Mac-1 Promotes FcγRIIA-Dependent Cell Spreading and Migration on Immune Complexes†

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ABSTRACT: The integrin Mac-1 plays a critical role in Fc receptor (FcR)-mediated antibody-dependent cellular cytotoxicity (ADCC). However, the mechanism by which Mac-1 facilitates the functions of FcγRIIA, a major FcR expressed on human leukocytes, is not fully understood. We report here that Mac-1 sustains cell adhesion, enhances cell spreading, and accelerates cell migration on preformed immune complexes (ICs) by directly interacting with FcγRIIA but not with the IC substrate. Coupling Mac-1 to FcγRIIA allows FcγRIIA to reside in the leading front of actin polymerization at the filopodial extension and thus could potentially enhance FcγRIIA-mediated cell spreading and migration. The direct interaction between Mac-1 and FcγRIIA is demonstrated by co-immunoprecipitation, by cell surface co-localization, and by solid-phase binding assays using recombinant α_MI-domain and soluble FcγRIIA. Further mutational analysis identifies the E^{253}-R^{261} sequence within the α_MI-domain as part of the FcγRIIA binding interface within Mac-1. Altogether, these results demonstrate that FcγRIIA recognizes Mac-1 via the α_MI-domain but not the lectin domain, a distinct feature from other FcRs, and that Mac-1 binding confers FcγRIIA with the ability to prolong cell adhesion as well as to spread and migrate on the ICs, leading to effective cell killing by ADCC.

Receptors recognizing the Fc fragment (FcR)[1] of the immunoglobulin (Ig) molecules are critical to host defense by mediating leukocyte migration toward ICs deposited within the site of infections and by eliciting potent cytolysis of IC-sensitized targets, such as malignant cells and invading pathogens (*1*−*8*). A number of clinical studies have demonstrated that FcγRIIA (CD32), a major FcR that recognizes the IgG subclass, is essential to the efficacy of several therapeutic antibody-based drugs, including Herceptin for HER-2/neu-positive breast cancer cells, Rituxan for non-hodgkins lymphoma, and the antibodies for melanoma (*1*, *2*, *9*). Yet, the inappropriate engagement of FcγRIIA also causes autoimmune diseases in patients undergoing treatment using these therapeutic antibodies (*10*) and in transgenic mice overexpressing FcγRIIA (*11*).

The FcγRIIA-mediated antibody-dependent cytotoxicity (ADCC) is dependent on both FcγRIIA and the integrin Mac-1 ($\alpha_M\beta_2$, CR3, CD11b/CD18) (*1*, *8*, *12*, *13*). Despite the importance of Mac-1 in FcγRIIA-mediated leukocyte functions and the extensive studies conducted on Mac-1 interaction with various FcRs, including FcγRIIIB (CD16), FcαRI (CD89), and FcϵRII (CD23), in addition to FcγRIIA (*2*−*8*, *14*−*16*), the molecular basis underlying the Mac-1 recognition of these FcRs is still less understood. Primarily on the basis of inhibition studies using lectin-domain-specific inhibitors such as *N*-acetyl-D-glucosamine (NADG) (*13*, *15*, *17*), the binding sites within Mac-1 for most of the FcRs are mapped to its lectin-domain in the α subunit. However, as Mac-1's ability to promote FcγRIIA-mediated phagocytosis is insensitive to NADG inhibition (*18*), the nature of the FcγRIIA binding site within Mac-1 remains elusive.

Given the importance of Mac-1 in both FcγRIIA-mediated immunotherapy and autoimmune diseases (*1*, *2*, *9*, *10*), we sought to elucidate the molecular basis underlying the critical role of Mac-1 in the FcγRIIA-mediated processes, including sustained adhesion, cell spreading, and cell migration on ICs. Here, we report the first biochemical evidence for a direct interaction between FcγRIIA and Mac-1, including (1) the co-localization of these two proteins on the cell surface, (2) co-immunoprecipitation (co-IP) as a complex, (3) the direct interaction between the recombinant α_MI-domain of Mac-1 and soluble FcγRIIA, and (4) the identification of the E^{253}-R^{261} sequence within Mac-1, which is critical for FcγRIIA recognition. Moreover, our data shows that Mac-1 binding to FcγRIIA changes the spatial organization of FcγRIIA relative to that of actin filaments and, thereby, potentially facilitates sustained cell adhesion, cell spreading, and cell migration toward IC-sensitized tumor cells, which in combination results in the effective cytolysis of the malignant cells via FcγRIIA-mediated ADCC.

EXPERIMENTAL PROCEDURES

Materials. Human kidney 293 cells and the expression vector pCIS2XN were gifts from Dr. F. J. Castellino (Notre

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[1] Abbreviations: FcR, receptor for the Fc fragment of immunoglobulin; IC, immune complex; ITAM, immunoreceptor tyrosine-based activation motifs; NIF, neutrophil inhibitory factor; PMA, phorbol 12-myristate-13-acetate.

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Dame, IN). NIF was kindly provided by Dr. E. F. Plow (Cleveland, OH). Dithiobis-succinimidylpropionate (DSP) was from Pierce (Rockford, IL). Fugene 6 was from Roche (Indianapolis, IN), and the β_2-specific activating mAb MEM48 was from Biodesign (Kennebunk, ME). The mAbs IV3, 44a, OKM1, M1/70, and IB4 as well as the HL60 cells were from ATCC (Rockville, MD). All other reagents were of the highest grade available from Sigma Chemical Co. (St Louis, MO) unless otherwise noted.

Establishment of Stable Cell Lines. The differentiation of HL60 was induced with 1% DMSO for 6 days in RPMI-1640 plus 10% fetal bovine serum at a starting cell density of 1×10^5 cells/mL. Maturation of HL60 to neutrophils was confirmed by FACS analysis using mAb OKM1 (for Mac-1) and mAb IV3 (for FcγRIIA) and by the morphological examination of Hema3 (Fisher Scientific)-stained Cytospin (Shandon) smears. To establish human 293 cell lines that express both FcγRIIA and Mac-1, the Mac-1-expressing 293 cells, which were established previously in the laboratory (*19*), were transfected with the expression vector pCIS2XN−FcγRIIA, where the cDNA of FcγRIIA was inserted into pCIS2XN at *Xba I* and *Not I* sites and pcDNA3.1(+)-hygro, a hygromycin selection marker, using Fugene 6. To remove the 75-residue cytoplasmic tail of FcγRIIA, the extracellular and transmembrane domains of FcγRIIA were amplified by PCR using primers 5′AATCTAGACCATGGCTATGGAG-ACCCAAATGTCTCAG 3′and 5′ACGTAGCGGCCGCT-TACCTGCAGTAGATCAACGCCACTACAGCAGCAA-CAAT 3′, and the amplified fragments were inserted into pCIS2XN as described above. The correctness of the inserted FcγRIIA sequence was verified by DNA sequencing. After hygromycin selection, the cell lines with similar surface expressions were established by dual-color cell sorting using an α_M-specific mAb (M1/70) and an FcγRIIA-specific mAb (IV3). To exclude possible clonal effects, multiple independent clones were used for each cell line in the study.

