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Differential Regulation of Neutrophil CD18 Integrin Function by Di- and Tri-Valent Cations: Manganese vs. Gadolinium

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Abstract—Affinity regulation of integrin function plays an important role during both leukocyte–endothelial and leukocyte–leukocyte interactions. We compared the roles of Mn^{2+} (Manganese) and Gd^{3+} (Gadolinium) in regulating leukocyte CD18-integrin function. We observed that: (i) Both cations prolonged neutrophil homotypic aggregation following chemoattractant IL-8 stimulation, with Gd^{3+} being effective at doses two orders of magnitude (10 μM range) lower that Mn^{2+}. (ii) While both Gd^{3+} and Mn^{2+} mediate homotypic cell aggregation via L-selectin and CD18 integrins, their effects on the integrin subunits, LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), was different. Gd^{3+} altered both LFA-1 and Mac-1 function, while the dominant effect of Mn^{2+} was on Mac-1. This effect of Gd^{3+} on LFA-1 function was confirmed in cell-free studies that measured the binding of recombinant ICAM-1 to LFA-1 immobilized on beads. (iii) Both ions augmented the binding of 327C, an antibody that recognizes active CD18 on human neutrophils, both in the presence and absence of exogenous IL-8. The effects of Mn^{2+} was more pronounced since it caused 3–4-fold increase in mAb 327C binding to neutrophils compared to Gd^{3+} which increased antibody binding by only ~80%. 327C binding was partially reduced by Ca^{2+}. Further, 327C binding induced by Mn^{2+} did not correlate tightly with cell adhesion function. (iv) In studies that monitored intracellular Ca^{2+} ($[Ca^{2+}]_i$), the addition of Mn^{2+} but not Gd^{3+} to neutrophils altered $[Ca^{2+}]_i$ levels. Overall, while both Gd^{3+} and Mn^{2+} stabilize high affinity CD18 mediated cell adhesion, Gd^{3+} affects integrin conformation while Mn^{2+} may also trigger other effects.

Keywords—LFA-1, Mac-1, Inflammation, Leukocyte, 327C, CBRM1/5, Endothelium, Cell aggregation.

INTRODUCTION

The molecular requirements for neutrophil homotypic aggregation and neutrophil–endothelial cell interaction are similar since adhesion molecules belonging to the selectin and integrin families mediate both processes.[22,38] In both cases, binding interactions between selectins and their glycoprotein ligands initiate transient cell–cell recognition while more stable, firm arrest is mediated by two members of the CD18/β_2 integrin family: LFA-1 (CD11a/CD18 or $\alpha_L\beta_2$) and Mac-1 (CD11b/CD18 or $\alpha_M\beta_2$). Both intercellular adhesion molecule-1 (ICAM-1) dependent and independent pathways regulate neutrophil adhesion. While ICAM-1 is a dominant LFA-1 ligand on endothelial cells, ICAM-3 is an important ligand for LFA-1 during neutrophil–neutrophil adhesion.[24,26] The ligands for Mac-1 are numerous and they include ICAM-1, factor X, heparan sulfate, fibrinogen, elastase, E-selectin, ICAM-2, GpIb, iC3b, JAM-C, LDL-receptor-related proteins, Thy-1 (CD90), vitronectin, matrix metalloproteinase MMP9 and denatured serum proteins. Evidence in literature further suggests that both protein–protein and protein–carbohydrate interactions may condition integrin mediated binding during neutrophil–neutrophil and neutrophil–endothelial cell adhesion.

The importance of divalent cations in regulating integrin function is well-established.[15,30] Both LFA-1 and Mac-1 have an insert (I)-domain in their α-chain which recognizes their ligands (Fig. 1). The binding of divalent cation magnesium (Mg^{2+}) to the Metal Ion Dependent Adhesion Site (MIDAS) in the I-domain is necessary for the function of this family of heterodimeric proteins. Alternatively, manganese (Mn^{2+}) may also bind the MIDAS site and augment ligand recognition.[2,6,8,14]

In order to further examine the role of cations in regulating integrin function, in this paper, we contrast the roles of Mn^{2+} and gadolinium (Gd^{3+}) in mediating neutrophil homotypic aggregation and LFA-1 ICAM-1 binding interactions. Neither reagent is found in substantial quantities in human blood though these are common reagents used in a variety of biological experiments. Gadolinium (Gd^{3+}) is a member of the lanthanide family with an ionic radius of 0.94 Å, which

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is comparable to the radius of Ca^{2+} (0.99 Å). In studies of homotypic neutrophil aggregation under defined fluid flow,[39] we observed that both Mn^{2+} and Gd^{3+} mediate irreversible neutrophil aggregation, though Gd^{3+} is effective at 50-fold lower doses. While Mn^{2+} played a dominant role by increasing Mac-1 function in the cell adhesion studies, Gd^{3+} affected both Mac-1 and LFA-1 function. Assays performed in a cell-free system confirm that Gd^{3+} increases the binding interaction between LFA-1 and ICAM-1. Activation dependent antibodies directed against CD11b and CD18 were applied to detect activation-specific conformations. This includes mAb CBRM1/5, which identifies conformation change in the ligand binding I-domain of CD11b. This reagent is reported to recognize a fraction of Mac-1 on activated neutrophils that participate in cell adhesion to fibrinogen and ICAM-1.[5] A second antibody used called 327C is directed against the CD18 integrin. With regard to this reagent, integrins are thought to fluctuate between a bent, low-affinity conformation and extended, high-affinity state.[37] Activation-specific antibodies recognize new sites that are exposed in the extended but not the bent conformation. While most activation-specific anti-CD18 mAbs bind the C-terminal region of this protein in the integrin EGF-like domain between amino acids 435–600,[37] 327C has been shown to map to the upstream C-terminal region between amino acids 23 and 411 of the CD18 integrin[3] (Fig. 1). This latter region contains the CD18 I-like domain and critical intra-chain disulfide bonds. Using these activation specific reagents, we show that both Mn^{2+} and Gd^{3+} promote the extended conformation of integrins compared to the bent structure, with Mn^{2+} causing 5–8-fold increase in 327C binding. Finally, studies performed to measure transmembrane signaling by detection of intra-cellular calcium levels suggest that, Mn^{2+} alters the intracellular Ca^{2+} levels of neutrophils in addition to promoting ligand recognition. Gd^{3+} has no such effect. Thus Gd^{3+} may prove to be a useful reagent for studies of integrin function in cell based assays.

MATERIAL AND METHODS

Reagents

Monoclonal antibodies (mAbs) used in this study were from mouse unless otherwise stated. These include anti-LFA-1 mAbs 38 (IgG_{2a}, Ancell, Bayport, MN), TS1/22 (IgG_1, Pierce Biotechnology, Rockford, IL), TS2/4 (IgG_1, Pierce), and humanized MHM24 (Raptiva, IgG_1, Genentech, San Francisco, CA); anti-Mac-1 mAbs 44 (IgG_{2a}, ATCC, Manassas, VA) and Phycoerythrin (PE) conjugated D12 (IgG_{2a}, BD Biosciences, San Jose, CA); anti-CD18/β_2-integrin mAbs R15.7 (IgG1, gift from Robert Rothlein, Boeringer Ingelheim, CN), IB4 (IgG_{2a}, ATCC), and TS 1/18 (IgG_1, Pierce); anti-CD54/ICAM-1 mAb R6.5 (IgG2a, Robert Rothlein) and FITC labeled anti-CD62L DREG-56 (IgG_1, ebiosciences, San Diego, CA). A mAb that recognized activated CD11b antigen (Clone CBRM1/5, IgG_1, eBioscience) was also used. Of the above, all antibodies used for blocking studies (mAbs 44, MHM24, and IB4) were dialyzed against Ca^{2+} free HEPES buffer (110 mM NaCl, 10 mM KCl, 10 mM glucose, 2mM $MgCl_2$, and 30 mM HEPES, pH 7.4) overnight at 4 °C before use. Recombinant LFA-1 and ICAM-1 were gifts from ICOS (Bothell, WA). LFA-1 was provided as a heterodimer with an inserted leucine zipper, and ICAM-1/Ig was produced as a dimeric, chimeric human IgG containing two full-length ICAM-1 units (molecular mass was 150 kDa as confirmed by native PAGE).[18,33] mAbs against the leucine zipper (Clone 324C), CD18 activation epitope (clone 327C), and LFA-1 I domain small molecule allosteric inhibitor IC487475 of the *p*-arylthio cinnamides series were also from ICOS.[3,33] Lovastatin sodium, which

