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Mac-1 promotes FcγRIIA-dependent cell spreading and migration on immune complexes

Ying Xiong¹, Chunzhang Cao¹, Alexandra Makarova², Brad Hyman², and Li Zhang^{1,*}

¹ Center for Vascular and Inflammatory Diseases, and Department of Physiology, University of Maryland School of Medicine, 800 W Baltimore Street, Baltimore, MD 21201, USA

² Alzheimer's Disease Research Laboratory, Harvard Medical School, Charlestown, MA 02129, USA

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 pre-formed 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 the FcγRIIA-mediated cell spreading and migration. 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 E²⁵³-R²⁶¹ 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 cytotoxicity of the 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, 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 (α_Mβ₂, CR3, CD11b/CD18) (1,8,12,13). Despite the importance of Mac-1 in the 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 Mac-1 recognition of these FcRs is still less understood. Based primarily on inhibition studies using

* Corresponding author: Li Zhang, Department of Physiology, University of Maryland School of Medicine, 15601 Crabbs Branch Way, Rockville, MD 20855; Telephone: 301-738-0657; FAX: 301-738-0465; E-Mail: LiZhang@SOM.UMARYLAND.EDU.

¹FcR, 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.

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 Fc γ RIIA-mediated phagocytosis is insensitive to NADG inhibition (18), the nature of the Fc γ RIIA binding site within Mac-1 remains elusive.

Given the importance of Mac-1 in both Fc γ RIIA-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 Fc γ RIIA-mediated processes, including sustained adhesion, cell spreading and cell migration on ICs. Here, we report the first biochemical evidence for a direct interaction between Fc γ RIIA and Mac-1, including 1) co-localization of these two proteins on the cell surface, 2) co-immunoprecipitation (co-IP) as a complex; 3) direct interaction between the recombinant α_M I-domain of Mac-1 and soluble Fc γ RIIA, and 4) identification of the E²⁵³-R²⁶¹ sequence within Mac-1 that is critical for Fc γ RIIA recognition. Moreover, our data shows that Mac-1 binding to Fc γ RIIA changes the spatial organization of Fc γ RIIA relative to actin filaments, and thereby potentially facilitates sustained cell adhesion, cell spreading, and cell migration toward IC-sensitized tumor cells, which in combination results in effective cytolysis of the malignant cells via Fc γ RIIA-mediated ADCC.

EXPERIMENTAL PROCEDURES

Materials

Human kidney 293 cells and the expression vector pCIS2XN were gifts from Dr. F. J. Castellino (Notre 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). mAbs IV3, 44a, OKM1, M1/70 and IB4, as well as HL60 cells were from ATCC (Rockville, MD). All other reagents were the highest grade available from Sigma Chemical Co. (St Louis, MO) unless otherwise noted.

Establishment of stable cell lines

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 Fc γ RIIA), and by morphological examination of Hema3 (Fisher Scientific)-stained Cytospin (Shandon) smears. To establish human 293 cell lines that express both Fc γ RIIA and Mac-1, the Mac-1-expressing 293 cells, which were established previously in the laboratory (19), were transfected with the expression vector pCIS2XN-Fc γ RIIA, where the cDNA of Fc γ RIIA 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 Fc γ RIIA, the extracellular and transmembrane domains of Fc γ RIIA were amplified by PCR using primers 5' AATCTAGACCATGGCTATGGAGACCCAAATGTCTCAG 3' and 5' ACGTAGCGGCCGCTTACCTGCAGTAGATCAACGCCACTACAGCAGCAACAAT 3', and the amplified fragments were inserted into pCIS2XN as described above. The correctness of the inserted Fc γ RIIA 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 Fc γ RIIA-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 non-tissue culture plates. Briefly, a 24-well polystyrene plate (BD Biosciences) was coated in the center with 70 μ l bovine serum albumin (BSA, 1mg/ml) and followed by 70 μ l anti-BSA IgG (150 μ g/ml in DPBS) (16). The plate was