Cell Adhesion and Spreading Assays. Cell adhesion to the ICs was conducted in 24-well nontissue culture plates. Briefly, a 24-well polystyrene plate (BD Biosciences) was coated in the center with 70 μL of bovine serum albumin (BSA, 1 mg/mL) followed by 70 μL of anti-BSA IgG (150 μg/mL in DPBS) (*16*). The plate was then blocked with 300μL of 0.5% poly(vinylpyrrolidone) (Sigma) in DPBS. A total of 5×10^5 293 cells or 2×10^6 phorbol 12-myristate-13-acetate (PMA)-stimulated HL60 cells in 200 μL of Hanks' balanced salt solution (HBSS) containing 1mM $CaCl_2$, 1mM $MgCl_2$, and 10 mM HEPES in the presence or absence of 10 μg/mL of β_2-specific activating mAb (MEM48), 25nM NIF, 150mM NADG, 150mM α-methylmannoside (mannoside), or 150mM glucose were added to each well and incubated at 37 °C. At different time points, 200 μL of 4% paraformaldehyde (PFA) was added to the plates to terminate cell adhesion/detachment, and the nonadherent cells were removed by washing with DPBS. The remaining adherent cells were quantified by 0.5% Crystal Violet staining as described previously (*19*). For cell spreading, 1×10^5 cells were added into each well and incubated at 37 °C for 30−60 min and then fixed with 4% PFA. Cell spreading was quantified under a phase contrast microscope (20× objectives) on the basis of four randomly picked fields in each well and calculated as the ratio of spread versus total cells. Cell spreading was further verified by 3D confocal fluorescence imaging as described below.

Immunoprecipitation. A single cell suspension, prepared from 293 cells expressing FcγRIIA or FcγRIIA/Mac-1 were incubated with NIF, NADG, or glucose in HBSS containing 10mM HEPES, 1mM $CaCl_2$, and 1mM $MgCl_2$ at room temperature for 15 min, and then, their surface receptors were cross-linked with a 12 Å-long thiol-cleavable and amine-reactive cross-linker DSP (2mM) for 30 min. After washing, the residual active cross-linker was inactivated with 100mM ethanolamine at pH8 in the above HBSS buffer, and then, the cells were lysed in a RIPA buffer (50 mM Tris-HCl at pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 2 mM Na_3VO_4, 10 μg/mL leupetin, 10 μg/mL aprotinin, and 1 mM PMSF). The cell lysates, prepared by centrifugation, were then incubated with an FcγRIIA-specific mAb (IV3) at 4 °C for 60 min and then with Protein A-Sepharose (Amersham, Piscataway, NJ). After washing, the immunoprecipitates were eluted in an SDS−PAGE loading buffer, separated by SDS−PAGE in the presence of β-mercaptoethanol, and transferred to PVDF membranes. The presence of Mac-1 in the immunoprecipitates was visualized by immunoblot using a rabbit anti-α_M cytoplasmic tail antibody (ARC23) and an HRP-conjugate of goat anti-rabbit IgG.

Live Cell Imaging. Cells expressing FcγRIIA with and without Mac-1 were allowed to adhere to the ICs on a 24-well nontissue culture plate at 37 °C for 25 min in F-12/DMEM containing media alone, NIF (100 nM), NADG (150 mM), or glucose (150 mM) as described above for the cell adhesion assays. After washing to remove the nonadherent cells, cell migration was monitored using the Neue LiveCell System (Camp Hill, PA) at 37 °C and 5% CO_2 for 10 h. Time lapse images were taken in 2-minute intervals, and image analysis was performed using MetaMorph (Universal Imaging).

Confocal Laser-Scanning Immunofluorescence Microscopy. Cells expressing FcγRIIA with and without Mac-1 were allowed to adhere to the IC-coated coverslips, as described for the cell adhesion assays. The adherent cells were fixed with 4% PFA, blocked with 10% calf serum plus 2% BSA, and then incubated with goat anti-human CD32 (R&D) and mouse anti-Mac-1 (OKM1). After washing, the cells were then stained with an Alexa 488 conjugate of donkey anti-goat IgG and an Alexa 568 conjugate of goat anti-mouse IgG or Alexa 488-phalloidin for actin filaments (Molecular probes) after permeabilization with 0.4% Triton X-100. The coverslips were mounted in Fluosave (Calbiochem), and confocal fluorescence images were obtained using Bio-Rad Radiance 2000 laser scanning confocal system equipped with a Nikon Eclipse E800 microscope (BioRad, Hercules, CA). Image analysis and 3D image reconstruction were performed using Volocity (Improvision).

Fluorescence Lifetime Imaging Microscopy (FLIM). The cells were stained with goat anti-human CD32, with or without mouse anti-Mac-1 (OKM1) or a control mouse IgG, followed by incubation with an Alexa 488 conjugate of anti-goat IgG and an Alexa 568 conjugate of anti-mouse IgG as described above. FLIM images were acquired using a BioRad Radiance 2000 multiphoton microscope equipped with a high-speed Hamamatsu MCP detector. Excitation at 800 nm was empirically determined to excite Alexa 488 but not

Alexa 568. The donor (Alexa488) fluorophore lifetimes were fit to two exponential decay curves to calculate the fraction of fluorophores within each pixel that interact with an acceptor. As a negative control, the Alexa 488 lifetime was measured in the absence of the acceptor (Alexa 568), which showed lifetimes equivalent to FITC−IgG alone or in solution (*20*).

FACS Analysis. A total of 10^6 cells expressing FcγRIIA, Mac-1, or FcγRIIA plus Mac-1 in HBSS were incubated with 1 μg of mAb IV3 (for FcγRIIA) or OKM1 (for Mac-1) for 30 min. A subtype-matched mouse IgG served as a control. After washing with PBS, the cells were mixed with RPE-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) for another 30 min. The cells were then washed and resuspended in 500 μL of DPBS. FACS analysis was performed using FACScan (BD Biosciences), counting 10 000 events. Mean fluorescence intensities were quantified using the FACScan program.

Preparation of Soluble FcγRIIA. The FcγRIIA extracellular domain was amplified by PCR using the following primers: 5′TATAGATCTGATGGCTATGGAGACCCAAATGTCTCAG3′ and 5′ACGTAGCGGCCGCTTAAGGTGAAGAGCTGCCCATGCT3′ and then cloned into the baculovirus transfer vector pAcGP67A within *Bgl II* and *Not I* sites. After confirmation of its correct sequence by DNA sequencing, pAcGP67A−sFcγRIIA was transfected, together with the Baculogold-linearized Baculovirus DNA (BD Biosciences), into the sf9 cells. Single colonies were picked from the plaque assay plates and expanded, and the presence of sFcγRIIA in the condition media was determined by immunoblot using goat anti-human CD32 antibody. For large scale production, sFcγRIIA was purified using an IV3 affinity column. The purity of the recombinant sFcγRIIA was verified by both SDS−PAGE and by immunoblot as described above.

Solid-Phase Binding Assays. To test the direct interactions between soluble FcγRIIA and the α_MI-domain, 96-well microtiter plates (Immulon 4B, Dynex Technologies Inc, Chantilly, VA) were coated with the ICs as described above, followed by incubation with soluble FcγRIIA. After washing and blocking with 2% BSA, GST, or GST-α_MI-domain (10 μg/mL; produced in *E. coli* (*21*)) in 1% BSA−TBST (20 mM Tris, 150 mM NaCl, 1mM Ca^{2+}, 1 mM Mg^{2+}, 0.05% Tween 20 at pH 7.4), was added in the presence or absence of different inhibitors and incubated for 2−3 h at RT. After washing with TBST, the bound GST-α_MI-domain was detected using anti-GST conjugated to horseradish peroxidase (HRP) and the HRP substrate 3,3,5,5,-tetramethylbenzidine (KPL, Gaithersburg, MD). Reciprocally, the plate was coated with either GST or GST-α_MI-domain and blocked with BSA. Soluble FcγRIIA was then added and incubated at RT for 2 h. After washing, bound FcγRIIA was detected using goat anti-human CD32, followed by HRP-donkey anti-goat IgG and its substrate as described above.