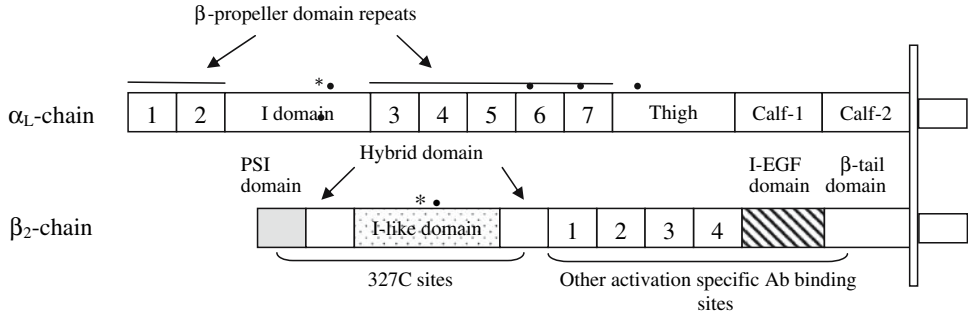

FIGURE 1. Primary structure of LFA-1 ($\alpha_L\beta_2$ integrin). Schematic shows various domains located within the hetrodimeric protein. * Denotes metal binding sites. This site on the α-chain is termed MIDAS (Metal-Ion Dependent Adhesion Site). This MIDAS forms part of the ligand binding epitope when LFA-1 binds ICAM-1. • Denotes putative Ca binding sites on integrins. CBRM1/5 binds the I-domain of α_M integrins (Mac-1). Common activation dependent antibody binding sites are located near the C-terminus of the β_2-integrins (resides 435–600). 327 binds amino acids between 23 and 411. (Figure adapted from[37] with permission from publisher).

targets the I-domain allosteric site (IDAS) and abrogates LFA-1 recognition of ICAM-1, was purchased from Calbiochem (San Diego, CA). Neutrophil stimulus Platelet Activating Factor (PAF) and N-Formyl-Met-Leu (fMLP) were purchased from Sigma and recombinant human chemokine IL-8 was from R&D systems (Minneapolis, MN). Cell permeable calcium binding dye Fluo-4-AM and Alexa Fluor® 488 $F(ab')_2$ fragments of goat anti-mouse IgG (H + L) were purchased from Molecular Probes-Invitrogen (Eugene, OR). Pluronic F-127 was from BASF (Mount Olive, NJ). Anhydrous gadolinium chloride ($GdCl_3$) powder and manganese chloride ($MnCl_2$) flake were purchased from Sigma. $GdCl_3$ was stored in anhydrous state in inert atmosphere prior to experimentation. In all runs, unless otherwise mentioned, experimental buffer was 30 mM HEPES with 0.1% human serum albumin (HSA). Gadolinum was dissolved in this buffer before use in this project. The review by Adding *et al.*[1] presents a detailed discussion on the use of gadolinium salts and chelates, including the reagent's *in vivo* pharmacology and toxicology properties. When required, FITC conjugation of mAbs was performed as described elsewhere.[41] Kits from Invitrogen were used for Alexa 488 conjugation of mAbs.

Neutrophil Homotypic Aggregation

Neutrophil homotypic aggregation experiments were performed using a cone and plate viscometer from Haake Inc. (Paramus, NJ) at 37 °C using protocols described previously.[39] Briefly, human neutrophils isolated from fresh human blood were stored in Ca^{2+} free HEPES buffer at 4 °C prior to the experiments. Just prior to each run, 200 μL neutrophil suspension at 2×10^6 cell/mL were stained with 10 ng/mL nuclear dye acridine orange either in the presence or absence of blocking antibodies (mAbs 44, MHM24, or IB4) at 15 μg/mL for 10 min on ice. Samples were then warmed to room temperature for 3 min 1.5 mM Ca^{2+} was then added to cell along with either Gd^{3+} (typically at 20 μM) or Mn^{2+} (at 1 mM). Cells were then incubated for an additional 2 min at 37 °C prior to being shear mixed in the viscometer. The time of shear initiation was termed $t = 0$ s. At fixed time points thereafter, 20 μL aliquots of the cell suspension were taken and immediately fixed in 100 μL of cold buffer containing 1% paraformaldehyde and 10 ng/mL acridine orange dye. Following overnight fixation, these samples were analyzed using a flow cytometer (FACSCalibur, Becton Dickinson). The extent of homotypic aggregation was expressed as the fraction of singlets recruited into larger aggregates according to: % Aggregation $= 100 \times (1 - S/(S + 2D + 3Tr + 4Q + 5P + 6Sx))$, where S = single neutrophils, D = doublets, Tr = triplets, Q = quadruplets, P = pentuplets, and Sx = sextuplets and larger unresolved aggregates.

LFA-1 Immobilization on Microspheres

LFA-1 was immobilized on 6 μm diameter amino microspheres (GE Biosciences, Piscataway, NJ) using cross-linker sulfosuccinimidyl maleimide-*N*-hydroxy-succinimide ester (SMCC, Pierce) as previously described.[33] Briefly, a sulfhydryl group was added to anti-leucine zipper 324C by incubating 324C with 0.125 mM SMCC according to manufacturer's instructions. Following this, 324C-SH was mixed with the amino cross-linker beads for 2 h at 8 °C, washed and then stored at 1.5×10^8 beads/mL at 4 °C. Directly prior to experimentation, LFA-1 (20 μg/mL) was mixed with 2×10^6 beads in 75 μL volume at 37 °C for 30 min. The bivalent binding structure of the antibody (324C) allows for clustering of the LFA-1 into a dimeric configuration on the bead surface. Thus, a cell-free system was created for specific detection of bivalent ICAM-1.

Cell-Free Assay for Measurement of ICAM-1 LFA-1 Interactions

Beads with immobilized LFA-1 prepared above were washed and resuspended in 75 μL HEPES buffer. HSA, $CaCl_2$, and $MgCl_2$ was absent in this buffer. Following equilibration at 37 °C for 30 min in the presence of uniform mixing using Eppendorf Thermomixer R (Brinkmann Instruments), either 1.5 μM $GdCl_3$ or 3 mM $MgCl_2$ were added to beads along with Alexa488 conjugated dimeric ICAM-1 at 20 μg/mL for an additional 20 min. Flow cytometry measurement of ICAM-1 fluorescence associated with beads was then performed. Mean fluorescent intensity values are reported. In runs were inhibitors or mAbs were added, these reagents were added at 37 °C, 10 min prior to addition of cations.

Antibody Binding to Neutrophils

L-selectin, LFA-1, Mac-1, CD11b active epitope, and CD18 I-like domain active epitope expressed on the neutrophil surface were detected using fluorescently conjugated mAbs FITC-DREG56, FITC-MHM24, PE-D12, FITC-CBRM1/5, and Alexa 488-327C respectively using flow cytometry. Neutrophil samples were prepared using protocols that are identical to that used in the cell adhesion assays described above. Unless otherwise mentioned, the protocol for these runs involved incubating 5 μL samples obtained at fixed time points with fluorescent mAbs for exactly 1 min at 37 °C. These samples were then diluted with

20-fold excess HEPES buffer and read immediately using the flow cytometry. Events were acquired for 30 s. Data are presented as mean fluorescence intensity (MFI) of individual sample divided by MFI of control cells at $t = 0$ (without Mn^{2+} or Gd^{3+}).

Intracellular Calcium

Measurement of intracellular calcium ($[Ca^{2+}]_i$) was carried out using the fluorescent reagent Fluo-4-AM. Here, neutrophils at 5×10^6 cell/mL were loaded with the indicator by incubating cells with 1 μM Fluo-4 AM and 0.02% F-127 for 30 min at room temperature in dark. Cells were then diluted in HEPES buffer with 1.5 mM Ca^{2+} to 0.05×10^6 cell/mL, and incubated at room temperature for an additional 3 min. Flow cytometry data collection was then initiated by monitoring intracellular Fluo-4 signal. At 2 min, in some runs, 20 μM Gd^{3+} or 1 mM Mn^{2+} was added to the sample. HEPES buffer with 1.5 mM Ca^{2+} was used as mock stimulus in control runs. 5 nM IL-8 was added at 4 min in some runs. Sample MFI values which are a measure of intracellular calcium levels ($[Ca^{2+}]_i$) are reported as a function of time.

