal Ca^{2+} plus Mg^{2+} condition. However, we find that agonist mAb 24 produced a lower increase in the level of cell adhesion as compared to the other two agonists - LA1 and KIM127. In all cases, the adhesion of K562 WT to immobilized fibrinogen was also significantly reduced by the anti-CD11b blocking mAb 44a, further confirming that the increased cell adhesion by all of these agonists was mediated by CD11b/CD18. As we have shown before, this agonistic effect of leukadherins is not limited to a single compound, as additional leukadherins LA2 and LA15 similarly enhanced CD11b/CD18-mediated cell adhesion (Figure S2).

Next, to examine if the chemical and biological agonists of CD11b/CD18 have a similar effect on ligand binding by CD11b/CD18 from different species, we used the murine macrophage cell line RAW 264.7 and the primary rat macrophages that have constitutive CD11b/CD18 surface expression. The small molecule compound LA1 binds to an allosteric pocket in the α A-domain of CD11b (referred to as Socket for Isoleucine (SILEN) [46] in CD11b or IDAS in CD11a [47]) that is highly conserved across various species [48], suggesting that LA1 should be able to activate CD11b/CD18 from various species. However, the biological agonists (e.g.; activating mAbs) are highly species-selective. Therefore, we used a well-characterized anti-mouse activating antibody (M18/2) [25] with the murine RAW 264.7 cells and the anti-rat anti-CD11b activating antibody ED7 [28, 34] with the primary rat macrophages. The anti-mouse CD18 mAb M18/2 is a non-blocking antibody that has been reported to simulate CD11b/CD18-dependent cell adhesion and rosetting [25–27]. The anti-rat CD11b antibodies ED7 (and another mAb ED8) have been shown to enhance CD11b/CD18-dependent granulocyte adhesion and homotypic aggregation under certain conditions, suggesting that they activate CD11b/CD18 [28].

The RAW 264.7 cells showed minimal level of adhesion to immobilized fibrinogen in the presence of physiologic 1mM Ca^{2+} and Mg^{2+} , which significantly increased (to maximal levels) with the agonist Mn^{2+} (Figure 3A). As with K562 WT cells, agonists LA1 and the activating mAb (M18/2) significantly and similarly increased the level of cell adhesion, as compared to the basal condition. Furthermore, in both cases, addition of blocking anti-CD11b mAb M1/70 [36] eliminated the increase in cell-adhesion due to the two agonists, again confirming that CD11b/CD18 mediated the increased cell adhesion by both types of agonists. Similarly, the primary rat macrophages showed almost no adhesion to immobilized fibrinogen in the presence of non-activating, physiologic Ca^{2+} and Mg^{2+} ions (Figure 3B), but bound significantly more upon activation with Mn^{2+} . Again, both types of agonists – the chemical LA1 and the activating mAb ED7 – significantly enhanced the level of macrophage adhesion as well, as compared to the non-activating condition, and, in both cases, addition of blocking anti-CD11b mAb OX42 [35] greatly reduced the effects of the two agonists, confirming that the increased rat macrophage cell adhesion by both agonists was mediated by CD11b/CD18. Surprisingly, in our hands, ED8, which has been reported as

an agonist similar to ED7 and having an overlapping epitope with ED7 [28, 34], did not produce any significant enhancement in the adhesion of the primary rat macrophages to immobilized fibrinogen (data not shown), suggesting that ED7 is a stronger agonist than ED8. Taken together, these results show that selective agonists of the integrin CD11b/CD18, be it chemical or biological, enhance CD11b/CD18-dependent cell adhesion. Additionally, it shows that while biologics have strong species dependence, leukadherins are equally effective on human, murine and rat CD11b/CD18.

3.2. Activation of CD11b/CD18 reduces macrophage cell migration and wound-healing

Wound-healing assays are routinely used as a classic method for studying cell migration and to assess the effects of various perturbations of the underlying biological processes on such a key cellular function [49, 50]. Macrophages play a significant role in wound-healing [51] and confluent monolayers of macrophages, when artificially wounded or scratched with a pipette tip, respond to the disruption of cell-cell contacts by healing the wound through a combination of proliferation and migration [49]. Macrophages and other leukocytes use CD11b/CD18 dependent cell adhesion (as well as other $\beta 2$ integrins) to migrate over two-dimensional surfaces [52]. We have previously shown that CD11b/CD18 agonist LA1, by freezing integrin in a ligand-bound state, impairs neutrophil chemotaxis [12]. However, how LA1 compares to a biological agonist of the integrin CD11b/CD18 in a head-to-head comparison is not known. Here, we used a murine macrophage cell-based wound-healing assay to compare the relative efficacy of LA1 with a biological agonist of CD11b/CD18 (the anti-CD18 activating mAb M18/2) using the RAW 264.7 cells [53].

We plated the RAW 264.7 cells to confluence and the cells were 'wounded' by scraping a marked location on the plate with a pipette tip. As shown in Figure 4, stimulation with either lipopolysaccharide (LPS) or with the phorbol ester phorbol-12-myristate-13-acetate (PMA) significantly induced the migration of macrophages back into the wound area, as compared to the unstimulated cells (DMSO). Expectedly, treatment with the agonists LA1 and M18/2 alone showed no significant increase in the migration of macrophages into the wound area, as compared to unstimulated cells (DMSO). However, both CD11b/CD18 agonists significantly, and to a similar extent, reduced the migration of LPS- or PMA-stimulated macrophages. Additionally, the compounds LA2 and LA15 similarly reduced the migration of LPS-stimulated macrophages (Figure S3), showing that the leukadherins family of compounds has a similar effect on macrophage migration. These results suggest that integrin CD11b/CD18 activation via either type of agonist equally impairs the migratory capacity of macrophages.

3.3. Activating antibodies, but not LA1, induce integrin clustering and intracellular signaling

Next, we evaluated if there were any significant differences in the integrin binding mediated signaling that is induced by the two types of agonists. While the activating antibodies and leukadherins both enhance CD11b/CD18-dependent cell adhesion and reduce cell migration, it is possible that integrin activation may induce undesirable intracellular signaling, such as those that have been described for the integrin antagonists, which may limit their effectiveness and utility in the clinic [54–60]. For example, α IIB $\beta 3$ and α V $\beta 3$ antagonists

induce outside-in signaling [54, 56]. Similarly, CD11b/CD18 ligation with ligands or blocking antibodies induces CD11b/CD18 clustering [31] and outside-in signaling via activation and phosphorylation of mitogen activated protein kinases (MAPKs), including the c-Jun NH₂-terminal kinase (JNK), the p38 MAPK and the extracellular signal-regulated kinase (ERK) [29, 31, 61, 62], thereby up-regulating the pro-inflammatory NF- κ B signaling [63–65]. Additionally, both inactive and active CD11b/CD18 can be induced to form macro clusters, but the two types of macro clusters transduce different intracellular signals [31], suggesting that an analysis of both clustering and intracellular signaling is needed to fully study the effects of the two types of CD11b/CD18 agonists.