RESULTS

Cell Adhesion to ICs Is Dependent on FcγRIIA but Not on Mac-1.

It has been reported that FcγRIIIB (CD16) and FcαRI (CD89), which do not contain an ITAM (*22*) sequence within their cytoplasmic domains, require Mac-1 for sustained cell adhesion and the formation of close cell contacts with the IC-coated targets (*3*−*5*, *23*). However, FcγRIIA, which possesses an ITAM motif within its cytoplasmic domain and can signal in the absence of other ITAM-containing subunits, still depends on Mac-1 for its ADCC activity toward the IC-tagged targets (*1*, *6*, *8*). To understand the role of Mac-1 in FcγRIIA-mediated functions, we first evaluated whether Mac-1 is required for cell adhesion to ICs, using human 293 cells (which do not express endogenous Mac-1 or FcγRIIA) that express either Mac-1 alone or Mac-1 plus FcγRIIA. Cell adhesion was conducted on IC-coated 24-well nontissue culture plates, and the adherent cells were quantified by staining with crystal violet as described previously (*19*). In addition, the specific contributions of FcγRIIA and Mac-1 to cell adhesion were further verified using their corresponding specific inhibitors mAb IV3 and NIF (*21*), respectively. We found that the 293 cells expressing either FcγRIIA alone or both FcγRIIA and Mac-1 adhered to the ICs (Figure 1A). In contrast, no specific adhesion to ICs was observed for Mac-1-expressing cells, even upon integrin activation by the addition of a β_2-activating antibody (MEM48) (*24*) (Figure 1A). Confirming the specificity of the assay, no adhesion was seen for mock-transfected cells. Moreover, adhesion by the FcγRIIA/Mac-1-expressing cells was blocked by the FcγRIIA-specific mAb IV3 but not by a control IgG. The addition of the Mac-1-specific inhibitor NIF had no effect (Figure 1A), although it completely blocked Mac-1-mediated cell adhesion to the Mac-1 ligand fibrinogen (data not shown). Together, these results demonstrated that the adhesion of the FcγRIIA/Mac-1-expressing cells to ICs is mediated by FcγRIIA and that Mac-1, with or without activation, does not directly support cell adhesion to this substrate.

Mac-1 Promotes Sustained Cell Adhesion by FcγRIIA. Involvement of the α_MI-Domain. To assess the possibility that Mac-1 facilitates the FcγRIIA-dependent ADCC activity by supporting sustained leukocyte adhesion to ICs, we evaluated cell detachment at different time points. In these experiments, cells expressing FcγRIIA with or without Mac-1 were added to IC-coated plates at 37 °C, and at different time points, PFA was added to stop cell adhesion/detachment and to fix the cells. The nonadherent cells were then removed by washing, and the adherent cells were quantified by crystal violet staining. We found that the FcγRIIA-expressing cells adhered rapidly to the ICs such that more than 90% of the input cells became adherent in 25 min (peak adhesion; assigned as time 0 of cell detachment), and thereafter, they gradually detached from the IC-coated plates. The expression of Mac-1 in FcγRIIA/293 cells had no effect on the initial phase of cell adhesion, evidenced by the similar number of adherent cells at peak adhesion (Figure 1B, time 0). However, cells expressing FcγRIIA alone showed rapid detachment from the IC-coated plates. At 40 min after peak adhesion, very few cells remained adherent (Figure 1B). In contrast, the FcγRIIA/Mac-1-expressing cells maintained adhesion and did not detach for an extended period of time (more than 60 min), indicating that Mac-1 is required for sustained cell adhesion (Figure 1B). Confirming the specific requirement of Mac-1 in this process, the treatment of the FcγRIIA/Mac-1-expressing cells with NIF, an α_MI-domain-specific inhibitor of Mac-1 (*21*), abolished sustained adhesion but did not affect initial cell

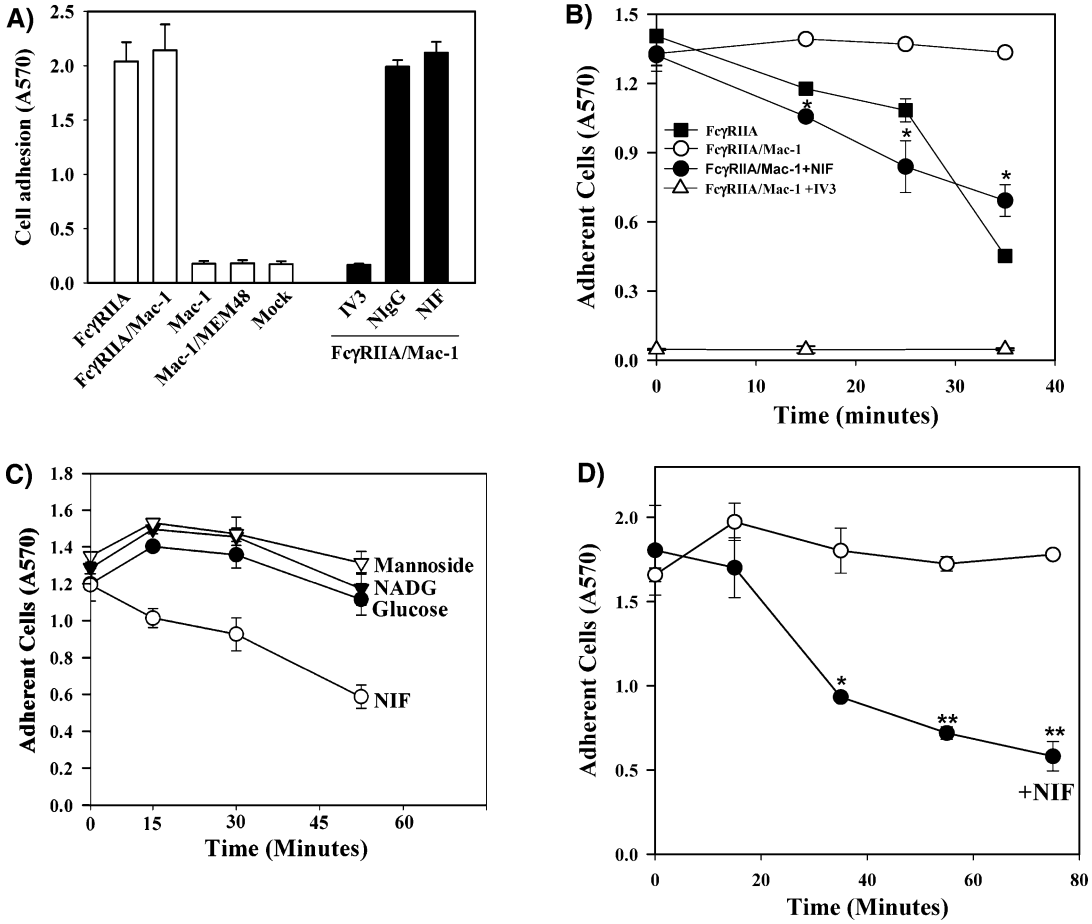

FIGURE 1: FcγRIIA but not Mac-1 is responsible for cell adhesion to ICs. (A) Cell adhesion is dependent on FcγRIIA. Human 293 cells expressing either FcγRIIA or Mac-1 alone or FcγRIIA plus Mac-1 were allowed to adhere to IC-coated 24-well nontissue culture plates in the presence or absence of a β_2-activating mAb (MEM48), an FcγRIIA-specific blocking mAb (IV3), a Mac-1-specific inhibitor (NIF), or a nonimmune IgG for 20 min at 37 °C. After washing, the adherent cells were quantified by the crystal violet method. When verifying specificity, no significant cell adhesion was observed for mock-transfected 293 cells. (B) Sustained cell adhesion is dependent on Mac-1. Human 293 cells expressing either FcγRIIA alone or both FcγRIIA and Mac-1 were allowed to adhere to IC-coated 24-well plates in the presence or absence of NIF or mAb IV3. At different time points, PFA was added to stop cell adhesion/detachment, the plates were washed, and the adherent cells were quantified. The time of peak cell adhesion (25 min post-cell addition) was designated as time zero of cell detachment; (*), $p < 0.01$ by Student's *t*-test. (C) Sustained cell adhesion is independent of the Mac-1 lectin-domain. The above study was conducted on the FcγRIIA/Mac-1-expressing cells in the presence of NADG, mannoside, or NIF. Glucose was used as a negative control. (D) Sustained neutrophil adhesion requires Mac-1. Differentiated HL60 cells were activated by PMA, washed, and then allowed to adhere to IC-coated plates in the presence or absence of NIF at 37 °C. At different time points, cell detachment was determined as mentioned above; (*), $p < 0.01$; (**), $p < 0.001$ by Student's *t*-test. Data shown are the means ± SD of three independent experiments.

