UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE MEDICINA

TESIS DOCTORAL

Activación y Señalización del Receptor del Complemento CR3

Signaling and activation of complement recepetor

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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SIGNALING AND ACTIVATION OF COMPLEMENT RECEPTOR CR3

A Dissertation Presented

by

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ABSTRACT

Integrins lie at the core of many critical immunobiological processes, ranging from regulating the formation of immunological synapses and antigenic presentation to phagocytosis. The Complement Receptors CR3/$\alpha_M\beta_2$ and CR4/$\alpha_X\beta_2$ are endowed with ability to engulf and dispose mainly of complement-opsonized particles, thereby being involved in pathogen elimination, apoptotic cell clearance and removal of cellular debris. Therefore, these receptors contribute to both immunity and tissue homeostasis. Research in the field of integrin-mediated phagocytosis has shed light on the molecular events controlling integrin activation and their effector functions. Activation of these receptors is dependent on signals derived from cytokine receptors as well as receptors sensing molecular patterns associated with either pathogens, cellular damage or stress. This process is termed inside-out signaling, and critically involves the recruitment of Talin to the cytoplasmic tails of the integrin β subunit, which is mediated by Rap1 activation and RIAM binding. In contrast to inside-out signaling, outside-in signaling describes the molecular events downstream integrin activation and remains less explored for these receptors.

The following thesis titled "Signaling and Activation of Complement Receptor CR3" sets out to establish the roles of components of the integrin adhesion complex to the process of phagocytosis and outside-in signaling, through the use of genetic tools to insert copies, silence or delete key proteins in a well-established human cell line model.

An initial work dissects the contribution of RIAM to the overall process of phagocytosis and integrin downstream signaling. The role of VASP is also analyzed. VASP is determined to be a critical mediator of actin dynamics during complement-

mediated phagocytosis, specifically during outside-in activation. and dysregulation in its expression abolishes particle engulfment due to an overactive cytoskeleton.

RIAM is identified as controlling cytoskeletal rearrangements necessary for particle engulfment through recruiting VASP to the phagocytic cup. RIAM also controlled VASP phosphorylation status, which informs its actin elongation activity. Therefore, RIAM appeared to work as a relay in integrin complement receptor outside-in signaling, since it was capable of coordinating integrin activation and cytoskeletal rearrangements via its interaction with VASP.

The requirement for expression of integrin proximal proteins for the acquisition of a phagocytic phenotype is also analyzed. RIAM, VASP and Vinculin expression are identified as specifically controlling the surface expression of integrin subunits α_M, α_X and β_2. A deficiency in these proteins prevents the increased expression of these subunits during neutrophilic differentiation. The expression of these proteins was found to be critical for the induction of these integrin subunit transcription, therefore linking the existence of functional integrin signaling to its transcriptional regulation.

This control over integrin expression was observed to be due to the impact that RIAM, VASP and Vinculin all have on actin polymerization, since α_M expression can be increased and almost renormalized through the use of actin stabilizing drugs, which increase actin polymerization. The fact that integrin expression could be rescued this way, pointed to the MRTF-A/SRF pathway. Induction of the transcriptional activity of the transcription factor SRF (Serum Response Factor) requires the nuclear translocation of MRTF-A, which is sequestered in the cytoplasm by monomeric G-actin. We observed that in knockouts MRTF-A remained in the cytoplasm and therefore could not activate SRF. Conversely, the reverse was

observed for the SRF corepressor FHL-2, which was able to inhibit SRF transcription in the absence of expression of RIAM, VASP and Vinculin, which are required for its sequestering in the plasma membrane.

Therefore, the following research gives an understanding of the roles that integrin adhesion complex proteins play both, directly during phagocytosis and during myeloid differentiation, in order for cells to acquire a phagocytic phenotype.

RESUMEN

Las integrinas se sitúan en el centro de muchos procesos inmunológicos críticos; desde la regulación de la sinapsis inmunitaria y la presentación antigénica hasta la fagocitosis. Los Receptores del Complemento CR3/$\alpha_M\beta_2$ y CR4/$\alpha_X\beta_2$ están dotados con la habilidad de engullir y eliminar partículas opsonizadas con el complemento, y por tanto están involucrados en la eliminación de patógenos, células apoptóticas y restos celulares. Estos receptores contribuyen tanto a procesos de inmunidad como de homeostasis tisular. La investigación en el campo de la fagocitosis mediada por integrinas ha permitido dilucidar los eventos moleculares que controlan la activación de integrinas y su función efectora. La activación de estos receptores es dependiente de señales derivadas de receptores de citoquinas, así como de receptores de patrones moleculares asociados a patógenos y daño celular o estrés. Este proceso se conoce como señalización "inside-out", e involucra el reclutamiento crítico de Talina a las colas citoplasmáticas de la subunidad β de las integrinas, en un proceso dependiente de la activación de Rap1 y la unión de Talina a RIAM. En contraposición a la señalización "inside-out", la señalización "outside-in" describe los eventos moleculares que se producen tras la activación de la integrina y han sido menos explorados en el contexto de las integrinas fagocíticas.

El siguiente proyecto de Tesis Doctoral titulado "Activación y Señalización del Receptor del Complemento CR3" se propone establecer el papel que ejercen proteínas del complejo de adhesión de integrinas en los procesos de fagocitosis y señalización "outside-in" mediante el uso de herramientas genéticas para insertar copias, silenciar o eliminar proteínas que se sospechan críticas para el proceso en un modelo de línea celular promielocitica bien establecido.

Un trabajo inicial disecciona la contribución de RIAM al proceso entero de fagocitosis y senalización "outside-in". También se analiza el papel de VASP, un mediador de la dinámica del citoesqueleto de actina, durante la fagocitosis mediada por el complemento. VASP controla la internalización de la partícula y una desregulación en su expresión abroga este proceso debido a un citoesqueleto sobreactivado.

Se identifica a RIAM como la responsable de controlar la reorganización del citoesqueleto, ya que es capaz de reclutar a VASP a la copa fagocítica. RIAM también controla el estado de fosforilación de VASP, determinando su actividad como elongador de actina. Por tanto, RIAM parece funcionar como un relé durante la fagocitosis, ya que es capaz de coordinar la activación de integrinas con la reorganización del citoesqueleto a través de su interacción con VASP.

Se analiza también el requerimiento de expresión de proteínas próximas a las integrinas para la adquisición de un fenotipo fagocítico. La expresión de RIAM, VASP y Vinculina controlan de manera específica la expresión en superficie de las subunidades α_M, α_X y β_2. La deficiencia en estas proteínas previene el incremento de expresión de estas subunidades durante la diferenciación celular hacia neutrófilos. La expresión de estas proteínas parece ser determinante en la inducción de la transcripción del mRNA de estas subunidades, por tanto conectando la existencia de señalización funcional con regulación de la transcripción.

Este control sobre la expresión de integrinas parece estar ligado al impacto que estas proteínas tienen sobre la polimerización de actina. Esto es debido a que la expresión de estas subunidades incrementa y casi llega a renormalizarse con el uso de compuestos estabilizadores del citoesqueleto que causan un incremento en la polimerización de actina. El hecho de que la expresión de integrinas pudiese

revertirse de esta manera indica la participación en el proceso de la vía MRTF-A/SRF. La inducción de la actividad transcripcional de SRF (Factor de Respuesta al Suero) requiere la translocación nuclear de su coactivador MRTF-A, el cual es secuestrado en el citoplasma por unirse a actina monomérica. Se observa que en células con deleción de la expresión de RIAM, VASP y Vinculina, MRTF-A se mantiene esencialmente en el citosol, y por tanto no puede activar a SRF. Una localización inversa se observa para FHL-2 (correpresor de SRF), concentrándose en el núcleo y por tanto inhibiendo la transcripción mediada por SRF. La ausencia de expresión de RIAM, VASP y Vinculina parece controlar esta translocación nuclear, ya que en células parentales FHL-2 se encuentra localizado principalmente en la región cortical sub-membrana.

Por tanto, el siguiente trabajo de investigación permite profundizar en el conocimiento de la función que desempeñan proteínas del complejo de adhesión de integrinas, tanto directamente sobre la fagocitosis como en la diferenciación mieloide y por tanto determinado la adquisición de un fenotipo fagocítico.

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CHAPTER 1

INTRODUCTION

1.1. Innate Immunity and Phagocytes

To defend against scores of pathogens (virus, bacteria, fungi, helminths, protozoans and arthropods) present in their environment, many species have evolved to acquire an immune system [1]. The immune system is a complex web of cells, proteins and specialized tissue structures which work synergistically to rid the host of the invading organism [2]. The innate immunity constitutes the first line of defense against infection and induces response from the acquired immunity components. The innate system is phylogenetically older than the acquired immune response, arising in early coelomata before the separation of invertebrates and vertebrates [3-5]. Due to its broad protection and faster response, most multicellular organisms depend exclusively on this system, [1, 6-9]. In the absence of an innate immune system, adaptive immunity confers weak or ineffective protection, as evidenced by the high propensity to infection patients with immunodeficiencies involving innate components [10, 11].

An important component of the innate immune system are the phagocytic cells termed phagocytes, including professional (monocytes/macrophages, neutrophils, dendritic and mast cells) [12] and non-professional phagocytes (fibroblasts, epithelial, endothelial and mesenchymal cells) [12-15]. These cells capture particulate material, and participate in antigen presentation, immunological tolerance, tissue remodeling and efferocytosis (removal of apoptotic or necrotic

bodies), and the resolution of inflammation [16, 17], thereby being able to modulate and influence the immune response. We will concentrate on professional phagocytes, many of which belong to a subgroup of leukocytes collectively known as myeloid cells.

Macrophages are derived from circulating blood monocytes, which take up residence in virtually every tissue, sometimes even amidst the parenchyma of vital organs [18]. These ensure that organs are immunologically surveyed. Macrophages are a morphologically diverse group, ranging from flat and irregular Kuppfer cells to spindle-shaped histiocytes [19]. All of them possess an extended Golgi apparatus, and abundant lysosomes and vesicles which house a plethora of lytic enzymes to perform their main phagocytic function.

Polymorphonuclear cells, named by their segmented nuclei are of special importance in infection containment due to their granules and secretion of proinflammatory cytokines as well as their phagocytic capability. Neutrophils are primarily geared towards the elimination of pathogens and are orders of magnitude more abundant in blood than the rest of the polymorphonuclear cells [20, 21]. Neutrophil cells are the first to be recruited to the site of the infection following inflammatory signals [22]. These can be of diverse origin, ranging from bacterial products like the bacterial wall lipopolysaccharide (LPS) and the bacterial tripeptide fMLP, to chemokines and like interleukin-8 (IL-8), IL-1β, and IL-17, passing through anaphylotoxins like C5a and C3a [23].

