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RESEARCH ARTICLE

RIAM (Rap1-interacting adaptor molecule) regulates complement-dependent phagocytosis

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Abstract Phagocytosis mediated by the complement receptor CR3 (also known as integrin $\alpha_M\beta_2$ or Mac-1) is regulated by the recruitment of talin to the cytoplasmic tail of the β_2 integrin subunit. Talin recruitment to this integrin is dependent on Rap1 activation. However, the mechanism by which Rap1 regulates this event and CR3-dependent phagocytosis remains largely unknown. In the present work, we examined the role of the Rap1 effector RIAM, a talin-binding protein, in the regulation of complement-mediated phagocytosis. Using the human myeloid cell lines HL-60 and THP-1, we determined that knockdown of RIAM impaired $\alpha_M\beta_2$ integrin affinity changes induced by stimuli fMLP and LPS. Phagocytosis of complement-opsonized RBC particles, but not of IgG-opsonized RBC particles, was impaired in RIAM knockdown cells. Rap1 activation via EPAC induced by 8-pCPT-2′-O-Me-cAMP resulted in an increase of complement-mediated phagocytosis that was abrogated by knockdown of RIAM in HL-60 and THP-1 cell lines and in macrophages derived from primary monocytes. Furthermore, recruitment of talin to β_2 integrin during complement-mediated phagocytosis was reduced in RIAM knockdown cells. These results indicate that RIAM is a critical component of the phagocytosis machinery downstream of Rap1 and mediates its function by recruiting talin to the phagocytic complement receptors.

Keywords RIAM · Rap1 · Talin · $\alpha_M\beta_2$ · Mac-1 · CR3 · Phagocytosis

Abbreviations

BSA	Bovine serum albumin
8CPT	8-pCPT-2-O-Me-cAMP
FCS	Fetal calf serum
HRP	Horseradish peroxidase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PMA	Phorbol myristate acetate
RA	Retinoic acid
FNG	Fibrinogen
mAb	Monoclonal antibody
TBS	Tris buffered saline
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction

Phagocytosis involves the recognition and engulfment of large particles (>0.5 μm) by a multi-step actin-driven process. Mammalian professional phagocytes such as macrophages,

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granulocytes, and dendritic cells, continuously survey the organism sensing for foreign particles and apoptotic cells. Particle recognition is mediated by a wide variety of cell-surface receptors that bind directly to molecular patterns present on the particle surface, or indirectly through opsonins recognized by opsonic phagocytic receptors [1]. Opsonization by IgG or the complement fragment iC3b provides a mechanism that increases and diversifies the recognition repertoire of phagocytes through a limited number of receptors. The complement receptor CR3 (also known as Mac1, CD11b/CD18, $\alpha_M ß_2$) and the complement receptor CR4 (also termed p150, 95, CD11c/CD18, $\alpha_X ß_2$) are members of the $ß_2$ integrin family. Activation of CR3 and CR4 is induced by inflammatory mediators such as bacterial products (LPS, fMLP) and pro-inflammatory cytokines such as TNFα or PAF (platelet activating factor) [2]. Binding of these mediators to their cognate receptors triggers signaling events in the cell known as "*inside-out*" signaling pathways that result in complement receptor activation and increased affinity of the integrins for their ligands [3]. Subsequently, binding of complement-opsonized particles to their integrin-type receptors initiates signaling cascades into the cell ("*outside-in*" signaling) that induce actin cytoskeleton reorganization, membrane remodeling, and finally engulfment of the opsonized particle [4–6].

Small GTPases of the Ras and Rho families are implicated in the regulation of complement-mediated phagocytosis. While RhoA is the main GTPase activated by integrin "*outside-in*" signaling, Rap1 is the main GTPase involved in "*inside-out*" signaling pathways [7]. Through this mechanism, Rap1 regulates the affinity of various integrins, including $\alpha_4 ß_1$, $\alpha_3 ß_1$, $\alpha_{IIb} ß_3$, $\alpha_L ß_2$ and $\alpha_M ß_2$ [8–13]. In macrophages, signals from several surface receptors induce a rapid and transient Rap1 activation followed by an increase in the binding and phagocytic uptake of complement-opsonized particles [13]. Expression of Rap1V12, a constitutive active mutant of Rap1A, in the murine macrophage cell line J774-A1 induces an increase in the phagocytic uptake of C3-opsonized particles. In contrast, expression of Rap1N17, a dominant negative mutant of Rap1, completely abrogates complement receptor-mediated phagocytosis induced by LPS, TNFα or PMA [2].

In addition to Rap1, the cytoskeletal protein talin is considered an essential mediator of integrin activation. Different reports demonstrate that talin binding to the cytoplasmic region of the $ß_1$, $ß_2$, or $ß_3$ integrin subunits induces a conformational change in the integrin that results in integrin activation [14–17]. Several lines of evidence point to talin as an important functional regulator of complement receptors. In macrophages, talin is recruited to the phagocytic cup induced by complement-opsonized targets [18]. Point mutations in the talin-binding motif of $ß_2$ integrin reduce the recruitment of talin to the phagocytic cup and the uptake of complement-opsonized particles. Furthermore, knockdown of talin in the macrophage cell line J774-A1 and in COS cells expressing $\alpha_M ß_2$, strongly impairs complement-mediated phagocytosis [17]. In $\alpha_{IIb} ß_3$ integrin binding of talin to the $ß_3$ subunit is regulated by active Rap1 [10] and a recent report suggests that active Rap1 is also required for talin recruitment to $\alpha_M ß_2$ integrin [18].

RIAM (Rap1-GTP-interacting adaptor molecule) is a molecular partner of active Rap1 first characterized as a VASP-binding protein that functions downstream of Rap1 in T lymphocytes [12]. RIAM overexpression induces increased T lymphocyte adhesion to fibronectin and ICAM-1 mediated by an increase in β_1 and β_2 integrin affinity, respectively. Conversely, knockdown of RIAM abrogates activation of $\alpha_4 \beta_1$ and $\alpha_L \beta_2$ integrins induced by the expression of Rap1E63, a constitutive active mutant of Rap1 [12]. RIAM has been implicated in the APC-T cell interaction where it mediates the recruitment of active Rap1 to the immune synapse via its interaction with the SKAPP55-ADAP module [19]. Furthermore, RIAM interacts with PLC-γ1 and regulates PLC-γ1 spatio-temporal distribution and activation in T cells upon TCR triggering [20]. In platelets, the complex formed by active Rap1 and RIAM promotes talin targeting to the cytoplasmic tail of the β_3 subunit of $\alpha_{IIb} \beta_3$ leading to integrin activation [10, 21]. This effect is mediated by the direct interaction of the N-terminal region of RIAM with the talin globular head domain [22]. By regulating β_1 integrin activity, RIAM also affects migration and invasion in melanoma cells [23].

Currently, it remains largely unknown how Rap1 regulates the recruitment of talin to complement receptors at the phagocytic cup. In the present study, we determined that RIAM is actively involved in complement-dependent phagocytosis by regulating the activation of $\alpha_M \beta_2$ integrin (the CR3 complement receptor). Knockdown of RIAM in the human myeloid cell lines HL-60 and THP-1 and in macrophages derived from primary human monocytes, abrogated the increased $\alpha_M \beta_2$ integrin affinity, and phagocytosis of complement-opsonized particles induced by Rap1 activation. Moreover, RIAM knockdown reduced the recruitment of talin to the β_2 integrin and to the phagocytic cup, upon complement-dependent phagocytosis as determined by co-immunoprecipitation assays and confocal microscopy, respectively. These results indicate that RIAM is an integral component of an "*inside*-out" signaling pathway mediated by Rap1 and regulates complement-dependent phagocytosis by recruiting talin to the cytoplasmic tail of $\alpha_M \beta_2$ integrin.

Materials and methods

Cell culture and generation of macrophages from peripheral blood monocytes (MDMs)

HL-60 and THP-1 cell lines were cultured routinely in RPMI 1640 media supplemented with 10 % FCS,

2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 units/ml). Culture was maintained without exceeding 10^6 cells/ml in order to avoid spontaneous differentiation [24]. Cells were induced to differentiate into macrophage-like cells by treatment with 20 ng/ml PMA for at least 48 h or, in the case of HL-60, into neutrophil-like cells by treatment with 1 μM retinoic acid for 4 days. Differentiation was monitored by surface expression of the differentiation markers CD11b and CD11c assessed by flow cytometry analysis using Bear-1 (anti CD11b) or HC1/1 (anti CD11c) mAbs. HEK-293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % FCS, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 units/ml). MDMs were obtained from human monocytes isolated from buffy coats by using Ficoll-paque PLUS gradient (GE Healthcare, Madrid, Spain) followed by incubation of the PBMC fraction with a CD14 antibody coupled to magnetic beads (MACS cell separation column, Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were differentiated into macrophages by culture in RPMI 1640 media supplemented with 10 % FCS, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 units/ml) in the presence of 1,000 IU/ml of human GM-CSF (Immunotools, Friesoythe; Germany) for 5 days.

shRNA silencing and siRNA transfections

shRIAM- and shControl-HL-60 and THP-1 polyclonal cell lines were generated using previously described lentiviral plasmids [12], encoding GFP in combination with a RIAM-specific shRNA or a control shRNA. HL-60 and THP-1 cells were transduced with shRIAM or shControl lentiviral particles and GFP-expressing cells were sorted by flow cytometry 48 h post-infection. In these cells, GFP expression and RIAM knockdown was stable for at least 1 month in culture. shRIAM-99 and shControl-TR3 polyclonal cell lines were generated by transducing HL-60 cells with the retroviral plasmids pRS-TI326599, containing a RIAM-specific shRNA sequence or with pRS-TR30003, containing a GFP-specific shRNA oligonucleotide as negative control (Hush shRNA Plasmid Panels 29-mer, OriGene Technologies, Rockville, MD, USA). Retroviral-transduced cells were selected with 0.6 μg/ml of Puromycin (Sigma-Aldrich, Madrid, Spain) to generate the corresponding polyclonal cell lines. MDM cells were transduced with the lentiviral plasmids expressing GFP and either a RIAM-specific shRNA or a control shRNA oligonucleotide, as described above [12]. GFP expression was monitored 48 h after transduction and subsequently cells were used for opsonophagocytosis, real-time RT-PCR or for integrin activation epitope exposure assay. Alternatively, MDM cells were transfected with a RIAM-specific siRNA oligonucleotide (5′GCACUUUGCUGGGAGAGAU3′) or the Mission siRNA universal negative control, both siRNAs from Sigma-Aldrich, Madrid, Spain. For this purpose, the cells were transfected with 300 nM of each siRNA by Nucleofection using the Nucleofector Kit for Human Macrophages according to Amaxa Optimized Protocols. At 48 h after transfection, RIAM expression was determined by immunoblot and the cells used for opsonophagocytosis.