then blocked with 300 μ l of 0.5% polyvinylpyrrolidone (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 μ l of Hanks' balanced salt solution (HBSS) containing 1mM CaCl₂, 1mM MgCl₂ and 10 mM HEPES in the presence or absence of 10 μ g/ml 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 μ l of 4% paraformaldehyde (PFA) was added to the plates to terminate cell adhesion/detachment, and the non-adherent 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 to 60 minutes, and then fixed with 4% PFA. Cell spreading was quantified under phase contrast microscope (20X objectives) based on four randomly picked fields in each well, and calculated as the ratio of spread vs total cells. Cell spreading was further verified by three-dimensional confocal fluorescence imaging as described below.

Immunoprecipitation

Single cell suspension, prepared from 293 cells expressing Fc γ RIIA or Fc γ RIIA/Mac-1, were incubated with NIF, NADG, or glucose in HBSS containing 10mM HEPES, 1mM CaCl₂ and 1mM MgCl₂ at room temperature for 15 min, and then their surface receptors were crosslinked with a 12Å-long thiol-cleavable and amine-reactive crosslinker DSP (2mM) for 30 min. After washing, the residual active crosslinker was inactivated with 100mM ethanolamine, pH8 in the above HBSS buffer and then the cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 2 mM Na₃VO₄, 10 μ g/ml leupetin, 10 μ g/ml aprotinin, and 1 mM PMSF). The cell lysates, prepared by centrifugation, was then incubated with an Fc γ RIIA-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 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 Fc γ RIIA with and without Mac-1 were allowed to adhere to the ICs on a 24-well non-tissue culture plate at 37°C for 25 min in F-12/DMEM containing media alone, NIF (100nM), NADG (150mM) or glucose (150mM), as described above for the cell adhesion assays. After washing to remove the non-adherent cells, cell migration was monitored using the Neue LiveCell System (Camp Hill, PA) at 37°C and 5% CO₂ for 10 hours. 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 Fc γ RIIA 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 filament (Molecular probes) after permeabilization with 0.4% Triton X-100. 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 three-dimensional image reconstruction were performed using Velocity (Improvision).

Fluorescence lifetime imaging microscopy (FLIM)

Cells were stained with goat anti-human CD32, with or without mouse anti-Mac-1 (OKM1) or a control mouse IgG, and 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 800nm was empirically determined to excite Alexa 488, but not Alexa 568. 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, Alexa 488 lifetime was measured in the absence of acceptor (Alexa 568), which showed lifetimes equivalent to FITC-IgG alone or in solution (20).

FACS analysis

A total of 10^6 cells expressing expressing Fc γ RIIA, Mac-1, or Fc γ RIIA plus Mac-1 in HBSS were incubated with 1 μ g mAb IV3 (for Fc γ RIIA) or OKM1 (for Mac-1) for 30 min. A subtype-matched mouse IgG served as a control. After washing with PBS, cells were mixed with RPE-conjugated Donkey anti-mouse IgG (Jackson ImmunoResearch) for another 30 min. Cells were then washed and resuspended in 500 μ l 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 Fc γ RIIA

The Fc γ RIIA 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-sFc γ RIIA 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 sFc γ RIIA in the condition media was determined by immunoblot using goat anti-human CD32 antibody. For large scale production, sFc γ RIIA was purified using an IV3 affinity column. The purity of the recombinant sFc γ RIIA was verified by both SDS-PAGE and by immunoblot as above.