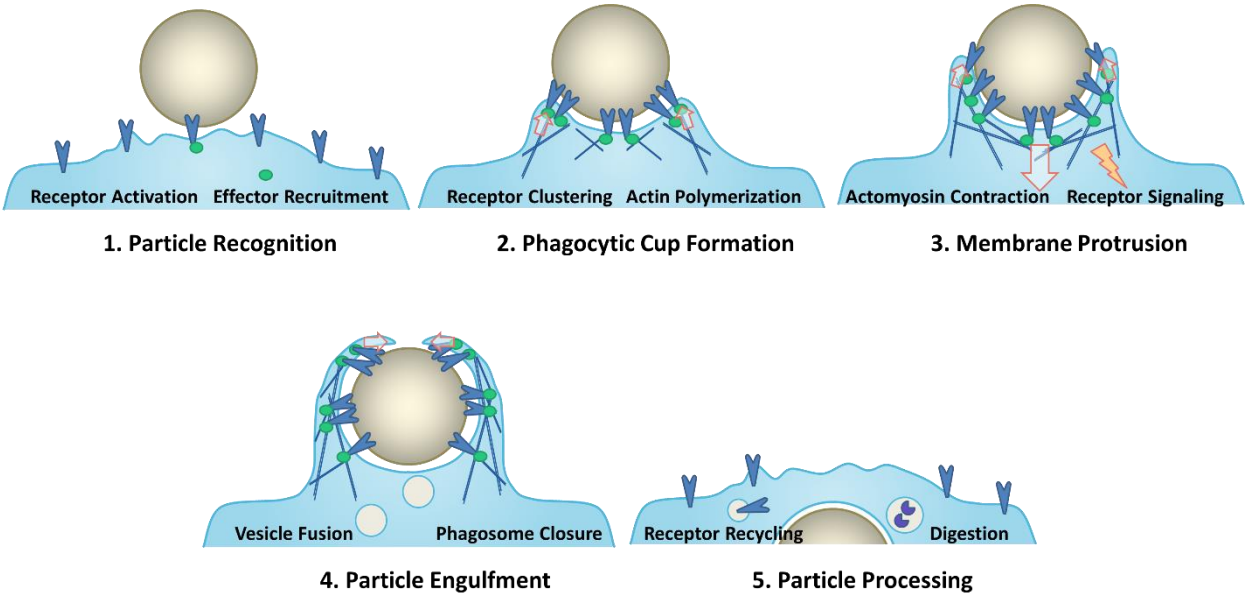
1.2. The Phagocytic Process

Phagocytosis is a fundamental and evolutionarily conserved process critical for functions which range from uptaking microbes as nutrients by single celled

organisms [24, 25], to tissue remodeling, removal of cellular debris and protein deposits, and clearance of pathogens as the first line of defense against infection [13, 26-31].

Phagocytosis entails the engulfment and disposal of particles in sequential steps (**Figure 1.1**). These include: 1) particle recognition and binding; 2) cytoskeletal remodeling and phagocytic cup formation; 3) membrane protrusion; 4) particle engulfment; 5) phagolysosomal digestion and component recycling [32-34].

Figure 1.1 The Early Steps in the Phagocytic Process.



Phagocytic receptors diffuse along the plasma membrane and bind ligands present on the surface of the particle. Ligand binding and clustering of the receptors elicits signaling that results in changes in membrane lipids, cytoskeletal remodeling and activates actin polymerization, generating a protrusive force against the plasma membrane. This actin protrusion is coupled with the activation of the actomyosin machinery and generates a pulling force. Mobilization of membrane reservoirs from surface folds of the plasma membrane and intracellular vesicles provides the required membrane surface area to envelop the particle. Once the particle is fully enveloped and the protrusions reach a meeting point, membrane fission enables the separation of the phagosome from the plasma membrane.

Particle recognition and phagocytic receptor binding is the first step in the phagocytic process and determined by several parameters: receptor affinity, lateral

diffusion of receptors in the plasma membrane and ligand accessibility [35]. Phagocytic receptors are diverse in terms of structure, ligand specificity, ligand affinity, avidity and signaling. Phagocytic receptors can be broadly categorized according to the method of particle recognition; those that require an intermediate molecule (opsonins), and those that don't (non-opsonic receptors). Opsonic receptors include Fc receptors (FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa, FcαRI, FcεRI), Complement receptors (CR1, and integrins CR3/$\alpha_M\beta_2$ and CR4/$\alpha_X\beta_2$), the TAM receptor Family (Tyro3, Axl, MerTK) and the apoptotic cell receptor integrins ($\alpha_5\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$)[36]. Non-opsonic receptors include the Scavenger Receptor family (Scavenger Receptor A/SR-A1, SR-A6/MARCO, SR-B2/CD36, SR-I3/SCARF-1, SR-L1/LRP1, SR-E2/Dectin-1, SR-H2/Stabilin-2, TIM-4, etc.) [37, 38], C-type lectins (Mannose Receptor/MMR, Dectin-1, Mincle, etc.) [39], other phosphatidylserine receptors (BAI1/ADGRB1)[39, 40] and CD14 [36]. Many phagocytic receptors cooperate in order to ensure efficient phagocytosis, and some non- receptors have been demonstrated to require integrin expression [36, 41-43].

Whilst most phagocytic receptors have a constant affinity for their ligands, this is not the case for integrins, which require conformational changes to reach a high affinity conformation [36, 44]. Receptor diffusion in the plasma membrane limits activation through regulating clustering and therefore receptor avidity [45]. Receptor clustering is dependent on the mechanical properties of the phagocytic particle and the generation of force, since an increased rigidity and ligand density results in a decrease in intermolecular distance (<60nm) between active integrins [46], whereas low-tensile loads permit stable adhesion formation with a lower degree of integrin clustering and a minimal interdistance of 200 nm [47]. More closely packed receptors may also favor an increased recruitment of dimeric proteins which may further potentiate activation and clustering. This is the case for phagocytic integrins

recruiting dimeric adaptors such as Talins and Kindlins [45, 48-51]. Phagocytic receptor movement in the plasma membrane is constrained by the cortical actin cytoskeleton into corrals, as described in the "picket and fence" model [33], and hence require cytoskeletal remodeling in order to form clusters. Additionally, receptor engagement may require the exclusion of the long and highly gylcosylated proteins that form the glycocalyx from the adhesion site, since these serve as a physical barrier to be overcome in order for phagocytosis to take place [52].

Therefore a successful engagement of the receptors requires a remodeling of the cytoskeleton and the generation of a protrusive force [53]. This process is cooperative, since active receptors signal downstream and induce the sequential activation of other receptors in what is termed as the "zipper mechanism" of phagocytosis [54]. This process leads to the formation of an actin-rich structure termed the phagocytic cup [55-57], which is governed by the protrusive force, membrane and cortical tension, as well as the physical parameters of the engulfed particle (rigidity, size and shape), which make this process highly mechanosensitive [53]. Membrane protrusion also requires the mobilization of membrane reservoirs (endocytic vesicles and granules) in order to provide the necessary surface to engulf the particle and depend on the exocytic SNARE pathway [58-60].

Finally, dynamin-2 mediated membrane fission terminates the entire process, and the phagosome undergoes maturation and phagolysosomal fusion, as well as receptor recycling [52, 61-63]. In this last process, actin cytoskeleton remodeling is also key, since membrane fission requires an initial burst of actin polymerization[64, 65] and subsequent depolymerization [66]. These processes are tightly spatiotemporally regulated by the recruitment of effectors to membrane domains enriched in phosphatidylinositol membrane lipids [44, 66, 67].

1.3. Integrins and Phagocytosis

The role of integrins in phagocytosis is evolutionarily conserved and can be traced from *Caenorhabditis elegans* INA-1/PAT-3, involved in clearance of apoptotic cells [68], and *Drosophila* αPS3/βν, which has roles in microbial defense and apoptotic cell removal [69, 70] (**Table 1.1**). In mammalians, the orthologues $\alpha_V\beta_3/\alpha_V\beta_5$ are expressed in professional and non-professional phagocytes (endothelial, epithelial, fibroblast, neuronal and mesenchymal cells) with a role in phosphatidylserine-rich apoptotic/necrotic body clearance. Professional phagocytes in mammals express complement receptors $\alpha_M\beta_2$/CR3 and $\alpha_X\beta_2$/CR4, which are phagocytic receptors involved in host defense and tissue homeostasis [71]. Other integrins with reduced phagocytic capacity ($\alpha_5\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$) are involved in phagocytosis of fibrillar or denatured extracellular matrix components (**Table 1.1**).

Table 1.1 Major Mammalian Phagocytic Integrins and their Invertebrate Orthologues.

Integrin	αI domain	Co-Receptors	Phagocytic Targets	Expression
$\alpha_M\beta_2$	+	-SR-A1/2 [41] -Dectin1 [72] -RAGE [73]	-iC3b-opsonized particles [74] -C3d-opsonized particles [75] -Denatured proteins [76, 77] -Bacteria (LPS, LBP) [78, 79] -Zymosan [80, 81] -Myelin sheaths [82] -Platelet factor 4 (PF4) [83] -LL-37 [84]	Professional phagocytes
$\alpha_x\beta_2$	+	-	-iC3b-opsonized particles [74] -Osteopontin [85] -Fibrillar α-synuclein (αSN) [86]	
$\alpha_2\beta_1$	+	-	-Collagen fibrils [87-89]	Non-professional phagocytes
$\alpha_3\beta_1$	-	-CD36/SCARB3 [90]	-Laminin [91]	
$\alpha_5\beta_1$	-	-	-Fibronectin aggregates [92] -Fibronectin-opsonized apoptotic bodies [93] -Vitronectin [94]	
$\alpha_6\beta_1$	-	-CD36/SCARB3 [90]	-Fibrillar β-amyloid [95, 96]	Professional phagocytes
$\alpha_v\beta_3$	-	-TIM4 [97], -CD36/SCARB3 [98], -MerTK[42, 99]	-Opsonized MFG-E8 [100, 101] -Opsonized Gas6 [102] -Opsonized ProS1[103, 104] -TSP-1 [105] -Apoptotic or necrotic bodies [106, 107]	Professional and non-professional phagocytes
$\alpha_v\beta_5$	-			
αPS3/βν	-	-	-Peptidoglycan [69, 108] -Apoptotic cells [69, 70]	*Drosophila* phagocytes.
INA-1/PAT-3	-	-	-Apoptotic cells [68]	*C. elegans* phagocytes

Here, we will focus on β_2 integrin mediated phagocytosis ($\alpha_M\beta_2$ and $\alpha_x\beta_2$) and in the events determining their activation and downstream signaling in relation to cytoskeletal remodeling and particle engulfment.