Statistical Analysis

Data are expressed as Mean ± SEM. To test statistical significance of data, paired Student's *t*-tests were performed in case of dual-comparisons, while ANOVA analysis followed by the Student Newmann Keuls test was performed for multiple comparisons. $p < 0.05$ was considered significant.

RESULTS

Irreversible Homotypic Neutrophil Aggregation in the Presence of Selected Cations

Homotypic neutrophil aggregation experiments were performed using a cone-plate viscometer at a constant shear rate of 650/s. The buffer for all runs contained 1.5 mM Ca^{2+} and 2 mM Mg^{2+}, while additional cations were added as noted below. Here, we observed that while shearing neutrophils in the cone-plate viscometer in the absence of stimulus did not result in cell aggregation, addition of 5 nM IL-8 caused reversible homotypic neutrophil aggregation (Fig. 2). Cells aggregated in the first minute and subsequently dissociated. Addition of 20 μM Gd^{3+} (panel A) or 1 mM Mn^{2+} (panel B) in the presence of IL-8 stimulation resulted in irreversible neutrophil aggregation. These cations alone in the absence of IL-8 did not mediate cell aggregation. The extent of neutrophil aggregate sustenance was dependent on the concentration of Gd^{3+} (panel C) and Mn^{2+} (panel D). Gd^{3+} mediated some irreversible aggregation at concentration >10 μM while Mn^{2+} was only effective above 0.5 mM. Based on these data, all subsequent runs with cations were performed with either 20 μM Gd^{3+} or 1 mM Mn^{2+} unless otherwise mentioned.

With the goal of determining if the irreversibility observed upon addition of cations was stimulus specific, additional studies were conducted with fMLP and PAF (Fig. 3). These studies were conducted at a higher shear rate (1785/s) than in Fig. 2, since stimulation of neutrophils with fMLP and PAF results in aggregates that are more stable at higher shear rates compared to IL-8 (data not shown). Here, we observed that with fMLP or PAF stimulation the reversibility of neutrophil homotypic aggregation was reduced to varying degrees upon addition of 20 μM Gd^{3+}. Experiments conducted at high shear rates also revealed that Gd^{3+} reduced the rates of aggregate formation in the first 60–120 s. Overall, while the cations tested themselves did not facilitate neutrophil aggregation in the absence of stimulus, they acted in synergy with chemotactic stimulus to both sustain homotypic neutrophil aggregation at later times ($t > 120$ s) and slow down the rate of initial aggregation at $t < 60$ s. Most significant was the finding that Gd^{3+} was effective at a ~50-fold lower concentration compared to Mn^{2+}.

Gd^{3+} and Mn^{2+} Differentially Regulate the Function of CD18-Integrin Subunits, LFA-1 and Mac-1

Antibody blocking experiments against different integrin subunits was performed in order to determine the role of the CD18 subunits, LFA-1 and Mac-1, in Mn^{2+} and Gd^{3+} induced neutrophil aggregate sustenance (Fig. 4). At 650/s, Mac-1 was the dominant integrin in the absence of either Mn^{2+} or Gd^{3+} with LFA-1 playing a supporting role (panel A). Blocking both receptors simultaneously abrogated neutrophil aggregation. This was observed upon using either an anti-CD18 blocking mAb (Fig. 4a) or upon addition of both anti-LFA-1 and anti-Mac-1 blocking mAbs together.[39] Consistent with our earlier reports,[24,26] anti-Mac-1 was also more effective than anti-LFA-1 at preventing the formation of homotypic aggregates at $t > 4$ min. This suggests that Mac-1 is the dominant receptor contributing to sustenance of neutrophil aggregates.

In experiments performed in the presence of Gd^{3+} (panel B) or Mn^{2+} (panel C), function blocking mAb against CD18 completely blocked cell aggregation, confirming that these ions together regulate CD18 function. Anti-Mac-1 was more effective at blocking prolonged aggregation induced by Mn^{2+}, and this indicates a dominant role of Mn^{2+} in sustaining Mac-1

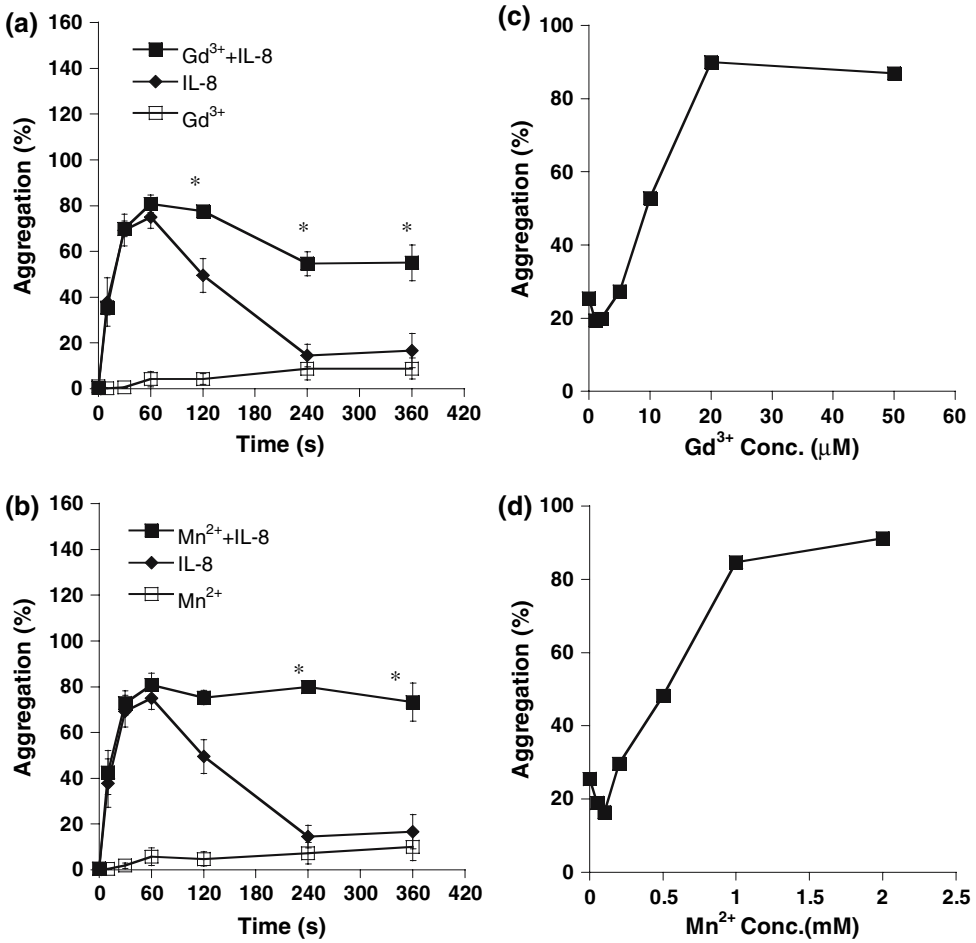

FIGURE 2. Kinetics of Gd^{3+} or Mn^{2+} regulated neutrophil homotypic aggregation. Neutrophils (2×10^6 cells/mL) were incubated with either (a) 20 μM Gd^{3+} or (b) 1 mM Mn^{2+} for 2 min at 37 °C before stimulation with 5 nM IL-8 (in some runs) and simultaneous mixing in a cone-plate viscometer at a shear rate of 650/s. Neutrophil homotypic aggregation kinetics was measured at different time points after addition of IL-8. Neither Gd^{3+} nor Mn^{2+} alone induced significant cell aggregation. However, in runs where IL-8 was added as stimulus, cell aggregation was prolonged irreversibly upon addition of Gd^{3+} or Mn^{2+}. Panel C and D show the percentage of neutrophil homotypic aggregation at 6 min after stimulation in the presence of varying dose of Gd^{3+} (panel C) or Mn^{2+} (panel D). 20 μM Gd^{3+} or 1 mM Mn^{2+} was sufficient to sustain the cell aggregates. Error bars represent SEM from 4 independent experiments. *$p < 0.05$ with respect to neutrophil aggregation run without Gd^{3+} or Mn^{2+}, but with IL-8.

function. In the presence of Gd^{3+}, however, anti-Mac-1 alone was not fully effective at abrogating cell adhesion at 4 min and this suggests that there are differences in the manner by which Gd^{3+} and Mn^{2+} regulate CD18 integrin function. One possibility is that Gd^{3+} may enhance adhesion via LFA-1 in addition to Mac-1.