Solid phase binding assays

To test direct interactions between soluble Fc γ RIIA and the α_M I-domain, 96-well microtiter plates (Immulon 4B, Dynex Technologies Inc, Chantilly, VA) were coated with the ICs as described above and followed by incubation with soluble Fc γ RIIA. After washing and blocking with 2% BSA, GST or GST- α_M I-domain (10 μ g/ml; produced in *E. coli* (21)) in 1% BSA-TBST (20 mM Tris, 150 mM NaCl, 1mM Ca²⁺, 1 mM Mg²⁺, 0.05% Tween 20, pH 7.4), was added in the presence or absence of different inhibitors and incubated for 2–3 hours at RT. After washing with TBST, bound GST- α_M I-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_M I-domain and blocked with BSA. Soluble Fc γ RIIA was then added and incubated at RT for 2 hours. After washing, bound Fc γ RIIA was detected using goat anti-human CD32, and 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 non-tissue culture plates, and the adherent cells were quantified by staining with crystal violet as we described previously (19). In addition, 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 (Fig. 1A). In contrast, no specific adhesion to ICs was observed for Mac-1-expressing cells, even upon integrin activation by addition of a β₂-activating antibody (MEM48) (24) (Fig. 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. Addition of the Mac-1-specific inhibitor NIF had no effect (Fig. 1A), although it completely blocked Mac-1-mediated cell adhesion to the Mac-1 ligand fibrinogen (data not shown). Together, these results demonstrated that 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 non-adherent 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 minutes (peak adhesion; assigned as time 0 of cell detachment), and thereafter they gradually detached from the IC-coated plates. 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 (Fig. 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 (Fig. 1B). In contrast, the FcγRIIA/Mac-1-expressing cells maintained adhesion and did not detach for an extended period of time (more than 60 minutes), indicating that Mac-1 is required for sustained cell adhesion (Fig. 1B). Confirming the specific requirement of Mac-1 in this process, treatment of the FcγRIIA/Mac-1-expressing cells with NIF, an α_MI-domain-specific inhibitor of Mac-1 (21), abolished the sustained adhesion, but did not affect the initial cell adhesion (Fig 1B). Furthermore, addition of the FcγRIIA-specific mAb IV3 completely blocked cell adhesion, even in the presence of Mac-1 (Fig 1B). Since Mac-1 is known to interact with the 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). Addition of NADG or α-methylmannoside (Mannoside) at 150mM, a concentration that is effective in blocking co-capping between Mac-1 and FcγRIII (17), had no effect on sustained cell adhesion. Similarly, addition of its control polysaccharide glucose did not have any effect either (Fig. 1C). These results suggest that the ability of Mac-1 to sustain

the Fc γ RIIA-mediated cell adhesion is dependent on the α_M I-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 Fc γ RIIA (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 into the plates to stop cell adhesion/detachment. The non-adherent cells were then removed by washing and the remaining adherent cells were quantified. Results from these experiments showed that similar to the 293 cells expressing recombinant Fc γ RIIA and Mac-1, the differentiated HL60 cells maintained adhesion to the ICs for an extended period of time and addition of NIF significantly accelerated cell detachment ($p=0.01$ at 35 min and $p<0.001$ at 55 min and 75 min) (Fig 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 Fc γ RIIA on the cell surface

Cell adhesion and detachment are controlled by actin polymerization and the re-organization of the cytoskeleton. To see if Mac-1 prolongs Fc γ RIIA-mediated cell adhesion by changing the spatial organization between Fc γ RIIA and actin polymerization, we visualized the distributions of Mac-1, Fc γ RIIA, and actin filaments by confocal laser scanning fluorescence microscopy. For these experiments, the Fc γ RIIA/Mac-1-expressing cells were allowed to adhere to the ICs on coverslip and then fixed with paraformaldehyde. Surface Fc γ RIIA and Mac-1 was stained with a goat anti-human CD32 (for Fc γ RIIA) and a murine mAb OKM1 (for Mac-1) respectively, followed by their corresponding secondary antibodies conjugated to either Alexa 488 or Alexa 568. The results showed that Fc γ RIIA (in green) co-localized well with WT Mac-1 (in red) on the cell surface (top panels). Verifying the specificity of the immunostaining, no fluorescence was observed for non-immune control IgG-stained samples (bottom panels) under the same instrument settings. To see if Mac-1 also colocalizes with Fc γ RIIA on DMSO-differentiated HL60 cells, confocal fluorescence microscopy experiments were conducted and representative confocal images were presented in Fig. 2A (Panel b). Overall, approximately 80% Mac-1 were colocalized with Fc γ RIIA, and strong colocalization was primarily found at focal adhesion sites and in filopodial extensions.