1.4. Integrin Structure and Activation

Phagocytic integrins are heterodimeric (α and β subunit) receptors. Subunits are divided into ectodomains, transmembrane helix and short cytoplasmic tails.

The extracellular portion of the α and β subunits are comprised of several subdomains organized into a globular ligand-binding N-terminal "head" and long extended C-terminal "legs". The α subunit head consists of a folded seven-bladed β-propeller, a thigh domain and two calf domains (**Figure 1.2**). The α-subunit ectodomains contain Mg^{2+}-binding metal-ion-dependent adhesive sites (MIDAS), and Adjacent to MIDAS (AdMIDAS), which binds inhibitory Ca^{2+} or activating Mn^{2+} [109, 110]. Ligand binding can occur either at the αI-domain (α-subunit) present in α_X, α_M and α_2, or at the α/β-chain interface in those integrins without the αI domain (**Figure 1.2, Table 1.1**).

The β subunit is composed of an I-like domain (βI), which is structurally similar to the αI-domain in α-subunits, a PSI (Plexin/Semaphorin/Integrin) domain, a hybrid domain, four EGF repeats, and a β-terminal domain (β-TD) domain (**Figure 1.2**).

Integrins are tightly regulated by conformational changes, a hallmark of which is cytoplasmic tail separation [111]. Integrin conformations are described according to the state of the headpiece (open/closed) and leg ectodomains (extended/bent) [112]. Resting integrins remain in an inactive (closed-bent) conformation with the lowest free energy (-4.0 kcal/mol for $\alpha_5\beta_1$), with respect to fully activated integrins [113]. This conformation is characterized by a closed ligand-binding site and clasped membrane proximal regions [114]. In activated integrins (extended-open), the hybrid domain (β-subunit) swings away from the α-chain, and

the membrane proximal regions unclasp. This correlates with the rearrangement of the MIDAS and opening of the ligand binding site [114].

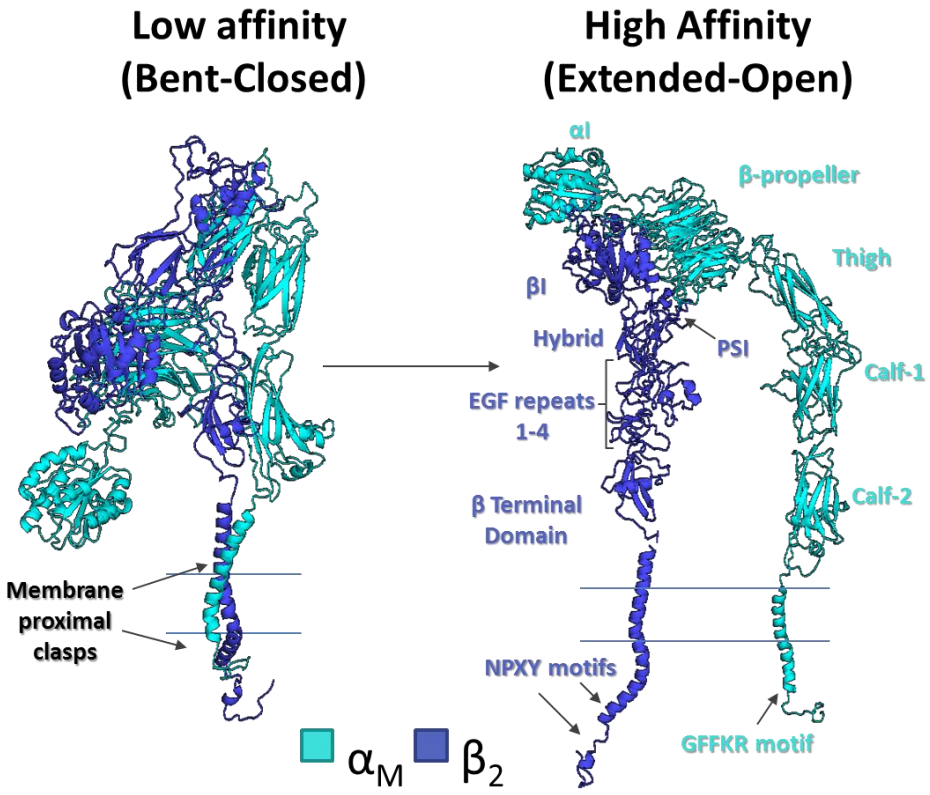


Figure 1.2. Phagocytic integrin $\alpha_M\beta_2$ structure and activation pathways.

3D structure model generated through homology modelling using Modeller 9.23. The following PBD entries served as templates: 1m8o, 2k9j, 2knc, 3k6s (low-affinity/bent conformation) and 1dpq, 2lkj, 2m3e, 2rn0, 2vdo, 3g9w, 3fcu, 5e6s, 6ckb, 6avu (high affinity conformation), and the sequences for α_M (NP_001139280.1) and β_2 (NP_000202.3). PSI: Plexin-Semaphorin-Integrin domain.

Structural and mutational studies have investigated models of integrin activation to probe whether integrin extension or leg separation occurs first. Mutations and deletions of the CD-loop (β-TD) proposed to keep integrins from extending, have shown no impact on $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ activation [115], and there is little proof that mutations in this region affects β_2 integrins [116], strongly indicating that releasing these constraints is not enough to induce activation.

Structural studies [117] have demonstrated that $\alpha_X\beta_2$ follows the 'switch-blade' model of activation, where leg separation occurs first, releasing constraints of the bent conformation and opening of the ligand-binding site resulting in an intermediate/low affinity conformation (extended-closed) [118]. This intermediate conformation has a free energy between 1.6 and 0.5 kcal/mol, meaning the high affinity conformation is thermodynamically favored [113, 119]. Mutations in the EGF3 repeat of the β_2-subunit have also been shown to induce a high affinity conformation through destabilizing the thermodynamically favorable bent conformation and facilitating leg separation [120]. It is noteworthy that a bent-open conformation has been described for $\alpha_L\beta_2$ and $\alpha_M\beta_2$, allowing integrins to bind ICAM in *cis* which may regulate neutrophil function [121], however the specifics of how this activation takes place remain unknown.

Integrin activity is regulated by changes in affinity and aggregation, with the latter affecting receptor avidity. Cytoplasmic proteins bind to α- or β-subunits causing tail separation, stabilizing their high affinity conformation [111, 122]. This can be triggered either through signaling from other receptors ('inside-out' signaling), or from direct ligand-binding or experimentally, using Mn^{2+} ('outside-in' signaling), which triggers downstream signaling pathways [123].

1.5. Inside-out Signaling

1.5.1. Rap1 as a Signaling Node

Early studies in complement-dependent phagocytosis using mutants of small GTPases, pointed to Rap1 as the main regulator of $\alpha_M\beta_2$ activity [124] and to it being required for β_1-mediated phagocytosis [125]. Rap1 acted as a node, connecting different signaling pathways (chemokines, fMLP, PAF, TNFα) to integrin activation [126].

Rap1 GTP loading is induced by specific Guanine-Nucleotide Exchange Factors (GEFs), being Epac1 (dependent on cyclic AMP; cAMP) and CalDAG-GEFs (dependent on Ca^{2+}/Diacylglycerol; DAG) the best characterized (**Figure 1.3**). Epac1 expression was found to increase during monocyte-macrophage differentiation, correlating with the acquisition of immunoregulatory functions [127], and in neutrophilic HL-60 cells, pharmacological activation of Epac1 increased Rap1-GTP and complement-dependent phagocytosis [128]. RasGRP3/CalDAG-GEFIII had similar effects promoting Rap1 activation and phagocytosis [129]. Mutations in CalDAG-GEF1 produced leukocyte adhesion deficiency syndrome (LADIII) with defective neutrophil-endothelial adhesion [130], and mouse *CalDAG-GEF1*$^{-/-}$ macrophages showed reduced integrin activation [52]. Rap1 activation can be induced by Toll-like receptors (TLRs) [131], however the signaling pathways remain poorly defined. In neutrophils, secreted myeloid-related proteins (MRPs) 8 and 14 bind to TLR4 causing Rap1 activation and β_2-dependent adhesion [132]. In macrophages, low concentrations of TLR3/4/9 agonists induced RasGRP3-dependent Rap1 activation [133]. Activation of $\alpha_M\beta_2$ by TLR2/TLR4 required Rac1-GTP loading, PI3K activity and cytohesin-1 binding to the β_2 subunit [134]. The role of cytohesin-1 is controversial, as the use of cytohesin-1 siRNAs and inhibitors results in an increase in the $\alpha_M\beta_2$ affinity conformation [135].