Cell activation and reagents

For activation experiments, cell lines or MDMs were left untreated or stimulated with 100 nM fMLP, 3 μ/ml LPS 100 μM 8-pCPT-2′-O-Me-cAMP or 100 ng/ml PMA at 37 °C for the indicated times. Cell activation was stopped with cold PBS, cells were washed twice with cold PBS and processed. fMLP, LPS, and PMA were obtained from Sigma-Aldrich (Madrid, Spain) and 8-pCPT-2′-*O*-Me-cAMP from Biolog (Bremen, Germany).

Integrin activation epitope exposure assay

Cells were incubated with HBS (20 mM HEPES pH 7.2, 150 mM NaCl, 1 mg/ml glucose) containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and Fc receptors were blocked by incubation with 100 μg/ml of human γ-Globulin (Sigma-Aldrich, Madrid, Spain). Cells were incubated either with CBRM1/5, an αM activation-dependent mAb, which also blocks binding of ligands to this integrin (Biolegend), αM expression mAb (Bear-1), or isotype control mAb, followed by staining with donkey anti-mouse Alexa-647 Ab (Invitrogen, Madrid, Spain). Cells were washed and resuspended in HBS with 1 mM appropriate cations and analyzed by flow cytometry. Activation epitope exposure was determined by assessing binding of CBRM1/5 relative to the binding of the activation-independent antibody Bear-1.

Cell adhesion to fibrinogen

Cells were stimulated as indicated and were subsequently incubated with 50 μg of soluble fibrinogen coupled to Alexa-647 (Invitrogen, Madrid, Spain) for 30 min at 37 °C. Cells were washed and fibrinogen binding was measured by flow cytometry. When indicated, the cells were incubated with fibrinogen in the presence of 1 μg of the anti $\alpha_M\beta_2$ blocking antibody CBRM1/5 (Biolegend, San Diego, CA, USA).

Opsonization and phagocytosis assay

Complement-opsonized RBCs were prepared and used for the phagocytosis assay as previously described [25]. Briefly, fresh sheep RBC (Oxoid, Thermo Fisher Scientific) labeled with 2 μM DDAO-AM (Molecular Probes, Invitrogen) were incubated with sub-agglutinating concentrations of rabbit IgM-anti sheep RBC cells (Chemical & Scientific

Corporation, Westbury, NY, USA), washed and incubated with 10 % C5-deficient human serum (Sigma). When FcγR phagocytosis was assessed, RBCs were labeled and incubated with sub-agglutinating concentrations of rabbit IgG anti-sheep RBC cells (Chemical & Scientific Corporation, Westbury, NY, USA) washed and used for the phagocytic assay. For the phagocytosis assay, cell lines or MDMs were starved for 3 h and were subsequently treated with the indicated stimuli. Cells were incubated in a 1:10 ratio with complement-opsonized RBCs (C-RBC) or unopsonized-RBCs as negative control, for 30 min at 37 °C and the unbound RBC were removed by washing three times with ice-cold PBS. When indicated, the cells were challenged with C-RBCs in the presence of 1 μg of the anti-$\alpha_M\beta_2$ blocking antibody CBRM1/5. To determine the phagocytic index, RBCs bound to cells were exposed to a hypotonic shock with H_2O during 15 s, followed by addition of 2× concentrated PBS to restore osmolarity. Cells were analyzed by flow cytometry and data were expressed as percent of association (%A) indicating percent of cells with one or more attached particles, or percentage of phagocytosis (%P) indicating percentage of cells with one or more internalized particles. The AI and PI indexes were calculated by multiplying the percentage of cells that bound and internalized or just internalized the DDAO-labeled RBCs with the GMFI of the DDAO-positive cells (AI = GMFI x %A), (PI = GMFI x %P) [26].

GST pull-down assays, Western blotting, immunoprecipitation, and real-time RT-PCR

For GST-pull-down assays, the cells were lysed in a buffer containing 50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 10 % glycerol, and 1 % NP40 supplemented with 2 mM sodium orthovanadate, 1 mM PMSF, and protease Inhibitor Cocktail (Sigma). An amount of 60 μg of cell lysates was separated by SDS-PAGE and analyzed by Western blotting with a polyclonal Rap1 antibody (SantaCruz Biotechnology Inc). Rap1 activation was assessed by pull-down assays using GST-RalGDS-RBD fusion protein as previously described [12]. For Western blotting, standard immunoblot methods were employed and the following antibodies were used: RIAM rabbit polyclonal antibody [12], Rap1 rabbit polyclonal antibody (SantaCruz Biotechnology Inc), and mouse mAb against tubulin (Sigma). Where indicated, densitometric analysis was performed and the relative band density quantification for each band was assessed using the Multi-Analyst Gel-Doc 2000 software (Bio-Rad).

For co-immunoprecipitation assays, shControl and shRIAM HL-60 cells were stimulated with 8CPT and challenged or not with C-RBC for 20 min. Unphagocytosed C-RBCs were removed by treatment with H_2O during 15 s, followed by restoration of osmolarity with 2× concentrated PBS. shControl and shRIAM HL-60 cells were lysed in 1 % Brij-97 buffer containing 50 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ supplemented with protease inhibitors. Protein of each cell type (500 μg) was incubated for 5 h at 4 °C with protein G-Sepharose beads coupled to 10 μg/ml of the anti-integrin β_2 mAb TS1/18. Then, the samples were washed with 1:10 diluted lysis buffer, boiled in reducing or nonreducing Laemmli buffer, resolved by 8 % SDS-PAGE, and transferred to nitrocellulose membranes. Blots were developed with anti-integrin β_2 mAb MEM-48 (Sigma) in nonreducing conditions or anti-talin mAb 8d4 and anti-RIAM polyclonal antibody [12] in reducing conditions.

Real-time RT-PCR was performed with a specific probe for RIAM (APBB1IP gene) using GAPDH probe for standardization; both probes were purchased from Applied Biosystems. All reactions were performed in triplicates. Results were expressed as percentage of detected mRNA transcripts relative to transduction efficiency and were the average of three independent experiments.

Immunostaining and fluorescence microscopy

For the analysis of complement-dependent phagocytosis by fluorescence microscopy, 250,000 shControl- and shRIAM-THP-1 cells were seeded onto Lab-Tek II Chamber slides (Nunc), and were differentiated to macrophages with PMA. Where indicated, cells were starved for 3 h, stimulated with 8CPT and challenged with C-RBC particles for 1 h at 37 °C. Cells were washed with TBS, fixed using 4 % (w/v) paraformaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, 120 mM sucrose, pH 7.3) and permeabilized with 0.1 % (v/v) Triton X-100 in TBS. Fc receptors were blocked by incubation with 100 μg/ml of human γ-Globulin (Sigma), cells were incubated with rabbit IgG against RBCs (Chemical & Scientific Corporation, Westbury, NY, USA) followed by donkey anti-rabbit Alexa-555 (Invitrogen). Images were obtained using an AXIO-Imager A1 microscope (Zeiss) and 15 independent fields were analyzed using AxioVision 4.6.3 software. Phagocytic index was determined by expressing percentage of bound- and internalized-RBCs per 100 efficiently transduced cells.

To assess RIAM translocation to the phagocytic cup, 1×10^6 neutrophilic shControl and shRIAM HL-60 cells were stimulated with 8CPT and then challenged with C-RBC for 10 min at 37 °C prior to seeding onto poly-Lysine coated slides (Sigma). Cells were washed to remove unbound C-RBC and subsequently fixed with 4 % (w/v) paraformaldehyde in PHEM buffer and permeabilized with 0.1 % (v/v) Triton X-100 in TBS. Fc receptors were blocked by incubation with 100 μg/ml of human γ-Globulin (Sigma) and cells were incubated with Alexa Fluor Phalloidin 350 (Life Technology), the anti-talin mAb 8d4 (Sigma)

followed by Alexa647-conjugated donkey anti-mouse IgG (Invitrogen) and rabbit anti-RBC IgG (Chemical & Scientific Corporation, Westbury, NY, USA) followed by Alexa555-conjugated donkey anti-rabbit IgG (Invitrogen). Samples were mounted with Fluoro-Gel (Fluoroprobes) and images were obtained with a confocal laser scanner microscope (Zeiss LSM 510). Talin recruitment to the phagocytic cup was defined as enrichment in colocalization signal with bound RBCs, and images were obtained by LSM 510 Fiji software (released under General Public license). Final image processing was performed with Adobe Photoshop Images converting 647-channel in green in order to improve image quality.

Statistical analysis

Data are expressed as mean values ± SEM. Statistical analysis for in vitro assays was determined by unpaired two-tailed Student's *t* test and differences were considered statistically significant for $p < 0.05$. In some experiments, statistical analysis was performed using VassarStats on-line resource (Vassar College, Poughkeepsie, NY, USA). A single asterisk indicates values of $p < 0.05$, while a double asterisk indicates values of $p < 0.01$.

Results

Stable interference of RIAM expression in HL-60-derived neutrophils

RIAM, the effector of the small GTPase Rap1 involved in integrin activation, was first described as RARP1 (Retinoic Acid Response Protein 1), a protein induced during neutrophil-like differentiation with ATRA (all-trans retinoic acid) in the promyeloleukemic cell line HL-60. In this cell line, RARP-1 was proposed to play a role in myeloid differentiation based on its capacity to regulate transcriptional activity [27]. The HL-60 cell line has been used in opsonophagocytic assays using bacteria or RBCs as phagocytic particles [28]. Hence, we decided to use this cell line as a model to study the role of RIAM in complement-mediated phagocytosis. For this purpose, we transduced HL-60 cells with a lentiviral vector encoding RIAM-specific shRNA or a control shRNA [12] and we established polyclonal HL-60 cell lines expressing shRIAM or shControl. We also used a retroviral vector containing a RIAM-specific shRNA targeting a different sequence to generate another stable RIAM knockdown polyclonal HL-60 cell line (shRIAM-99) and its relevant control (shControl-TR3).