To further corroborate the above finding, we measured molecular proximity between Mac-1 and Fc γ RIIA on the HL60 cell surface by fluorescence lifetime imaging microscopy (FLIM) (20). This high resolution imaging technique is based on fluorescence resonance energy transfer (FRET) that occurs if two fluorophores are in very close proximity ($<100\text{\AA}$), resulting in the shortening of the donor fluorescence lifetime. Accordingly, we labeled DMSO-differentiated HL60 cells simultaneously with a goat anti-Fc γ RIIA 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-Fc γ RIIA 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 Fc γ RIIA resided $<100\text{\AA}$ from each other on the cell surface. Fig. 2B shows a representative pseudocolor-coded images of surface co-localization between Mac-1 and Fc γ RIIA (i.e. the z-section is set at the height of the cell surface), where uniformly bluish image (thus long fluorescence lifetime) was obtained in the absence of the acceptor (Panel b) or in the presence of a non-immune control IgG (Panel d), and a red to green-filled image representing 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 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 two dimensional (x-y) confocal fluorescence images at different z-positions were collected, and these images were then used to construct three-dimensional images by deconvolution using Volocity, which allows us to better visualize the spatial distributions of FcγRIIA and the actin filament. As shown in Fig. 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, co-expression 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). Taking 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-immunoprecipitation 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 (Fig. 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 crosslinked by a short-length thiol-cleavable crosslinker (spacer length=12Å). 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 immunoprecipitates 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 (Fig. 3A), thus verifying the specificity of the co-IP. Further confirming the specificity, the 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 less Mac-1 was obtained (Fig. 3C). Immunoblot with an anti-actin antibody demonstrated equal loading (data not shown), and stripping and re-probing of the above PVDF membrane with a goat anti-CD32 antibody confirmed the presence of FcγRIIA in the immunoprecipitation mixtures (Fig. 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 (Fig. 3) and blocked Mac-1's ability to sustain the FcγRIIA-mediated cell adhesion to the ICs (Fig. 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 as all other FcRs interact with the lectin-like domain of Mac-1 (13,15, 17). To determine if 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 pre-coated onto a 96-well plate. After blocking with BSA, GST or GST- α_{MI} -domain was added in the presence of 1mM Ca^{2+} and 1mM Mg^{2+} to the IC-coated wells, and bound α_{MI} -domain was detected using a 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 addition of NIF or an α_M -specific mAb 44a (Fig. 4). Verifying

specificity, GST- α_M I-domain did not bind to ICs in the absence of Fc γ RIIA, and a non-immune control IgG or a β_2 -specific mAb (IB4) had no effect on the α_M I-domain binding to Fc γ RIIA. Furthermore, no inhibition on α_M I-domain binding to Fc γ RIIA was observed for either NADG or its control glucose at 150mM.

The E²⁵³-R²⁶¹ sequence within the α_M I-domain is critical to Mac-1 recognition of Fc γ RIIA

If the α_M I-domain is the major binding interface between Mac-1 and Fc γ RIIA, we anticipated that mutations within the α_M I-domain should abolish Mac-1 recognition of Fc γ RIIA. In addition, since NIF competed effectively with Fc γ RIIA for binding to the α_M I-domain (Fig. 4), we expected that the Fc γ RIIA binding site may partially overlap the NIF binding site, which was localized previously by us to E²⁵³-R²⁶¹ and its surrounding sequences (29). Thus, we assessed if α_M (E²⁵³-R²⁶¹) β_2 , a mutant Mac-1 in which the E²⁵³-R²⁶¹ sequence (EDVIPEADR) has been replaced with its homologous sequence from the α_L I-domain, could form a complex with Fc γ RIIA in the co-IP experiments. Our previous work confirmed that mutating E²⁵³-R²⁶¹ did not alter the gross conformation of the α_M (E²⁵³-R²⁶¹) β_2 mutant receptor, based on studies using a panel of conformation-dependent mAbs (29). Fig. 5A shows that unlike WT Mac-1 (lane 2), significant less amount of α_M (E²⁵³-R²⁶¹) β_2 was co-immunoprecipitated by Fc γ RIIA (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_M I-domain, especially the E²⁵³-R²⁶¹ sequence, in Fc γ RIIA binding to Mac-1.