Cell Free Assay Demonstrates that Gd^{3+} Promotes LFA-1 Binding to ICAM-1

To confirm that Gd^{3+} affects LFA-1 function, we quantified the binding of dimeric ICAM-1 to recombinant LFA-1 that was immobilized on polystyrene microspheres, in the presences of this cation (Fig. 5). Mg^{2+} was used as a positive control since we have previously shown that this cation promotes LFA-1 ICAM-1 binding at dose >3 mM.[33] 1.5 mM Ca^{2+} alone increases ICAM-1 binding to LFA-1 by only 20% in this cell-free system.[33] In the presence of Gd^{3+} LFA-1 bound dimeric ICAM-1/Ig at ~3-fold higher levels compared to the absence of cations (no stimulus control). In these studies, 1.5 μM Gd^{3+} and 3 mM Mg^{2+} were the lowest concentrations required to elicit significant LFA-1 activation and ICAM-1 binding (data not shown[33]). The binding in these studies is specific since, ICAM-1 binding was inhibited allosterically by stabilizing a lower affinity state in the presence of small molecules IC487475 and lovastatin. The specificity of the interaction was also confirmed? by effectively competing binding to near background levels in the presence of anti-LFA-1 mAb TS1/22, anti-ICAM-1 mAb R6.5, and allosteric anti-CD18 mAbs TS1/18 and R15.7. Taken together, these

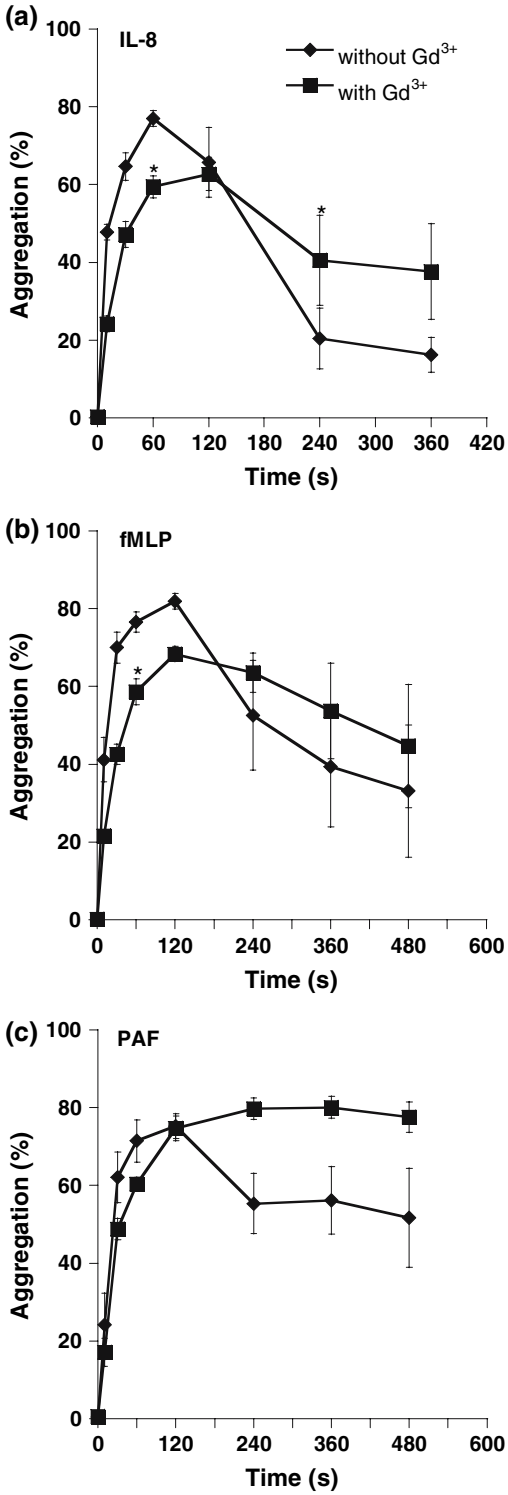

FIGURE 3. Effect of Gd^{3+} on chemoattractant induced neutrophil homotypic aggregation. Neutrophils (1×10^6 cells/mL) were incubated with 20 μM Gd^{3+} for 2 min at 37 °C before stimulation with (a) 5 nM IL-8, (b) 10 nM fMLP or (c) 5 nM PAF. Viscometer shear rate was 1785/s. Gd^{3+} not only sustained cell aggregation at later times it also decreased the initial rate of cell aggregation at $t < 60$ s in all three panels. Error bars represent SEM from 4 to 5 independent experiments. *$p < 0.05$ with respect to neutrophil aggregation run that lacked Gd^{3+}.

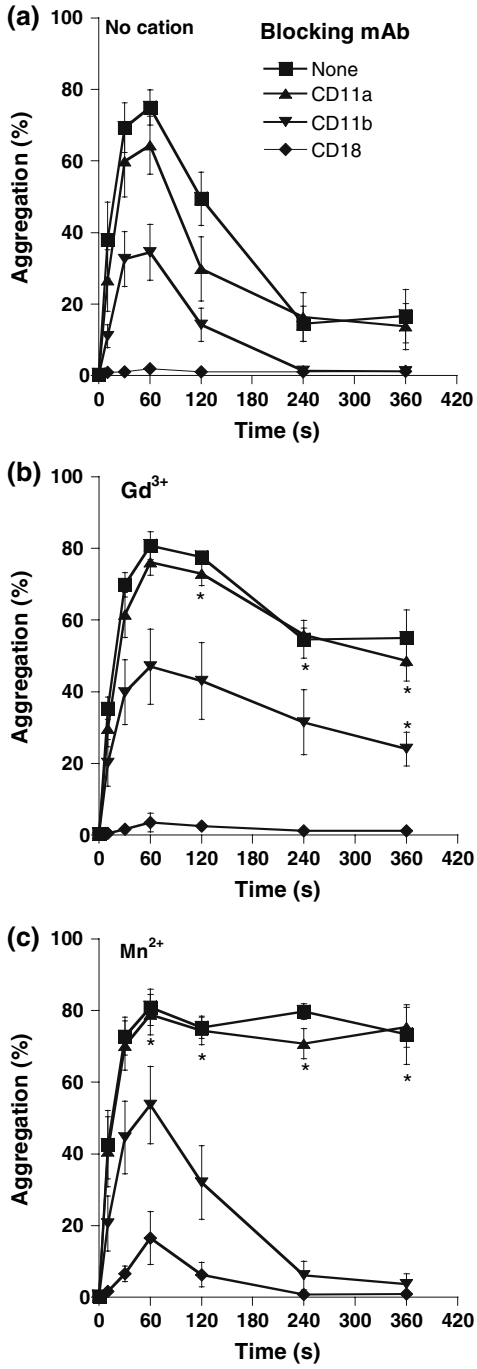

FIGURE 4. Role of integrin subunits in neutrophil homotypic aggregation. (a) Neutrophils (2×10^6 cells/mL) were stimulated with 5 nM IL-8. Fluid shear at 650/s was applied immediately after stimulation either in the absence of any antibody (None), or in the presence of 15 μg/mL function blocking antibody MHM24 (anti-LFA-1/CD11a), Mab44 (anti-Mac-1/CD11b), or IB4 (anti-CD18/β_2-integrin). Cell aggregation kinetics was measured. In some runs, neutrophils were incubated with (b) 20 μM Gd^{3+} or (c) 1 mM Mn^{2+} for 2 min at 37 °C before IL-8 stimulation using the protocol identical to that in panel A. Error bars represent SEM from 4 independent experiments. *$p < 0.05$ with respect to neutrophil aggregation with same antibody treatment but without Gd^{3+} or Mn^{2+} (i.e., data in panel A).