adhesion (Figure 1B). Furthermore, the addition of FcγRIIA-specific mAb IV3 completely blocked cell adhesion, even in the presence of Mac-1 (Figure 1B). Because Mac-1 is known to interact with FcRs via its lectin-like domain (*17*), we evaluated the role of this domain in supporting sustained adhesion by FcγRIIA using the lectin-domain specific inhibitor NADG (*17*). The addition of NADG or α-methyl-mannoside (mannoside) at 150 mM, a concentration that is effective in blocking co-capping between Mac-1 and FcγRIII (*17*), had no effect on sustained cell adhesion. Similarly, the addition of its control polysaccharide glucose also did not have any effect (Figure 1C). These results suggest that the ability of Mac-1 to sustain FcγRIIA-mediated cell adhesion is dependent on the α_MI-domain but not the lectin-like domain.

To determine if sustained leukocyte adhesion to ICs is similarly dependent on Mac-1, we used DMSO-differentiated HL60 cells as representatives of human neutrophils, which express both Mac-1 and FcγRIIA (*25*). The differentiated HL60 cells were activated with PMA and washed. The activated cells were then allowed to adhere to immobilized ICs in the presence or absence of NIF at 37 °C, and at different time points, PFA was added to the plates to stop cell adhesion/detachment. The nonadherent cells were then removed by washing, and the remaining adherent cells were quantified. The results from these experiments showed that similar to the 293 cells expressing recombinant FcγRIIA and Mac-1 the differentiated HL60 cells maintained adhesion to the ICs for an extended period of time, and the addition of NIF significantly accelerated cell detachment ($p = 0.01$ at 35 min and $p < 0.001$ at 55 and 75 min) (Figure 1D). Together, these experiments demonstrated that sustained neutrophil adhesion to the ICs, a process that is important for ADCC (*3*, *4*, *6*) and for neutrophil activation (*26*), is dependent on Mac-1.

Co-Localization between Mac-1 and FcγRIIA on the Cell Surface. Cell adhesion and detachment are controlled by actin polymerization and reorganization of the cytoskeleton. To see if Mac-1 prolongs FcγRIIA-mediated cell adhesion by changing the spatial organization between FcγRIIA and actin

polymerization, we visualized the distributions of Mac-1, FcγRIIA, and actin filaments by confocal laser scanning fluorescence microscopy. For these experiments, the FcγRIIA/Mac-1-expressing cells were allowed to adhere to the ICs on coverslips and then fixed with paraformaldehyde. Surface FcγRIIA and Mac-1 were stained with a goat anti-human CD32 (for FcγRIIA) and a murine mAb OKM1 (for Mac-1), followed by their corresponding secondary antibodies conjugated to either Alexa 488 or Alexa 568. The results showed that FcγRIIA (shown in green in Figure 2A) co-localized well with WT Mac-1 (in red) on the cell surface (top panels). Verifying the specificity of immunostaining, no fluorescence was observed for nonimmune control IgG-stained samples (Panel d) under the same instrument settings. To see if Mac-1 also co-localizes with FcγRIIA on DMSO-differentiated HL60 cells, confocal fluorescence microscopy experiments were conducted, and the representative confocal images are presented in Figure 2A (Panel b). Overall, approximately 80% of Mac-1 was colocalized with FcγRIIA, and strong co-localization was primarily found at focal adhesion sites and in filopodial extensions.

To further corroborate the above finding, we measured the molecular proximity between Mac-1 and FcγRIIA on the HL60 cell surface by fluorescence lifetime imaging microscopy (FLIM) (*20*). This high-resolution imaging technique is based on the fluorescence resonance energy transfer (FRET) that occurs if two fluorophores are in very close proximity (<100 Å), resulting in the shortening of the donor fluorescence lifetime. Accordingly, we labeled DMSO-differentiated HL60 cells simultaneously with a goat anti-FcγRIIA IgG and a mouse anti-Mac-1 (OKM1) or a control IgG, followed by staining with an Alexa 488-anti-goat and an Alexa 568-anti-mouse antibody, and the fluorescence lifetimes were determined using a multiphoton confocal microscope. The results showed that the presence of Mac-1 (i.e., the acceptor Alexa 568) significantly shortened the average fluorescence lifetime of the anti-FcγRIIA fluorophore (i.e., the donor Alexa 488) from 2293 ± 285 to 1324 ± 504 ps (1899 ± 237 ps with a control IgG; OKM1 vs IgG, $p = 0.00187$, $n = 11$), indicating that Mac-1 and FcγRIIA resided <100 Å from each other on the cell surface. Figure 2B shows representative pseudocolor-coded images of surface co-localization between Mac-1 and FcγRIIA (i.e., the *z*-section is set at the height of the cell surface), where a uniformly bluish image (thus long fluorescence lifetime) was obtained in the absence of the acceptor (Panel b) or in the presence of a nonimmune control IgG (Panel d), and a red-to-green-filled image representing the short fluorescence lifetime (therefore close proximity) was obtained in the presence of the acceptor Mac-1 fluorophore (Panel c).

Expression of Mac-1 Changes the Relative Spatial Organization between Actin and FcγRIIA. To determine if the expression of Mac-1 changes the relative spatial distribution of FcγRIIA and the actin filaments, the above cells were first stained with goat anti-CD32 (for FcγRIIA), fixed, and permeabilized with 0.4% Triton X-100. The cells were then stained with Alexa 488-labeled phalloidin for actin filaments and Alexa 568-anti goat IgG. A series of 2D ($x-y$) confocal fluorescence images at different *z*-positions were collected, and these images were then used to construct 3D images by deconvolution using Volocity, which allows us to better visualize the spatial distributions of FcγRIIA and the actin filament. As shown in Figure 2C, without Mac-1, the cells spread poorly with short cell extensions, where FcγRIIA (in red) stayed behind the actin filament staining (in green) (Panel a). In contrast, the coexpression of FcγRIIA and Mac-1 allowed the cells to spread on ICs, and exhibited a flat cell body and long filopodial extensions. Most importantly, when Mac-1 is present, FcγRIIA (in red) resided at the front of the actin filament (Panel b). Taken together, these data suggest that Mac-1 binding to FcγRIIA alters its relative spatial distribution to actin polymerization and thus potentially facilitates FcγRIIA-mediated cell adhesion and spreading on the ICs.