Mac-1 is critical to Fc γ RIIA-mediated cell spreading on ICs

To test the role of the α_M I-domain in Mac-1's ability to promote Fc γ RIIA-mediated cell spreading on ICs, we conducted cell spreading experiments in the presence or absence of different inhibitors, including NIF (for the α_M I-domain), NADG and α -methylmannoside (for the lectin-domain), and glucose. Cell spreading was initially assessed by phase contrast microscopy and then confirmed based on three-dimensional confocal fluorescence images as described in Fig. 2B. No cell spreading was observed for cells expressing Fc γ RIIA or Mac-1 alone after incubation at 37 °C for 50 minutes (Fig. 5B). In contrast, a significant number (~23%) of the cells that expressed both Fc γ RIIA and Mac-1 spread, which could be inhibited by addition of NIF, but not NADG, α -methylmannoside or the control glucose, indicating that the α_M I-domain but not the lectin-domain plays a major role in promoting cell spreading. In further support of this conclusion, the α_M I-domain mutant α_M (E²⁵³-R²⁶¹) β_2 , that did not interact well with Fc γ RIIA (Fig. 5A), failed to support Fc γ RIIA-mediated cell spreading (Fig. 5B). Altogether, these data demonstrated that the α_M I-domain but not the lectin-domain of Mac-1 functions critically in the Fc γ RIIA-mediated cell spreading on ICs.

Mac-1 functions to promote Fc γ RIIA-dependent cell migration on ICs: an essential role of the α_M I-domain

Recent studies show that leukocyte migration to the site of inflammation is also mediated by the 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_M I-domain, binds Fc γ RIIA and changes its relative spatial organization to the actin filaments, we anticipated that the α_M I-domain may also be critical to the Fc γ RIIA-dependent cell migration on ICs. To examine this possibility, we performed cell migration experiments using human 293 cells that express Fc γ RIIA or Mac-1 alone, Fc γ RIIA plus Mac-1, and different combinations of Fc γ RIIA and Mac-1 mutants. The migration assay was performed on IC-coated 24-well plates at 37°C and 5% CO₂ for 10 hours with time-lapse images captured at 2-minute intervals, and 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 Fc γ RIIA-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 Fc γ RIIA and Mac-1 spread and formed filopodial extensions, and exhibited enhanced cell migration (Fig. 6). To help study the role of Mac-1 in Fc γ RIIA-dependent migration, and to exclude potential contributions of the Fc γ RIIA-ITAM-mediated intracellular signaling to cell migration, we established additional cell lines that express the cytoplasmic domain-truncated Fc γ RIIA, Fc γ RIIA(CT-), with or without Mac-1. Removal of the cytoplasmic tail in the absence of Mac-1 slightly enhanced cell migration (Fig. 6). In further support of Mac-1 binding to Fc γ RIIA via its extracellular α _MI-domain, cytoplasmic domain deletion of Fc γ RIIA had no effect on its colocalization with Mac-1 (Fig. 2A, panel c). Most importantly, Fc γ RIIA(CT-) was still capable of relocating to the focal adhesion sites/filopodial extensions (Fig. 2C, panel c) in a manner similar to that of Mac-1 (Fig. 2C, panel d). These results suggest that Fc γ RIIA linkage to the cytoskeleton is likely mediated by Mac-1 and not via its own cytoplasmic domain. Interestingly, cells expressing Fc γ RIIA(CT-) plus Mac-1 spread very well and migrated actively on ICs (Fig. 6; $p < 0.01$, 269 cells counted). Most importantly, we found that migration of the cells expressing Fc γ RIIA(CT-) and Mac-1 was inhibited by addition of NIF (Fig. 6; $p < 0.01$, 102 cells counted), and by mutating the E²⁵³-R²⁶¹ sequence within the α _MI-domain (Fig. 6; $p < 0.04$, 124 cells counted), indicating that the α _MI-domain plays a critical 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 Fc γ RIIA via its α _MI-domain, a mechanism that is unique among the many FcRs; 2) the Fc γ RIIA binding interface is located within a region surrounding residues E²⁵³-R²⁶¹; and 3) Mac-1 binding to Fc γ RIIA changes the spatial distribution of Fc γ RIIA relative to the actin filaments, and thereby potentially contributes to the Fc γ RIIA-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 Fc γ RIIA and provides direct evidence for a physical interaction between Mac-1 and Fc γ RIIA, which may be useful to our understanding of the critical role of Mac-1 in Fc γ RIIA-mediated tumor cell killing by ADCC (1).