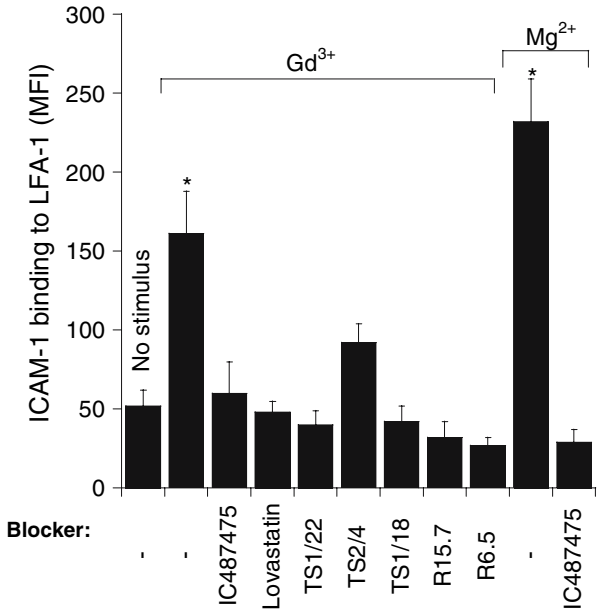

FIGURE 5. Gd^{3+} and Mg^{2+} alter LFA-1 ICAM-1 binding affinity. Alexa488 conjugated dimeric ICAM-1/Ig binding to LFA-1 was quantified in a cell-free assay. In some runs, various blocking and non-blocking reagents were added 10 min prior to addition of either 1.5 μM Gd^{3+} or 3 mM Mg^{2+} [Ca^{2+} was absent]. These reagents include small molecule inhibitors directed against LFA-1 I-domain (1 μM IC487475) and LFA-1 IDAS domain (100 μM lovastatin), anti-LFA-1 blocking mAb TS1/22 (20 μg/mL) and non-blocking mAb TS2/4 (20 μg/mL), anti-CD18 blocking mAbs TS1/18 (10 μg/mL) and R15.7 (40 μg/mL), and anti-ICAM-1 blocking mAb R6.5 (20 μg/mL). *$p < 0.01$ for ICAM-1 binding to beads in the presence of 1.5 μM Gd^{3+} and 3 mM Mg^{2+} compared to all conditions except TS2/4. Data are mean ± SEM, $n \geq 3$.

cell-free experiments demonstrate that Gd^{3+} can directly augment LFA-1 ICAM-1 binding interactions by upregulating integrin affinity.

Effect of Mn^{2+} and Gd^{3+} on Adhesion Molecule Expression, Activation, and Topography

Upon neutrophil activation, L-selectin is proteolytically shed to low levels, whereas Mac-1 expression is upregulated by 2–20-fold, and LFA-1 levels do not change.[39] Cell stimulation also results in increased binding of an activation specific antibody, CBRM1/5, to a small sub-population of Mac-1 that is competent in mediating cell adhesion.[5] We undertook flow cytometry studies to quantify the role of Gd^{3+} and Mn^{2+} in regulating selectin and integrin expression and integrin activation (Fig. 6). Directly conjugated antibodies against LFA-1 (FITC-MHM24, panel A), Mac-1 (PE-D12, panel B), active Mac-1 (FITC-CBRM1/5, panel C), and L-selectin (FITC-DREG56, panel D) were applied to isolated human neutrophils either in the presence or absence of 5 nM IL-8 stimulus. We observed that neither 20 μM Gd^{3+} nor 1 mM Mn^{2+} alone altered the levels of L-selectin, LFA-1, Mac-1, or Mac-1 active epitope in the absence of exogenous stimulus. Upon IL-8 addition, these cations also did not alter the response of neutrophils in terms of LFA-1 expression, Mac-1 upregulation or induction of the activation epitope (Figs. 6a–6c). While 20 μM Gd^{3+} significantly increased the rate of L-selectin shedding after stimulation, 1 mM Mn^{2+} had no such effect (Fig. 6d). The patterns of receptor expression change observed in Figs. 6a and 6b were qualitatively confirmed using fluorescence microscopy in conjunction with mAbs against LFA-1 and Mac-1 (data not shown). In these studies we did not observe changes in the topography of receptors between samples with these cations, with respect to mock controls performed in the absence of Gd^{3+}/Mn^{2+}. Thus, the di- (Mn^{2+}) and tri- (Gd^{3+}) valent ions do not induce receptor clustering which may affect both the avidity of adhesive interactions mediated by CD18 and the rates of cell adhesion.

CD18 Activation Epitope is Regulated by Mn^{2+} and Gd^{3+}

The above findings suggest a role of Mn^{2+} and Gd^{3+} in regulating integrin affinity. With the goal of further examining this possibility we monitored CD18 integrin function using mAb 327C, which reports on the active conformation of CD18 I-like domain.[3,18] This reagent binds to the amino-terminal portion of CD18 between amino acid residues 23–411.[3] Two different protocols were used to label active CD18 (Fig. 7). In the first method, neutrophils were stimulated with IL-8 and samples withdrawn at fixed time points were incubated with Alexa-488 conjugated 327C for exactly 1 min prior to cytometry analysis. These data indicate that 327C binding doubles within 2 min of activation after which there is a gradual decrease, albeit not to the base levels prior to cell stimulation. Similar observations as this are observed using fluorescence microscopy.[17] The 1 min incubation prior to cytometry analysis was sufficient to report on ~80–90% of active CD18 since doubling the concentration of 327C (to 20 μg/mL) only resulted in a 10% increase in antibody binding at any given time. In the second method, 327C was present through the course of the experiment. Here, a monotonic increase in 327C binding was observed with time and this suggests that the binding of this mAb to CD18 was irreversible. Taken together, the results indicate that the epitope recognized by 327C appears reversibly after IL-8 stimulation and that this mAb once bound to CD18 may effectively stabilize the high affinity conformation.

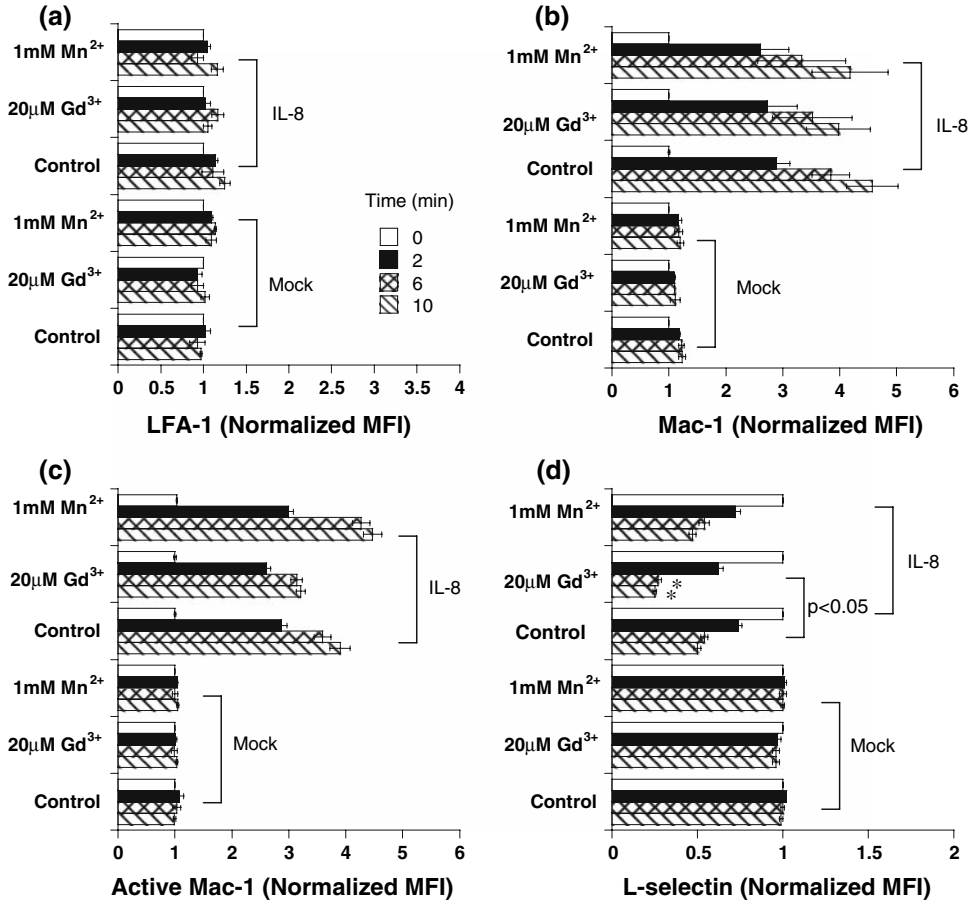

FIGURE 6. Effect of cations on expression of neutrophil cell-surface adhesion molecules. 2×10^6 neutrophils/mL were stimulated with 5 nM IL-8 either in the presence or absence of 20 μM Gd^{3+} or 1 mM Mn^{2+} using protocols identical to that in the cell adhesion studies. While many runs were performed with IL-8 stimulation, control runs with mock stimulus were also applied to confirm that cations themselves do not alter cell physiology. Samples at fixed time points were labeled for expression of: (a) LFA-1, (b) Mac-1, (c) Mac-1 active epitope measured using CBRM1/5, and (d) L-selectin. Neither Gd^{3+} nor Mn^{2+} altered integrin expression, neutrophil degranulation rates, or the rate of expression of Mac-1 activation epitope following stimulation. Slight but significant decrease in the rate of L-selectin shedding following IL-8 stimulation was observed in the presence of Gd^{3+}. Error bars represent SEM from 2–3 independent experiments. *$p < 0.05$ with respect control run without cation.