Compared to the relevant control cells, RIAM expression was diminished by 85 % in shRIAM cells (Fig. 1a) and by 70 % in shRIAM-99 cells (Supplementary Fig. 1a). RIAM expression was upregulated by treatment with RA in the HL-60 cell lines. However, RIAM levels remained diminished in shRIAM cells compared to shControl cells (Fig. 1a). This finding was observed also in the polyclonal shRIAM-99 cell line, albeit to a different magnitude (Supplementary Fig. 1a). RARP1/RIAM was proposed to play a role in differentiation [27]. Because expression of CR3 and CR4 receptors is upregulated during myeloid differentiation [29, 30], first we examined whether RIAM might have a role in the induction of these phagocytic receptors. Expression of CD11b, the alpha subunit of the CR3 receptor, and expression of CD11c, the alpha subunit of the CR4 receptor, displayed comparable upregulation in response to treatment with RA in shRIAM and shControl cells, as quantitated by the MFI and percentage of positive cells flow cytometric parameters (Fig. 1b and Supplementary Fig. 1b). These results indicate that RIAM is not involved in the upregulation of the phagocytic complement receptors upon differentiation of these myeloid cells with RA.

RIAM regulates the affinity of the myeloid integrin $\alpha_M\beta_2$

The bacterial products fMLP and LPS activate complement receptors and induce Rap1 activation [2]. To determine whether RIAM is involved in the regulation of CR3 ($\alpha_M\beta_2$) activation, we performed soluble fibrinogen-binding assays using the RIAM-KD neutrophilic HL-60 cells, stimulated or not with fMLP or LPS. Under these conditions, shRIAM cells stimulated with fMLP or LPS displayed a 20–30 % reduction of fibrinogen binding compared to shControl cells (Fig. 1c and Supplementary Fig. 1c). This reduction was noteworthy since $\alpha_M\beta_2$ activation appears to account only for approximately 40 % of total fibrinogen binding to these cells, as revealed by the use of CBRM1/5, an anti-$\alpha_M\beta_2$ blocking antibody (Fig. 1c). These experiments suggested that RIAM might regulate β_2 integrin activity, similarly to its effects in other cell types [12]. Changes in the binding of soluble ligands by integrins reflect changes in integrin affinity more than changes in integrin avidity [11, 31, 32]. To evaluate whether the interference in RIAM expression affected $\alpha_M\beta_2$ affinity, we used the anti-$\alpha_M\beta_2$ activation reporter antibody CBRM1/5 that binds to α_M only when it is in an active conformation state [33]. As shown in Fig. 1d, shControl cells increased the binding of mAb CBRM1/5 upon activation with fMLP and LPS. In contrast, the basal activation level of $\alpha_M\beta_2$ integrin on shRIAM cells was lower than on shControl cells, as detected by binding of mAb CBRM1/5, and no further increase in activation of this integrin was induced by LPS or fMLP in RIAM silenced cells. Notably, under these conditions, fMLP and LPS induced activation of Rap1 as determined by pull-down assays (Supplementary Fig. 1d). These results strongly suggest that the defect in integrin affinity regulation observed in these cell

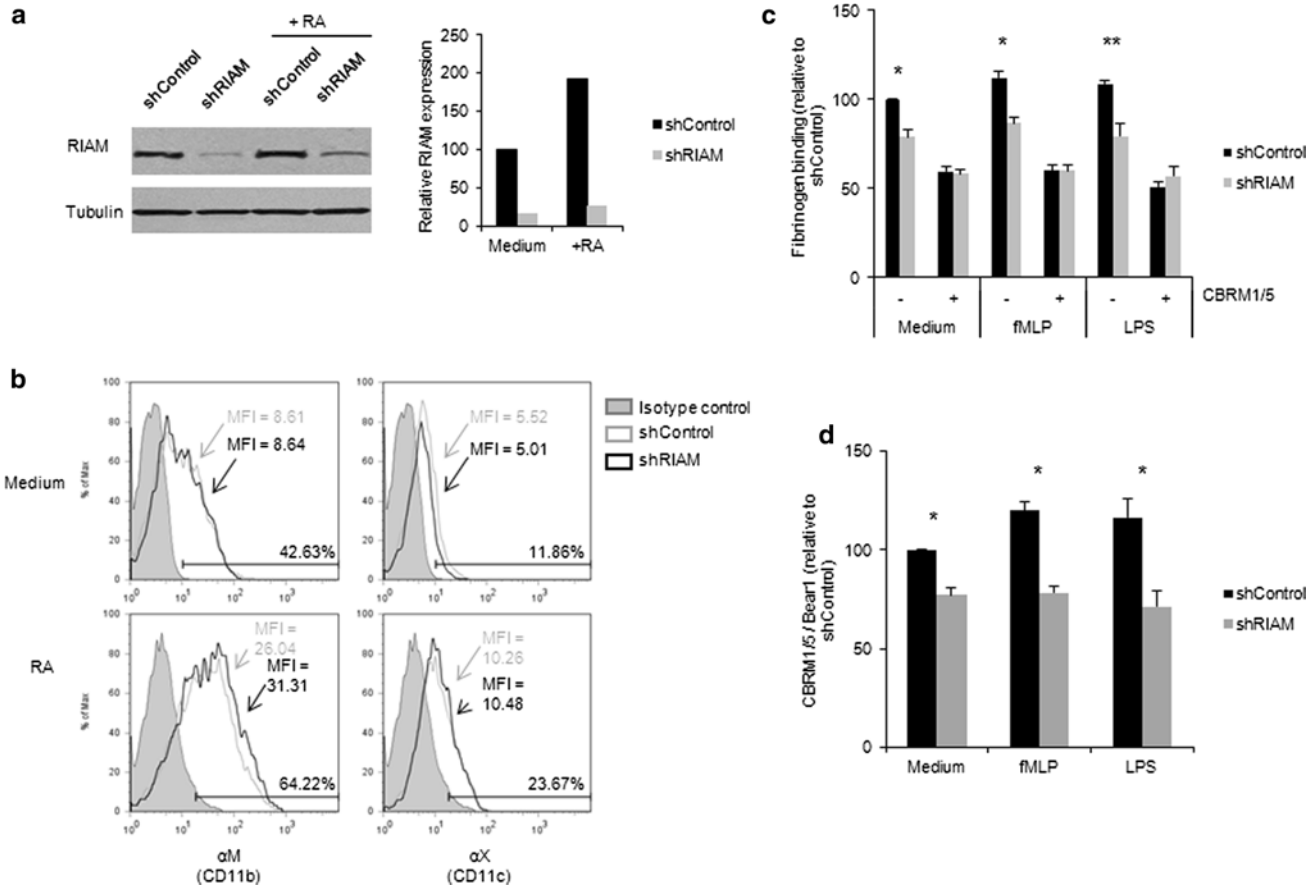

Fig. 1 RIAM expression regulates $\alpha_M\beta_2$ integrin affinity in the promyelocytic HL-60 cell line. **a** HL-60 cell line stably transduced with a lentiviral construct containing RIAM shRNA (shRIAM) or a control shRNA (shControl) [12], were left untreated or differentiated toward the neutrophilic lineage by RA treatment. RIAM expression was analyzed by Western blot and the quantification of RIAM knockdown was determined by densitometry and presented as relative to tubulin intensity. **b** Surface expression of myeloid differentiation markers α_M (CD11b) and α_X (CD11c) was analyzed in shControl and shRIAM cell lines by flow cytometry. *Grey-filled histograms* represent isotype control, *black line histograms* represent expression of the indicated molecules on shRIAM cell line, and *grey lines* correspond to the expression on shControl cell line. The % of fluorescent-positive cells and the MFI for each cell line are indicated. **c** Flow cytometric determination of soluble fibrinogen binding to the indicated neutrophilic HL-60 cell lines, either unstimulated (medium) or stimulated with 100 nM fMLP or 3 μg/ml LPS in the presence or absence of mAb CBRM1/5, a blocking antibody for $\alpha_M\beta_2$. Data are expressed as relative to fibrinogen binding to the unstimulated (medium) shControl cell line and correspond to seven independent experiments. **d** Activation of the $\alpha_M\beta_2$ integrin in neutrophilic-differentiated shControl and shRIAM HL-60 cells, either unstimulated (medium) or stimulated with 100 nM fMLP or 3 μg/ml LPS. Activation was assessed by the MFI of mAb CBRM1/5 binding relative to total expression of $\alpha_M\beta_2$ integrin detected by the conformation-independent mAb Bear-1. Data are expressed as MFI relative to unstimulated (medium) shControl cell line and correspond to 12 independent experiments

lines is downstream of Rap1 in the "*inside-out*" signaling pathway that activates $\alpha_M\beta_2$.

RIAM regulates complement-dependent phagocytosis in neutrophil-like cells

Since $\alpha_M\beta_2$ (CR3) is a receptor of complement-opsonized particles, we sought to determine whether RIAM had an effect on the phagocytic activity of this receptor. For this purpose, we performed an opsonophagocytic assay using as particles complement-opsonized RBCs (C-RBC) labeled with the fluorescent cell tracker DDAO-AE. shRIAM and shControl HL-60 cells were neutrophil-like differentiated, subsequently stimulated with fMLP or LPS and challenged with C-RBC. Cells were analyzed by flow cytometry and the association index (AI) and phagocytic index (PI) were calculated as indicated in the "Materials and methods". A low level of constitutive phagocytosis of RBC particles was observed in the absence of complement opsonization labeled as RBC in the graphs (Fig. 2a). Upon incubation with C-RBC under basal conditions of culture in medium alone, there was a significant increase in phagocytosis in both shRIAM and shControl HL-60 cells, indicating that this process was indeed mediated