Mac-1 and the FcRs are the two major receptors involved in innate immunity against foreign pathogens, including phagocytosis, 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. Based primarily on the 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, since NADG does not block Mac-1's ability to promote Fc γ RIIA-mediated phagocytosis (18), the molecular basis underlying Fc γ RIIA recognition of Mac-1 is unknown. In this work, we found that Fc γ RIIA interacts primarily with the α _MI-domain, but not the lectin-domain of Mac-1, which agrees well with this earlier study (18). In addition to our study, binding of soluble Fc γ RIIIA (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 the active integrin exists in an extended conformation (31), where the α_M I-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 Fc γ RIIA (33), it is not clear how these two receptors interact with each other above the cell membrane. One possibility is that Fc γ RIIA on one cell interacts with Mac-1 on another cell, which we consider unlikely, based on the following observations: 1) the Fc γ RIIA- and Mac-1-expressing cells did not undergo heterotypical aggregations (unpublished observation); 2) cell spreading and migration requires co-expression of Mac-1 with Fc γ RIIA on the same cell (Fig. 5B and Fig. 6); and 3) Mac-1 colocalizes with Fc γ RIIA at the cell extensions of individual cells (Fig. 2A). Another possibility is that, analogous to the interaction between uPAR and integrins (34), Fc γ RIIA may interact with the integrins that exist in the bent conformation, where the α_M I-domain would reside very close to the membrane (32). Should this be the case, Fc γ RIIA, 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 Fc γ RIIA alters the spatial distribution of Fc γ RIIA relative to actin filaments (Fig. 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 Fc γ RIIA but not by Fc γ RIII, and requires the presence of Mac-1 (35). An alternative mechanism is that Fc γ RIIA 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 (Fig. 1A); 2) the Fc γ RIIA-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_M I-domain did not interact directly with the ICs in solid-phase binding assays (Fig. 4) suggest that the ICs are not a ligand of Mac-1. Thirdly, it is possible that Fc γ RIIA may initiate intracellular signaling, such as activation of Ca^{2+} influx, PI-3K, and MAPK (14), via its ITAM sequence or via engagement of Mac-1 (“outside-in” signaling), and thereby contribute to the sustained cell adhesion, spreading and migration. In this regard, our observation that deletion of the entire cytoplasmic tail of Fc γ RIIA, including the ITAM sequence, did not abrogate, but rather promoted cell migration (Fig. 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 the Fc γ RIIA-mediated functions, including cell adhesion, spreading and migration by binding directly to Fc γ RIIA and modulating its surface distribution. We identified a novel binding site within Mac-1 for Fc γ RIIA that is located within the α_M I-domain but not the lectin domain. We have for the first time demonstrated a direct interaction between Mac-1 and Fc γ RIIA, based on 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 the Fc γ RIIA-mediated functions, and thus may be useful in the design of therapeutic agents that can either enhance leukocyte killing of invading bacteria and fungi as well as malignant cells, or block the deleterious FcR-induced autoimmune diseases.