Co-Immunoprecipition between Mac-1 and FcγRIIA from Total Cell Lysates. In agreement with the above co-localization experiments, Mac-1 has been shown to co-cap with a number of FcRs on the cell surface (*7*, *14*, *27*). To determine if Mac-1 and FcγRIIA co-localize by direct associations, we conducted co-IP experiments (Figure 3A) using 293 cells that express only Mac-1 (lane 1), FcγRIIA (lane 2), or both receptors (lanes 3−5). Given the dynamic nature of the Mac-1/FcγRIIA interaction (*28*), the potentially transient protein complex on the cell surface was cross-linked by a short-length thiol-cleavable cross-linker (spacer length = 12 Å). The resulting cell lysates were then incubated with either an FcγRIIA-specific mAb (IV3) or a control IgG at 4 °C, and the Mac-1/FcγRIIA complex was then pulled down with Protein A-Sepharose. The presence of Mac-1 in the immpunoprecipitates was detected by immunoblot using a polyclonal antibody specific for the 165 kDa α_M subunit (ARC23). The results showed that immunoprecipitation with the FcγRIIA-specific mAb IV3 (lane 4) but not a control IgG (lane 3) specifically precipitated Mac-1 from the total cell lysates (Figure 3A), thus verifying the specificity of the co-IP. Further confirming specificity, FcγRIIA-specific mAb IV3 failed to precipitate Mac-1 from lysates prepared from the cells expressing only Mac-1 (lane 1). In addition, when co-IP was conducted in the presence of the Mac-1-specific antagonist NIF (lane 5), significantly lower quantities of Mac-1 was obtained (Figure 3C). Immunoblot with an anti-actin antibody demonstrated equal loading (data not shown), and the stripping and re-probing of the above PVDF membrane with a goat anti-CD32 antibody confirmed the presence of FcγRIIA in the immunoprecipitation mixtures (Figure 3B).

Mac-1 Binding to FcγRIIA Is Mediated by Its α_MI-Domain. The observations that the α_MI-domain-specific inhibitor of Mac-1, NIF (*29*), inhibited the co-IP between Mac-1 and FcγRIIA (Figure 3) and blocked Mac-1's ability to sustain the FcγRIIA-mediated cell adhesion to the ICs (Figure 1) suggested that the α_MI-domain may be involved in Mac-1 binding to FcγRIIA. Such a requirement of the α_MI-domain in Mac-1 binding to the FcRs is unexpected because all other FcRs interact with the lectin-like domain of Mac-1 (*13*, *15*, *17*). To determine whether the α_MI-domain interacts directly with FcγRIIA, we prepared a recombinant GST fusion of the α_MI-domain in *E. coli* and a soluble FcγRIIA in sf9 cells. To mimic the conditions used in the cell adhesion experiments, recombinant soluble FcγRIIA was immobilized onto the ICs, which were precoated onto a 96-well plate. After blocking with BSA, GST or GST-α_MI-domain was added in the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+} to the IC-coated wells, and bound α_MI-domain was detected using a

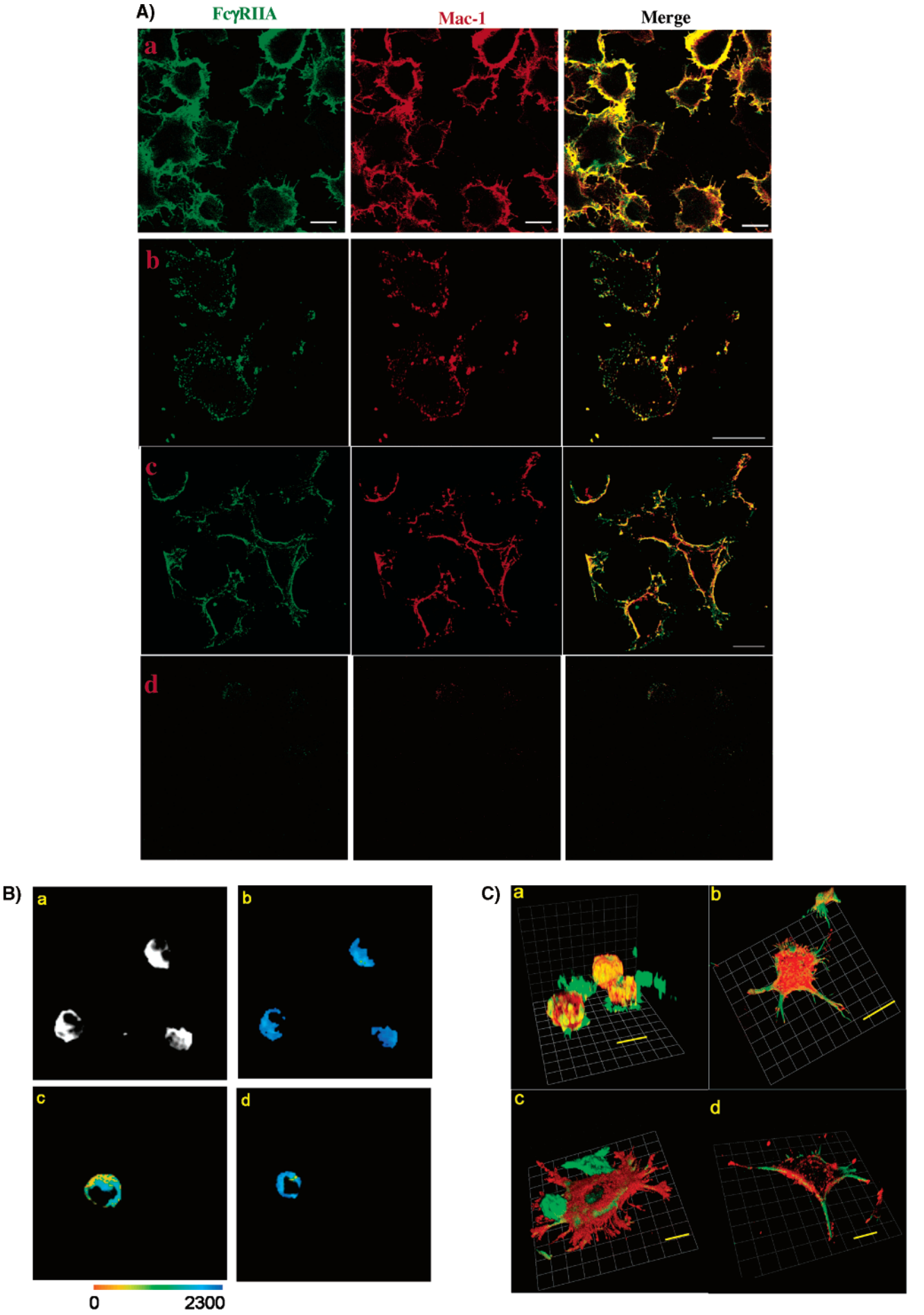
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FIGURE 2: Expression of Mac-1 alters the spatial distribution of FcγRIIA. (A) co-localization between Mac-1 and FcγRIIA. Human 293 cells expressing Mac-1/FcγRIIA (a and d) or Mac-1/ FcγRIIA(CT-) (c) and DMSO-differentiated HL60 cells (b) were allowed to adhere to ICs, fixed with 2% PFA, and then stained for FcγRIIA (goat anti-human CD32) and Mac-1 (mAb OKM1) (a−c) or control goat and mouse IgG (d), followed by staining with Alexa 488-donkey anti-goat IgG and Alexa 568 goat anti-mouse IgG. Confocal fluorescence images were acquired using a Bio-Rad Radiance 2000 system, and co-localization is shown in yellow. The bars shown represent 10 μm. The images shown are representative of 40 images taken from three independent experiments. (B) FLIM between Mac-1 and FcγRIIA on the cell surface. DMSO-differentiated HL60 cells were stained with the donor antibody (goat anti-FcγRIIA) in the absence (a, b) or presence of the acceptor antibodies specific for Mac-1 (OKM1) (c) or a control mouse IgG (d), followed by Alexa 488-anti-goat IgG and Alexa 568-anti-mouse IgG. (a) Surface fluorescence staining for FcγRIIA. (b−d) The FLIM images were taken at the *z*-section of the cell surface and presented in pseudocolors from red to blue, showing the fluorescence lifetimes (in ps) of Alexa 488 in the absence (b) or presence (c and d) of acceptor Alexa 568. (C) Mac-1 expression alters the spatial distribution of FcγRIIA relative to that of actin filaments. The adherent and fixed human 293 cells expressing either FcγRIIA alone (a), Mac-1 and FcγRIIA (b) or Mac-1 and FcγRIIA(CT-) (c, d) were stained with goat anti-human CD32 (a, b, and c) or mouse anti-Mac-1 (OKM1) (d), washed, and then permeabilized with 0.4% Triton X-100. These cells were then stained with Alexa 568-conjugated donkey anti-goat (a, b, and c) or goat anti-mouse IgG (d), respectively, and Alexa 488-phalloidin (for actin filaments). A series of 2D confocal fluorescence images at different *z*-sections were collected as in A, and 3D reconstruction was performed by deconvolution using the Volocity program. The bars shown represent 10 μm. The images shown are representative of approximately 20 images taken for each cell type. These experiments were repeated twice with similar results.