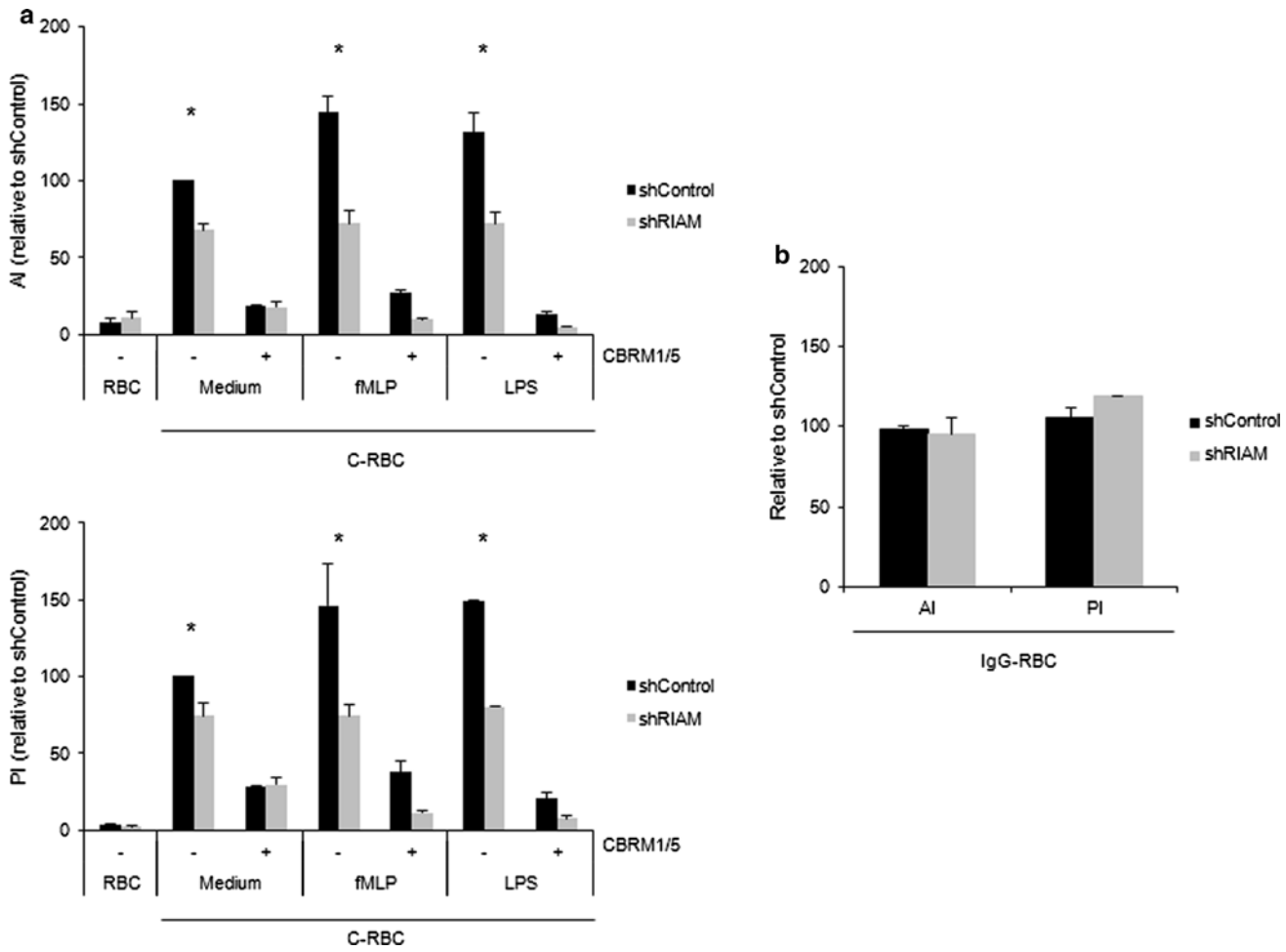

Fig. 2 RIAM regulates complement-dependent phagocytosis in neutrophilic-HL-60 cell lines. **a** Neutrophilic shControl and shRIAM cell lines unstimulated (medium) or stimulated with 100 nM fMLP or 3 μg/ml LPS were challenged with unopsonized red blood cells (RBC) or complement-opsonized red blood cells (C-RBC) in the presence or absence of mAb CBRM1/5, a blocking antibody for $\alpha_M\beta_2$. The association index (AI) and phagocytic index (PI) are represented as relative values to unstimulated shControl and correspond to at least five independent experiments. **b** A FcγR-mediated phagocytosis assay was performed using neutrophilic shControl and shRIAM cell lines. AI and PI are represented as relative to shControl and correspond to at least three independent experiments

by the complement phagocytic receptors. This was corroborated by the use of blocking antibody for $\alpha_M\beta_2$ that reduced the binding of C-RBC particles by 80–90 %. Upon cell stimulation with fMLP or LPS, there was a significant increase in phagocytosis in shControl HL-60 cells, but such an increase was undetectable in shRIAM HL-60 cells, which exhibited an overall 50 % reduction in phagocytosis compared to shControl cells under these stimulatory conditions (Fig. 2a). A similar pattern of results was observed using a different polyclonal RIAM-KD cell line, shRIAM-99, generated using a different RIAM shRNA sequence (Supplementary Fig. 2a). These results indicate that a reduction in RIAM expression leads to impaired activation of complement-mediated phagocytosis induced by "*inside-out*" signaling. Notably, there was a positive correlation between the quantitative RIAM expression level and the activation of complement-dependent phagocytosis, because the shRIAM cell line expressing lower RIAM protein level displayed more prominent suppression of phagocytosis as compared to the shRIAM-99 cell line, which expresses higher RIAM protein level and showed a less pronounced defect in phagocytosis (Fig. 2a vs. Supplementary Fig. 2a).

To determine whether this effect of RIAM expression was specific for phagocytic complement receptors, we performed the same opsonophagocytic assay using RBC particles opsonized with IgG. In these experiments, the AI and PI parameters were almost identical for both shRIAM and shControl HL-60 cell lines (Fig. 2b), indicating that the effect of RIAM on phagocytosis was specific for complement receptors and not for FcγR-dependent phagocytosis.

RIAM is required for the Rap1-induced activation of complement-mediated phagocytosis in neutrophil-like cells

Rap1 has a principal role regulating the "*inside-out*" signaling pathway that activates complement-mediated phagocytosis [12]. Likewise, our experiments indicated that RIAM is required to induce activation of complement-dependent phagocytosis. Since RIAM is an effector of Rap1, we next examined whether RIAM might be required for the Rap1-mediated activation of complement receptors. To induce activation of Rap1, we used the cAMP analogue 8CPT-2′-Me-O-AMPc (8CPT), which specifically activates EPAC, a GTP exchange factor specific for Rap1 [34]. EPAC proteins are expressed in neutrophils and macrophages and both cell types respond to 8CPT by transiently activating Rap1 [35–37]. Using HL-60 cells after neutrophilic differentiation, we determined that 8CPT indeed induced activation of Rap1, which peaked at 5 min of stimulation and gradually declined (Fig. 3a). To determine whether RIAM was required for the regulation of $\alpha_M\beta_2$ integrin affinity induced by Rap1 activation in these cells, we tested their capacity to bind soluble fibrinogen and the binding of the activation reporter antibody CBRM1/5 in cells stimulated with 8CPT. Treatment with 8CPT upregulated fibrinogen binding to shControl cells and this increase was abrogated by depletion of RIAM by shRNA. Again, the use of anti-$\alpha_M\beta_2$ blocking antibody only inhibited the binding of fibrinogen by 40 % (Fig. 3b), suggesting that in addition to the integrin $\alpha_M\beta_2$ other cell surface receptors also contribute to the binding of this extracellular ligand. Similarly, treatment of the shControl HL-60 cells with 8CPT or PMA induced an increase in the binding of mAb CBRM1/5, indicating increased affinity of the $\alpha_M\beta_2$ integrin. However, this increase in affinity was abrogated in RIAM knockdown cells (Fig. 3c). Finally, assessment of complement-mediated phagocytosis in shControl and shRIAM cells showed that increased phagocytosis of C-RBC particles was induced by 8CPT treatment and this effect was abrogated in RIAM knockdown cells (Fig. 3d). The crucial implication of the integrin $\alpha_M\beta_2$ in these opsonophagocytic assays was revealed by the important reduction of C-RBC phagocytosis (approximately 80 %) observed after blocking this integrin with mAb CBRM1/5 (Fig. 3d). Similar results were observed using the shRIAM-99 cell line and the relevant control in complement-dependent phagocytosis (Supplementary Fig. 2b). Overall, these results indicated that direct activation of Rap1 induces an increase in $\alpha_M\beta_2$ integrin affinity, in fibrinogen binding, and in complement-mediated phagocytosis that are all dependent on RIAM expression.

Complement-mediated phagocytosis is dependent on RIAM expression in macrophages

Our results in neutrophilic-like cells indicated that RIAM regulates $\alpha_M\beta_2$ affinity and the phagocytic activity downstream of Rap1. To determine whether RIAM also plays a role in regulating this integrin in macrophages, we differentiated the HL-60 cell lines towards the monocytic lineage using PMA. During incubation with PMA, RIAM was upregulated in shControl and shRIAM cells, but the significant difference in RIAM expression between both cell lines was not only retained but rather enhanced (Fig. 4a). PMA treatment upregulated CD11b and CD11c surface expression to a comparable degree on shControl and shRIAM cells (Fig. 4b). To assess complement-mediated phagocytosis, we performed opsonophagocytic assays of C-RBC upon stimulation with fMLP, LPS, or 8CTP. Similarly to neutrophilic-derived HL-60 cell lines, knockdown of RIAM resulted in a reduced basal (unstimulated) complement-mediated phagocytosis as well as in an almost complete lack of response to the different stimuli employed (Fig. 4c).

To further analyze the role of RIAM in complement-mediated phagocytosis in macrophages, we used the human promonocytic cell line THP-1 to knockdown RIAM expression using the GFP-expressing lentiviral plasmids encoding a RIAM shRNA or a control shRNA [12], establishing the shControl-THP1 and shRIAM-THP-1 polyclonal cell lines. RIAM expression was upregulated in shControl and shRIAM-THP-1 cells after differentiation with PMA treatment, but the RIAM protein level remained significantly lower in shRIAM-THP-1 cells compared to the shControl cells (Fig. 5a). The knockdown of RIAM expression was stable during cell differentiation (Fig. 5a) and the expression of CD11b and CD11c was similar in shRIAM- and shControl-THP-1 cell lines (Supplementary Fig. 3). By using the C-RBC opsonophagocytic assay described above, we detected a 50 % reduction in the AI and PI indexes of shRIAM-THP-1 cells compared to shControl-THP-1 when the cells were stimulated with fMLP, LPS, or 8CPT (Fig. 5b). Alternatively, to visualize the phagocytic capacity of these THP-1 cell lines differentiated to macrophages by PMA treatment, we stimulated them with 8CPT, challenged them with C-RBC particles, and analyzed their binding and uptake by confocal fluorescence microscopy. shRIAM-THP-1 cells clearly displayed a reduced binding and uptake of C-RBC particles compared to shControl-THP-1 cells (Fig. 5c), also showing a 50 % reduction in the phagocytic index (PI) calculated from the analysis of the fluorescence microscopy images (Fig. 5d).