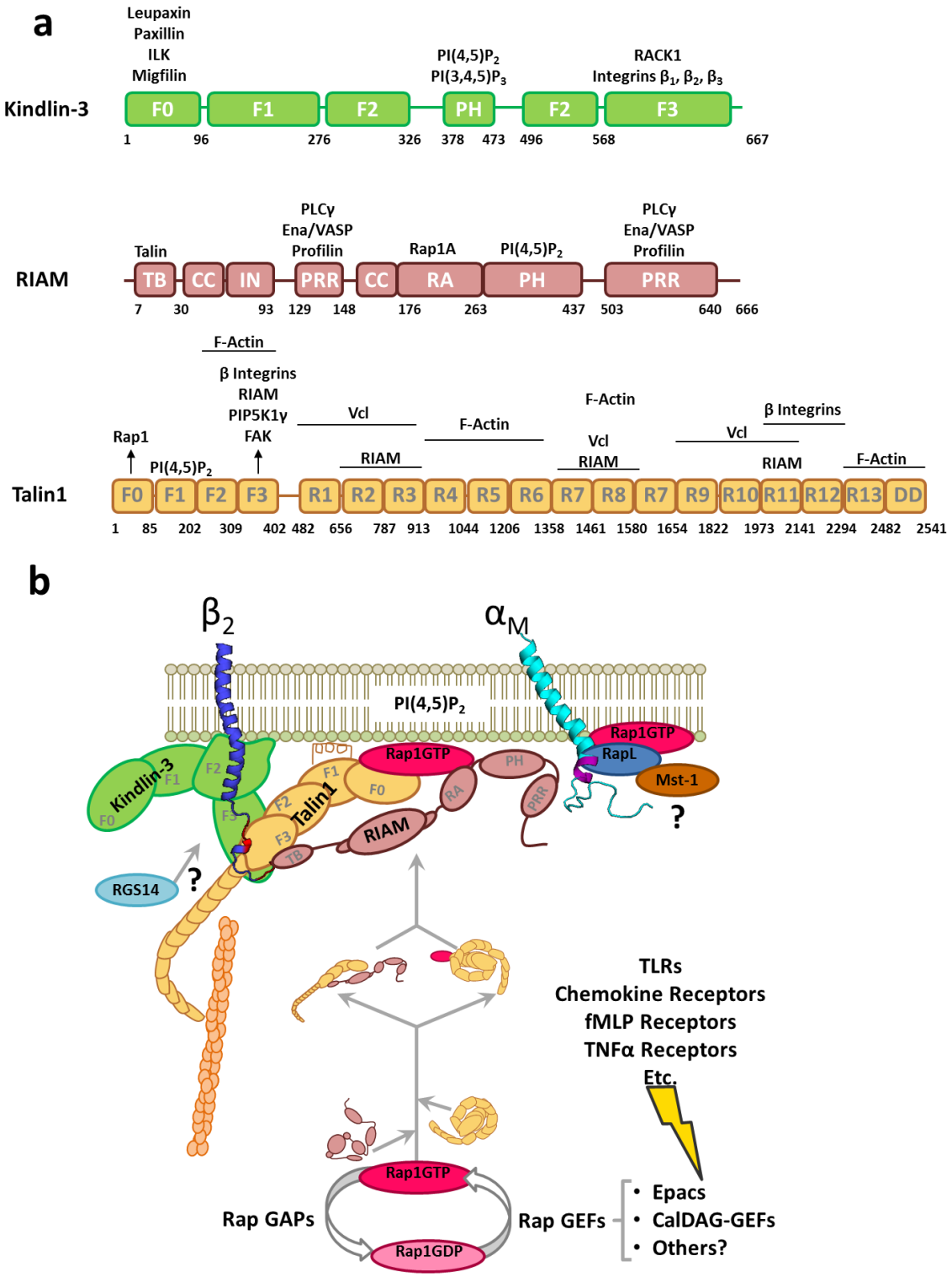


Figure 1.3. Inside-out pathway of integrin αMβ2 activation.

a) Domain structure and binding partner sites for Kindlin-3, RIAM and Talin1. This image is not to scale. For size reference, the aminoacid positions have been labelled. **b)**

Signals stemming from multiple receptors induce Rap1-GTP loading and RIAM-mediated recruitment of Talin1 to integrin tails, with possible contributions by other pathways. Protein-binding motifs in the integrin tails are shown in red (NPXY) and in purple (GFFKR). FERM domains are highlighted for Kindlin-3 and Talin1 (F0-F3). Talin domains are labelled R1-R13, and includes a dimerization domain (DD). Highlighted RIAM domains are as follows; TB: Talin1 Binding domain, RA: Rap Association domain, PH: Pleckstrin Homology domain, PRR: Prolin Rich Region. For simplicity, some proteins are shown as monomers. Question marks denote unsolved or hypothetical signaling steps.

1.5.2. Talin1 and Kindlin-3

Talin1 and Kindlin-3 are the best characterized integrin activators. Both belong to the FERM family but interact with distinct NPXY motifs in the cytoplasmic tails of β_1, β_2 and β_3, and thus contribute differently to activation [136]. Although Talin-binding is required for efficient β_5 activation during adhesion, it is dispensable for phagocytosis [137]. $\alpha_V\beta_5$ requires an unknown mediator that recognizes a YEMAS motif proximal to the NPXY. A candidate could be the FERM family FRMD5, as it promotes β_5-Kindlin-2 interaction and induces ROCK activation during adhesion [138] yet there is no information of its relevance in phagocytosis.

Talin1 contains an N-terminal globular head with a linear FERM domain and a C-terminal rod domain organized in 13 subdomains (R1-R13), which contains a dimerization domain, an integrin binding site, three F-actin binding sites and several Vinculin and RIAM binding sites [139, 140]. The FERM domain has 4 subdomains (F0-F1-F2-F3), where F3 contains the primary integrin-binding site (IBS) that interacts with the membrane-proximal NPXY motif conserved in β-integrin tails [122, 141, 142] (**Figure 1.3 a**). In resting leukocytes, Talin1 remains auto-inhibited due to an interaction between F2F3 and R9 subdomains, which mask the primary IBS [143]. Several Talin1 activation mechanisms have been proposed. By binding to PIP5Kγ, Talin1 is recruited to the plasma membrane where F2F3 domain binds to phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$), disrupting the head–tail

interaction and exposing the IBS [144, 145]. Additionally, RIAM-Talin1 interaction was described as necessary for Talin1 activation and recruitment to integrin tails (**Figure 1.3**) [146].

Hematopoietic cell-specific Kindlin-3, is mutated in Leukocyte Adhesion Deficiency type III (LADIII) causing $\beta_1/\beta_2/\beta_3$ activation defects [147, 148], and preventing neutrophils adhesion to iC3b and ICAM-1 [149]. Kindlin-3 binds to the membrane-distal NPKF sequence in the β_2 subunit tail without excluding Talin1 binding (**Figure 1.3**) [149]. Studies of individual contributions to activation revealed that Kindlin-3 is not sufficient to induce the high-affinity state of $\alpha_L\beta_2$ whereas Talin1 promotes full activation [150]. Whether binding of Talin1 and Kindlin-3 is sequential or simultaneous and their exact contribution to integrin activation remains to be explored. The signaling events directing Kindlin-3 to integrins also remain elusive, as in T cells, Kindlin-3 localization at immune synapses depends on Rap1 and Mst-1/RapL signaling [151], whereas no such interaction has been described for phagocytic cells.

1.5.3. RIAM-Talin1 Interaction

RIAM (Rap1-Interacting Adaptor Molecule or APBB1IP) was identified as a Rap1 effector that promoted β_2 and β_1 high affinity state, increasing T cell adhesion and spreading [152]. Protein homology studies revealed the existence of several RIAM homologous genes, collectively termed the MRL (Mig-10/RIAM/Lpd) family [153].

RIAM binds to Rap1-GTP through a central Ras-association domain (RA), to $PI(4,5)P_2$ through a Pleckstrin-Homology (PH) domain and to VASP, Profilin and PLCγ1 via proline-rich regions [152, 154-157] (**Figure 1.3 a**). RIAM also interacts with

Talin1 through its N-terminal and Talin1 has several RIAM-binding sites located at F3, R2, R3, R8 and R11 subdomains [139]. Binding of RIAM to Talin1-F3 releases Talin1 from autoinhibition (**Figure 1.3 b**), since it disrupts the intramolecular interaction between its F3 and R9 domains (**Figure 1.3 a**), resulting in the unmasking of the integrin binding site on F3 [158, 159].

RIAM binding to Talin protects Talin R2-R3 from stretching and unfolding, therefore making RIAM dissociation a prerequisite for vinculin binding to Talin R2-R3 [139, 160]. This effect seems to result from a RIAM-mediated stabilization of R3, which prevents the mechanosensitive exposure of Vinculin binding sites in Talin [160, 161]. Vinculin binding to Talin R3 triggers a conformational change which induces actin binding to Talin at R4 and R8 [140, 162], thereby increasing actin bundling and force generation (**Figure 1.3 a**).

Talin R11 binds vinculin in response to force but this is not affected by RIAM binding [160], whilst displacing RIAM from Talin R7-R8 requires a higher force (15 pN) [163] than that observed for R2-R3 (~5pN) [164] (**Figure 1.3 a**). The stoichiometry of vinculin binding to open-conformation Talin in solution is 1:1, suggesting all 11 Vinculin binding sites are occupied[143] and this locks Talin in an unfolded conformation[164].

These data suggest that Vinculin sequentially displaces RIAM from Talin [160], correlating with adhesion maturation and actomyosin force generation [139].

The Rap1-RIAM-Talin1-Integrin pathway also operates in complement-dependent phagocytosis. Studies in Talin1-silenced THP-1 cells revealed that Rap1 and Talin1 regulated each other's localization at phagocytic cups [165]. Reduced RIAM expression in human monocyte-derived macrophages (MDM), neutrophilic HL-60 cells and THP-1 cells diminished levels of high affinity $\alpha_M\beta_2$, reduced

complement-dependent phagocytosis and Talin1 recruitment to phagocytic cups [128]. Complement-dependent phagocytosis, cell adhesion to ICAM and ROS production was also impaired in mouse RIAM$^{-/-}$ macrophages and neutrophils [166]. Additionally, RIAM deficiency in vivo had a profound effect on β_2 activity but a moderate effect on β_1- or β_3-dependent functions [167].

Besides RIAM, Rap1 effectors RapL and RGS14 (Regulator of G-Protein Signalling-14) are proposed to regulate $\alpha_M\beta_2$ activation by inside-out signaling (**Figure 1.3 b**). The former is proposed to interact with α_M-subunit inducing integrin tail separation and integrin activation [168], however RapL has only been shown to interact with a GFFKR motif in α_L cytoplasmic tail, and there is no direct evidence that it plays a role in $\alpha_M\beta_2$ activation [169]. For RGS14, the integrin activation mechanism is unknown but seems to be dependent on Talin1-binding to β_2 [170].

Recently, a direct interaction between Rap1-GTP and Talin1 was described at Talin1 F0 and F1 subdomains [171-174]. Synergistic interaction between this region and an F1 lipid-interacting helix facilitates relocation of Talin1 and integrin-activating function (**Figure 1.3 b**) [174, 175]. This pathway could be relevant for fast cell responses, as disruption in mice impaired platelet aggregation, neutrophil adhesion, extravasation and phagocytosis, but had no effect on macrophage adhesion and migration [173].

1.6. Outside-in Signaling

Outside-in signaling during phagocytosis initiates upon ligand interaction, stabilizing the active conformation, separating integrin tails, allowing binding of actin cytoskeletal linkers (Talin1 and/or Kindlin-3), and reorganizing cytoskeletal constraints as described in the picket-fence model [33]. This generates the force

needed to drive membrane extension and particle engulfment/internalization (**Figure 1.4**). Regulators have been described in focal complex-like formations at the phagocytic cup [53].

1.6.1. Clustering and Tyrosine Kinases

One of the earliest events in outside-in signaling could be ligand-induced clustering, a process requiring Talin1 and/or Kindlin-3 [136, 176]. Kindlin-3-induced clustering is reported to activate Src family kinases (SFKs) [177, 178], by exclusion of tyrosine phosphatases such as CD45 [52]. CD45 is a critical modulator of SFK activity, since it can both dephosphorylate the inhibitory tyrosine residues of SFK, leading to hyperactivation, or dephosphorylate the activating residues, resulting in decreased activity [179]. Since CD45$^{-/-}$ macrophages present a defect when binding to β_2 integrin ligands [180], this implies that the presence of CD45 is required in the initial steps of the adhesion, but requires exclusion in order to maintain SFK activity.

