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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 promyelocytic cell lines HL-60 and THP-1, we determined that knockdown of RIAM impaired $\alpha_M\beta_2$ integrin affinity changes induced by fMLP. Phagocytosis of complement-opsonized RBC particles, but not 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 the phagocytic cup 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 phagocytic complement receptors.

Keywords

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

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INTRODUCTION

Phagocytosis involves the recognition and the engulfment of large particles ($>0.5\ \mu\text{m}$) by a multi-step actin-driven process. Mammalian professional phagocytes such as macrophages, 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 surface of the particle, or indirectly through opsonins recognized by opsonic phagocytic receptors [1]. Opsonization by IgG or by 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 (known as Mac1, CD11b/CD18, $\alpha_M\beta_2$) and the complement receptor CR4 (also termed p150, 95, CD11c/CD18, $\alpha_X\beta_2$) are members of the β_2 integrin family. Activation of CR3 and CR4 is induced by inflammatory mediators such as bacterial products (LPS, fMLP), pro-inflammatory chemokines and cytokines such as TNF α or PAF (the platelet activating factor) among others [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 $\alpha_4\beta_1$, $\alpha_3\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_L\beta_2$ and $\alpha_M\beta_2$ [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, TNF α 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 β_1 , β_2 or β_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 formed by complement-opsonized targets [18]. Point mutations of the talin binding motif of β_2 integrin reduce the recruitment of talin to the phagocytic cup and uptake of complement-opsonized particles. Furthermore, knockdown of talin in the macrophage cell line J774-A1 and in COS cells expressing $\alpha_M\beta_2$, strongly impairs complement-mediated phagocytosis [17]. In $\alpha_{IIb}\beta_3$ integrin binding of talin to the β_3 subunit is regulated by active Rap1 [10], and a recent report suggests that active Rap1 is also required for talin recruitment to $\alpha_M\beta_2$ integrin [18].

RIAM (Rap1-GTP-interacting adaptor molecule) is a molecular partner of active Rap1 that was 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. 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 APC-T cell interaction by regulating recruitment of active Rap1 to the immune synapse via its interaction with the SKAPP55-ADAP module [19]. Furthermore, RIAM interacts with PLC- γ 1 and regulates PLC- γ 1 spatio-temporal distribution and activation upon T cell activation via the TCR [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 via 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 the phagocytic cup and to complement receptors. 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 promyelocytic 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 phagocytic cup. These results indicate that RIAM is an integral component of an 'inside-out' signaling pathway mediated by Rap1 and regulates complement-mediated 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, 2mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μ g/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 hours or in the case of HL-60, into neutrophil-like cells by treatment with 1 μ M 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, 2mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μ g/ml). MDMs were obtained from human monocytes isolated from buffy coats by using Ficoll-paque PLUS gradient (GE Healthcare) followed by incubation of the PBMC fraction with a CD14 antibody coupled to magnetic beads (MACS^{TM} cell separation column, Miltenyi Biotec). Monocytes were differentiated into macrophages by culture in RPMI 1640 media

supplemented with 10% FCS, 2mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml) in presence of 1000 IU/ml of human GM-CSF (Immunotools) for 5 days.

shRNA silencing and transfections

HL-60, THP-1 or MDM-1 cells were transduced with lentiviral plasmids expressing RIAM shRNA specific and control shRNA oligonucleotides, as previously described [12]. For some experiments, cells were transduced with the retroviral plasmid pRS-TI326599 containing a RIAM specific shRNA sequence or with pRS-TR30003, a plasmid containing a GFP shRNA as negative control (Hush™ shRNA Plasmid Panels 29-mer, OriGene Technologies) according to manufacturer's instructions. 48 hours after transduction, the efficiency was monitored and lentiviral-transduced GFP-expressing cell lines were sorted and retroviral-transduced cell lines were selected with 0.6 µg/ml of Puromycin (Sigma). In these cells GFP expression and RIAM knockdown was stable for at least one month in culture. In MDMs, efficiency was monitored 48 hours after transduction and subsequently cells were used for opsonophagocytosis or real time RT-PCR.