Therefore, to investigate, we used a combination of confocal microscopy (to study clustering) and western blotting assays (to study intracellular signaling) using K562 WT cells. *First*, we studied CD11b/CD18 cell-surface distribution and macro clustering under various conditions using confocal microscopy. K562 WT cells in suspension were incubated with various agonists, fixed with paraformaldehyde and the surface expressed CD11b/CD18 was fluorescently stained with anti-CD11b/CD18 mAb IB4. The results are presented in Figure 5. K562 WT cells showed uniform distribution of CD11b/CD18 on the cell surface, with minimal macro clustering, in the absence of any stimulus, ligands, agonists or antibodies (Figure 5A, top panel, DMSO). As has been reported before, incubation of cells with ligand fibrinogen (Fg) resulted in significant CD11b/CD18 macro clustering (Figure 5A, bottom panel, DMSO) when quantitated using ImageJ [39] (Figure 5B). The agonist Mn²⁺, which has been shown to also induce CD11b/CD18 clustering [31], reproduced the clustering phenotype here (Figures 5A and 5B). Incubation of cells with the activating antibodies KIM127 and mAb 24 also resulted in significant CD11b/CD18 macro clustering on the cells surface and this increased level of integrin macro-clustering did not significantly change upon addition of the physiologic ligand fibrinogen, suggesting that activating anti-CD11b/CD18 antibodies induce CD11b/CD18 activation and clustering even in the absence of a ligand. Previous studies have also shown that other activating anti-CD11b antibodies, including VIM12 (which binds near the extracellular C-terminal region of CD11b [14]) and the full-length immunoglobulin (IgG) as well as the Fab-fragment of LFA-1/2 (which binds the EGF3 domain of CD18 [15, 16]), induce CD11b/CD18 clustering [29, 31]. However, incubation of the cells with the agonist LA1 alone did not induce any significant CD11b/CD18 macro clustering (Figure 5A, top panel, LA1) in the absence of ligand fibrinogen, but did so in the presence of fibrinogen [12]. The leukadherins LA2 and LA15 showed similar results (Figure S4). These data suggest that the two types of integrin agonists have very different effects on the integrins on the distribution and clustering of CD11b/CD18 on the surface of live cells.

Second, an effect of integrin ligation, macro clustering and redistribution in the plasma membrane is the triggering of outside-in intracellular signaling in cells via various MAPKs [66]. Triggering of such MAPK signals also rapidly stimulates transcription and secretion of a variety of pro-inflammatory cytokines and chemokines in leukocytes [64]. To test, we investigated two such MAPK pathways – the ERK pathway and the c-Jun NH₂-terminal kinase (JNK) pathway – by measuring the relative levels of phosphorylated ERK (pERK) and phosphorylated JNK (pJNK) using western blotting. K562 WT cells incubated with

vehicle (DMSO) alone showed minimal ERK and JNK activation (Figure 6) as compared to cells that were incubated with ligand fibrinogen or were activated with the protein kinase C (PKC) agonist phorbol ester PMA (positive control, [67]). Incubation of cells with agonist Mn^{2+} or the activating antibodies KIM127 or mAb 24 showed a significant activation of both ERK and JNK. Previously published studies have also shown that incubation of CD11b/CD18 expressing cells with either the agonist Mn^{2+} [62], blocking mAbs, ligand ICAM-1 or other anti-integrin activating mAbs [29], similarly induces ERK phosphorylation and MAPK signaling. Incubation of the cells with the agonist LA1 alone did not induce any significant pERK or pJNK in the absence of ligand fibrinogen. Additionally, the leukadherins LA2 and LA15 showed similar results (not shown). CD11b/CD18 ligation with fibrinogen showed high levels of both pERK and pJNK under all conditions, suggesting that such MAPK signaling may mimic a ligand-bound state of the cell. Thus, we conclude that CD11b/CD18 binding to activating antibodies mimics its ligand-bound state and induces intracellular MAPK signaling, whereas CD11b/CD18 binding to leukadherins does not. It is worth noting that leukocytes from knock-in animals expressing constitutively active integrins CD11a/CD18 (LFA-1) or $\alpha_4\beta_7$ do not show MAPK signaling in the absence of ligand [68–70], similar to the results obtained with the leukadherins.

Collectively, these results raise the possibility that while the two types of integrin agonists may have some common functional effects on CD11b/CD18 expressing cells (that they enhance ligand binding and cell adhesion), they may also have significant differences that would suggest that one may be more beneficial for *in vivo* use over the other.

3.4. LA1 does not induce global conformational changes in CD11b/CD18

Ligand binding induces extension of the integrin heterodimer and large, global conformational changes leading to outside-in signaling [27, 29, 31]. Binding of activating antibodies also induces global conformational changes in the integrin heterodimer and such large conformational changes mimic those induced upon ligand binding [29, 30]. For example, activating antibody KIM127 recognizes a region in the EGF2 domain of CD18 that is buried in the inactive, bent conformation of the integrin CD11/CD18 heterodimers and stabilizes an extended conformation upon binding [15, 19, 24], which also leads to the separation of the cytoplasmic tails of the heterodimer [29, 71]. Thus, mAb KIM127 is often used as a reporter for the extended conformation of β_2 integrins [28, 38, 43]. Similarly, mAb 24 binds to an activation-dependent epitope in the βA domain that mimics the ligand-bound conformation of the various CD11/CD18 heterodimers [17]. To determine the mechanistic basis for the lack of outside-in signaling by LA1 in the treated cells, we investigated the extent of the conformational changes caused by LA1 binding to cell-surface expressed CD11b/CD18 in K562 cells, using mAbs KIM127 and 24 as reporters of large/global conformational changes. Incubation of K562 WT cells in physiologic buffer alone showed basal level of KIM127 and mAb 24 binding, as measured by flow cytometry (Figure 7). Expectedly, addition of integrin agonist Mn^{2+} ions was sufficient to induce maximal increase in the amount of bound KIM127 and mAb 24, mimicking the ligand binding induced global changes in the CD11b/CD18 heterodimer as has been reported in the literature [17, 28, 38, 43]. However, incubation of these cells with small molecule agonist LA1 produced a very small increase in the KIM127 binding (Figure 7B), indicating that the

LA1-mediated αA activation does not expose the KIM127 epitope in the EGF2 of CD18 and suggesting that LA1 binding leads to domain-limited conformational changes, but not large global conformational changes in the CD11b/CD18 heterodimer on live cell surfaces. The global conformational changes in integrins trigger cytoplasmic leg-separation and generate outside-in signaling [29, 71]. LA1 binding showed increased binding of the second conformational reporter probe, the mAb 24, although the mAb 24 binding was less than the Mn^{2+} -induced change (maximal stimulus), further suggesting that LA1 binding leads to limited, local conformational changes in the CD11b/CD18 heterodimer on live cell surfaces.

3.5. While LA1 significantly reduces mechanical vascular injury in rats, the anti-CD11b activating antibody ED7 is ineffective

Percutaneous coronary intervention (PCI) is one of the most effective methods to unblock occluded arteries and facilitates coronary revascularization in humans [72]. Despite significant technological advances in PCI, restenosis (re-narrowing) secondary to neointimal hyperplasia remains a major complication limiting the success of coronary interventions in 5–25% of patients [73–75]. The recruitment of inflammatory macrophages and other leukocytes precedes neointimal hyperplasia [2]. The denudation of endothelial cell lining at the site of mechanical vascular injury during a PCI procedure leads to the deposition of fibrin and platelets, where selective binding between the platelet cell surface receptor GP Iba and leukocytic CD11b/CD18 leads to the migration and recruitment of these inflammatory cells [76]. Indeed, in experimental models, it has been shown that the genetic ablation of CD11b (CD11b^{-/-} animals) or its blockade (using biological antagonists) can reduce neointima formation after angioplasty or stent implantation [2, 77]. We have also previously demonstrated that LA1 significantly reduces macrophage accumulation at the site of mechanical vascular injury, resulting in significantly reduced neointimal hyperplasia [12]. Here, we used this experimental arterial balloon injury model to examine the relative efficacy of the two types of agonists, in a head-to-head comparison, on inflammatory responses *in vivo*.

First, we determined the dose-dependence of LA1 in this model system. We administered LA1 at two different doses (0.05 or 1 mg/kg/d, intraperitoneal (*i.p.*)), or the vehicle (DMSO), in a saline solution to SD male rats 30 min prior to injury and continued daily injections for the next three weeks and subsequently measured the extent of vascular injury via tissue histology (Figure S5). The injured arteries of LA1-treated rats developed significantly less neointimal hyperplasia (Figure S5B-S5C) as compared to those of animals receiving vehicle alone (Figure S5A). Additionally, LA1 showed a dose-dependent reduction in the extent of balloon catheter-induced mechanical vascular injury, with the higher dose producing a more significant reduction in it as compared to the vehicle treated control.