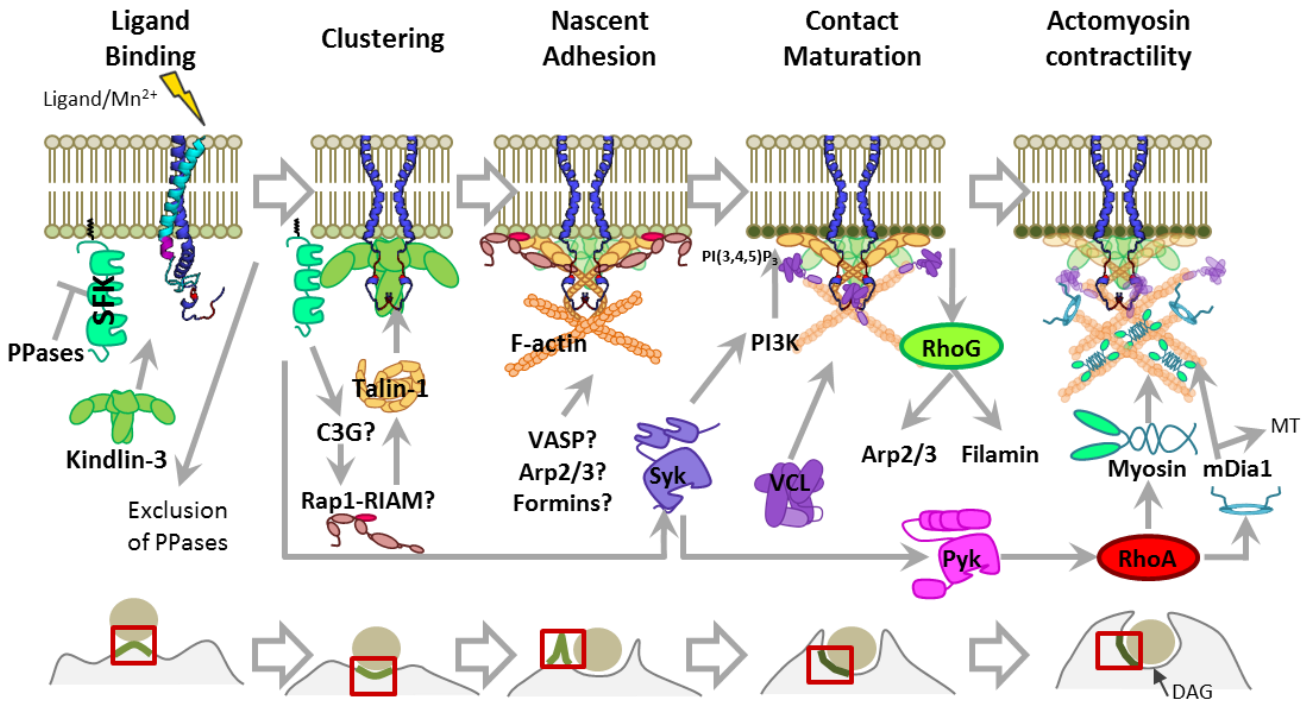


Figure 1.4. Outside-in pathway in the context of phagocytosis through $\alpha_M\beta_2$.

Src Family Kinases remain inhibited by membrane-bound tyrosine phosphatases. Kindlin-3 mediated clustering facilitates Src Family Kinase activation, which then triggers activation, contact maturation and contractility necessary for phagocytic engulfment. PPases: Phosphatases, SFK: Src Family Kinases, MT: Microtubules. Protein-binding motifs in the integrin tails are shown in red (NPXY) and in purple (GFFKR). For simplicity, some proteins are shown as monomers. Question marks denote unsolved or hypothetical signaling steps.

Size exclusion of these membrane-bound phosphatases with large extracellular domains seems to be a common feature of integrin-mediated close-contact immune processes such as Dectin-1 and FcγRIII phagocytosis and immune synapse formation [52, 181, 182]. This process does not exclude SFKs but favors their activation due to removing the inhibitory effect of these phosphatases [177, 178]. However, there are as of yet only indirect evidences [177, 178] that phosphatases such as CD45 are excluded during integrin-mediated phagocytosis.

SFKs appear to be exclusively involved in 'outside-in' signaling as SFK-deficient cells produced reduced ROS after integrin clustering [183], whereas ICAM-1 adhesion and complement-dependent phagocytosis were normal in pre-activated SFK-deficient cells [184, 185].

A requirement for SFK activation has been described for β_1, β_2 and β_3 integrins [177, 184, 186]. Hck, Fgr and Lyn are the representative SFKs in myeloid cells. Hck co-localized with $\alpha_M\beta_2$ at phagocytic cups of complement-opsonized zymosan [187, 188], and Hck knockout phenocopied the α_M knockout [189]. However, in U937 macrophage-like cells, Hck and Fgr siRNA, unlike Lyn, had no effect on particle internalization [190] and genetic restitution of Fgr-deficient cells inhibited adhesion, spreading and Syk activation [191]. In contrast, *Hck*$^{-/-}$ *Fgr*$^{-/-}$ *Lyn*$^{-/-}$ triple knockout showed no inhibition in CR3-mediated phagocytosis [192], which may point to compensatory roles of other ubiquitously expressed SFKs. Despite the

research into outside-in activation of SFKs, the exact mechanism and individual contribution of each SFK have yet to be dissected.

SFK activity precedes activation of tyrosine kinases Syk and FAK family member Pyk2. Syk is necessary for phagocytosis of iC3b-opsonized beads/zymosan and localizes at phagocytic cups [53, 193], whereas Pyk2 contributes to clearance of complement-opsonized bacteria [194]. Clustering of β_2 integrins results in Syk activation [195] which in turn triggers Pyk2 signaling [196]. Pharmacological inhibition of Syk and FAK kinases points to non-redundant functions during phagocytosis and to a possible sequential activation [53].

1.6.2. Phosphoinositides coordinate GTPases and cytoskeletal rearrangements

Phagocytosis requires sequential enrichment of phosphoinositides (PIPs) in the inner leaflet of the plasma membrane [67]. PIP enrichment recruits GEFs for small GTPases, which are sequentially activated [197], and other components of integrin adhesion complexes.

$PI(4,5)P_2$ enrichment can be induced by lipid redistribution due to particle-induced plasma membrane deformation [198] and/or by SFK or Talin1-induced PIP5Kγ activity [145, 199, 200]. $PI(4,5)P_2$ enrichment strengthens Talin1 anchoring [143] and recruits different factors involved in F-actin dynamics, like the actin-depolymerizing-factor ADF/Cofilin, whose activity is inhibited by $PI(4,5)P_2$ [201], or the formin mDia [202, 203]. Additionally, RIAM binds $PI(4,5)P_2$ and may recruit VASP and Profilin, which could also contribute to actin polymerization [152, 156] (**Figure 1.4**).

$PI(3,4)P_2$ recruits and induces Vinculin activation through disrupting an auto-inhibitory interaction [204]. This is dependent on Syk activity and to a lesser extent

on FAK/Pyk2 and is upstream from ROCK activation [53]. In focal complexes, RIAM contributes to Vinculin binding to Talin1, as RIAM-Talin1 interaction unmasks a Vinculin binding site in Talin1 [139]. Afterwards, Vinculin binding to F-actin and α-actinin favors filament bundling and force generation [205, 206].

Increased PI(3,4,5)P_3 at CR3-phagocytic cups [207] depends on PI3K [208] and Syk [196], and both are activated downstream of Kindlin-induced clustering [209]. PI(3,4,5)P_3 enrichment recruits Vav1/3 which are GEFs for the RhoA family GTPases [197]. Complement-dependent phagocytosis requires Vav1 to activate RhoA [124, 210] but also RhoG with no participation from Cdc42 and Rac1 [211]. However, expression of constitutively active Rac1 rescues the defective engulfment of Vav1-3 knockouts [212]. This discrepancy could be explained by the overlapping roles of RhoG and Rac1 [213, 214] (**Figure 1.4**).

In the final steps leading to engulfment, RhoA-GTP initiates the ROCK-MLCK-myosin signaling pathway and actomyosin contractility [215]. RhoA is enriched at phagocytic cups and its localization is modulated by motifs in β_2-integrin tails [210]. Premature activation of RhoA is inhibited by Rap-GTP through ARAP3, a dual GAP for Rho and Arf GTPases which is recruited by PI(3,4,5)P_3 and PI(3,5)P_2 [216]. Finally, mDia contributes to phagosome closure [53, 202] and particle engulfment by connecting the actin cytoskeleton to microtubules [217] (**Figure 1.4**) .

1.7. Integrins, Myelopoiesis and Myeloid Disorders

Myeloid cells require the correct expression of integrins and other surface molecules in order to perform their functions [218]. Myeloid immunodeficiencies can be caused by compromised mobility, inadequate phagocytic capacity and/or respiratory burst, or inadequate myelopoiesis (neutropenias or monocytopenias)

[219-222]. **Table 1.2** contains several myeloid immunodeficiencies. More comprehensive reviews can be found elsewhere [223-225]. The accumulation of non-functional cells may also be accompanied by an increased risk of myeloproliferative disorders due to dysregulated myelopoiesis [224, 226, 227].

Table 1.2 Myeloid Immunodeficiencies.

Type	Disease	Gene/s	Defects	Effect on Myelopoiesis	Refs
Migratory and chemotactic defects	Leukocyte Adhesion Deficiency I (LAD I)	*ITGB2* (integrin β_2)	Loss of integrin β_2 and its mediated functions. Recurrent infections.	Myeloid hyperplasia, Neutrophilia.	[228-230]
	LAD II	*SLC35C1* (FUCT1)	Aberrant GDP-Fucose transporter 1. Incorrect glycosylation and lack of selectins needed for leukocyte rolling.	Neutrophilia.	[231]
	LAD III	*FERMT3* (Kindlin-3)	Loss of integrin β_2 family mediated adhesion. Defective integrin activation and signaling.	Neutrophilia.	[232-235]
		RASGRP2 (CalDAG-GEF1)	Decreased Rap1 activation and signaling, and defective inside-out integrin activation	Neutrophilia.	[236, 237]
	Rac2 deficiency	*RAC2* (Rac2 GTPase)	Chemotactic and adhesive defects. Severe phagocytic immunodeficiency and defective respiratory burst.	Neutrophilia (immature band precursors).	[238, 239]
	Serum Response Factor (SRF) related actinopathies [223]	*WAS* (Arp2/3 regulator WASp)	Wiskott–Aldrich syndrome (WAS). WASp deficiency. Defective actin dynamics, migration and adhesion.	Neutrophilia (immature precursors).	[240, 241]
		MRTFA (SRF cofactor)	Impaired expression of SRF-controlled cytoskeletal genes. Defective neutrophil motility.	Neutropenia.	[223]
		WDR1 (Actin disassembly factor)	Defects in WDR1/AIP1, a negative regulator of F-actin. Defective depolymerization. Neutrophil nuclei herniated.	Mild neutropenia.	[242]
		ACTB	Reduced chemotaxis and decreased ROS production	Neutropenia.	[226]