polyclonal antibody specific for GST. We found that GST-α_MI-domain but not the control GST bound soluble FcγRIIA, which could be blocked by the addition of NIF or an α_M-specific mAb 44a (Figure 4). When verifying specificity, GST-α_MI-domain did not bind to ICs in the absence of FcγRIIA, and a nonimmune control IgG or a β_2-specific mAb (IB4) had no effect on the α_MI-domain binding to FcγRIIA. Furthermore, no inhibition on α_MI-domain binding to FcγRIIA was observed for either NADG or its control glucose at 150 mM.

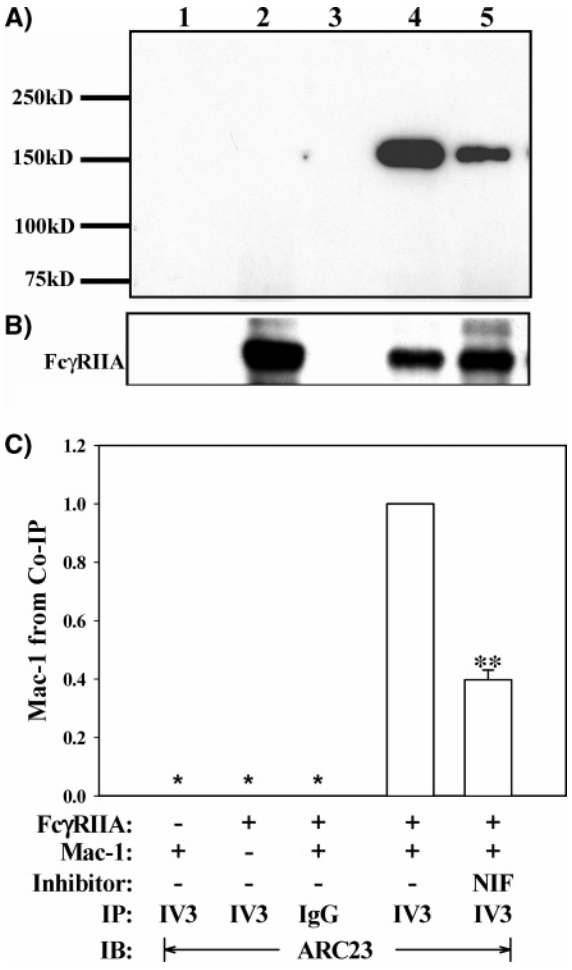

FIGURE 3: Mac-1 co-immunoprecipitates with FcγRIIA. Human 293 cells expressing Mac-1, FcγRIIA, or both in the presence or absence of NIF were cross-linked with DSP and then lysed in a cell lysis buffer. Co-IP was conducted using mAb IV3 against FcγRIIA, and the immunoprecipitated complex was separated by SDS−PAGE and immunoblotted with a polyclonal antibody (ARC23) against the cytoplasmic tail of the Mac-1 α subunit (A), and the membrane was stripped and re-probed with an anti-FcγRIIA antibody (B). Equal loading was confirmed by immunoblot with anti-actin antibody (not shown). The quantification of Mac-1 in co-IP was performed using an Image Station 2000R (Kodak) and expressed in arbitrary units (C). Data shown are the means ± SD of two independent experiments; (*), indicates a value below 0.001; (**), $p < 0.01$ by Student's *t*-test.

E^{253}-R^{261} Sequence within the α_MI-Domain Is Critical to Mac-1 Recognition of FcγRIIA. If the α_MI-domain is the major binding interface between Mac-1 and FcγRIIA, we anticipated that mutations within the α_MI-domain should abolish Mac-1 recognition of FcγRIIA. In addition, because NIF competed effectively with FcγRIIA for binding to the α_MI-domain (Figure 4), we expected that the FcγRIIA binding site may partially overlap the NIF binding site, which we localized previously to E^{253}-R^{261} and its surrounding sequences (*29*). Thus, we assessed if $\alpha_M(E^{253}$-$R^{261})\beta_2$, a mutant Mac-1 in which the E^{253}-R^{261} sequence (EDVI-PEADR) has been replaced with its homologous sequence from the α_LI-domain, could form a complex with FcγRIIA in the co-IP experiments. Our previous work confirmed that mutating E^{253}-R^{261} did not alter the gross conformation of

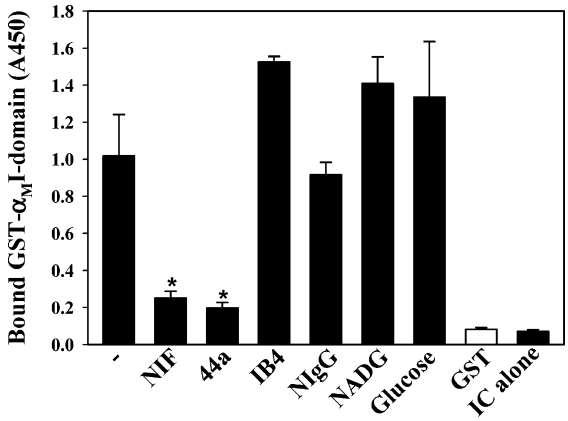

FIGURE 4: Direct binding of the α_MI-domain to soluble FcγRIIA. Soluble FcγRIIA was immobilized onto IC-coated 96-well microtiter plates, followed by blocking with BSA. GST or GST-α_MI-domain was then added in the presence or absence of NIF, mAb 44a (recognizing the α_MI-domain), mAb IB4 (recognizing the β_2 subunit), or nonimmune control IgG (NIgG) as well as NADG or its control glucose for 2 h at RT. The bound GST-α_MI-domain was detected using anti-GST conjugated to horseradish peroxidase (HRP) and the HRP substrate 3,3,5,5,-tetramethylbenzidine. The specificity of the assay was verified by the lack of GST binding to FcγRIIA. Data shown are the means ± SD of two independent experiments; (*), $p = 0.04$.