Next, to determine whether either type of agonist (chemical LA1 and biologic activating antibody) has a therapeutic advantage over the other, we performed a head-to-head comparison using this *in vivo* model. As the study involves use of rats as the experimental model system, we chose the anti-rat CD11b activating mAb ED7 for comparison with LA1. Previous reports show that administration of ED7 reduced kidney and inflammatory bowel

disease in rats [78, 79]. The mAb ED7 was obtained with high purity from ascites fluid according to published protocols [80] (Figure S6A). Staining and flow cytometric analysis showed high degree of binding by the anti-CD11b mAb ED7, but not the control mAb mIgG1, to the purified primary rat macrophages (Figure S6B) and a selective enhancement in macrophage cell adhesion to immobilized fibrinogen by ED7 vs the control mAb IgG1 (Figure S6C). To examine the effects of the two types of agonists on migration and influx of inflammatory macrophages into injured arteries in this model of vascular injury, we administered LA1 or ED7 to wild type rats 30 min prior to balloon injury and continued daily for the next 7 days. The dosage of ED7 used was based on the published studies [78, 79, 81]. Next, we analyzed the arteries to quantify the number of infiltrated macrophages in the presence of agonists LA1 or ED7 and compared it to vehicle treated animals. The arteries were harvested from animals 7 days after balloon injury and single cell suspensions were analyzed by flow cytometry (using anti-rat CD11b antibody WT.5) to quantitate CD11b⁺ macrophages in each sample. The flow cytometry data, shown in Figure 8, reflects the distribution of macrophages within the arterial wall and the surrounding adventitia. Consistent with previous findings, injured arteries of LA1 treated animals contained significantly less CD11b/CD18 macrophages than those in the control vehicle treated rats. We also found that ED7 treatment also similarly and significantly decreased the amount of infiltrated macrophages in the injured artery and in a dose dependent fashion (we tested two doses of 1mg/kg/d and 3.3 mg/kg/d, Figure S7), suggesting the doses of ED7 used here to be highly effective *in vivo*. These results suggest that both types of agonists can similarly reduce macrophage influx into the tissue.

Next, in order to examine the relative efficacy of LA1 and ED7, we administered LA1 (1mg/kg/d) or ED7 (4mg/kg/d) to SD male rats 30 min prior to balloon injury and continued daily for the next 21 days. We administered an isotype control antibody (mIgG1, 4mg/kg/d) to the control group of animals. Histochemical analysis of the injured rat arteries showed reduced neointimal thickening in ED7- and LA1-treated animals, as compared to the control mAb IgG1-treated group (Figure 9). However, only LA1 showed a significant reduction in neointimal hyperplasia (NIH), as compared to the control group and also showed significantly less neointima formation as compared to the ED7 group, suggesting that LA1 is more efficacious than the CD11b-activating mAb ED7 *in vivo* in the rat balloon injury model.

Together, these results suggest that leukadherin LA1 dose-dependently reduces vascular injury (as measured by neointimal thickening) and shows higher efficacy than anti-CD11b activating mAb ED7 *in vivo* and thus, has a clear therapeutic advantage.

4. Discussion

Leukadherins are a family of small molecule compounds that are novel integrin CD11b/CD18 agonists. Leukadherins increase CD11b/CD18-dependent cell adhesion, reduce leukocyte migration and inflammatory injury *in vivo*. The *in vitro* and *in vivo* efficacy of leukadherins suggested that many agents that activate CD11b/CD18 and enhance cell adhesion could be developed into pharmacologically useful therapeutics to treat inflammation. However, in this study we find that not all integrin agonists are equal and

that, as compared to the small molecule chemicals (leukadherins), biological agonists of CD11b/CD18, such as anti-CD11b activating antibodies, have additional effects on intracellular signaling by CD11b/CD18 that may reduce their potential as therapeutics. Such effects have also been reported with a number of ligand-mimetic integrin antagonists that have shown worsened clinical outcomes, perhaps due to induction of outside-in integrin signaling [9, 82]. We find that CD11b/CD18 activation via both types of reagents (the chemical leukadherins and the biologic activating mAbs) increases integrin-mediated cell adhesion and decreases cell migration and wound healing *in vitro*. However, unlike leukadherins, the biologic anti-CD11b/CD18 activating antibodies induced clustering of the cell surface expressed CD11b/CD18 and the phosphorylation of key intracellular signaling proteins, including the JNK and ERK MAP kinases, suggesting that CD11b/CD18 engagement with activating antibodies mimics ligand-bound state. Figure 6 clearly shows that while both types of CD11b/CD18 agonists, the chemical LA1 and the activating antibodies, bind and allosterically activate the integrin receptors, the two types of agonists clearly induce different outside-in signaling in cells in the absence of a ligand (fibrinogen). Figure 6 also shows that at a high concentration of ligand, the two types of agonists do not interfere with the typical ligand-binding induced outside-in signaling, and such signaling is the same under both cases. However, clearly an activating antibody alone is sufficient to induce significant outside-in signaling whereas LA1 binding *per se* did not induce any such signaling. It is important to note here that fibrinogen becomes appreciable for leukocyte adhesion and activation only after surface deposition [83–85]. Fibrinogen deposition on the endothelial surface exposes the cryptic $\gamma^{390-396}$ residues in fibrinogen that is recognized by the integrin CD11b/CD18 on leukocytes for high-affinity engagement, which leads to recruitment of leukocytes from circulation into the injured arteries. Furthermore, we used conformation-specific antibody probes to illustrate a potential reasoning behind these observed differences in signaling between the two types of agonists. Leukadherin binding did not induce large global conformational changes in the integrin CD11b/CD18, whereas the activating antibodies have been shown to induce such changes that mimic a ligand-bound integrin [29, 30]. Thus, while binding activating antibodies induce ligand-mimetic outside-in signaling in cells, binding of leukadherins seems to induce more modest, local changes in the integrin, which are likely not enough to induce outside-in signaling in cells (Figure 9). It has also been previously reported in literature that constitutively active integrin mutants, where the ligand-binding α A-domain of CD11b is locked in its active conformation, do not induce intracellular signaling [30]. Similarly, knock-in mice expressing constitutively active integrin mutants ($\alpha 4\beta 7$ [69], CD11a/CD18 [70]) do not show any increase in outside-in signaling in leukocytes expressing constitutively active integrins. This suggests that α A-domain activation, by itself, is likely not sufficient for inducing ligand-binding mimetic conformational changes and outside-in signaling, whereas binding of large, antibody agonists, certainly induces global conformational changes in integrins, thereby producing ligand-binding mimetic outside-in signaling. Thus, it further suggests that the use of biologic activating antibodies may have additional unforeseen consequences, while the chemical leukadherins seem to have limited negative effects on leukocyte function. Additionally, when compared in a head-to-head study in a vascular injury model *in vivo*, while both types of agents similarly reduced the influx of inflammatory macrophages into the injured arteries, leukadherin LA1 dose-dependently reduced vascular

injury and showed significantly higher efficacy than the anti-CD11b activating antibody ED7, suggesting that this small molecule agonist of CD11b/CD18 has a clear therapeutic advantage over the biologic activating antibody. However, more detailed mechanistic studies in the future will also be needed to provide more insights into the differences between the *in vivo* functions of the two types of agonists. Taken together, our data presented here shows that leukadherins, which are our newly discovered novel small molecule agonists of CD11b/CD18, are effective anti-inflammatory agents *in vivo*, and have the potential to be developed into novel therapeutics, whereas biological agonists of CD11b/CD18, in the form of anti-CD11b/CD18 activating antibodies, are sub-optimal and may require significant amount of optimization before they are ready for *in vivo* use.