Migratory and chemotactic defects (*cont.*)	TLR signaling defects	*MyD88/ IRAK4*	Altered TLR2/4 and IL-1R signaling. Susceptibility to bacterial infections.	Disrupted myelopoiesis, neutropenia.	[243-245]
		CARD9 (caspase family CARD9)	Susceptibility to fungal diseases. Defective TLR signaling, and Dectin and CR3 phagocytosis. Impaired Rac1 activation, F-actin remodeling, and phagosome formation.	-	[246-248]
Neutropenias	Severe congenital neutropenias (SCN)	*ELA2* (Neutrophil elastase)	Hyperactivation of the unfolded protein response. Increased apoptosis in myeloid progenitors.	Myelodysplasia and cyclic neutropenia.	[249]
		GFI1 (transcription factor)	Inhibits proliferation. Required for differentiation of myeloid progenitors.	Neutropenia.	[250]
		HAX1	Defect in Hax1 apoptosis repressor. Increased progenitor apoptosis.	Neutropenia.	[251]
		CSF3R (G-CSF receptor)	Defective expression of the GCSF receptor required for myelopoiesis.	Neutropenia.	[252]
		G6PC3	Altered glucose-6-phosphatase expression. Defective Myeloid differentiation, chemotaxis and ROS production	Neutropenia.	[253]
		VPS45	Altered veicle protein sorting. Defective Myeloid differentiation and migration	Neutropenia.	[254]
		JAGN1 (Unknown function JAGN1 protein)	Defective JAGN1 expression (tetraspanin and Erv protein sorting homologous protein). Required for G-CSFR signaling. Increased apoptosis in progenitors.	Neutropenia.	[255-257]
		WAS (Arp2/3 regulator WASp)	X-linked neutropenia. Hyperactive WASp. Aberrant actin polymerization. Increased apoptosis in myeloid progenitors.	Monocytopenia, neutropenia, myeloid maturation arrest.	[258]

Impaired granule formation, respiratory burst or killing	Neutrophil-specific granule deficiency	*CEBPE* (C/EBPε transcription factor)	Lack of critical myelopoiesis transcription factor. Altered secondary granule formation, and abnormal chemotaxis.	Lack of myeloid differentiation.	[259]
	Chronic Granulomatous Disease	*CYBA/B, NCF1/2/4* (NADPH oxidase complex)	Defective NADPH oxidase function and ROS production. Immune dysregulation.	-	[260]
	Myeloperoxidase deficiency	*MPO* (MPO enzyme)	Defective production of hypochlorous acid for microbial killing.	-	[261]
	Chediak–Higashi Syndrome	*LYST* (lysosomal trafficking regulator)	LYST deficiency. Abnormally large neutrophil azurophilic granules. Impaired chemotaxis. Increased progenitor apoptosis.	Neutropenia.	[262]

1.7.1. Overview of Myelopoiesis

Myeloid precursors can differentiate into either mononuclear phagocytes (monocytes and tissue macrophages), or polymorphonuclear granulocytes such as neutrophils [263]. The generation of mature monocytes and neutrophils from bone marrow precursors in hematopoietic stem cell niches is termed myelopoiesis [264]. Lineage commitment is dependent on the activation of transcriptional networks and common myeloid progenitors dynamically regulate the expression of CCAAT-enhancer binding protein-α (C/EBPα), C/EBPε, and PU.1 [265-269]. Induction of these transcription factors is regulated by cytokine growth factors GM-CSF, which activates hematopoietic progenitors and directs them towards myelopoiesis [270, 271], and M-CSF, which has been shown to activate PU.1, a master regulator of myelopoiesis, instructing myeloid lineage decision [272]. PU.1 is capable of interacting with other transcription factors of this network, such as GATA-1, Runx1 or C/EBPα, in order to orchestrate myeloid differentiation [273].

Both PU.1 and C/EBPα induce the upregulation of receptors for myeloid-lineage growth factors M-CSF (CD115), G-CSF (CD114), and GM-CSF (CD116) [274-279]. PU.1 binds and regulates its own promoter [280], as well as inducing the expression of integrin β_2 (CD18) and α_M (CD11b) [281, 282]. PU.1 upregulates α_M expression in promyelocytic cell lines (HL-60) and primary AML blasts in response to all-*trans* retinoic acid [273, 282-285] and this effect has been shown to be in response to G-CSF-Stat3 signaling during myelopoiesis [286]. Knockout analysis suggested Gfi-1 and PPARγ were critical to the all-*trans* retinoic acid-induced differentiation program of HL-60 cells [287].

The ratio of C/EBPα to PU.1 expression is crucial in determining monocytic or granulocytic cell commitment. A high expression of PU.1 favors monopoiesis, but

reduced levels of PU.1 allows C/EBPα to carry out a granulopoiesis-specific program, which is accompanied by the suppression of monocyte differentiation [288, 289]. C/EBPα initiates a signaling cascade that results in the activation of Serum response factor, SRF [290], which also drives the expression of phagocytic integrins like α_2, α_M and β_2 [291], and whose activity is controlled by MRTF-A, a sensor of G:F-actin ratios [292-295] and FHL-2, an MRTF-A antagonist [296].

Later-stage neutrophil precursors show increased levels of C/EBPε [297-299], as well as Runx1 and Gfi-1 [300], induce cell cycle arrest [289] and the formation of specific and gelatinase granules [301, 302]. Monopoiesis requires an IRF8-KLF4 transcription factor cascade dependent on PU.1 expression and downstream of ERK signaling [303, 304]. IRF8 controls the expression of lysosomal proteins critical for macrophage function such as Lysozyme M, Cystatin C and cathepsins [305, 306], and has also been shown to regulate the monocytic differentiation of promyelocitic HL-60 cells in response to PMA [307-309].

THESIS STRUCTURE AND OBJECTIVES

1.8. Thesis Structure

This thesis is structured in chapters that are thematically linked. Chapter 1 is a general introduction; Chapter 2 outlines the methods used for all studies presented here; Chapters 3-4contain results obtained during the course of the thesis; Chapter 8 presents an overall general discussion of the significance of the results obtained and outlines several avenues to continue the research; Chapter 9 states the conclusions of the thesis.

1.9. Objectives

The objectives of this thesis are presented here in the context of the chapters where they were addressed**:**

Chapter 3:

- Determine the role of RIAM in the outside-in pathway during complement-mediated phagocytosis.
- Assess whether a reduction in RIAM expression controls integrin downstream signaling.
- Examine whether VASP participates in complement-mediated phagocytosis and if it is recruited to phagocytic cups.
- Analyze whether the role of VASP is dependent on RIAM expression.
- Explore whether VASP phosphorylation status is controlled by RIAM expression.

Chapter 4:

- Establish the effect of RIAM, VASP and Vinculin deletion in complement-mediated phagocytosis.
- Investigate whether deletion of these proteins affect the expression of other integrin-associated molecules.
- Uncover the mechanism through which RIAM, VASP and Vinculin expression regulate phagocytic capacity.

- Revert the observed phenotype for all knockouts using genetic tools or pharmacological treatments.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell Cultures

Human promyelocytic HL-60 (ATCC: CCL-240) and derived cell lines were cultured in 10 ml RPMI 1640 medium with 10% (v/v) fetal-calf serum (FCS), 1% (v/v) glutamine and 1% (w/v) penicillin-streptomycin (Lonza) [128, 310, 311] using Nunc™ 100 mm dishes (Thermo Scientific). Cells were passed every two days and seeded at a cell density of 2.5x105 cells/ml. Cells were differentiated into neutrophil-like HL-60 using 1 μM retinoic acid (Sigma) during at least 2 days. Finally, HEK 293T cells (ATCC: CRL-3216) were also cultured in the same media in Nunc™ 100 mm dishes (Thermo Scientific) for adherent cells. 293T cells were passaged according to confluency every 2-3 days.

Cells were routinely tested for mycoplasma contamination and only mycoplasma-negative cells were used.

2.2. Phagocytosis Assays

Phagocytosis assays were carried out as previously described [128, 311]. Fresh sheep red blood cells (RBCs) (Thermo Scientific) were labelled with 2 μM DDAO (Invitrogen) at 37ºC for 20 minutes. Excess DDAO was removed by washing with RPMI 10% FCS, reserving an aliquot of labelled unopsonized RBCs. Opsonization was carried out by incubation with sub-agglutinating concentrations of polyclonal rabbit IgM anti-sheep RBC cells (MyBioSource) and later treated with 10% C5-deficient human serum (Sigma) for complement opsonization. Differentiated

HL-60 cells were harvested and starved for 3h in serum-free RPMI, subsequently treated with either 320 nM LPS (Sigma) or 1 mM $MnCl_2$ and incubated for 30 or 5 min respectively. Cells were then incubated in a 1:10 ratio with complement-opsonized RBCs (C3-RBC) or unopsonized RBCs as negative control, for 30 min at 37°C and unbound RBC were removed by washing thrice with ice-cold PBS. To determine particle internalization, cell-bound RBCs were exposed to a 30s hypotonic shock with distilled H_2O, and isotonicity restored through addition of an equal volume of twice-concentrated PBS.

Cells were analyzed using a BD FACSCalibur II flow cytometer (BD Biosciences), data analyzed using the FlowJo package (BD Biosciences) and expressed as Association Index (AI), indicating the amount of cells with attached and engulfed particles, or Phagocytic Index (PI) indicating cells with internalized particles [128]. These indexes are all normalized with respect to the AI for unstimulated control cells. The Binding Index (BI) was determined by subtracting internalization counts from total counts [311, 312] and Phagocytic Efficiency (PE) was defined as the ratio PI/AI. It is to be noted that an increase in BI constitutes a decrease in efficiency. These indexes are all normalized with respect to the AI for unstimulated control cells.

Phagocytosis was also carried out as described above on poly-L-lysine (PLL) coated glass slides obtained by incubation with 0.005% PLL (Sigma) for 30 minutes at 37°C. Excess PLL was washed with PBS and slides were left to dry. Once dry, the phagocytosis assay was carried out.