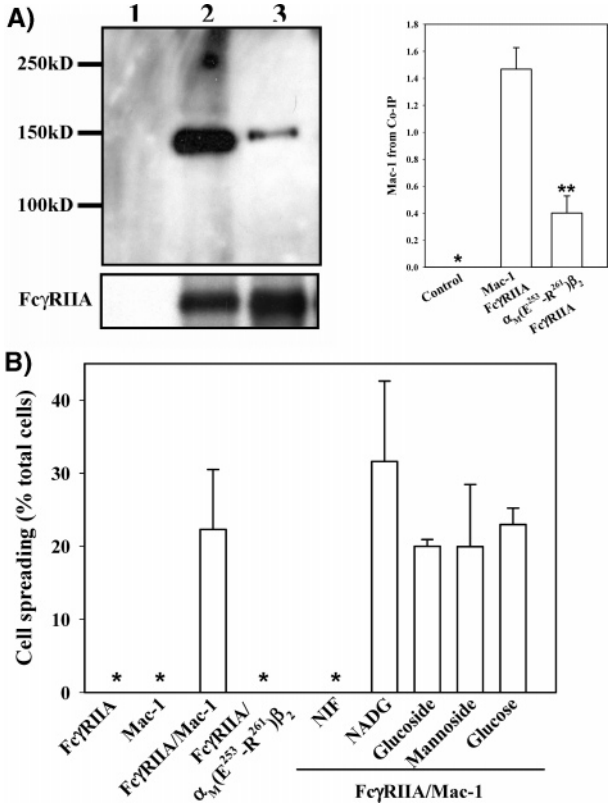

FIGURE 5: Critical role of segment E^{253}-R^{261} within the α_MI-domain in Mac-1/FcγRIIA recognition and in FcγRIIA-mediated cell spreading. (A) Co-IP between FcγRIIA and either WT or a mutant Mac-1, $\alpha_M(E^{253}$-$R^{261})\beta_2$, was conducted and quantified as mentioned above. The amount of FcγRIIA present in the co-IP was verified by stripping the membrane and re-probing with an anti-FcγRIIA antibody (bottom panel). The quantification of Mac-1 in co-IP was performed using an Image Station 2000R (Kodak) and expressed in arbitrary units (right panel). Data shown are the means ± SD of two independent experiments; (*), indicates a value below 0.001; (**), $p < 0.01$ by Student's *t*-test. (B) Cell spreading. The cells expressing FcγRIIA, Mac-1, FcγRIIA/Mac-1, or FcγRIIA/$\alpha_M(E^{253}$-$R^{261})\beta_2$ were allowed to adhere to IC-coated plates as described in Figure 1, in the presence or absence of NIF, NADG, β-methyl D-glucoside (gulcoside), α-methylmannoside (mannoside), or glucose at 37 °C for 50 min. Cell spreading was quantified manually under the phase-contrast microscope and confirmed by 3D confocal fluorescence microscopy; (*), indicates less than 0.5%. Data shown are the means ± SD of duplicate experiments and are representative of three independent experiments.

the $\alpha_M(E^{253}$-$R^{261})\beta_2$ mutant receptor, on the basis of studies using a panel of conformation-dependent mAbs (*29*). Figure 5A shows that unlike WT Mac-1 (lane 2) the significantly lower amount of $\alpha_M(E^{253}$-$R^{261})\beta_2$ was co-immunoprecipitated by FcγRIIA (lane 3). No protein was precipitated using an irrelevant control IgG, thus confirming the specificity of the assay (lane 1). Together, these data confirm a critical role of the α_MI-domain, especially the E^{253}-R^{261} sequence, in FcγRIIA binding to Mac-1.

Mac-1 Is Critical to FcγRIIA-Mediated Cell Spreading on ICs. To test the role of the α_MI-domain in Mac-1's ability to promote FcγRIIA-mediated cell spreading on ICs, we conducted cell spreading experiments in the presence or absence of different inhibitors, including NIF (for the α_MI-domain), NADG and α-methylmannoside (for the lectin-domain), and glucose. Cell spreading was initially assessed by phase contrast microscopy and then confirmed on the basis of 3D confocal fluorescence images as described in Figure 2B. No cell spreading was observed for cells expressing FcγRIIA or Mac-1 alone after incubation at 37 °C for 50 min (Figure 5B). In contrast, a significant number (~23%) of the cells that expressed both FcγRIIA and Mac-1 spread, which could be inhibited by the addition of NIF but not NADG, α-methylmannoside, or the control glucose, indicating that the α_MI-domain but not the lectin-domain plays a major role in promoting cell spreading. In further support of this conclusion, the α_MI-domain mutant $\alpha_M(E^{253}$-$R^{261})\beta_2$ that did not interact well with FcγRIIA (Figure 5A) failed to support FcγRIIA-mediated cell spreading (Figure 5B). Altogether, these data demonstrated that the α_MI-domain but not the lectin-domain of Mac-1 functions critically in the FcγRIIA-mediated cell spreading on ICs.

Mac-1 Functions to Promote FcγRIIA-Dependent Cell Migration on ICs. An Essential Role of the α_MI-Domain. Recent studies show that leukocyte migration to the site of inflammation is also mediated by FcRs. In addition, these investigators found that such an FcR-dependent leukocyte migration requires Mac-1 (*4*). Given our above observations that Mac-1, via its α_MI-domain, binds FcγRIIA and changes its relative spatial organization to the actin filaments, we anticipated that the α_MI-domain may also be critical to FcγRIIA-dependent cell migration on ICs. To examine this possibility, we performed cell migration experiments using human 293 cells that express FcγRIIA or Mac-1 alone, FcγRIIA plus Mac-1, and different combinations of FcγRIIA and Mac-1 mutants. The migration assay was performed on IC-coated 24-well plates at 37 °C and 5% CO_2 for 10 h with time-lapse images captured at 2-minute intervals, and the migration of these different cells were quantified by randomly picking four view fields and calculating the net linear distances migrated by the cells within these fields using MetaMorph. The results showed that the FcγRIIA-expressing cells did not spread or form cell extensions on ICs. These cells oscillated randomly at their original positions, and no significant movement was observed. The cells expressing both FcγRIIA and Mac-1 spread and formed filopodial extensions and exhibited enhanced cell migration (Figure 6). To help study the role of Mac-1 in FcγRIIA-dependent migration and to exclude potential contributions of the FcγRIIA-ITAM-mediated intracellular signaling to cell migration, we established additional cell lines that express the cytoplasmic domain-truncated FcγRIIA, FcγRIIA(CT-), with or without Mac-1. The removal of the cytoplasmic tail in the absence of Mac-1 slightly enhanced cell migration (Figure 6). In further support of Mac-1 binding to FcγRIIA via its extracellular α_MI-domain, the cytoplasmic domain deletion of FcγRIIA had no effect on its co-localization with Mac-1 (Figure 2A, Panel c). Most importantly, FcγRIIA-(CT-) was still capable of relocating to the focal adhesion sites/filopodial extensions (Figure 2C, Panel c) in a manner similar to that of Mac-1 (Figure 2C, Panel d). These results suggest that FcγRIIA linkage to the cytoskeleton is likely mediated by Mac-1 and not via its own cytoplasmic domain. Interestingly, the cells expressing FcγRIIA(CT-) plus Mac-1 spread very well and migrated actively on ICs (Figure 6; $p < 0.01$, 269 cells counted). Most importantly, we found that the migration of the cells expressing FcγRIIA(CT-) and Mac-1 was inhibited by the addition of NIF (Figure 6; $p < 0.01$, 102 cells counted) and by mutating the E^{253}-R^{261} sequence within the α_MI-domain (Figure 6; $p < 0.04$, 124