Over the last >15 years, there have been at least three published reports on the use of anti-CD11b activating antibody ED7 in reducing inflammatory injury *in vivo* [78, 79, 81]. Thus, it was quite surprising for us to find that it was not more effective in reducing the balloon catheter induced vascular injury in rats here. There could be several reasons for this lack of efficacy of ED7. *First*, it is conceivable that the dose of ED7 used here (4mg/kg/d) was inadequate. We do not believe that this was an issue here because it has previously been shown (in multiple studies) that similar doses of ED7 effectively reduced - a) the mobilization of leukocytes into the peritoneal cavity after thioglycollate-injection [86], b) intestinal damage in a model of acute colitis [79], and c) structural and functional injury in rats with Adriamycin-induced nephrosis [78]. Previous literature also shows that an even lower dose of ED7 (1–3mg/kg) is effective even when administered every other day or even weekly [78, 79, 81] and that such amounts of anti-CD11b antibodies are sufficient enough to completely coat circulating leukocytes in the blood within 1h after injection and maintain it for at least 48 hrs [79, 87]. Activating antibodies at such high level of integrin receptor binding on cells have also been previously reported in literature to have an effect on ligand binding – that they increase ligand binding at these doses [29]. Therefore, we chose to use this “effective” reported ED7 dose in animals with injured arteries to prevent inflammation and diseases development. *Second*, ED7 is a mouse anti-rat antibody and one could argue that immune-clearance by the rodent system limited its efficacy. However, as ED7 has previously been shown to have at least some *in vivo* efficacy in wild type rats and intraperitoneal administration of ED7 was shown to reduce Adriamycin induced nephropathy in animals [78, 79, 81], we believe this to be less of an issue as well. *Third*, as we have tested only ED7 as a model test agent representing activating antibodies, it could be reasoned that other activating antibodies may be more efficacious biological agents *in vivo*. While not necessarily so (see the next point) we believe that future studies with additional anti-CD11b activating antibodies would be a logical step to investigate such a possibility. A limitation here is that there aren’t very many anti-CD11b/CD18 activating antibodies available for use in rodents, and that they do not have cross-reactivity between species, thus limiting their validation in multiple species. *Fourth*, the full IgG molecule of ED7 was used in the studies presented here. Since the ED7 IgG would, in addition to activating CD11b/CD18, dimerize CD11b/CD18 upon binding the integrin on the leukocyte cell surface, it is also plausible that a reason for the differences observed between the two types of agonists was due to the IgG induced dimerization, suggesting that a non-dimerizing Fab fragment of the activating antibodies may be better. However, Lefort *et al* recently showed that non-

dimerizing Fab-fragments of activating anti-CD11b mAbs are sufficient to induce outside-in signaling in cells, whereas the Fab of non-activating, blocking anti-CD11b mAb 44 are not [29]. They also showed that CD11b/CD18 activation using activating mAbs, but not clustering, was sufficient to induce outside-in signaling in cells. Recent reports have suggested that activating antibodies can induce global conformational changes in the integrin heterodimer and that such changes mimic ligand binding induced outside-in signaling [29, 30]. It has also been previously reported that constitutively active integrin mutants, where the ligand-binding α A-domain of CD11b is locked in its active conformation, do not affect intracellular signaling [30]. These observations and our data suggests that LA1 binding to the α A-domain of CD11b may induce only local, domain-limited conformational changes in CD11b/CD18, whereas activating antibodies (whether IgG or Fab) and ligands induce a more global conformational activation of the entire integrin heterodimer and the concomitant outside-in signaling (Figure 10). It is likely that small molecule agonists, like LA1, prime integrin CD11b/CD18 and induce intermediate affinity conformations [28]. This might be relevant in the setting of vascular repair where our findings would suggest that ED7-bound leukocytes could be more “pro-inflammatory” in nature and enhance disease as compared to the LA1-bound cells. Future structural and functional studies will further define the actual activation state of LA1-bound CD11b/CD18. Thus, while it is plausible that Fab fragments of other biological agonists of CD11b/CD18 may show efficacy in a select few inflammatory models, it is also quite possible that anti-integrin activating antibodies in general, due to their display of some of the undesirable side-effects that have been described for the integrin antagonists [54–60], may have limited effectiveness and utility as a therapeutic. This suggests that small molecules are better suited for the development of integrin agonists as therapeutics.

In conclusion, small molecule based agonists of CD11b/CD18 represent a preferred and effective pharmacological approach for the design of novel agents to treat a variety of inflammatory and autoimmune diseases in mammals.

Supplementary Material

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References

1. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell*. 2002; 110:673–687. [PubMed: 12297042]
2. Simon DI, Dhen Z, Seifert P, Edelman ER, Ballantyne CM, Rogers C. Decreased neointimal formation in Mac-1(–/–) mice reveals a role for inflammation in vascular repair after angioplasty. *J Clin Invest*. 2000; 105:293–300. [PubMed: 10675355]

3. Arnaout MA, Todd RF 3rd, Dana N, Melamed J, Schlossman SF, Colten HR. Inhibition of phagocytosis of complement C3- or immunoglobulin G-coated particles and of C3bi binding by monoclonal antibodies to a monocyte-granulocyte membrane glycoprotein (Mol). *J Clin Invest.* 1983; 72:171–179. [PubMed: 6874946]
4. Li R, Arnaout MA. Functional analysis of the beta 2 integrins. *Methods Mol Biol.* 1999; 129:105–124. [PubMed: 10494561]
5. Rieu P, Sugimori T, Griffith DL, Arnaout MA. Solvent-accessible residues on the metal ion-dependent adhesion site face of integrin CR3 mediate its binding to the neutrophil inhibitory factor. *J Biol Chem.* 1996; 271:15858–15861. [PubMed: 8663417]
6. Yonekawa K, Harlan JM. Targeting leukocyte integrins in human diseases. *J Leukoc Biol.* 2005; 77:129–140. [PubMed: 15548573]
7. Shimaoka M, Springer TA. Therapeutic antagonists and conformational regulation of integrin function. *Nat Rev Drug Discov.* 2003; 2:703–716. [PubMed: 12951577]
8. Harlan JM, Winn RK. Leukocyte-endothelial interactions: clinical trials of anti-adhesion therapy. *Crit Care Med.* 2002; 30:S214–219. [PubMed: 12004238]
9. Cox D, Brennan M, Moran N. Integrins as therapeutic targets: lessons and opportunities. *Nat Rev Drug Discov.* 2010; 9:804–820. [PubMed: 20885411]
10. Dove A. CD18 trials disappoint again. *Nat Biotechnol.* 2000; 18:817–818. [PubMed: 10932141]
11. Molloy ES, Calabrese LH. Therapy: Targeted but not trouble-free: efalizumab and PML. *Nat Rev Rheumatol.* 2009; 5:418–419. [PubMed: 19648939]
12. Maiguel D, Faridi MH, Wei C, Kuwano Y, Balla KM, Hernandez D, Barth CJ, Lugo G, Donnelly M, Nayer A, Moita LF, Schurer S, Traver D, Ruiz P, Vazquez-Padron RI, Ley K, Reiser J, Gupta V. Small molecule-mediated activation of the integrin CD11b/CD18 reduces inflammatory disease. *Sci Signal.* 2011; 4:ra57. [PubMed: 21900205]
13. Arnaout MA. Integrin structure: new twists and turns in dynamic cell adhesion. *Immunol Rev.* 2002; 186:125–140. [PubMed: 12234368]
14. Stockl J, Majdic O, Pickl WF, Rosenkranz A, Prager E, Gschwandler E, Knapp W. Granulocyte activation via a binding site near the C-terminal region of complement receptor type 3 alpha-chain (CD11b) potentially involved in intramembrane complex formation with glycosylphosphatidylinositol-anchored Fc gamma RIIB (CD16) molecules. *J Immunol.* 1995; 154:5452–5463. [PubMed: 7730647]
15. Lu C, Ferzly M, Takagi J, Springer TA. Epitope mapping of antibodies to the C-terminal region of the integrin beta 2 subunit reveals regions that become exposed upon receptor activation. *J Immunol.* 2001; 166:5629–5637. [PubMed: 11313403]
16. Petruzzelli L, Maduzia L, Springer TA. Activation of lymphocyte function-associated molecule-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) mimicked by an antibody directed against CD18. *J Immunol.* 1995; 155:854–866. [PubMed: 7608563]
17. Dransfield I, Hogg N. Regulated expression of Mg²⁺ binding epitope on leukocyte integrin alpha subunits. *EMBO J.* 1989; 8:3759–3765. [PubMed: 2479549]
18. Andrew D, Shock A, Ball E, Ortlepp S, Bell J, Robinson M. KIM185, a monoclonal antibody to CD18 which induces a change in the conformation of CD18 and promotes both LFA-1- and CR3-dependent adhesion. *Eur J Immunol.* 1993; 23:2217–2222. [PubMed: 7690325]
19. Stephens P, Romer JT, Spitali M, Shock A, Ortlepp S, Figdor CG, Robinson MK. KIM127, an antibody that promotes adhesion, maps to a region of CD18 that includes cysteine-rich repeats. *Cell Adhes Commun.* 1995; 3:375–384. [PubMed: 8640375]
20. Orzechowski RP, Plescia J, Altieri DC, Bajt ML. AlphaMbeta2 (CD11b/CD18, Mac-1) integrin activation by a unique monoclonal antibody to alphaM I domain that is divalent cation-sensitive. *Journal of leukocyte biology.* 2000; 68:641–649. [PubMed: 11073102]
21. Beals CR, Edwards AC, Gottschalk RJ, Kuijpers TW, Staunton DE. CD18 activation epitopes induced by leukocyte activation. *J Immunol.* 2001; 167:6113–6122. [PubMed: 11714770]
22. Huth JR, Olejniczak ET, Mendoza R, Liang H, Harris EA, Lupher ML Jr, Wilson AE, Fesik SW, Staunton DE. NMR and mutagenesis evidence for an I domain allosteric site that regulates lymphocyte function-associated antigen 1 ligand binding. *Proc Natl Acad Sci U S A.* 2000; 97:5231–5236. [PubMed: 10805782]