2.3. Fluorescence Microscopy

Cells attached on PLL coated glass slides were fixed with 4% paraformaldehyde for 10 minutes and permeabilized with PBS 0.5% Triton X-100 for

15 min. Cells were incubated with the indicated primary antibodies: rabbit polyclonal anti-sheep RBC (MP Biomedicals), rabbit IgG anti-VASP (Cell Signaling), mouse IgG anti-pSer^{157}-VASP (Santa Cruz), mouse polyclonal anti-iC3b antibody (kindly donated by Dr. Santiago Rodriguez de Cordoba, CIB, CSIC, Madrid, Spain), mouse anti-MRTF-A (G8 clone, SantaCruz), rabbit anti-FHL-2 (Abcam), mouse BEAR-1 (hybridoma supernatant), biotin-labelled mouse Ts2/16 (prepared in house using the a Biotin labelling kit purchased from Sigma, as described elsewhere [313]. Primary antibodies were used as per the manufacturer's instructions (1:20 anti-iC3b, 1:20 anti-VASP, 1:100 anti-pSer^{157}-VASP, 1:1000 anti-sheep RBC, 1:20 anti-MRTF-A, and 1:100 anti-FHL-2), or using 50µl of supernatant or 10 µg/ml of biotin-labelled purified antibody.

After thorough washing, staining with secondary antibodies (donkey anti-rabbit or donkey anti-mouse Alexa Fluor® 488, 555 or 647 conjugated antibodies, Life Technologies) and TRITC, Alexa Fluor® 647 or Alexa Fluor® 488 conjugated phalloidins (Life Technologies) was performed. All antibodies were used as per the manufacturer's instructions and in the presence of excess human gamma globulin (100µg/ml) and 1% BSA as blocking agents. When tandem staining was required, RBCs were stained either using the rabbit polyclonal anti-sheep RBC or the mouse polyclonal anti-iC3b, depending on the compatibility with the other antibodies used. Stained cells were then observed using a confocal LSM510 META Axiovert200 microscope (Zeiss) microscope and image analysis was performed using the ImageJ software package. Live imaging of phagocytosing cell lines was similarly performed in a LSM510 META Axiovert200 microscope (Zeiss) and cells were maintained at 37ºC in an atmosphere of 5% CO_2.

2.4. Western Blotting

To analyze protein expression, 10^7cells were harvested by centrifugation and cell lysis was carried out for 10 min at 0ºC in 10µl of GST Buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 2 mM MgCl2, 10 % v/v glycerol, 1 % v/v NP-40) supplemented with 1 mM PMSF, 25 mM NaF, 1 mM Na3VO4, and a protease inhibitor cocktail (Sigma). Protein concentration was determined using the RC DC™ Protein Assay kit (Bio-Rad).

SDS-PAGE was carried out as described by Laemmli, loading 50µg of total protein per lane from cell lysates. Prestained protein molecular weight standards (Bio-Rad) were used. Proteins were electrotransferred to a nitrocellulose membrane (300 mA, constant amperage, 2h), blocked and incubated overnight at 4ºC with the following anti-human primary antibodies: rabbit IgG anti-VASP, (Cell Signaling), mouse IgG anti-pSer157-VASP, (AbCAM), mouse IgG anti-α-Tubulin, (Sigma), IgG anti-phospho-ERK, (SantaCruz), and IgG anti-ERK, (BD Trans Lab) mouse IgG anti-Vinculin (H-10 clone, Santa Cruz), sheep IgG anti-RIAM (R&D Systems), mouse IgG anti-Talin (8D4 clone, Sigma). These were prepared in a blocking buffer containing 5% Bovine Serum Albumin (BSA) in TN-Tween (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20). After washes, membranes were later incubated (40 min, RT) with a secondary IRDye® IgG anti-rabbit, anti-goat/sheep or anti-mouse fluorescent antibodies (Li-Cor). All antibodies were used as per the manufacturer's instructions. The signal was then measured in a Li-Cor Odyssey imaging system and quantified using the ImageStudio software (Li-Cor).

2.5. Gene Silencing

Knock-down of RIAM expression using siRNA from Sigma was performed using the X-tremeGENE reagent (Roche). Briefly, $2x10^6$ cells in a 6-well plate were differentiated towards macrophage-like cells, transferred to serum-free media and incubated for 4h with X-tremeGENE polyplexes. These consisted of 110 pmol of either target or MISSION® siRNA Universal Negative Controls (Sigma) as per the manufacturer's recommendations. After the 4h incubation period, cell media was substituted for regular RPMI 1640 containing 10% serum. Transfection was assessed through both Western blot and functional assays.

2.6. Gene Knockout

Protein knockout lines were obtained using a CRISPR-CAS9 system and a double nickase strategy. Pairs of sgRNAs were designed using the Optimized CRISPR Design tool (Zhang Lab, MIT, 2013) [314], and the highest scoring pairs were selected. To ensure the truncated proteins were non-functional, sgRNAs were directed towards the first common exon for all isoforms of VASP (exon 2), RIAM (exon 3) and Vinculin (exon 3).

The corresponding pairs of sgRNAs (**Table 2.**) and their complementary oligonucleotide chains were ordered (Sigma), annealed and ligated into a previously BbsI-digested PX458 plasmid [314].Competent TOP10 Escherichia coli were transformed with the ligation mixture, and plasmids were harvested using a Wizard® Plus SV Miniprep DNA purification system (Promega) or a Plasmid Maxi kit (Qiagen), as per the manufacturer's instructions.

Table 2.1 sgRNA Sequences Used for CRISPR-CAS9 silencing.

Gene	Exon	Forward sgRNA (5'-3')	Reverse sgRNA (5'-3')
VASP	2	CACCGGTAGATCTGGACGCGGCTGA	CACCGGCCAATTCCTTTCGCGTCGT
APBB1IP **(RIAM)**	3	CACCGATTTGTTCCATAACCAAGAG	CACCGCACTGGTATCAGCCAATATG
Vcl	3	CACCGTCAATTAGATAATCTCGAGC	CACCGGGGTCAAGGGGCATCCTCTC

Cell transfection was carried out using the Neon Transfection System (ThermoFisher). Cells were plated the day prior to obtain 70%-90% confluency at the day of transfection. For each nucleofection, 250 000 cells and mixture of 3µg of the two sgRNA plasmids were employed. Cells were then transfected in a 10µl volume using a single 35ms and 1350V pulse, and left to recuperate for 24h in RPMI 1640 10% FCS media without antibiotics. Cells were then sorted according to transient EGFP fluorescence using a FACS Aria Fusion cell sorter (BD Biosciences). EGFP-positive cells were diluted and cloned into p96 wells. Protein expression was then assessed through Western blot and negative clones were selected.

Sanger sequencing was used to analyzed DNA editing in the selected clones, and sequences compared to their respective genomic sequences. RIAM KO clone H9D2 presented 2 nt deletion (650 Del and 689 Del), VASP KO clone F6, presented a 46 nt deletion (394_439 Del), VASP KO clone F10 presented a 44 nt deletion (397_441 Del), Vcl KO clone A3 presented a 38nt deletion (400_438 Del) and Vcl KO clone C4 presented a 35 nt deletion (405-440 Del).

2.7. Gene Transfection

The cell lines HL-60 VASP-EGFP, HL-60 EGFP, and VASP re-expressing lines F6-KI and F10-KI, were generated through retroviral transduction using a pMSCV-EGFP-VASP plasmid kindly donated by Matthias Krause (King's College, London).

To generate the pMSCV-EGFP plasmid, pMSCV-EGFP-VASP was cut and ligated to remove the VASP sequence. Correct ligation was assessed by DNA sequencing.

Retroviral particles were produced in packaging HEK 293T cell line through transfection using 9 μg polyethylenimine or PEI (Sigma) complexes with 3 μg of total DNA per 200 000 cells. Packaging, envelope and vector plasmid proportions were maintained as per the manufacturer's recommendations (2:1:3; pCMV-GP:pCMV-VSV-G:vector). The supernatants containing retroviral particles were harvested and added to HL-60 cells grown to log phase and treated with polybrene (Sigma) to a final concentration of 8μg/ml. Cells were then centrifuged (2200 rpm, 90 min), grown overnight and afterwards cultured normally. Transfection was monitored through fluorescence microscopy. Afterwards, cells were sorted according to fluorescence at the cell sorting service at CBMSO (CSIC, Madrid, Spain) and cultured normally until a stable cell line had been established. Characterization of these cells was done via western blot.

2.8. Integrin Cell Surface Expression Analysis and Jasplakinolide Treatment

Integrin expression was monitored through flow cytometry, as described in the previous section, and Geometric Mean Fluorescence Intensity (GMFI) was determined and normalized against the GMFI obtained for isotype controls. Staining was performed in a p96 U-bottom plate (Thermo Scientific), using 500 000 cells per well and staining volumes of 30 μl per well, unless otherwise stated.

Cells were fixed with 2% formaldehyde for 10 minutes, and blocked for 30 minutes with a PBS buffer containing 1% BSA and 100μg/ml human gamma globulin (Sigma). The following hybridoma-derived monoclonal mouse antibodies were used

to detect protein expression: BEAR-1 (integrin α_M) [315], Ts1/11 (integrin α_L) [316], HC1/1 (integrin α_X) [317], Ts2/16 (integrin β_1) [318], Lia2/3 (integrin β_2) [319], PAINS-10 (tetraspanin CD9) [313], Vj1/12 (CD59) [320] and 5A6 (tetraspanin CD81) [321]. For these, 100 µl per well of cell culture supernatants were used and supplemented with 100µg/ml human gamma globulin (hγG) in order to block Fcγ receptors, and cells were incubated for 1.5h at room temperature. After this, cells were washed and stained with a donkey anti-mouse Alexa Fluor® 488 antibody (Life Technologies), as per the manufacturer's instructions. To determine total cellular F-actin, Phalloidin-Alexa Fluor® 647 (Life Technologies) was used.

In order to induce F-actin polymerization a jasplakinolide treatment was employed. Cells initiated differentiation using retinoic acid as described previously. After 24h in the presence of RA, cells were washed and a 2h 1µM jasplakinolide (Santa Cruz) stimulation was performed. Cells were then washed and left to continue with retinoic acid differentiation for 24h. After this time period, α_M integrin expression was assessed.

2.9. Integrin Transcript Analysis Through RT-qPCR

Quantification of mRNA levels of integrin transcripts were performed using the RT-qPCR service offered by the Genomics unit of the Parque Científico de Madrid, Madrid, Spain. Briefly, $6x10^6$ retinoic acid differentiated HL-60 cells per assay were used. Quantification was performed with both triplicate biological replicates and technical replicates. mRNA was extracted using a *miRNAEasy* kit (Thermo Scientific). cDNA and RT-minus samples were prepared as per the service's standardized protocols. PCR primers and TaqMan probes were acquired from Applied Biosystems (Thermo Fisher) for the following genes: *ITGAM* (α_M integrin subunit), *ITGAX* (α_X integrin subunit), *ITGAL* (α_L integrin subunit), *ITGB2* (β_2 integrin

subunit), *APBB1IP* (RIAM), and *GAPDH y 18S rRNA* were used as reference genes. Since calculated efficiencies for amplification of the gene of interest and reference genes were similar and close to 100%, fold change in mRNA expression was calculated using the Livak-Schmittgen $\Delta\Delta C_T$ method [322].