Cell activation and reagents

For activation experiments, cell lines or MDMs were left untreated or stimulated with 100 nM fMLP, 3 µg/ml LPS or 100 µM 8-pCPT-2'-O-Me-cAMP at 37°C for the indicated times. Cell activation was stopped with cold PBS, cells were washed twice with cold PBS and processed. fMLP and LPS were obtained from Sigma 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 either 1 mM MgCl₂ and 1 mM CaCl₂ or 1 mM MnCl₂ and Fc receptors were blocked by incubation with 100 µg/ml of human γ-Globulin (Sigma). Cells were incubated either with αM activation-dependent (CBRM 1/5) mAb, αM expression mAb (Bear-1), or isotype control mAb, followed by staining with donkey anti-mouse Alexa-647 Ab (Invitrogen). Cells were washed and resuspended in HBS with 1 mM cations, and analyzed by flow cytometry. Activation epitope exposure was determined by assessing binding of CBRM 1/5 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) for 30 minutes at 37°C. Cells were washed and fibrinogen binding was measured by flow cytometry.

Opsonization and phagocytosis assay

Complement-opsonized RBCs were prepared and used for the phagocytosis assay as previously described [25]. Briefly fresh sheep RBC (Oxoid, Termofisher) labeled with 2µM DDAO-AM (Molecular Probes, Invitrogen) were incubated with sub-agglutinating concentrations of rabbit IgM-anti sheep RBC cells (Chemical & Scientific Corporation, Westbury, NY), washed and incubated with 10% C5 deficient human serum (Sigma). When

Fc γ R phagocytosis was assessed, RBCs were labeled and incubated with sub-agglutinating concentrations of rabbit IgG anti sheep RBC cells (Chemical & Scientific Corporation, Westbury, NY) washed and used for the phagocytic assay. For the phagocytosis assay, cell lines or MDMs were starved for 3 hours 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 minutes at 37°C and the unbound RBC were removed by washing 3 times with ice-cold PBS. To determine the phagocytic index, RBCs bound to cells were exposed to a hypotonic shock with H₂O during 15 seconds, followed by addition of 2X 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 percent of phagocytosis (%P) indicating percent of cells with one or more internalized particles. The AI and PI indexes were calculated by multiplying the percentage of cells bound and internalized or just internalized the DDAO labeled RBCs with the MFI of the DDAO positive cells (AI= GMFI x %A), (PI = GMFI x %P) [26].

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

Cell lysates were prepared by incubating the cells during 15 minutes in lysis buffer containing 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 10% glycerol and 1% NP40 supplemented with 2 mM Sodium Orthovanadate, 1 mM PMSF and protease Inhibitor Cocktail (Sigma). 60 μ g of cell lysates were 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). 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 average of three independent experiments.

Immunostaining and fluorescence microscopy

For the analysis of complement-dependent phagocytosis by fluorescence microscopy, 250000 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 hours, stimulated with 8CPT and challenged with C-RBC particles for 1 hour 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 MgCl₂, 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 μ g/ml of human γ -Globulin (Sigma), cells were incubated with rabbit IgG against RBCs (Chemical & Scientific Corporation, Westbury, NY) 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 neutrophil-like shControl and shRIAM HL-60 cells were stimulated with 8CPT and were challenged with C-RBCs for 10 minutes at 37 C prior to seeding onto poly-Lysine coated slides (Sigma). Cells were 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 μ g/ml of human γ -Globulin (Sigma) and cells were incubated with the anti-talin mAb, 8d4 (Sigma) and rabbit anti-RBC IgG (Chemical & Scientific Corporation, Westbury, NY) followed by Alexa647-conjugated donkey anti mouse IgG (Invitrogen) and Alexa555-conjugated donkey anti rabbit IgG (Invitrogen). Samples were mounted with Fluoro-Gel (Fluoroprobes) and images were obtained with 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 $\pm$ SEM. Statistical analysis for *in vitro* assays was determined by unpaired two-tailed Student *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), 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 a RIAM shRNA [12] and we established HL-60 cell lines expressing shRIAM or shControl. We also used a second RIAM shRNA sequence to generate a stable RIAM knockdown 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 (Figure 1a) and by 70% in shRIAM-99 cells (Supplemental Figure S1a). RIAM expression was upregulated by treatment with RA in the HL-60 cell lines, however RIAM levels remained diminished in shRIAM cell line compared to shControl (Figure 1a).