Due to the latter effect of 327C in regulating CD18 conformation, the first method was applied in all subsequent assays. Thus, our experiments focus on measuring leukocyte response at a given time while excluding features associated with the time dependent binding kinetics of 327C.

Experiments were next performed to determine the role of cations in regulating 327C binding to CD18, in the absence (Figs. 8a and 8b) and presence of IL-8 (Figs. 8c and 8d). Some runs were performed with HEPES buffer lacking the cations Ca^{2+} and Mg^{2+} unless otherwise mentioned (Figs. 8a and 8c). Here, each di/trivalent ion was added one at a time at the indicated concentration and their affect on 327C binding was measured. In other cases, the HEPES buffer in all runs contained 1.5 mM Ca^{2+} and 2 mM Mg^{2+} (Figs. 8b and 8d). Here, the effect Gd^{3+} and Mn^{2+} was examined under conditions identical to that in previous adhesion studies.

When HEPES buffer lacking divalent ions was used, we observed that Gd^{3+} and Mn^{2+} alone, even in the absence of IL-8, augmented 327C binding to CD18 (Fig. 8a). 1.5 mM Ca^{2+} decreased mAb binding by 20% while 1.0 mM Mg^{2+} had no significant effect. Increased mAb binding was observed even at low Mn^{2+} concentrations (0.2 mM) that were insufficient to sustain neutrophil aggregation irreversibly (see inset to Fig. 2b). Further, the level of 327C binding was markedly higher in the case of Mn^{2+} treatment compared to all other cations. In studies conducted in the presence of IL-8 (Fig. 8c), Mn^{2+} and Gd^{3+} were observed to cause significantly higher levels of 327C binding at later time points following IL-8 stimulation, compared to runs with Ca^{2+} and Mg^{2+} alone. Overall,

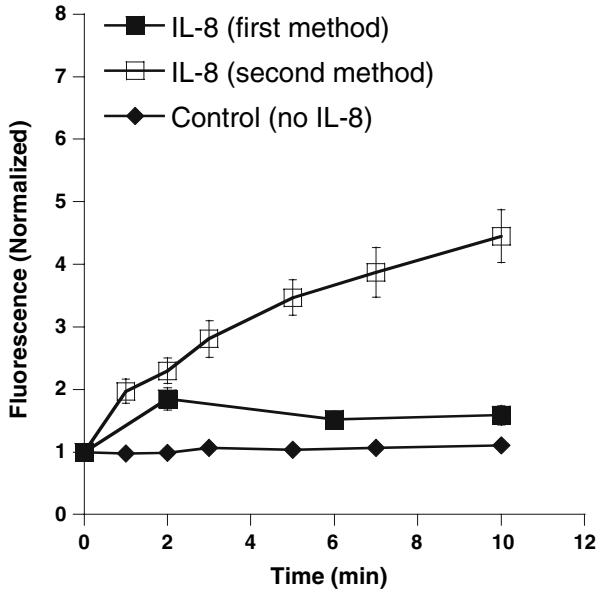

FIGURE 7. Flow cytometry detection of neutrophil CD18 activation using 327C. Neutrophil binding of Alexa 488 labeled mAb 327C was monitored using two methods. In the first method, neutrophils (1×10^6 cells/mL) were incubated in HEPES buffer at room temperature for 5 min, 1.5 mM Ca^{2+} was then added and the sample was placed at 37 °C for 2 min. 5 nM IL-8 was stimulus in this run and samples withdrawn at specific time points were incubated with 10 μg/mL 327C-Alexa-488 at 37 °C for exactly 1 min prior to cytometry analysis. Samples in the second method were treated identically to the first, except that 10 μg/mL 327C-Alexa-488 was present in the sample during the duration of the run. In this case, additional 327C was not added after sample withdrawal. Control runs (without IL-8) were performed using the first method. 327C geometric mean fluorescence intensity at each time was normalized with respect to unstimulated cell levels at $t = 0$.

since 327C binding was upregulated even in the absence of IL-8, which is necessary for cell adhesion, the results suggest that 327C binding may not strictly correlate with cell adhesion function. The observation that Gd^{3+} augments 327C binding upon IL-8 addition suggests that this trivalent ion may stabilize the extended conformation of CD18.

Studies performed in the presence of Ca^{2+} and Mg^{2+} (Figs. 8b and 8d) were similar to corresponding data in the absence of these ions (Figs. 8a and 8c). Here also, Mn^{2+} and Gd^{3+} augmented 327C binding both in the absence of IL-8 and upon IL-8 stimulation. In comparison to studies conducted with Ca^{2+}/Mg^{2+}-free HEPES buffer, the presence of these divalent ions decreased the binding of 327C by ~50% upon addition of Mn^{2+} and Gd^{3+}. This result taken together with the observation that Ca^{2+} alone reduced 327C binding suggests that Ca^{2+} may competes with Mn^{2+} and Gd^{3+} either directly or allosterically to regulate CD18-integrin structure.

Mn^{2+}, But Not Gd^{3+}, Alters Neutrophil Intracellular Ca^{2+} Levels

Differences in the action of Mn^{2+} vs. Gd^{3+} on LFA-1 and Mac-1 function, the observation that low Mn^{2+} (0.2 mM) augments 327C binding, and the exaggerated response of 327C to Mn^{2+} compared to either IL-8 or Gd^{3+}, suggests that Mn^{2+} and Gd^{3+} may exert there effects via distinct mechanisms. To examine this possibility in greater detail, studies were performed with Fluo-4 to monitor intracellular calcium levels ($[Ca^{2+}]_i$) in response to the cations (Fig. 9). With regard to these studies, we observed that the fluorescence of Fluo-4 in modified both in the presence of Mn^{2+} and Gd^{3+} in cell-free experiments, at dose down to 1 μM (i.e., no neutrophils were present in these fluorimeter studies where Fluo-4 and cation were titrated and resulting fluorescence was measured). In these runs, Mn^{2+} binding to Fluo-4 quenched the fluorescence signal while Gd^{3+} enhanced Fluo-4 fluorescence (data not shown). Experiments with neutrophils were performed both in the absence (panel A) or presence (panel B) of IL-8. In Fig. 9a, 20 μM Gd^{3+} alone did not elicit intracellular calcium flux, while 1 mM Mn^{2+} increased Fluo-4 signal. The later observation suggests that Mn^{2+} may alter the $[Ca^{2+}]_i$ via yet unidentified mechanisms. Upon addition of 5 nM IL-8 (Fig. 9b), a transient changes in $[Ca^{2+}]_i$ was observed. Neither Gd^{3+} nor 0.2 mM Mn^{2+} affected this calcium flux. However, 1.0 mM Mn^{2+} decreased the Fluo-4 signal suggesting that this cation either penetrates cells in appreciable amounts or alters cellular $[Ca^{2+}]_i$ response. Such a suggestion that agonists can promote Mn^{2+} influx across cell membrane is supported by another report in literature.[29] Overall, while the exact mechanism by which Mn^{2+} affects Fluo-4 signal is not clarified in the current report, our observations support the notion that Mn^{2+} besides altering CD18 conformation also affects signal transduction in cell-based assays.