23. Zhang H, Liu JH, Yang W, Springer T, Shimaoka M, Wang JH. Structural basis of activation-dependent binding of ligand-mimetic antibody AL-57 to integrin LFA-1. *Proc Natl Acad Sci U S A*. 2009; 106:18345–18350. [PubMed: 19805116]
24. Ortlepp S, Stephens PE, Hogg N, Figdor CG, Robinson MK. Antibodies that activate beta 2 integrins can generate different ligand binding states. *Eur J Immunol*. 1995; 25:637–643. [PubMed: 7535698]
25. Sanchez-Madrid F, Simon P, Thompson S, Springer TA. Mapping of antigenic and functional epitopes on the alpha- and beta-subunits of two related mouse glycoproteins involved in cell interactions, LFA-1 and Mac-1. *J Exp Med*. 1983; 158:586–602. [PubMed: 6193226]
26. Sharfuddin AA, Molitoris BA. Pathophysiology of ischemic acute kidney injury. *Nat Rev Nephrol*. 2011; 7:189–200. [PubMed: 21364518]
27. Varga G, Balkow S, Wild MK, Stadtbauer A, Krummen M, Rothoeft T, Higuchi T, Beissert S, Wethmar K, Scharffetter-Kochanek K, Vestweber D, Grabbe S. Active MAC-1 (CD11b/CD18) on DCs inhibits full T-cell activation. *Blood*. 2007; 109:661–669. [PubMed: 17003381]
28. Draskovic-Pavlovic B, Van Der Laan LJ, Pejnovic N, Dijkstra CD, Colic M. Differential effects of anti-rat CD11b monoclonal antibodies on granulocyte adhesiveness. *Immunology*. 1999; 96:83–89. [PubMed: 10233681]
29. Lefort CT, Hyun YM, Schultz JB, Law FY, Waugh RE, Knauf PA, Kim M. Outside-in signal transmission by conformational changes in integrin Mac-1. *J Immunol*. 2009; 183:6460–6468. [PubMed: 19864611]
30. Pluskota E, Soloviev DA, Szpak D, Weber C, Plow EF. Neutrophil apoptosis: selective regulation by different ligands of integrin alphaMbeta2. *J Immunol*. 2008; 181:3609–3619. [PubMed: 18714035]
31. Whitlock BB, Gardai S, Fadok V, Bratton D, Henson PM. Differential roles for alpha(M)beta(2) integrin clustering or activation in the control of apoptosis via regulation of akt and ERK survival mechanisms. *J Cell Biol*. 2000; 151:1305–1320. [PubMed: 11121444]
32. Wright SD, Rao PE, Van Voorhis WC, Craigmyle LS, Iida K, Talle MA, Westberg EF, Goldstein G, Silverstein SC. Identification of the C3bi receptor of human monocytes and macrophages by using monoclonal antibodies. *Proc Natl Acad Sci U S A*. 1983; 80:5699–5703. [PubMed: 6225125]
33. Hogg N, Stewart MP, Scarth SL, Newton R, Shaw JM, Law SK, Klein N. A novel leukocyte adhesion deficiency caused by expressed but nonfunctional beta2 integrins Mac-1 and LFA-1. *J Clin Invest*. 1999; 103:97–106. [PubMed: 9884339]
34. Damoiseaux JG, Dopp EA, Neefjes JJ, Beelen RH, Dijkstra CD. Heterogeneity of macrophages in the rat evidenced by variability in determinants: two new anti-rat macrophage antibodies against a heterodimer of 160 and 95 kd (CD11/CD18). *Journal of leukocyte biology*. 1989; 46:556–564. [PubMed: 2572659]
35. Robinson AP, White TM, Mason DW. Macrophage heterogeneity in the rat as delineated by two monoclonal antibodies MRC OX-41 and MRC OX-42, the latter recognizing complement receptor type 3. *Immunology*. 1986; 57:239–247. [PubMed: 3512425]
36. Springer T, Galfre G, Secher DS, Milstein C. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. *Eur J Immunol*. 1979; 9:301–306. [PubMed: 89034]
37. Park JY, Arnaout MA, Gupta V. A simple, no-wash cell adhesion-based high-throughput assay for the discovery of small-molecule regulators of the integrin CD11b/CD18. *J Biomol Screen*. 2007; 12:406–417. [PubMed: 17438069]
38. Gupta V, Gylling A, Alonso JL, Sugimori T, Ianakiev P, Xiong JP, Arnaout MA. The beta-tail domain (betaTD) regulates physiologic ligand binding to integrin CD11b/CD18. *Blood*. 2007; 109:3513–3520. [PubMed: 17170130]
39. Szczur K, Zheng Y, Filippi MD. The small Rho GTPase Cdc42 regulates neutrophil polarity via CD11b integrin signaling. *Blood*. 2009; 114:4527–4537. [PubMed: 19752396]
40. Xiong JP, Stehle T, Goodman SL, Arnaout MA. New insights into the structural basis of integrin activation. *Blood*. 2003; 102:1155–1159. [PubMed: 12714499]