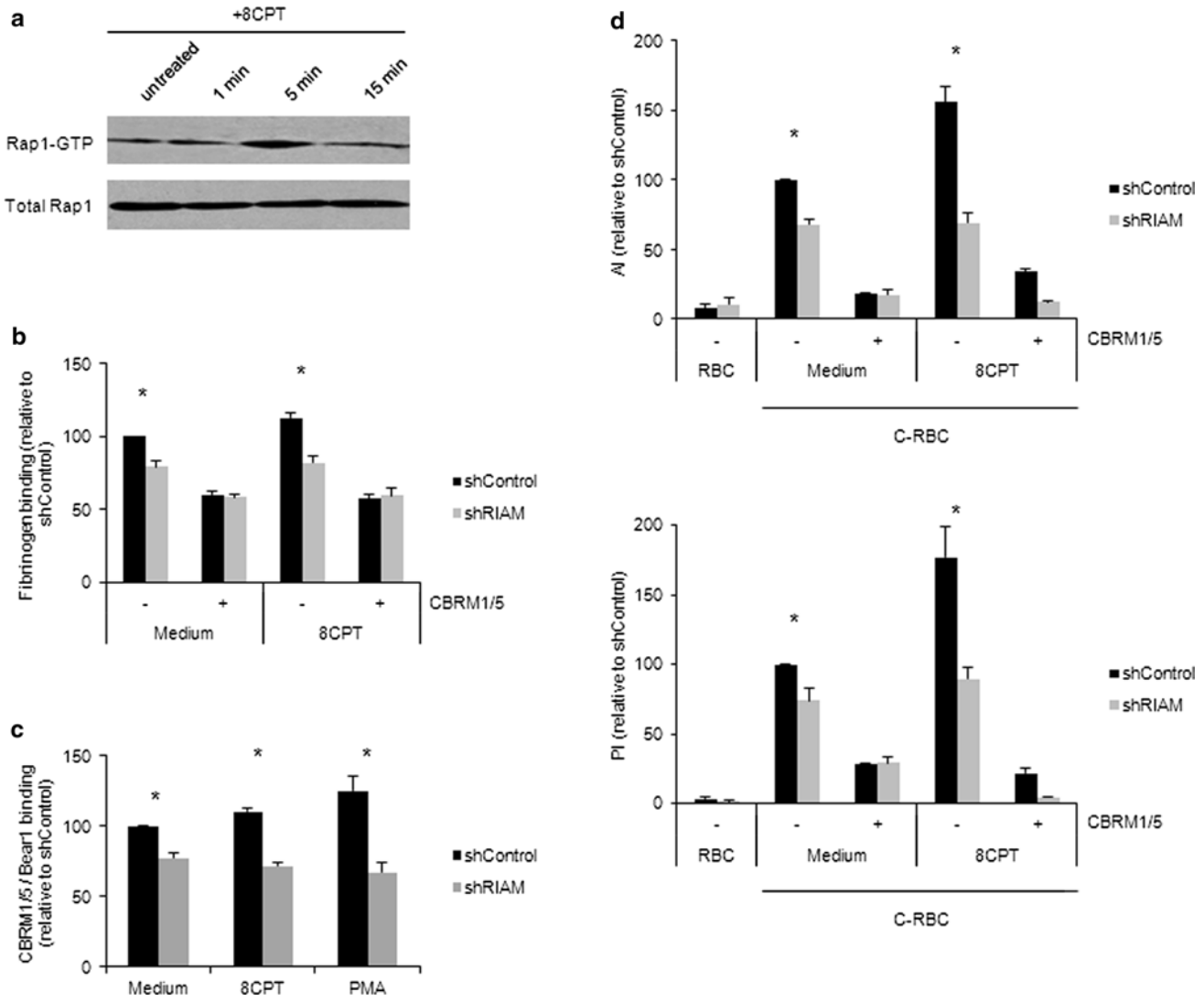

Fig. 3 RIAM expression is required for Rap1-induced complement-mediated phagocytosis. **a** Rap1 activation in neutrophilic HL-60 cell line, either untreated or treated with 100 μM 8CPT for the indicated times, was determined by a pull-down assay using GST-RalGDS-RBD followed by Western blot with Rap1-specific antibody. **b** The binding of fibrinogen to neutrophilic shControl and shRIAM cell lines, either unstimulated (medium) or stimulated with 100 μM 8CPT in the presence or absence of the $\alpha_M\beta_2$ blocking mAb CBRM1/5, was analyzed by flow cytometry. Data are expressed as relative to fibrinogen binding to unstimulated shControl cells and correspond to seven independent experiments. **c** Activation of the $\alpha_M\beta_2$ integrin in neutrophilic shControl and shRIAM cells either unstimulated (medium) or stimulated with 100 μM 8CPT or with 100 ng/ml PMA. Activation was determined by the MFI of mAb CBRM1/5 binding relative to total expression of $\alpha_M\beta_2$ integrin detected by the conformation independent mAb Bear-1. Data are expressed as relative to unstimulated shControl cells and correspond to 12 independent experiments. **d** A complement-dependent phagocytosis assay was performed in neutrophilic shControl and shRIAM HL-60 cell lines either untreated or stimulated with 100 μM 8CPT and challenged with complement opsonized RBCs (C-RBC) or unopsonized red blood cells (RBC) in the presence or absence of mAb CBRM1/5, a blocking antibody for $\alpha_M\beta_2$. The AI and PI indexes are represented as relative values to unstimulated shControl values and correspond to at least five independent experiments

RIAM is required for complement-dependent phagocytosis in primary human monocyte-derived macrophages (MDM)

Our studies described above indicated that RIAM plays a role in regulating complement-dependent phagocytosis both in neutrophils and in macrophages. For these studies, we used cell lines with well-established adequacy as experimental tools for this purpose. To determine the direct biological relevance of our findings, we examined the role of RIAM in $\alpha_M\beta_2$ integrin activation and the phagocytic properties of primary human macrophages. For this purpose, we generated human monocyte-derived macrophages (MDM) and subsequently we transduced these cells with GFP-expressing lentiviral shRIAM and shControl constructs

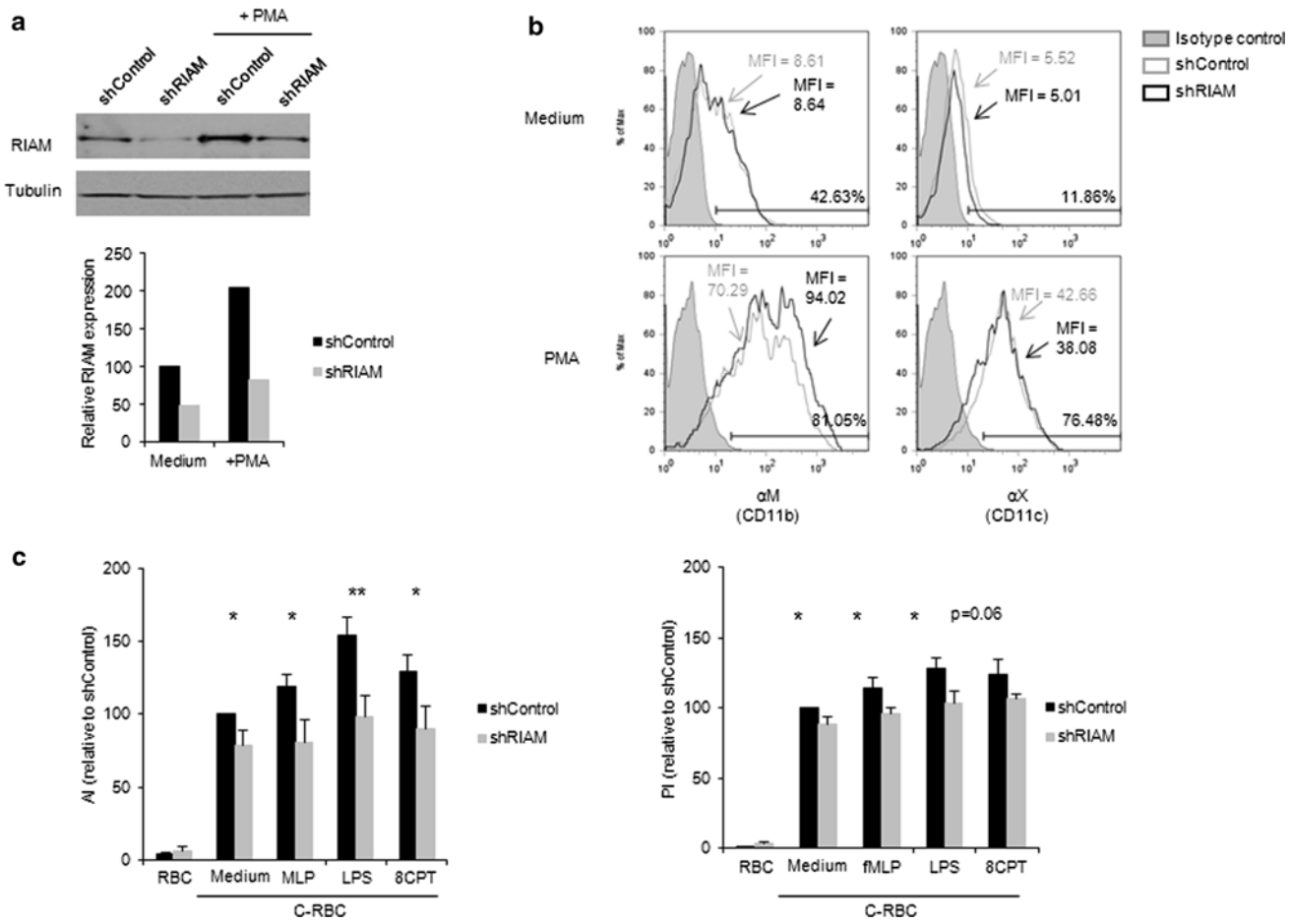

Fig. 4 RIAM regulates complement-dependent phagocytosis in macrophage-like HL60 cells. **a** shControl and shRIAM HL-60 cell lines undifferentiated or differentiated with PMA towards the macrophage lineage were analyzed for RIAM expression by Western blot. Quantification of RIAM knockdown was determined by densitometry and the value is expressed as signal intensity relative to tubulin. **b** Surface expression of myeloid differentiation markers α_M (CD11b) and α_X (CD11c) was analyzed by flow cytometry in macrophage-like shControl and shRIAM HL-60 cell lines. *Grey-filled histograms* represent isotype control, *black lines* represent expression of the indicated proteins on shRIAM cell line, and *grey lines* correspond to the expression on shControl cell line. The percentage of positive cells and the MFI for each cell line are indicated. **c** Macrophage-like shControl and shRIAM cells either unstimulated or stimulated with 100 nM fMLP, 3 μg/ml LPS or 100 μM 8CPT were challenged with complement-opsonized red blood cells (C-RBC) or unopsonized red blood cells (RBC) as control. The AI (*left graph*) and PI (*right graph*) were expressed as values relative to unstimulated shControl, and correspond to five independent experiments