DISCUSSION

Regulation of Molecular Affinity by Cations

The affinity and avidity states of integrins vary with time and applied stimulus. In solution and on the cell surface, integrins can be stabilized in at least three distinct conformation states: (i) bent, closed, low-affinity conformation; (ii) extended, intermediate-affinity conformation, and (iii) extended, open, high-affinity state.[37] In the bent state, the head and tail portions of the protein are in close proximity to the cell wall and this conformation is not favorable for ligand binding. Quiescent integrins are in this low affinity or

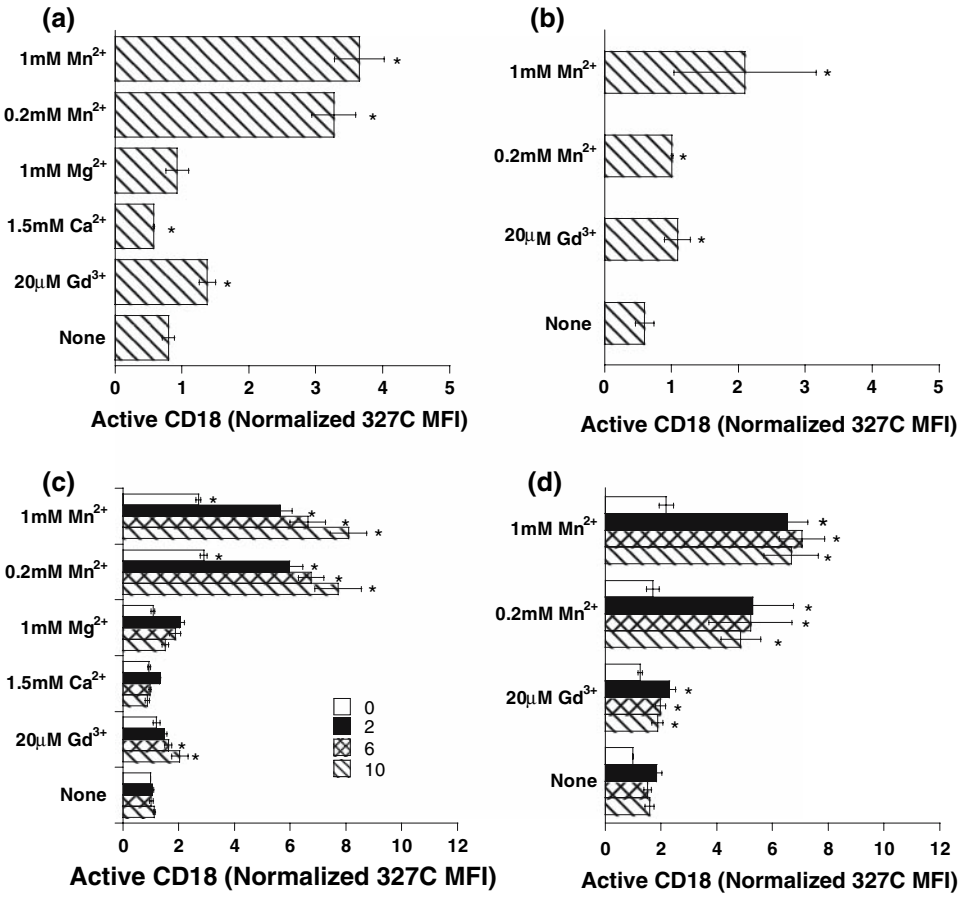

FIGURE 8. Cation mediated regulation of CD18 integrin conformation. (a) Neutrophils were treated in the same way as the control run (no IL-8) in Fig. 7, except that the HEPES buffer used was free of multivalent cations including Ca^{2+} and Mg^{2+} unless otherwise mentioned. Here, cations at indicated dose were added to the sample just prior to the 37 °C incubation step. (b) Data in panel B are identical to panel A, only the HEPES buffer used here for all runs contained 1 mM Ca^{2+} and 2 mM Mg^{2+}. In all runs, samples were withdrawn after 12 min incubation at 37 °C, and the extent of Alexa 488 conjugated mAb 327C binding was quantified after 1 min incubation using flow cytometry. (c) Experiments identical to panel A were performed only 5 nM IL-8 was used as stimulus and samples were withdrawn at indicated times, instead of a single sample at 12 min. (d) Samples were treated identically to panel B, only 5 nM IL-8 stimulus was applied. Error bars represent SEM from 2 to 5 independent experiments. *$p < 0.05$ with respect to "None" at indicated time. Data in all panels represent geometric mean fluorescence intensity normalized with respect to unstimulated cell fluorescence at $t = 0$.

'closed' conformation. Once stimulus is added, dramatic switch-blade like rearrangement in the quaternary structure of the protein results in a change of protein conformation from a compact to an extended structure. In the extended state, the head of the protein is oriented away from the cell surface. Further rearrangement in the ligand binding domain (α-chain I-domain in the case of LFA-1 and Mac-1) results in the 'open' conformation. This open conformation then binds ligands with high-affinity. Dissociation in the non-covalent interaction between the α- and β-cytoplasmic tails and increased separation between these cytoplasmic units is also thought to accompany this change in affinity. Thus, integrins are thought to oscillate between these three states as leukocytes respond to chemotactic stimulus (outside-in signaling) or changes in heterodimer chemical environment. While high calcium doses stabilize the low-affinity conformation, both ligand binding and cell activation favor the high-affinity state. In addition to changes in protein conformation that control molecular affinity, the clustering of the integrins following cell stimulation alters the avidity or valency of the molecular interactions and this further regulates cellular adhesivity.[11]

During homotypic neutrophil aggregation, following an initiate phase of rapid activation and firm cell binding, the strength of cell adhesion mediated by both LFA-1 and Mac-1 decreases gradually over a period of minutes.[10,24] This decrease in integrin function was reflected in the reversible dissociation of homotypic neutrophil aggregates as observed in our data. In studies of leukocyte–endothelial cell interaction, this decrease in the contribution of integrins with time is thought to be a prerequisite for cell detachment from

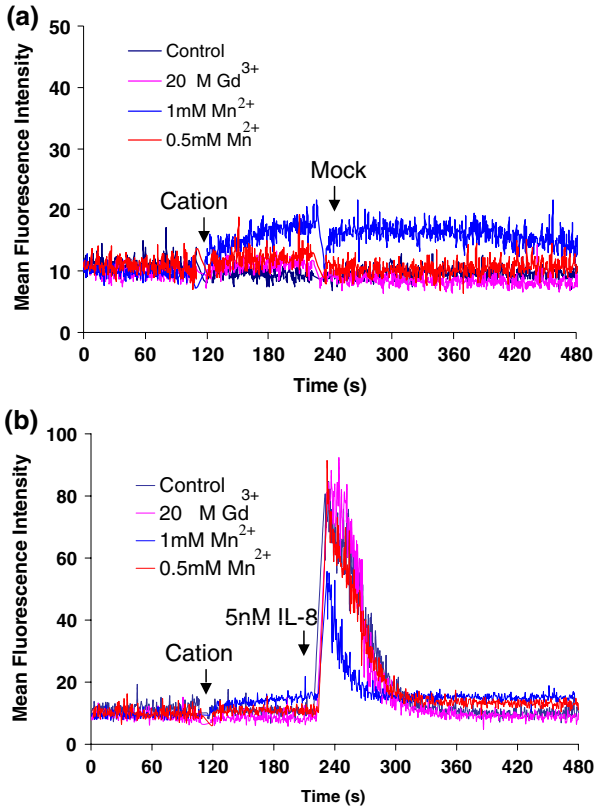

FIGURE 9. Flow cytometry detection of intracellular calcium flux. Neutrophil intracellular calcium level ($[Ca^{2+}]_i$) was measured based on fluorescence signal due to Fluo-4-AM. IL-8 stimulus is absent in panel A, while it is added in panel B. Mean fluorescence intensity (MFI) recorded using flow cytometry are reported. Arrows indicate time of addition of 10 μM Gd^{3+} or 0.5–1 mM Mn^{2+}, or 5 nM IL-8 stimulus. 1 mM Mn^{2+} itself increased cellular Fluo-4 signal in the absence of stimulus. 1 mM Mn^{2+} also decreased the measured MFI following addition of IL-8.

the endothelium prior to transmigration.[34,36] Our studies that monitor mAb 327C binding to neutrophils suggest that Mn^{2+} and Gd^{3+} promote the extended conformation of CD18 integrins that is associated with increased 327C binding. Microscopy investigations reveal that neither cation affected the distribution of CD18 integrin subunits, LFA-1 and Mac-1, either prior to chemokine stimulation or after stimulation. Based on these observations we conclude that it is the increased affinity of integrins mediated upon cation addition rather than alterations in the avidity of interaction that results in the sustained, irreversible aggregation of neutrophils reported here. Indeed, similar results have been noted in the case of CD29/β_1-integrins, where Mn^{2+} simultaneously both enhanced the binding of an antibody mAb 15/7 to lymphocytes and the affinity of $\alpha_4\beta_1$ binding to VCAM-1 and fibronectin.[23,43]

Comparison of Mn^{2+} vs. Gd^{3+}

Differences were observed in the mechanism by which Mn^{2+} and Gd^{3+} affect neutrophil function. First, the dose of Mn^{2+} required for irreversible neutrophil aggregation was 1 mM, and this was 50-fold higher than that required for Gd^{3+} (20 μM). This difference may be due to the trivalent nature of Gd^{3+} and the large coordination number of this cation, which may allow it to either bind integrins with lower dissociation constant, or which may allow stronger interactions within the ligand binding site compared to Mn^{2+}.