41. Gabeler EE, van Hillegersberg R, Statius van Eps RG, Sluiter W, Gussenhoven EJ, Mulder P, van Urk H. A comparison of balloon injury models of endovascular lesions in rat arteries. *BMC Cardiovasc Disord.* 2002; 2:16. [PubMed: 12350231]
42. Faridi MH, Maignel D, Barth CJ, Stoub D, Day R, Schurer S, Gupta V. Identification of novel agonists of the integrin CD11b/CD18. *Bioorg Med Chem Lett.* 2009; 19:6902–6906. [PubMed: 19879752]
43. Nishida N, Xie C, Shimaoka M, Cheng Y, Walz T, Springer TA. Activation of Leukocyte beta(2) Integrins by Conversion from Bent to Extended Conformations. *Immunity.* 2006; 25:583–594. [PubMed: 17045822]
44. Altieri DC. Occupancy of CD11b/CD18 (Mac-1) divalent ion binding site(s) induces leukocyte adhesion. *J Immunol.* 1991; 147:1891–1898. [PubMed: 1890307]
45. Dransfield I, Cabanas C, Craig A, Hogg N. Divalent cation regulation of the function of the leukocyte integrin LFA-1. *J Cell Biol.* 1992; 116:219–226. [PubMed: 1346139]
46. Xiong JP, Li R, Essafi M, Stehle T, Arnaout MA. An isoleucine-based allosteric switch controls affinity and shape shifting in integrin CD11b A-domain. *J Biol Chem.* 2000; 275:38762–38767. [PubMed: 11034990]
47. Weitz-Schmidt G, Welzenbach K, Brinkmann V, Kamata T, Kallen J, Bruns C, Cottens S, Takada Y, Hommel U. Statins selectively inhibit leukocyte function antigen-1 by binding to a novel regulatory integrin site. *Nat Med.* 2001; 7:687–692. [PubMed: 11385505]
48. Weitz-Schmidt G, Welzenbach K, Dawson J, Kallen J. Improved lymphocyte function-associated antigen-1 (LFA-1) inhibition by statin derivatives: molecular basis determined by x-ray analysis and monitoring of LFA-1 conformational changes in vitro and ex vivo. *J Biol Chem.* 2004; 279:46764–46771. [PubMed: 15304496]
49. Yarrow JC, Perlman ZE, Westwood NJ, Mitchison TJ. A high-throughput cell migration assay using scratch wound healing, a comparison of image-based readout methods. *BMC Biotechnol.* 2004; 4:21. [PubMed: 15357872]
50. Lampugnani MG. Cell migration into a wounded area in vitro. *Methods in molecular biology* (Clifton, N J). 1999; 96:177–182.
51. Martin P, Leibovich SJ. Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends in cell biology.* 2005; 15:599–607. [PubMed: 16202600]
52. Lammermann T, Bader BL, Monkley SJ, Worbs T, Wedlich-Soldner R, Hirsch K, Keller M, Forster R, Critchley DR, Fassler R, Sixt M. Rapid leukocyte migration by integrin-independent flowing and squeezing. *Nature.* 2008; 453:51–55. [PubMed: 18451854]
53. Suen PW, Ilic D, Cavegion E, Berton G, Damsky CH, Lowell CA. Impaired integrin-mediated signal transduction, altered cytoskeletal structure and reduced motility in Hck/Fgr deficient macrophages. *Journal of cell science.* 1999; 112(Pt 22):4067–4078. [PubMed: 10547366]
54. Alghisi GC, Ponsonnet L, Ruegg C. The integrin antagonist cilengitide activates alphaVbeta3, disrupts VE-cadherin localization at cell junctions and enhances permeability in endothelial cells. *PLoS One.* 2009; 4:e4449. [PubMed: 19212436]
55. Cox D. Oral GPIIb/IIIa antagonists: what went wrong? *Current pharmaceutical design.* 2004; 10:1587–1596. [PubMed: 15134557]
56. Peter K, Schwarz M, Ylanne J, Kohler B, Moser M, Nordt T, Salbach P, Kubler W, Bode C. Induction of fibrinogen binding and platelet aggregation as a potential intrinsic property of various glycoprotein IIb/IIIa (alphaIIb beta3) inhibitors. *Blood.* 1998; 92:3240–3249. [PubMed: 9787160]
57. Mahalingam B, Ajroud K, Alonso JL, Anand S, Adair BD, Horenstein AL, Malavasi F, Xiong JP, Arnaout MA. Stable Coordination of the Inhibitory Ca²⁺ Ion at the Metal Ion-Dependent Adhesion Site in Integrin CD11b/CD18 by an Antibody-Derived Ligand Aspartate: Implications for Integrin Regulation and Structure-Based Drug Design. *Journal of immunology.* 2011; 187:6393–6401.
58. Zhu J, Negri A, Provasi D, Filizola M, Collier BS, Springer TA. Closed headpiece of integrin alphaIIb beta3 and its complex with an alphaIIb beta3-specific antagonist that does not induce opening. *Blood.* 2010; 116:5050–5059. [PubMed: 20679525]

59. Chew DP, Bhatt DL, Sapp S, Topol EJ. Increased mortality with oral platelet glycoprotein IIb/IIIa antagonists: a meta-analysis of phase III multicenter randomized trials. *Circulation*. 2001; 103:201–206. [PubMed: 11208677]
60. Gao C, Boylan B, Bougie D, Gill JC, Birenbaum J, Newman DK, Aster RH, Newman PJ. Eptifibatide-induced thrombocytopenia and thrombosis in humans require FcγRIIa and the integrin β3 cytoplasmic domain. *The Journal of clinical investigation*. 2009; 119:504–511. [PubMed: 19197137]
61. Giancotti FG, Ruoslahti E. Integrin signaling. *Science*. 1999; 285:1028–1032. [PubMed: 10446041]
62. de Bruyn KM, Rangarajan S, Reedquist KA, Figdor CG, Bos JL. The small GTPase Rap1 is required for Mn(2+)- and antibody-induced LFA-1- and VLA-4-mediated cell adhesion. *J Biol Chem*. 2002; 277:29468–29476. [PubMed: 12171996]
63. Kettritz R, Choi M, Rolle S, Wellner M, Luft FC. Integrins and cytokines activate nuclear transcription factor-κB in human neutrophils. *J Biol Chem*. 2004; 279:2657–2665. [PubMed: 14613935]
64. Rezzonico R, Imbert V, Chicheportiche R, Dayer JM. Ligation of CD11b and CD11c β(2) integrins by antibodies or soluble CD23 induces macrophage inflammatory protein 1α (MIP-1α) and MIP-1β production in primary human monocytes through a pathway dependent on nuclear factor-κB. *Blood*. 2001; 97:2932–2940. [PubMed: 11342414]
65. Walzog B, Weinmann P, Jeblonski F, Scharffetter-Kochanek K, Bommert K, Gaetgens P. A role for β(2) integrins (CD11/CD18) in the regulation of cytokine gene expression of polymorphonuclear neutrophils during the inflammatory response. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 1999; 13:1855–1865.
66. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science*. 2002; 298:1911–1912. [PubMed: 12471242]
67. Yu H, Suchard SJ, Nairn R, Jove R. Dissociation of mitogen-activated protein kinase activation from the oxidative burst in differentiated HL-60 cells and human neutrophils. *J Biol Chem*. 1995; 270:15719–15724. [PubMed: 7797573]
68. Semmrich M, Smith A, Feterowski C, Beer S, Engelhardt B, Busch DH, Bartsch B, Laschinger M, Hogg N, Pfeffer K, Holzmann B. Importance of integrin LFA-1 deactivation for the generation of immune responses. *J Exp Med*. 2005; 201:1987–1998. [PubMed: 15955836]
69. Park EJ, Mora JR, Carman CV, Chen J, Sasaki Y, Cheng G, von Andrian UH, Shimaoka M. Aberrant activation of integrin α4β7 suppresses lymphocyte migration to the gut. *J Clin Invest*. 2007; 117:2526–2538. [PubMed: 17786243]
70. Park EJ, Peixoto A, Imai Y, Goodarzi A, Cheng G, Carman CV, von Andrian UH, Shimaoka M. Distinct roles for LFA-1 affinity regulation during T-cell adhesion, diapedesis, and interstitial migration in lymph nodes. *Blood*. 2010; 115:1572–1581. [PubMed: 20023213]
71. Kim M, Carman CV, Springer TA. Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science*. 2003; 301:1720–1725. [PubMed: 14500982]
72. Stroupe KT, Morrison DA, Hlatky MA, Barnett PG, Cao L, Lyttle C, Hynes DM, Henderson WG. Cost-effectiveness of coronary artery bypass grafts versus percutaneous coronary intervention for revascularization of high-risk patients. *Circulation*. 2006; 114:1251–1257. [PubMed: 16966588]
73. Ajani AE, Yan BP, Clark DJ, Eccleston D, Walton A, Lew R, Meehan A, Brennan A, Reid C, Duffy SJ. Contemporary treatment of in-stent restenosis and the incidence of recurrent in-stent restenosis in the era of drug-eluting stents. *Heart Lung Circ*. 2007; 16:269–273. [PubMed: 17419097]
74. Chen M, John J, Derek D, Lee D, Ellis S, Bhatt D. Bare metal stent restenosis is not a benign clinical entity. *American heart journal*. 2006; 151:1260–1264. [PubMed: 16781233]
75. Kaltoft A, Kelbaek H, Thuesen L, Lassen JF, Clemmensen P, Klovgaard L, Engstrom T, Botker HE, Saunamaki K, Krusell LR, Jorgensen E, Tilsted HH, Christiansen EH, Ravkilde J, Kober L, Kofoed KF, Terkelsen CJ, Helqvist S. Long-term outcome after drug-eluting versus bare-metal stent implantation in patients with ST-segment elevation myocardial infarction: 3-year follow-up