[12]. shRIAM and shControl transduced MDMs displayed comparable transduction efficiencies (Fig. 6a). Under these conditions, we detected a 30 % reduction in RIAM expression in shRIAM-MDM compared to shControl-MDM cells, as determined by RT-PCR (Fig. 6b). To determine whether RIAM knockdown could affect the activation of $\alpha_M\beta_2$ integrin in these cells, we analyzed the binding of the activation reporter mAb CBRM1/5 to MDM cells treated or not with 8CPT. We observed that treatment with 8CPT increased by 50 % CBRM1/5 binding to GFP-positive shControl-MDM cells. However, GFP-positive shRIAM-MDM cells treated with 8CPT did not increase CBRM1/5 binding, suggesting that RIAM expression is required for the Rap1-mediated activation of the $\alpha_M\beta_2$ integrin (Fig. 6c). Next, we wanted to determine the role of RIAM in phagocytosis in MDM cells. For this purpose, we performed an opsonophagocytic assay using C-RBC as particles and treating or not the cells with 8CPT. The cells were analyzed by flow cytometry gating on the GFP-positive cells, and the percentage of bound particles (%A) and the AI parameters were determined in shRIAM-MDM and shControl-MDM cells. Under basal conditions, GFP-positive shControl-MDM and shRIAM-MDM cells bound a small amount of non-opsonized RBC particles (Fig. 6d). When MDM cells were incubated with C-RBC particles, shControl- and shRIAM-MDM cells displayed a comparable constitutive activation level of complement receptors (17 and 25 % of the cells bound C-RBCs, respectively). However, when the cells were treated with

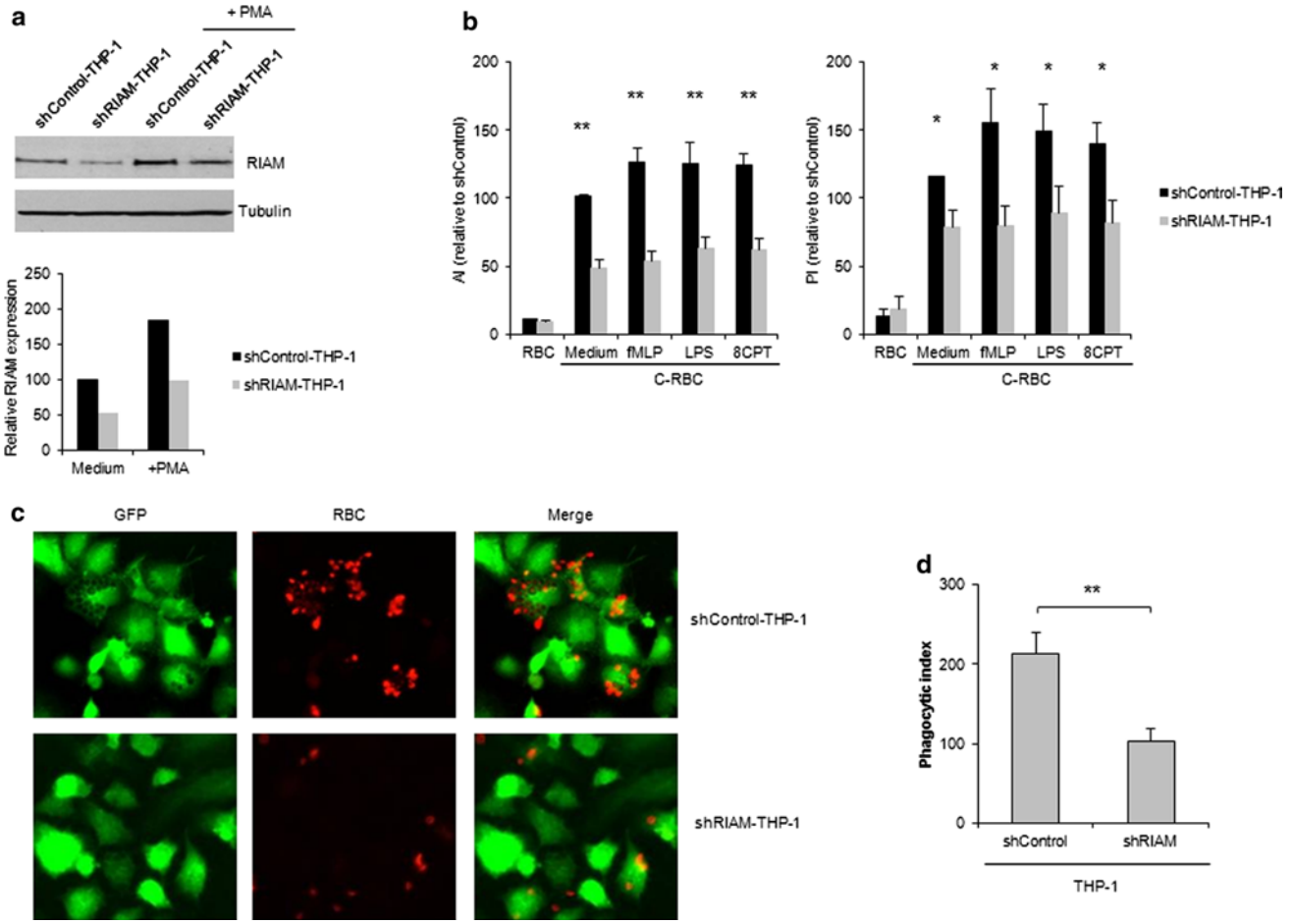

Fig. 5 RIAM regulates complement-dependent phagocytosis in macrophage-like THP-1 cells. **a** shControl-THP1 and shRIAM-THP-1 cells, either untreated or differentiated toward the macrophage lineage with PMA were analyzed by Western blot for RIAM expression. Quantification of RIAM knockdown was determined by densitometry and is presented as relative to tubulin intensity. **b** Macrophage-like shControl- and shRIAM-THP-1 cells were left untreated (medium) or stimulated with either 100 nM fMLP, 3 μg/ml LPS or 100 μM 8CPT. Cells were subjected to an opsonophagocytic assay with complement-opsonized red blood cells (C-RBC), and with unopsonized red blood cells (RBC) used as control. Association index (AI) and phagocytic index (PI) are presented as values relative to unstimulated shControl-THP-1 cells. **c** Complement-mediated phagocytosis assay was performed with macrophage-like shControl- and shRIAM-THP-1 cells activated with 100 μM 8CPT and cells were analyzed by immunofluorescence. GFP-positive cells express shControl or shRIAM constructs, whereas RBC were detected using an anti-sheep RBC antibody coupled to Alexa-555. **d** The phagocytic index was calculated as number of bound or internalized RBCs per 100 GFP-positive phagocytes counted in 15 independent fields

8CTP, shControl-MDM displayed a significant increase of C-RBC binding to approximately 80 %, whereas shRIAM-MDM cells showed no increase of C-RBC binding, indicating a lack of complement receptor activation compared to untreated cells (Fig. 6d). Assessment of AI and % A parameters in five independent experiments confirmed this observation (Fig. 6e). To corroborate these results, we transfected the MDM cells with a siRNA control and a commercially available siRNA that targets a different sequence in RIAM mRNA. siRIAM transfection in MDMs caused a 35 % reduction in RIAM protein expression as determined by immunoblotting (Supplementary Fig. 2c). In siControl-MDM cells, treatment with 8CPT increased complement-dependent phagocytosis, as determined by the AI parameter. However, in the RIAM knockdown cells (siRIAM-MDM), this increase was undetectable, corroborating the data shown in Fig. 6d and e. Together, these results strongly indicate that RIAM regulates activation of Rap1-dependent complement-mediated phagocytosis in primary human macrophages.

RIAM regulates the recruitment of talin to complement receptors

Talin binding to the cytoplasmic tail of integrin β chains appears to be a general mechanism for integrin activation [15]. Partial depletion of talin in murine macrophages and human THP-1 cells differentiated to macrophages has been shown to impair complement-dependent phagocytosis [17].