Second, cell adhesion studies performed in the presence of blocking antibodies to LFA-1 and Mac-1 suggest that Mn^{2+} primarily affects neutrophil Mac-1 function while Gd^{3+} affects both Mac-1 and LFA-1. Cell free experiments where ICAM-1 binds LFA-1 clearly demonstrate that Gd^{3+} enhances the affinity of LFA-1 during recognition of ICAM-1.[33] Here, by immobilizing LFA-1 on beads we eliminate possible activation due to inside-out signaling and changes in the avidity of interaction during the course of the assay. Further, use of the reconstituted system allows us to monitor binding interactions between ICAM-1 and LFA-1 in the absence of other CD18 integrins. The Gd^{3+} concentration used in the cell-free assay is ~15-fold lower than in the cell-based studies (1.5 vs. 20 μM). This difference may be due to the nature of LFA-1 immobilization, surface immobilized vs. membrane-bound. Alternatively, differences in the level of Ca^{2+} in the two assay systems may affect the Gd^{3+} concentration applied. In this regard, Ca^{2+} was absent in the buffer used in the cell-free assay while it was present in the cellular assays. In this context, as shown in Fig. 8, Ca^{2+} reduced the binding of 327C to neutrophils suggesting that it partially inhibited the formation of the extended integrin conformation. Another reason for the observed difference may be the density of LFA-1 in both systems since this is 5–10-fold higher in the reconstituted-bead assay system compared to the cellular assay.

Third, the addition of Mn^{2+} caused a 3–4-fold increase in the extent of mAb 327C binding to neutrophils compared to Gd^{3+} which increased binding of the antibody by only ~80%. The increased binding of 327C in these assays not only took place when Mn^{2+} was added at 1 mM but also at lower concentrations of the cation (0.2 mM) that was insufficient to sustain irreversible neutrophil aggregation. These findings suggest that 327C binding in some cases does not strictly correlate with alterations in integrin binding properties.

Fourth, intracellular calcium ($[Ca^{2+}]_i$) is a key secondary messenger that participates in signal

transduction and it regulates integrin function.[15] Further, neutrophils alter their intracellular calcium levels following chemokine stimulation.[35] In studies of neutrophil intracellular calcium levels, we observed that Mn^{2+} but not Gd^{3+}, appears to elicit a calcium flux in cells in the absence of IL-8 stimulus. Further, it appeared to affect the cell's calcium response upon stimulation. This effect on calcium response was most evident at the 1 mM dose. Taken together, the results suggest that Mn^{2+} and Gd^{3+} may affect the rates of homotypic neutrophil aggregation via distinct pathways. Gd^{3+} may act by decreasing the turnover rates of β_2-integrins while Mn^{2+} appears to also alter the intracellular pathways in cells. The later observations are consistent with another report which shows that Mn^{2+} affects cellular ATP levels in addition to integrin function.[19] We and others have noted that neutrophil aggregation is reversible following a variety of treatments including fMLP, PAF, and IL-8, but not the protein kinase C activator, PMA.[32] Stimulation of neutrophils with PMA results in irreversible cell aggregation and increased amounts of CD18 cytoplasmic tail phosphorylation.[7,20] Such changes in CD18 phosphorylation may alter integrin–cytoskeletal interactions and contribute to cellular adhesivity differences.[12,40] The effect of Mn^{2+} and Gd^{3+} on CD18 phosphorylation and integrin–cytoskeletal interactions was not examined in this paper and is ripe for future investigation.

Finally, Gd^{3+}, but not Mn^{2+}, enhanced the rate of neutrophil L-selectin shedding after stimulation with IL-8. One possibility is that Gd^{3+} may regulate the activity of a neutrophil surface metalloprotease, ADAM-17/TACE (Tumor necrosis factor-α-converting enzyme), which catalyzes L-selectin shedding.[16] The faster L-selectin shedding rate in the presence of Gd^{3+} may contribute to the reduced rate of cell aggregate in the first 60–120 s at the shear rate of 1785/s (Fig. 3). This suggestion is supported by our previous studies where we demonstrated that inhibition of L-selectin shedding using a metalloprotease inhibitor, KD-IX-73-4, increased the efficiency of cell adhesion at a shear rate of 1600/s.[25]

Cation Effects on Integrin Function

Numerous studies have examined the binding sites of cations on integrins and the regulation of integrin affinity and structure as a result of cation engagement. These studies show that both Mg^{2+} and Mn^{2+} bind the MIDAS site in the Inserted (I) domain of integrins and induce conformation changes that are detected by 'activation' reporter antibodies.[15] In addition to this site, the β-propellar domain of the α-subunit contains three cation binding motifs for Ca^{2+}, and the I-like domain of the β-chain contains additional sites that bind Mg^{2+} and Ca^{2+}[15,37] (Fig. 1). The observation that low levels of calcium are required for LFA-1 ICAM-1 interactions and that high concentrations of this cation inhibit molecular recognition[13,21,31] has lead to the concept that there are two classes of Ca^{2+} binding sites on integrins. These include a high affinity site that promotes integrin heterodimer formation and ligand binding, and an inhibitory low affinity site that appears to allosterically compete with the Mg^{2+} in the I-domain.[13,27] While the exact site(s) of Gd^{3+} binding is not resolved in the current paper evidence from measurement of 327C binding in the presence or absence of Ca^{2+} suggests that Gd^{3+} may compete with Ca^{2+} binding site on integrins in addition to binding the MIDAS site. Future crystal structure studies may address the exact mechanism of Gd^{3+} action.

Differences were observed between the effects of Mg^{2+} in cell-free vs. cell-based assays. In this regard, while 3 mM Mg^{2+} alone supports LFA-1 ICAM-1 binding in cell-free assays, 1 mM Mg^{2+} was not sufficient to support neutrophil aggregation in the absence of exogenous stimulation. In this regard, it is important to note that the cell-free system specifically detects changes in receptor activating conformation but these do not measure cell adhesion properties. Besides a shift in integrin conformation, other features like a sufficient increase in molecular avidity, cell signaling that leads to phosphorylation of integrins, and integrin–cytoskeletal interactions play an important role in modulating cell adhesion function. Thus, it is possible that while the cell-free assay provides an efficient readout of integrin conformation, cell-adhesion measure a more complex process that includes changes in molecular conformation, affinity, and avidity. Mg^{2+} may thus alter integrin conformation without supporting adhesion function.

Besides its affect on leukocyte integrin function two other common applications of Gd^{3+} require mention. First, chelated Gd^{3+} is commonly used for imaging applications and thus studies of the effect of Gd^{3+} on human leukocyte function are relevant in this context, particularly if sufficient free-Gd^{3+} is released from these reagents.[28] Second, Gd^{3+} is an inhibitor of mechanosensitive stretch-activated ion channels (SACs).[4,42] In this regard, Gd^{3+} has been shown to block intracellular Ca^{2+} elevation via SAC.[9,42] Since SAC channels have not been reported to exist in leukocytes and since Gd^{3+} in our experiments did not affect intracellular Ca^{2+} levels, we suggest that our experiments do not currently support the possibility that shear dependent forces regulate neutrophil activity and function.

Overall, our studies compare the effects of Mn^{2+} and Gd^{3+} in regulating integrin function, and the

potential use of Gd^{3+} in future cell-based studies of integrin biology.

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