- of the randomized DEDICATION (Drug Elution and Distal Protection in Acute Myocardial Infarction) Trial. *J Am Coll Cardiol*. 2010; 56:641–645. [PubMed: 20688033]
76. Wang Y, Sakuma M, Chen Z, Ustinov V, Shi C, Croce K, Zago AC, Lopez J, Andre P, Plow E, Simon DI. Leukocyte engagement of platelet glycoprotein Iba1 via the integrin Mac-1 is critical for the biological response to vascular injury. *Circulation*. 2005; 112:2993–3000. [PubMed: 16260637]
 77. Rogers C, Edelman ER, Simon DI. A mAb to the beta2-leukocyte integrin Mac-1 (CD11b/CD18) reduces intimal thickening after angioplasty or stent implantation in rabbits. *Proc Natl Acad Sci U S A*. 1998; 95:10134–10139. [PubMed: 9707613]
 78. Wang Y, Mahajan D, Tay YC, Bao S, Spicer T, Kairaitis L, Rangan GK, Harris DC. Partial depletion of macrophages by ED7 reduces renal injury in Adriamycin nephropathy. *Nephrology (Carlton)*. 2005; 10:470–477. [PubMed: 16221097]
 79. Palmen MJ, Dijkstra CD, van der Ende MB, Pena AS, van Rees EP. Anti-CD11b/CD18 antibodies reduce inflammation in acute colitis in rats. *Clin Exp Immunol*. 1995; 101:351–356. [PubMed: 7648720]
 80. Palmen MJ, Dijkstra CD, van der Ende MB, Pena AS, van Rees EP. Anti-CD11b/CD18 antibodies reduce inflammation in acute colitis in rats. *Clin Exp Immunol*. 1995; 101:351–356. [PubMed: 7648720]
 81. Huitinga I, Damoiseaux JG, Dopp EA, Dijkstra CD. Treatment with anti-CR3 antibodies ED7 and ED8 suppresses experimental allergic encephalomyelitis in Lewis rats. *European journal of immunology*. 1993; 23:709–715. [PubMed: 8449218]
 82. Quinn MJ, Plow EF, Topol EJ. Platelet glycoprotein IIb/IIIa inhibitors: recognition of a two-edged sword? *Circulation*. 2002; 106:379–385. [PubMed: 12119257]
 83. Lishko VK, Kudryk B, Yakubenko VP, Yee VC, Ugarova TP. Regulated unmasking of the cryptic binding site for integrin alpha M beta 2 in the gamma C-domain of fibrinogen. *Biochemistry*. 2002; 41:12942–12951. [PubMed: 12390020]
 84. Flick MJ, Du X, Degen JL. Fibrin(ogen)-alpha M beta 2 interactions regulate leukocyte function and innate immunity in vivo. *Exp Biol Med (Maywood)*. 2004; 229:1105–1110. [PubMed: 15564436]
 85. Flick MJ, Du X, Witte DP, Jirouskova M, Soloviev DA, Busuttill SJ, Plow EF, Degen JL. Leukocyte engagement of fibrin(ogen) via the integrin receptor alphaMbeta2/Mac-1 is critical for host inflammatory response in vivo. *J Clin Invest*. 2004; 113:1596–1606. [PubMed: 15173886]
 86. Huitinga I, Damoiseaux JG, Dopp EA, Dijkstra CD. Treatment with anti-CR3 antibodies ED7 and ED8 suppresses experimental allergic encephalomyelitis in Lewis rats. *Eur J Immunol*. 1993; 23:709–715. [PubMed: 8449218]
 87. Ahn GO, Tseng D, Liao CH, Dorie MJ, Czechowicz A, Brown JM. Inhibition of Mac-1 (CD11b/CD18) enhances tumor response to radiation by reducing myeloid cell recruitment. *Proc Natl Acad Sci U S A*. 2010; 107:8363–8368. [PubMed: 20404138]
 88. Lee JO, Bankston LA, Arnaout MA, Liddington RC. Two conformations of the integrin A-domain (I-domain): a pathway for activation? *Structure*. 1995; 3:1333–1340. [PubMed: 8747460]

Highlights

- CD11b/CD18 regulates leukocyte adhesion, migration and inflammatory functions
- Increasing adhesion by activating CD11b/CD18 is a novel therapeutic approach
- We compared two types of CD11b/CD18 agonists using in vitro and in vivo assays
- Small molecules, leukadherins, induced no outside-in signaling Leukadherins were superior to activating mAbs in reducing inflammation

Figure 1.

LA1 and its binding site in the αA -domain **A**. Chemical structure of the agonist LA1. **B**. Ribbon diagram showing a computational model for the binding of LA1 in an activation-sensitive region of the αA -domain. The αA -domain (green ribbon) is shown in its open conformation [88]. A metal ion at the MIDAS site is shown as a red sphere. The docked LA1 (purple CPK model) binds in the activation-sensitive F- $\alpha 7$ region, such that its most hydrophobic moiety interacts with the hydrophobic pocket between helices $\alpha 7$ and $\alpha 1$ and the F-strand.

Figure 2.

Cells expressing CD11b/CD18 show increased adhesion in the presence of various agonists. A histogram showing percentage adhesion of K562 WT cells to immobilized fibrinogen in the presence of physiologic 1 mM Ca^{2+} and 1mM Mg^{2+} ions (Control), the non-selective integrin agonist Mn^{2+} (1mM), or the agonists LA1 (15 μ M), activating mAb KIM127 (1:100 dilution of ascites fluid) and the activating mAb 24 (20 μ g/mL) in the absence or presence of blocking anti-CD11b antibody 44a (1:100 dilution of ascites fluid). Data shown are mean + the standard error of the mean (SEM) (n = 3 to 6 replicates per condition) and are from one of at least three independent experiments. **p<0.001, *** p<0.0001.

Figure 3.