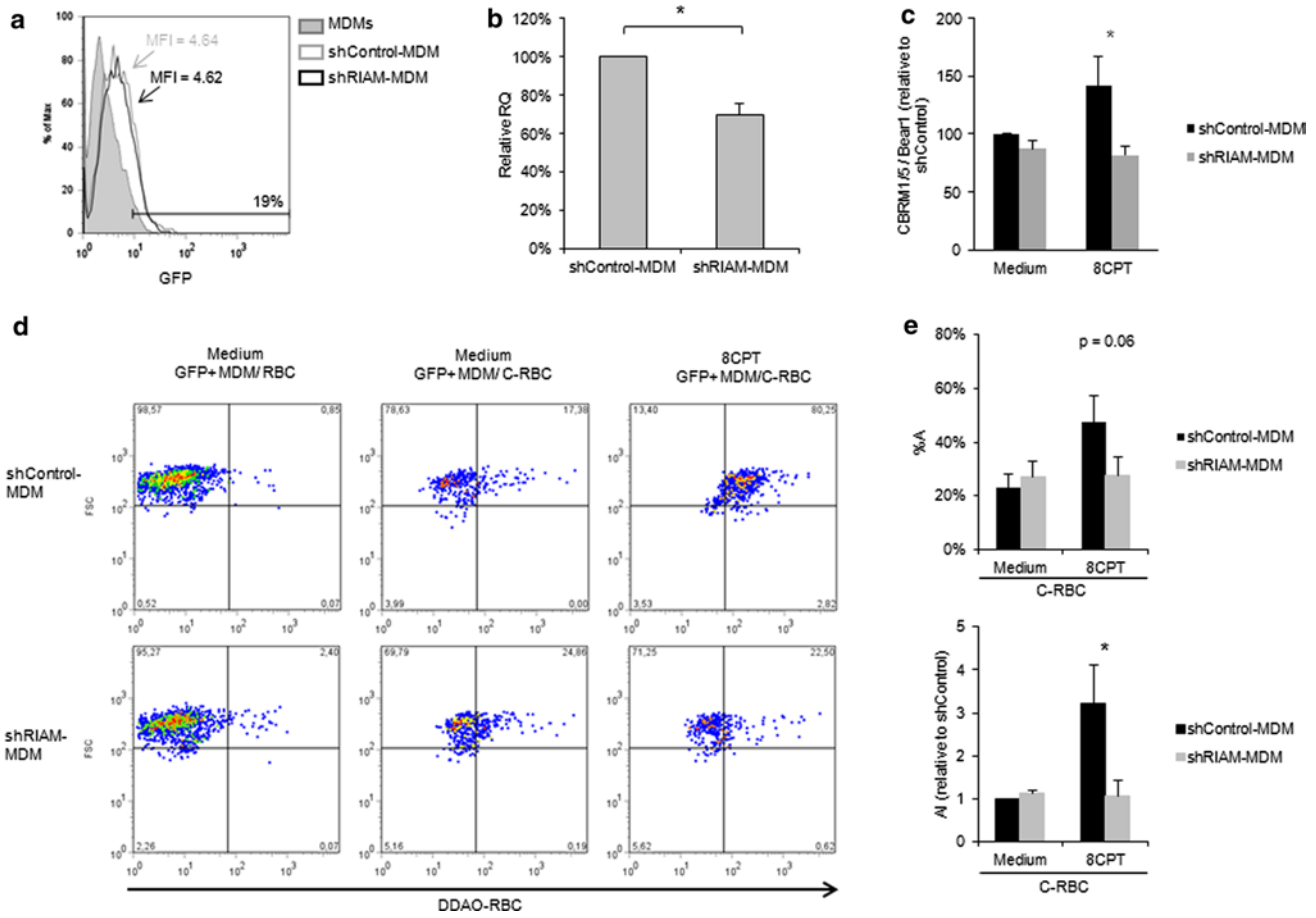

Fig. 6 RIAM regulates complement-mediated phagocytosis in peripheral blood monocyte-derived macrophages (MDM). **a** MDMs were transduced with GFP-expressing lentiviral plasmids containing shControl or shRIAM sequences [12], and the percentage of GFP-positive cells was assessed by flow cytometry 48 h post-infection. Results are representative of five independent experiments. **b** RIAM expression was determined by RT-PCR in shControl- and shRIAM-MDM cells. Relative RQ represents the percentage of RIAM mRNA detected in each cell type relative to transduction efficiency. Results are representative of five independent experiments. **c** Activation of $\alpha_M\beta_2$ integrin in shControl-MDM and shRIAM-MDM cells, either unstimulated (medium) or stimulated with 100 μM 8CPT, was determined as the MFI of mAb CBRM1/5 binding relative to total expression of $\alpha_M\beta_2$ integrin detected by the conformational independent mAb Bear-1. Data are expressed as relative to unstimulated shControl-MDM cells and correspond to five independent experiments. **d** One representative complement-dependent opsonophagocytic experiment in shControl- and shRIAM-MDM cells is shown. *Dot plots* represent GFP-positive MDM cells. *Left panels* show shControl- and shRIAM- MDMs challenged with unopsonized red blood cells (RBC) as control. *Middle panels* show shControl- and shRIAM-MDMs incubated with complement-opsonized red blood cells (C-RBC) in medium and *right panels* show these cells stimulated with 100 μM 8CPT and challenged with C-RBCs. **e** An opsonophagocytic assay was performed using shControl and shRIAM-MDMs either untreated or stimulated with 100 μM 8CPT and challenged with C-RBCs. The % A (*upper graph*) and the AI (*lower graph*) were expressed as values relative to unstimulated shControl-MDM GFP-positive cells. Results are representative of five independent experiments

In primary megakaryocytes and in $\alpha_{IIb}\beta_3$-expressing CHO cells, a mechanism has been proposed for talin recruitment to the cytoplasmic tail of β_3 integrin that involves Rap1 activation and RIAM [10, 21, 22]. To determine whether RIAM also regulates the recruitment of talin to the β_2 integrin chain of phagocytic complement receptors downstream of Rap1, we performed C-RBC phagocytosis in shRIAM and shControl neutrophilic HL-60 cells, and analyzed talin recruitment to β_2 integrin by co-immunoprecipitation assays. As shown in Fig. 7a and b, both talin and RIAM were found to co-immunoprecipitate with integrin β_2 subunit in lysates from unstimulated cells and this co-immunoprecipitation increased when the cells were stimulated with 8CPT and challenged with C-RBC. Noteworthy, in shRIAM HL-60 cells, the amount of talin recruited to the β_2 integrin was reduced by 60 % compared to shControl cells, as determined by co-immunoprecipitation with β_2 subunit (Fig. 7a and b). These results strongly indicate that RIAM regulates talin recruitment to the integrin β_2 subunit during complement-dependent phagocytosis.

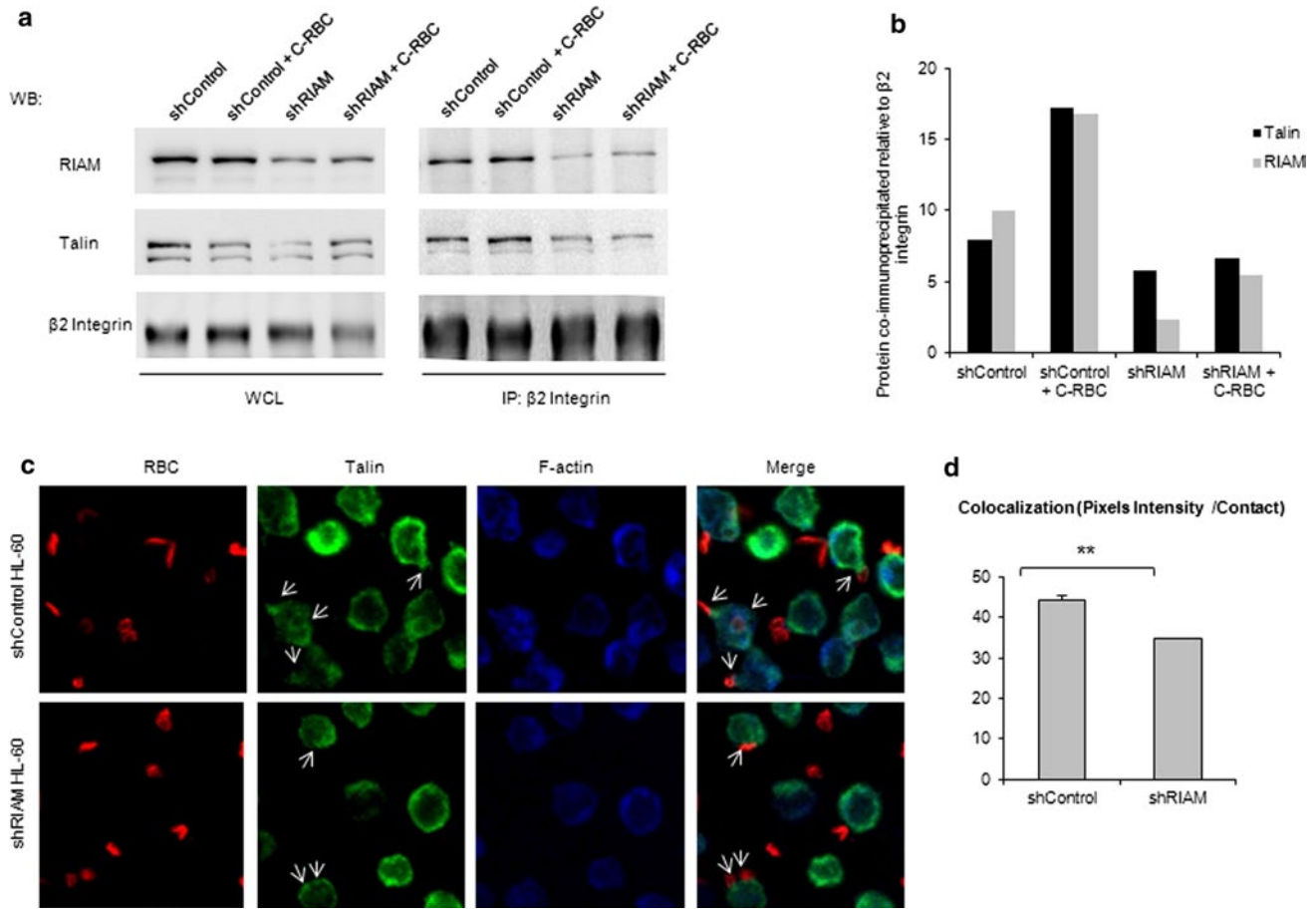

Fig. 7 RIAM participates in talin recruitment to the integrin β_2 during complement-mediated phagocytosis. **a** Neutrophilic shControl and shRIAM HL-60 cells were left untreated or treated with 100 μM 8CPT and challenged with complement-opsonized red blood cells (C-RBC) for 20 min. Cells were lysed and co-immunoprecipitation was performed using the anti-β_2 integrin mAb TS1/18 coupled to protein-G-Sepharose. Proteins were resolved by SDS-PAGE under reducing or non-reducing conditions, transferred to nitrocellulose membranes, and immunoblotted with anti-integrin β_2, under non-reducing conditions or anti-talin and anti-RIAM under reducing conditions. **b** Quantification of protein co-immunoprecipitated with β_2 integrin was determined by densitometry and presented as relative to the amount of β_2 integrin in each lane. **c** shControl and shRIAM HL-60 cells were treated with 100 μM 8CPT and challenged with C-RBC for 10 min. Samples were fixed, permeabilized, stained with phalloidin, an anti-Talin mAb and anti-RBC polyclonal antibody and analyzed by confocal microscopy. A representative field of five analyzed fields is shown. *Arrows* show colocalization between talin, phalloidin, and C-RBCs (merge) where the phagocytic cup is formed. **d** Enrichment in colocalization signal at the cell-RBC contact site was used to determine the average pixel intensity/contact in shControl and shRIAM cells. In total, 555 shControl cells with 340 bound C-RBCs and 725 shRIAM cells with 350 bound C-RBCs, were analyzed

To corroborate these results by confocal microscopy, we performed complement-dependent phagocytosis in shRIAM and shControl neutrophilic HL-60 cells treated with 8CPT. Talin recruitment to the phagocytic cup was determined as enrichment of the talin colocalization signal with F-actin and the surface bound C-RBCs. A significant reduction in talin colocalization signal at individual contacts was observed in shRIAM HL-60 cells compared to shControl cells (Fig. 7c and d). Taken together, these data indicate that RIAM regulated complement-mediated phagocytosis by recruiting talin to the β_2 integrin subunit of the phagocytic receptors at the phagocytic cup.