This finding was observed also in the shRIAM-99 cell line albeit to a different magnitude (Supplemental Figure S1a). 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 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 (Figure 1b and Supplemental Figure S1b). These results indicate that RIAM is not involved in the upregulation of the phagocytic complement receptors upon differentiation of myeloid cells.

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

The bacterial products fMLP and LPS are reported to 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 neutrophil-like HL-60 cell lines 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, suggesting that RIAM might regulate β_2 integrin activity, similarly to its effects in other cell types [12], (Figure 1c and Supplemental Figure 1c). 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 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 this is in an active conformation state [33]. As shown in Figure 1d, both shControl and shRIAM cells increased the binding to CBRM1/5 upon activation with fMLP. However in shRIAM cells the increase in CBRM1/5 binding was less prominent than in shControl cells. Notably, under these conditions fMLP induced activation of Rap1 as determined by pull down assay (Supplemental figure 1d). These results strongly suggest that the defect in integrin affinity regulation observed in these cell 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-differentiated and were 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 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 (Figure 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 effect was mediated by the complement phagocytic receptors CR3 and/or CR4. However, shRIAM cells showed a 20% reduction in AI and PI indexes compared to shControl cell lines. Upon cell stimulation with fMLP or LPS, there was a significant increase in phagocytosis in shControl HL-60 cells, but such increase was undetectable in shRIAM HL-60 cells which

exhibited a 50% reduction in phagocytosis compared to shControl (Figure 2a). A similar pattern of results was observed using shRIAM-99 cell line (Supplemental Figure 2a). These results indicate that a reduction in RIAM expression leads to impaired activation of CR3- and/or CR4-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 shRIAM-99 cell line, which expressed higher RIAM protein level and less prominent defect in phagocytosis (Figure 2a and Supplemental Figure 2a).

To determine whether this effect of RIAM expression was specific for the phagocytic complement receptors, we performed the same opsonophagocytic assay using RBC particles opsonized with IgG. For these experiments, the AI and PI were comparable between shRIAM and shControl HL-60 cell lines (Figure 2b), indicating that the effect of RIAM on phagocytosis was specific for complement receptors and not for Fc γ R 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), 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 induced activation of Rap1, which peaked at 5 minutes of stimulation and gradually declined (Figure 3a). To determine whether RIAM was required for the regulation of phagocytosis induced by Rap1 activation in these cells, we tested the binding capacity of soluble fibrinogen. Treatment with 8CPT upregulated fibrinogen binding to shControl cells and this effect was abrogated by depletion of RIAM by shRNA (Figure 3b). Assessment of complement mediated phagocytosis, by challenging 8CPT-treated cells with C-RBC, showed that increased phagocytosis of C-RBC was induced by 8CPT treatment and this effect was abrogated in RIAM knockdown cells (Figure 3c). Similar results were observed using shRIAM-99 cell line and the relevant control (Supplemental Figure 2b). These results indicated that direct activation of Rap1 induces an increase in fibrinogen binding and complement-mediated phagocytosis that is 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 (Figure 4a). PMA treatment upregulated CD11b and CD11c expression to a comparable degree in shControl and shRIAM cells (Figure 4b). To assess complement-mediated phagocytosis, we performed opsonophagocytic assay 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 complement-mediated phagocytosis (Figure 4c).