Agonists enhance macrophage cell adhesion. **A.** A histogram showing percentage adhesion of murine RAW 264.7 macrophages to immobilized fibrinogen in the presence of physiologic Ca^{2+} and Mg^{2+} ions (1mM each) (Control), the agonist Mn^{2+} (1mM), the agonist LA1 (15 μM), or the activating mAb M18/2 (20 $\mu\text{g}/\text{mL}$) and in the absence or presence of blocking anti-CD11b antibody M1/70 (20 $\mu\text{g}/\text{mL}$). Data shown are mean + SEM ($n = 3$ to 6 replicates per condition) and are from one of at least three independent experiments. *** $p < 0.0001$. **B.** A histogram showing percentage adhesion of primary rat macrophages to immobilized fibrinogen in the presence of physiologic Ca^{2+} and Mg^{2+} ions (1mM each) (Control), the agonist Mn^{2+} (1mM), the agonist LA1 (15 μM) or the activating mAb ED7 (20 $\mu\text{g}/\text{mL}$) and in the absence or presence of blocking anti-CD11b antibody OX42 (20 $\mu\text{g}/\text{mL}$). Data shown are mean + SEM ($n = 3$ to 6 replicates per condition) and are from one of at least three independent experiments. *** $p < 0.0001$.

Figure 4.

Increasing macrophage adhesion decreases cell migration and *in vitro* wound healing. **A.** A histogram showing quantitation of the wound-healing data presented in **(B)** below. The plot shows percentage of wound area that is healed via migration of RAW 264.7 macrophages into the wound under various conditions 48h after the pipette tip induced injury. ** $p < 0.001$, *** $p < 0.0001$, ns=not significant **B.** RAW 264.7 cells were plated in 24-well tissue culture plates (2×10^6 cells/well) and allowed to adhere in complete media for 12h. The cell monolayers were treated with vehicle (DMSO, agonist LA1 (15 μ M), activating antibody M18/2 (20 μ g/mL) and with LPS (100 ng/mL) or PMA (100 nM) that accelerate cell migration and wound-healing, for 1 h prior to wounding with a pipette tip. Furthermore, cell monolayers in additional wells were treated with LPS (100 ng/mL) or PMA (100 nM) in the presence of LA1 (15 μ M) or M18/2 (20 μ g/mL) to investigate whether increasing CD11b/CD18-dependent cell adhesion will impact the wound-healing process under these conditions. Subsequently, the wounded monolayer cell cultures were incubated for 48h. Images of the cells in culture were obtained immediately prior to (0h) and after the completion of the experiment (48h) using an inverted phase contrast microscope attached to a video camera. Representative images shown here are from one well of a triplicate well experiment from one of at least two to three independent experiments. Dotted lines represent the wound margins at the beginning of the experiment (0h). Black scale bar represents 50 μ m.

Figure 5.

Activating antibodies, but not LA1, induce CD11b/CD18 clustering on live cells. **A.** Confocal microscopy images of CD11b distribution on the surface of K562 WT cells. Cell suspensions were incubated for 45 min at 37°C with DMSO (vehicle), non-selective agonist Mn²⁺ (1 mM), agonist LA1 (15 μM), activating mAb KIM127 (1:100 dilution of ascites fluid), or the activating mAb 24 (20 μg/mL) in the absence (top panel, -Fg) or the presence of the ligand fibrinogen (50 μg/mL) (bottom panel, +Fg) and the cells were fixed with paraformaldehyde (4%) prior to labeling the surface expressed CD11b/CD18 mAb IB4 and imaged. The fluorescence images shown are representative of three independent experiments. White scale bar represents 25 μm. Also shown below each image is a 3D representation of CD11b fluorescence intensity for selected cells, analyzed in

ImageJ. **B.** A histogram showing ImageJ based quantitation of the number of CD11b macro clusters per cell from each of the conditions shown in (**A**) above. Data shown are mean + SEM from >40 cells per condition and from >3 independent experiments for each condition. *** $p < 0.0001$, ns=not significant.

Figure 6.

Activating antibodies induce intracellular MAPK signaling that mimics ligand-bound state, but LA1 does not. Immunoblots of whole cell lysates from K562 WT cells treated in suspension for 45 min at 37°C with either PKC agonist PMA (positive control), DMSO (vehicle), non-selective agonist Mn²⁺ (1 mM), activating mAb KIM127 (1:100 dilution of ascites fluid), activating mAb 24 (20 µg/mL) or the agonist LA1 (15 M) were probed for (A) phosphorylated ERK (pERK; pThr202/pTyr204) and total ERK or (B) phosphorylated JNK (pJNK; pThr183/pTyr185) and total JNK. Immunoblot results from two independent experiments were quantified using ImageJ software and ratio of phosphorylated protein to total protein was determined. Histograms showing this quantitation for each set are also shown. Data shown are mean + SEM. * p<0.05, *** p<0.0001, ns=not significant. C-D. Immunoblots of whole cell lysates from K562 WT cells treated as in (A) and (B) but in the

presence of ligand fibrinogen (50 $\mu\text{g/mL}$). Immunoblot results from two independent experiments were quantified and are also shown, as in (A) and (B).

Figure 7.

Agonist LA1 does not induce global conformational changes in CD11b/CD18. **A.** Histograms showing level of CD11b/CD18 expression on the surface of live K562 WT cells using heterodimer specific antibody IB4 (right) and the isotype IgG2a control mAb (left), as measured using flow cytometry. **B.** FACS analysis on live K562 WT cells showing the reactivity of CD11b/CD18 conformation reporter probe antibodies KIM127 (1:50 dilution of ascites) and mAb 24 (15 μ g/mL) under various conditions. The binding by isotype IgG1 control antibody (15 μ g/mL) is shown in the topmost panels. Cells were incubated with mAbs KIM127 or 24 in the absence of agonists (Control), in the presence of LA1 (15 μ M) or Mn²⁺ ions (1mM). Data shown are representative of at least three independent experiments.

Figure 8.

Agonists LA1 and ED7 similarly reduce the influx of macrophages in injured arteries. **A-C.** Representative FACS analyses plots of single cell suspensions for CD11b⁺ macrophages (based on binding with anti-rat CD11b antibody WT.5) in arteries 7 days after balloon injury from rats treated post-surgery with a vehicle control (**A**), activating anti-CD11b mAb ED7 (3.3 mg/kg/d) (**B**) or LA1 (1mg/kg/d) (**C**). Data shown are representative of at least 4–6 independent measurements. **D.** A bar graph showing quantitation of CD11b⁺ macrophages in the injured arteries from multiple animals (n=4–6/group). Data shown are mean+SEM. *p<0.05, ** p<0.001, ns=not significant.

Figure 9.

Agonist LA1 is better at ameliorating vascular injury as compared to activating anti-CD11b antibody ED7. **A-C.** Representative photomicrographs of arteries 21 days after balloon injury from rats treated post-surgery with a control irrelevant mouse IgG (mIgG1, 4mg/kg/d) (**A**), activating anti-CD11b mAb ED7 (4mg/kg/d) (**B**) or LA1 (1mg/kg/d) (**C**). Arrows point to the neointimal thickening. **D.** A bar graph showing the neointima to media ratio determined by morphometric analysis of the injured arteries from the treated animals (n=6–7/group). Data shown are mean+SEM. *p<0.05, *** p<0.0001, ns=not significant.

Figure 10.

Model of integrin activation and outside-in signaling by various agonists. CD11b/CD18 activation by binding with biological agonists (activating antibodies or ligands) induces large, global conformation changes in the heterodimer such that the changes are relayed to the integrin cytoplasmic tails and induce outside-in signaling in the cells. Activation of the ligand-binding αA -domain by small molecule agonist LA1 induces local conformational changes, without relaying it to distal domains of the heterodimer and, hence, do not generate outside-in signaling mimicking the ligand-bound integrin.