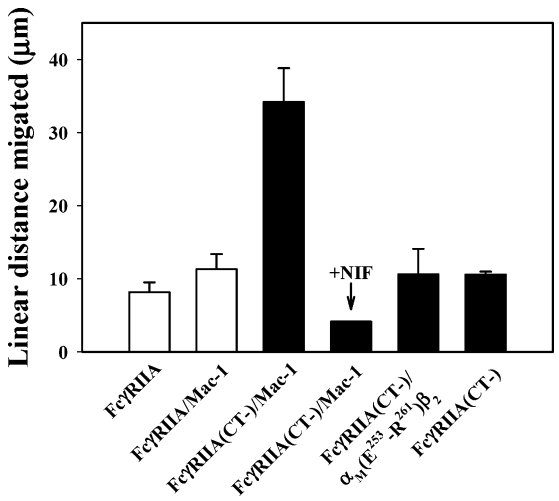

FIGURE 6: Mac-1 promotes FcγRIIA-dependent cell migration. Cells expressing different combinations of FcγRIIA or its mutant FcγRIIA (CT-) with Mac-1 or its mutant $\alpha_M(E^{253}\text{-}R^{261})\beta_2$ were allowed to adhere to ICs on a 24-well nontissue culture plate at 37 °C with or without NIF. After washing to remove the nonadherent cells, cell migration was monitored using the Neue LiveCell System at 37 °C and 5% CO_2. Time lapse images were taken with a 10× objective every 2 min. To quantify cell migration, three to four random fields with a minimum of 70 cells were selected for each experiment, and the net linear distance migrated was calculated for each individual cell using Metaview (Universal Imaging) and then averaged. Data shown are the means ± SD of three independent experiments.

cells counted), indicating that the α_MI-domain plays a critical role in Mac-1's ability to promote cell migration on ICs.

DISCUSSION

The major findings of this study are (1) Mac-1 binds directly to FcγRIIA via its α_MI-domain, a mechanism that is unique among the many FcRs; (2) the FcγRIIA binding interface is located within a region surrounding residues E^{253}-R^{261}; and (3) Mac-1 binding to FcγRIIA changes the spatial distribution of FcγRIIA relative to that of actin filaments and, thereby, potentially contributing to FcγRIIA-mediated sustained cell adhesion, cell spreading, and cell migration on IC-deposited targets. Thus, our study represents the first molecular characterization of Mac-1 binding to FcγRIIA and provides direct evidence for a physical interaction between Mac-1 and FcγRIIA, which may be useful to our understanding of the critical role of Mac-1 in FcγRIIA-mediated tumor cell killing by ADCC (*1*).

Mac-1 and FcRs are the two major receptors involved in innate immunity against foreign pathogens including phagocytosis, the production of reactive oxygen intermediates, and various cytolytic enzymes as well as ADCC. Although each of the receptors recognizes its own ligands (C3bi and the ICs, respectively) and elicits distinct biological responses, numerous studies have demonstrated that Mac-1 and FcRs function cooperatively to promote cell killing (*2*−*8*, *14*−*16*). Interactions between FcRs and Mac-1 are mostly studied indirectly by their ability to co-cap on the cell surface (*15*) and by the ability of Mac-1 to promote FcR-mediated leukocyte functions, including phagocytosis (*6*, *18*), activation (*5*), and ADCC (*6*, *8*). However, no biochemical studies demonstrating a direct interaction between Mac-1 and FcRs have been reported. Primarily on the basis of inhibition studies with the lectin-domain-specific inhibitors such as NADG, it is generally believed that FcRs interact with Mac-1 via its lectin domain (*15*). However, because NADG does not block Mac-1's ability to promote FcγRIIA-mediated phagocytosis (*18*), the molecular basis underlying FcγRIIA recognition of Mac-1 is unknown. In this work, we found that FcγRIIA interacts primarily with the α_MI-domain but not the lectin-domain of Mac-1, which agrees well with an earlier study (*18*). In addition to our study, the binding of soluble FcγRIIIA (CD16) to surface Mac-1 and CD11C/CD18 based exclusively on FACS analysis has been reported (*14*). However, CD16 binding to Mac-1 can be inhibited by antagonists that are specific for either the α_MI-domain (mAb 904) or the lectin-domain (NADG) (*14*). Furthermore, no direct interaction between Mac-1 and murine CD16 was found in a recent study (*30*). Thus, more studies are needed to clarify this confusion and to definitively localize the CD16 binding site within Mac-1.

The switchblade model of integrin activation predicts that active integrin exists in an extended conformation (*31*), where the α_MI-domain likely resides approximately ∼250 Å above the cell membrane (*32*). Given the relatively small size (a total of ∼170 residues in D1 and D2) of FcγRIIA (*33*), it is not clear how these two receptors interact with each other above the cell membrane. One possibility is that the FcγRIIA on one cell interacts with the Mac-1 on another cell, which we consider unlikely on the basis of the following observations: (1) the FcγRIIA- and Mac-1-expressing cells did not undergo heterotypical aggregations (unpublished observations); (2) cell spreading and migration requires the coexpression of Mac-1 with FcγRIIA on the same cell (Figures 5B and 6); and (3) Mac-1 colocalizes with FcγRIIA at the cell extensions of individual cells (Figure 2A). Another possibility is that, analogous to the interaction between uPAR and integrins (*34*), FcγRIIA may interact with the integrins that exist in the bent conformation, where the α_MI-domain would reside very close to the membrane (*32*). Should this be the case, FcγRIIA, like uPAR, might also be capable of modulating the activity of Mac-1. Further experiments will be required to test these different hypotheses.

Our data shows that Mac-1 binding to FcγRIIA alters the spatial distribution of FcγRIIA relative to that of actin filaments (Figure 2B) and, thereby, promotes its sustained cell adhesion, spreading, and migration on ICs (Figs. 1B, 5B, and 6). Consistent with our observations, Kusunoki et al. reported that sustained neutrophil adhesion to ICs is mediated by FcγRIIA but not by FcγRIII and requires the presence of Mac-1 (*35*). An alternative mechanism is that FcγRIIA functions indirectly to promote cell spreading and migration by activating Mac-1, which in turn binds to ICs. However, our observations that (1) Mac-1 did not support cell adhesion to ICs with or without activation (Figure 1A); (2) the FcγRIIA-specific blocking antibody IV3 inhibited Mac-1-mediated cell adhesion by differentiated HL60 in the presence of PMA (data not shown); and (3) the recombinant α_MI-domain did not interact directly with ICs in solid-phase binding assays (Figure 4) suggest that ICs are not ligands of Mac-1. It is possible that FcγRIIA may initiate intracellular signaling, such as the activation of Ca^{2+} influx, PI-3K, and MAPK (*14*), via its ITAM sequence or via the engagement of Mac-1 (outside-in signaling) and, thereby, contribute to sustained cell adhesion, spreading, and migration. In this

regard, our observation that the deletion of the entire cytoplasmic tail of FcγRIIA including the ITAM sequence did not abrogate but rather promoted cell migration (Figure 6) is intriguing. It is likely that the ITAM sequence, which has been reported to recruit both SHP-1 and SHIP, may actually inhibit sustained cell adhesion, spreading, and migration. Further experiments to investigate these possible mechanisms are underway.

In summary, we demonstrated that Mac-1 promotes FcγRIIA-mediated functions, including cell adhesion, spreading, and migration, by binding directly to FcγRIIA and modulating its surface distribution. We identified a novel binding site within Mac-1 for FcγRIIA, which is located within the α_MI-domain but not the lectin domain. We have for the first time demonstrated a direct interaction between Mac-1 and FcγRIIA on the basis of both co-immunoprecipitation experiments using total cell lysates and solid-phase binding assays using purified proteins. The information provided in this study may help us better understand how Mac-1 contributes to FcγRIIA-mediated functions and, thus, may be useful in the design of therapeutic agents that can either enhance the leukocyte killing of invading bacteria and fungi as well as malignant cells or block the deleterious FcR-induced autoimmune diseases.

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