Discussion

In the present study, we examined the role of the Rap1 effector RIAM, a talin-binding protein, in the regulation of complement-mediated phagocytosis. Our results indicated that RIAM is actively involved in complement-dependent phagocytosis mediated by Rap1 and regulates this effect by recruiting talin to the cytoplasmic tail of the β_2 subunit of the $\alpha_M\beta_2$ integrin, the CR3 complement receptor. Previous reports have identified Rap1 as the main small GTPase involved in the "*inside-out*" signaling pathway that activates CR3 [2]. The cytoskeletal actin-binding protein talin, another

molecule involved in the "*inside-out*" signaling pathway to integrins [15], is also known to regulate the activity of complement receptors [17]. Talin is recruited to the phagosome by various phagocytic receptors [5], and talin knockdown in mammalian phagocytes specifically abrogates the ingestion of complement-opsonized particles [17]. A previous report demonstrated that Rap1 works upstream of talin in the "*inside-out*" signaling pathway that activates $\alpha_M\beta_2$ and that the region of the β_2 integrin subunit spanning amino acids 732 and/to 761 seems to be essential for the $\alpha_M\beta_2$ activation mediated by Rap1 [38]. Although the roles of Rap1 and talin in regulating $\alpha_M\beta_2$ activation are well established, the mechanism that links this small GTPase to talin recruitment into the phagocytic cup and to complement-mediated phagocytosis via the $\alpha_M\beta_2$ integrin remains elusive.

Our present studies provide mechanistic insight to these previous observations and reveal a molecular pathway, which links active Rap1, talin recruitment, and functional activation of complement-mediated phagocytosis via affinity regulation of the $\alpha_M\beta_2$ integrin, the CR3 complement receptor. Our findings demonstrated that the Rap1 effector RIAM is mandatory for active Rap1 to mediate the increase of $\alpha_M\beta_2$ affinity and complement-mediated phagocytosis. We used two different RIAM shRNA constructs, which induced depletion of endogenous RIAM in the human myeloid cell lines HL-60 and THP-1. We determined that in HL-60 neutrophilic-like cells stimulated with fMLP or LPS the binding of soluble fibrinogen, a ligand for $\alpha_M\beta_2$, was reduced following RIAM knockdown with specific shRNAs. Similarly, the binding of CBRM1/5, an activation reporter antibody for this integrin, was diminished in these cell lines after RIAM silencing, supporting an active role of RIAM in regulating $\alpha_M\beta_2$ affinity. This finding is consistent with the function of RIAM in megakaryocytes and in CHO cells expressing $\alpha_{IIb}\beta_3$ in which RIAM knockdown results in a reduced binding of PAC-1, an activation reporter mAb for $\alpha_{IIb}\beta_3$ and in reduced fibrinogen binding through this integrin [21]. Consistent with these effects of RIAM on activation of $\alpha_{IIb}\beta_3$ integrin, T cells overexpressing RIAM showed an increase in $\alpha_L\beta_2$ and $\alpha_4\beta_1$ integrin affinity, measured as increased binding of the activation reporter antibodies KIM127 and HUTS-4, respectively [12]. Our results presented here also showed that interference of RIAM expression in neutrophilic-like HL-60 and macrophage-like THP-1 cells resulted in significant impairment of phagocytosis of complement-opsonized RBCs particles. Treatment of these cells with the pro-inflammatory stimuli fMLP or LPS increased complement-mediated phagocytosis, consistently with previous observations [2]. This increase in the phagocytic capacity was completely abrogated in shRIAM cell lines, indicating an essential role for RIAM in the "*inside-out*" signaling pathway that induces activation of complement phagocytic receptors.

Our observations are in contrast to the findings of a previous study that reported that RIAM was not involved in the regulation of $\alpha_M\beta_2$ activity [18]. These contrasting conclusions possibly reside in the distinct experimental approaches employed by these studies. In the previous study, fluorescence microscopy was employed for the assessment of opsonophagocytic activity [18]. In our work we used, in addition to fluorescence microscopy, biochemical assays, and flow cytometry, a robust and sensitive method that allows the analysis of large numbers of cells, the quantification of cells involved in phagocytosis, and the fluorescence intensity of the bound and/or ingested RBCs. We also used fMLP and LPS to activate our myeloid cell lines, thereby overcoming a potential cell refractory state to full integrin activation when the same pharmacological compound, PMA, is used to induce both the differentiation and the activation of these cells [39, 40]. This might explain why we were able to detect a potent effect of RIAM knockdown in complement-dependent phagocytosis, although this effect was not as prominent as that observed by knocking-down talin expression [18]. We further supported the significance and biological relevance of our findings by assessing the role of RIAM in complement-mediated phagocytosis using primary MDM cells. These cells display lower basal phagocytic activity than myeloid cell lines and do not require pharmacological differentiation with PMA. In MDM cells, direct activation of Rap1 induced by 8-pCPT-2′-O-Me-cAMP resulted in increased complement-dependent phagocytosis that was abrogated by depletion of endogenous RIAM with RIAM shRNA or RIAM siRNA. Consistent with this observation, shRIAM-MDM cells treated with 8CPT presented a defect in $\alpha_M\beta_2$ integrin affinity activation compared to shControl-MDM cells. Together, our studies in HL-60 and THP-1 cell lines and in primary MDM cells provide evidence that RIAM has a mandatory role in an "*inside-out*" signaling pathway downstream of Rap1, which activates phagocytic integrins. Because RIAM N-terminus contains a talin-binding site [22], we hypothesized that RIAM might regulate the recruitment of talin to the β_2 subunit of the phagocytic integrins. In fact, by using co-immunoprecipitation studies, we observed an increased recruitment of talin and RIAM to β_2 integrin in cells stimulated with 8CPT and challenged with C-RBC. However, the recruitment of talin to β_2 integrin was strongly reduced in RIAM knockdown cells. These results are in agreement with our confocal microscopy findings showing that RIAM knockdown cells displayed diminished recruitment of talin at the contact site of the cell with the C-RBC.

Our results are consistent with a model of complement-mediated phagocytosis in which an "*inside-out*" signaling pathway to phagocytic integrins induces Rap1 activation and binding to RIAM, which subsequently interacts with talin and regulates its recruitment to the integrin β_2 subunit

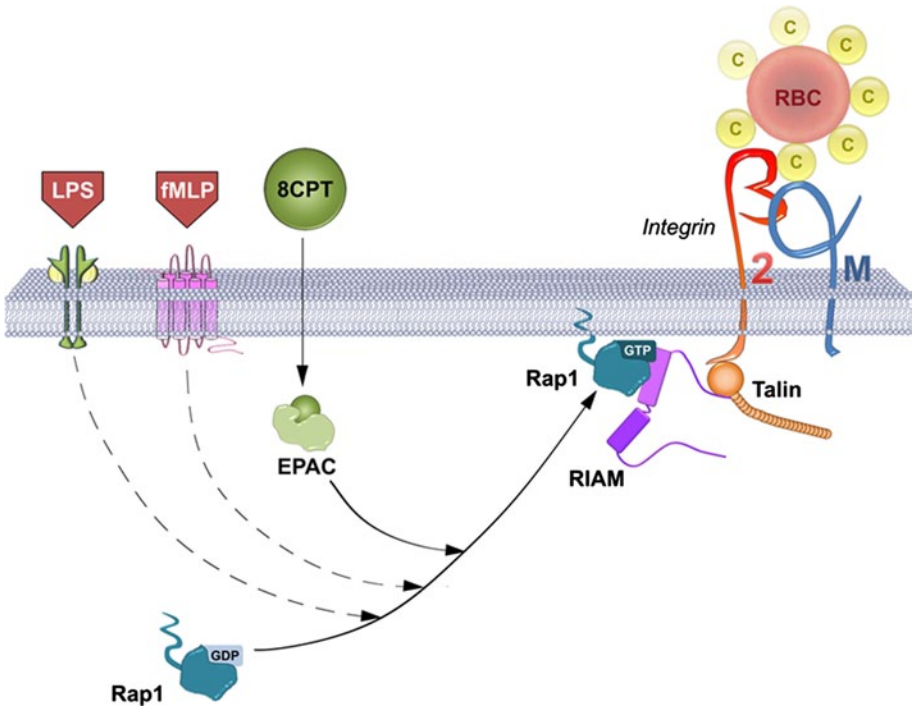

Fig. 8 Model for the "*inside-out*" signaling pathway downstream of Rap1 leading to $\alpha_M\beta_2$ integrin activation in complement-mediated phagocytosis. Agonist receptor activation by the bacterial products fMLP or LPS initiates signaling cascades that promote the exchange of GDP for GTP in the small GTPase Rap1. Rap1-GTP interacts with its effector RIAM, which in turn interacts with talin recruiting this protein to the proximity of the $\alpha_M\beta_2$ integrin. Talin head binding to the cytoplasmic tail of the integrin β_2 chain triggers structural changes in $\alpha_M\beta_2$ integrin that lead to an increased affinity for its ligand, the iC3b complement fragment that coats phagocytic preys. Pharmacological activation of Rap1 by treatment with 8CPT, an AMPc analogue that binds directly to EPAC, recapitulates these biochemical events

of complement receptors to induce integrin activation and to initiate adhesion and internalization of complement-opsonized particles (Fig. 8). Although our present experimental data provide evidence that RIAM functions as a crucial link for the molecular and functional integration of these signaling components, it is intriguing to speculate that other properties of RIAM such as regulation of integrin clustering and reorganization of the actin cytoskeleton at the phagocytic cup might also contribute to complement-dependent phagocytosis. Further studies will be required to address these additional aspects of RIAM function. In conclusion, our present studies provide evidence that in addition to regulating adaptive immunity by affecting lymphocyte function, RIAM can regulate a central function of the innate immune system, which involves complement-dependent phagocytosis.

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