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A)

B)

C)

D)

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 non-tissue 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 non-immune IgG for 20 min at 37°C. After washing, the adherent cells were quantified by the crystal violet method. 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 the 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 Fc γ RIIA/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 the IC-coated plates in the presence or absence of NIF at 37°C. At different time points, cell detachment was determined as above. *, $p < 0.01$; **, $p < 0.001$ by Student's t-test. Data shown are the means $\pm$ SD of three independent experiments.

A)

Figure 2. Expression of Mac-1 alters spatial distribution of Fc γ RIIA

A) Co-localization between Mac-1 and Fc γ RIIA. Human 293 cells expressing Mac-1/Fc γ RIIA (a and d) or Mac-1/Fc γ RIIA(CT-) (c), and DMSO-differentiated HL60 cells (b) were allowed to adhere to ICs, fixed with 2% PFA and then stained for Fc γ RIIA (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 Bio-Rad Radiance 2000 system and co-localization was shown in yellow. Bars shown represent 10 μ m. The images shown are representative of 40 images taken from three independent experiments. B) FLIM between Mac-1 and Fc γ RIIA on the cell surface. DMSO-differentiated HL60 cells were stained with the donor antibody (goat anti-Fc γ RIIA) in

the absence (a, b) or the presence of the acceptor antibodies specific for Mac-1 (OKM1) (c) or a control mouse IgG (d), followed with Alexa 488-anti-goat IgG and Alexa 568-anti-mouse IgG. a) Surface fluorescence staining for Fc γ RIIA. b–d) 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 picoseconds) of Alexa 488 in the absence (b) or presence (c and d) of the acceptor Alexa 568. C) Mac-1 expression alters the spatial distribution of Fc γ RIIA relative to actin filaments. The adherent and fixed human 293 cells expressing either Fc γ RIIA alone (a), Mac-1 and Fc γ RIIA (b) or Mac-1 and Fc γ RIIA(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 two dimensional confocal fluorescence images at different z-sections were collected as in A), and three-dimensional reconstruction was performed by deconvolution using the Volocity program. Bars shown represent 10 μ m. The images shown are representative of approximately 20 images taken for each cell type. These experiments were repeated twice with similar results.

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 crosslinked with DSP and then lysed in 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). 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 $\pm$ SD of two independent experiments. * indicates a value below 0.001; **, $p < 0.01$ by Student's t-test.

Figure 4. Direct binding of the α_M I-domain to soluble Fc γ RIIA

Soluble Fc γ RIIA was immobilized onto IC-coated 96-well microtiter plates, followed by blocking with BSA. GST or GST- α_M I-domain was then added in the presence or absence of NIF, mAb 44a (recognizing the α_M I-domain), mAb IB4 (recognizing the β_2 subunit), or non-immune control IgG (NIGG), as well as NADG or its control glucose for 2 hours at RT. The bound GST- α_M I-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 lack of GST binding to Fc γ RIIA. Data shown are the means $\pm$ SD of two independent experiments. *, $P=0.04$.

A)

B)

Figure 5. A critical role of segment E²⁵³-R²⁶¹ within the α_M I-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 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). 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 $\pm$ SD of two independent experiments. * indicates a value below 0.001; **, $p < 0.01$ by Student's t-test. B) Cell spreading. 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 Fig. 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 three-dimensional confocal fluorescence microscopy. * indicates less than 0.5%. Data shown are the means $\pm$ SD of duplicate experiments and are representative of three independent experiments.

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 α_M(E²⁵³-R²⁶¹)β₂ were allowed to adhere to the ICs on a 24-well non-tissue culture plate at 37°C with or without NIF. After washing to remove the non-adherent cells, cell migration was monitored using the Neue LiveCell System at 37°C and 5% CO₂. Time lapse images were taken with a 10X objective every 2 minutes. 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.