To further analyse the role of RIAM in complement-mediated phagocytosis in macrophages, we used the human promonocytic cell line THP-1 to knockdown RIAM expression establishing shControl-THP1 and shRIAM-THP-1 cell lines. RIAM expression was upregulated in shControl and shRIAM-THP-1 cells after differentiation with PMA treatment, but RIAM protein level remained significantly lower in shRIAM-THP-1 cells compared to the shControl cells (Figure 5a). The knockdown of RIAM expression was stable during cell differentiation (Figure 5a) and the expression of CD11b and CD11c was similar in shRIAM- and shControl-THP-1 cell lines (Supplemental Figure 3). By using the 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 (Figure 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, and analyzed by fluorescence microscopy. shRIAM-THP-1 cells clearly displayed a reduced binding and uptake of C-RBC particles compared to shControl-THP-1 cells (Figure 5c), also showing a 50% reduction in the phagocytic index (PI) calculated from the analysis of the fluorescence microscopy images (Figure 5d).

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 sought to determine the role of RIAM in 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 [12]. shRIAM and shControl transduced MDMs displayed comparable transduction efficiency (Figure 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 (Figure 6b). To determine the role of RIAM in phagocytosis in MDM cells we performed an opsonophagocytic assay using C-RBC as particles and treating or not the cells with 8CPT to induce Rap1 activation. The cells were analyzed by flow cytometry gating on the GFP positive cells, and the %A and the AI 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 (Figure 6c). When MDM cells were incubated with C-RBCs in media, shControl and shRIAM 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 8CTP, shControl-MDM displayed a significant increase of C-RBC binding to approximately 80%, whereas shRIAM-MDM cells displayed no increase of C-RBC binding indicating a lack of complement receptor activation compared to untreated cells (Figure 6c). Assessment of AI and %A indexes in five independent experiments corroborated this observation (Figure 6d). 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]. 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-HL60 cells stimulated with 8CPT and we analysed talin recruitment to the phagocytic cup by confocal microscopy. To allow the cells to form the phagocytic cup with minimal internalization of the particles, the cells were seeded on poly-L-lysine slides during the phagocytic reaction and were incubated for 10 minutes at 37°C before fixation. Talin recruitment to the phagocytic cup was determined as enrichment of the talin colocalization signal with 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 (Figure 7). Taken together these data indicate that RIAM regulated complement-mediated phagocytosis by recruiting talin to 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], however, 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 promyelocytic 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 by the expression of RIAM specific shRNAs. Similarly, the binding of CBRM1/5, an activity reporter antibody for this integrin was abrogated in these cell lines after expression of a RIAM shRNA, 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 interaction with 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 HL-60 neutrophilic-like and THP-1 macrophage-like cells resulted in significant impairment of phagocytosis of complement-opsonized RBCs particles. Treatment of these cells with the proinflammatory 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, which 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. Instead of fluorescence microscopy that was employed for the assessment of opsonophagocytic activity by the previous study [18], in our work we used flow cytometry, a robust and sensitive method, which 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 promyelocytic cell lines, thereby overcoming a potential cell refractory state to full integrin activation when the same pharmacologic compound PMA is used to induce both the differentiation and activation of the cell [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 promyelocytic 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. Together our studies in HL-60 cell lines, 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 phagocytic integrins. Using confocal microscopy, we observed robust recruitment of talin to the phagocytic cup formed upon incubation with the C-RBC particles, which was abrogated in RIAM knockdown cells.

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 of complement receptors to induce integrin activation and to initiate adhesion and internalization of complement-opsonized particles (Figure 8). Although our present experimental data provide evidence that RIAM functions as a crucial link for the molecular and functional integration of these signalling 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BSA	Bovine Serum Albumin
8-CPT	8-pCPT-2-O-Me-cAMP
FCS	Fetal Calf Serum
HRP	Horse Radish 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

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Figure 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) were left untreated or were 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 value 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.** Soluble fibrinogen binding was determined in the indicated neutrophilic-HL-60 cell lines cultured in medium, 100nM fMLP or 3 μ /ml LPS and analyzed by flow cytometry. Data are expressed as relative to fibrinogen binding to shControl cell line cultured in medium. **d.** Binding of CBMR1/5 to the neutrophilic shControl and shRIAM cell lines cultured in medium alone (grey line histograms) or stimulated with 100nM fMLP (black line histograms). Grey-filled histograms represent binding of secondary antibody on cells cultured in medium alone, used as negative control. The % of fluorescent positive cells and the MFI for each cell line are indicated.

Figure 2. RIAM regulates complement-dependent phagocytosis in neutrophilic-HL-60 cell lines
a. Neutrophilic shControl and shRIAM cell lines unstimulated (medium) or stimulated with 100nM fMLP or 3 μ g/ml LPS were challenged with unopsonized red blood cells (RBC) or complement-opsonized red blood cells (C-RBC). The association index (AI) and phagocytic index (PI) are represented as relative values to unstimulated shControl. **b.** A Fc γ R-mediated phagocytosis assay was performed using neutrophilic shControl and shRIAM cell lines. AI and PI are represented as relative to shControl.

Figure 3. RIAM expression is required for Rap1-induced complement-mediated phagocytosis
a. Rap1 activation in neutrophilic HL-60 cell line, either untreated or treated or not with 100 μ M 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 (FNG) to neutrophilic shControl (black line) and shRIAM (grey-filled) cell lines cultured in medium alone or stimulated with 100 μ M 8CPT was analyzed by flow cytometry. The signal obtained from shControl (grey lines) and shRIAM (dotted grey lines) in the absence of fibrinogen was used as negative control. **c.** A complement-dependent phagocytosis assay was performed in neutrophilic shControl and shRIAM HL-60 cell lines either untreated or stimulated with 100 μ M 8CPT and challenged with complement opsonized RBCs (C-RBC) or unopsonized red blood cells (RBC). The AI and PI indexes are represented as relative values to unstimulated shControl values.

Figure 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 % of positive cells and the MFI for each cell line are indicated. **c.** Macrophage-like shControl and shRIAM cells either unstimulated or stimulated with 100nM fMLP, 3 μ g/ml LPS or 100 μ M 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 unstimulate shControl.

Figure 5. RIAM regulates complement-dependent phagocytosis in macrophage-like THP-1 cells
a. shControl-THP1 and shRIAM-THP-1 cell lines, 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 100nM, fMLP, 3 μ g/ml LPS or 100 μ M 8CPT. Cells were subjected to 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 THP-1 shControl cells. **c.** Complement-mediated phagocytosis assay was performed with macrophage-like shControl- and shRIAM-THP-1 cell lines activated with 100 μ M 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. Phagocytic index was calculated as number of bound or internalized RBCs per 100 GFP positive phagocytes counted in 15 independent fields.

Figure 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, and the % of GFP positive cells was assessed by flow cytometry 72 hours after 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 line relative to transduction efficiency. Results are representative of five independent experiments. **c.** One representative complement-dependent opsonophagocytic experiment is shown. Dot plots represent GFP positive shControl and shRIAM-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 C-RBCs in medium and right panels show these cells stimulated with 100 μ M 8CPT and challenged with C-RBCs. **d.** Opsonophagocytic assay was performed using shControl-and shRIAM-MDMs either untreated or stimulated with 100 μ M C-RBC. The %A (upper graph) and the AI (lower graph) were expressed as values relative to unstimulated shControl-MDM GFP+ cells. Results are representative of five independent experiments.

Figure 7. RIAM participates in talin recruitment to the phagocytic cup

a. Neutrophilic shControl and shRIAM HL-60 cells were treated with 100 μM 8CPT and challenged with C-RBC for 10 minutes. Samples were fixed, permeabilized, stained with an anti-Talin mAb and anti-RBC polyclonal antibody and were analyzed by confocal microscopy. A representative field of 5 analyzed fields is shown. Arrows show colocalization between talin and C-RBCs (merge) where the phagocytic cup is formed. **b.** 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.

Figure 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 turns 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. Pharmacologic activation of Rap1 by treatment with 8CPT, an AMPc analogue that binds directly to EPAC, recapitulates these biochemical events.