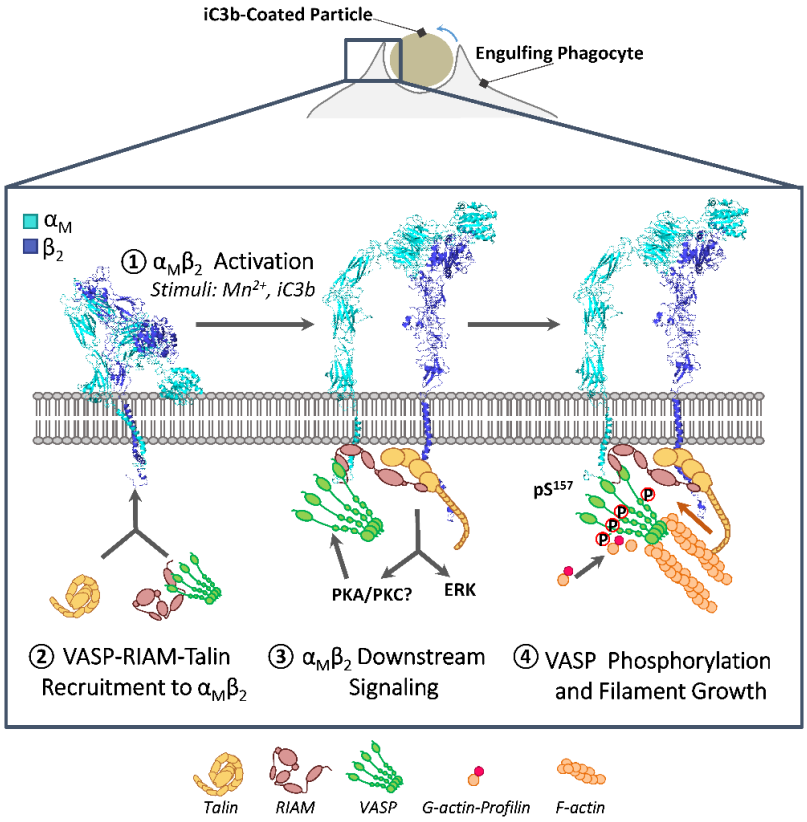
2.10. Statistical Analysis

Figures show either representative results or mean ± standard deviation (SD) of at least 3 independent experiments (repetitions are stated in the figure legends). For Chapter 3, significance between means was determined using two-tailed Student's t-test for independent samples (Welch test). Normality was assessed by using the Shapiro-Wilk test. For Chapter 4, Significance between means was determined using a multiple comparisons ANOVA followed by a *post-hoc* Dunnett's test to identify differences between groups.

To signal the degree of significance, asterisks were used as followed: a single asterisk denotes a significance of $p<0.05$; a double asterisk, $p<0.01$, a triple asterisk $p<0.005$ and a quadruple asterisk, $p<0.0001$. Statistical calculations, data handling and graphing were performed on Microsoft Excel 2016 (Microsoft, Redmond, WA, USA) and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

CHAPTER 3

RESULTS I: RIAM CONTROLS CYTOSKELETAL DYNAMICS NECESSARY FOR PHAGOCYTOSIS VIA ITS INTERACTION WITH VASP



Abstract: Activation of the phagocytic integrins and complement receptors $\alpha_M\beta_2$/CR3 and $\alpha_X\beta_2$/CR4 during complement-dependent phagocytosis by inside-out involves the Rap1-RIAM-Talin-1 pathway. Here, we analyze the implication of RIAM and its binding partner VASP, in the signaling events occurring downstream of β_2 integrins (outside-in) during complement-mediated phagocytosis. To this end we used HL-60 cell lines deficient in RIAM or VASP or overexpressing EGFP-tagged VASP to determine VASP dynamics at phagocytic cups. Our results indicate that RIAM deficient HL-60 cells presented impaired particle internalization and altered integrin downstream signaling during phagocytosis. Similarly, VASP deficiency completely blocked phagocytosis while VASP overexpression increased the random movement of phagocytic particles at the cell surface, with reduced internalization. Moreover, recruitment of VASP, the amount of pSer^{157}-VASP and formation of actin-rich phagocytic cups were dependent on RIAM expression. Our results suggested that RIAM worked as a relay in integrin complement receptors outside-in signaling coordinating integrin activation and cytoskeletal rearrangements via its interaction with VASP.

3.1. Background and Aims

Outside-in and inside-out signaling processes may involve different intracellular partners and/or protein interactions, but Talin recruitment to $\alpha_M\beta_2$ seems to be important for both pathways [323]. Since Talin recruitment is a limiting step in integrin activation, RIAM emerges as a key molecule in phagocytosis.

Previous results demonstrated that RIAM played a role in inside-out activation of complement receptors during phagocytosis [128]. RIAM knockdown in promyelocytic cell lines and monocyte-derived macrophages reduces phagocytosis in response to LPS and fMLP, diminishes expression of the activation-reporter epitope CBRM1/5 and impairs Talin1 recruitment to β_2 integrins [128]. RIAM-null polymorphonuclear leukocytes display reduced uptake of serum-opsonized bacteria and diminished free reactive oxygen species production, pointing to a role of RIAM in β_2 integrin-mediated outside-in signaling [166]. However, the involvement of RIAM in outside-in signaling had not been evaluated previously.

Outside-in signaling promotes actin cytoskeleton remodeling required for particle engulfment. Previously, the recruitment of actin elongation factors was relatively unknown, and Vasodilator-stimulated phosphoprotein (VASP) emerged as potential candidate involved in cytoskeletal dynamics leading to particle engulfment [324]. VASP interacts with RIAM [44, 152, 156], localizes at dynamic structures, such as filopodia and lamellipodia, and has a processive actin polymerase activity producing long linear actin filaments and subsequent membrane protrusions [325].

VASP function is differentially modulated by phosphorylation at several residues [326]. In vitro actin polymerization is blocked by phosphorylation in Ser^{239} and Thr^{278}, whilst $pSer^{157}$ has no impact on that process but dictates VASP membrane

localization [327, 328]. In cellular systems however, pSer^{157} promotes membrane ruffling suggesting actin polymerization [326, 329, 330].

Taking into account these precedents we hypothesized that during complement-mediated phagocytosis, RIAM may link integrin activation to the actin cytoskeleton via outside-in signaling involving VASP. To explore whether VASP plays a role in complement-mediated phagocytosis, whether RIAM participates in outside-in signaling, and if it is able to recruit and favor VASP phosphorylation, thereby linking integrin activation and cytoskeletal dynamics, we have used RIAM deficient HL-60 cells, EGFP-VASP expressing cells, as well as VASP knockouts.

3.2. Results

3.2.1. Efficient particle internalization during complement-mediated phagocytosis required RIAM

Since prior reports indicated that RIAM played a role in inside-out signaling during complement mediated phagocytosis [128], we tested whether RIAM was also involved in the downstream signaling (outside-in pathway). In order to determine the efficiency of particle internalization during complement-dependent phagocytosis, we used HL-60 cell lines stably expressing RIAM shRNA (shRIAM) or control shRNA (shCtrl) [128] differentiated towards neutrophil-like cells. In order to activate integrins, we employed a treatment with extracellular Mn^{2+}, which binds to the metal-ion-dependent adhesive site (MIDAS), and Adjacent to MIDAS (AdMIDAS) sites in the α_M I-domain. This induces a conformational change that leads to the acquisition of the high affinity conformation state of $\alpha_M\beta_2$. Cells were challenged with either complement opsonized sheep red blood cells (C-RBCs) or unopsonized RBCs and allowed to phagocytose for 20 minutes (**Figure 3.1 a-d**). Phagocytic capacity was measured using previously described indexes [128].

Association Index (AI) was determined by fluorescence from both attached and engulfed RBCs and Phagocytic Index (PI) represented only florescence from internalized particles, determined after a short hypotonic shock pretreatment. Binding Index (BI) was calculated as the difference between AI (total counts) and PI (internalized counts) to represent the quantity of bound non-internalized RBCs [312]. Indexes are represented normalized with respect to AI from unstimulated control cells. We also defined Phagocytic Efficiency (PE), as the relation between PI and AI for each cell line to determine the efficiency of particle internalization.

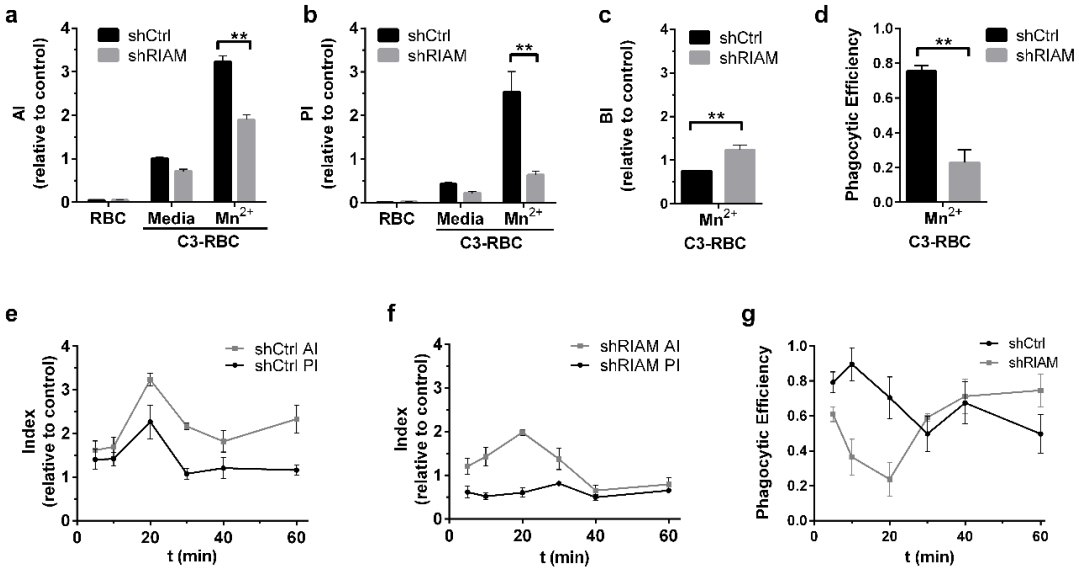


Figure 3.1. RIAM knockdown reduced phagocytosis after Mn^{2+} stimulation.

a-d) shCtrl and shRIAM HL-60 cells stimulated or not with 1 mM $MnCl_2$ (Mn^{2+} and Media, respectively) were challenged with unopsonized (RBC) or complement-opsonized erythrocytes (C3-RBC) for 20 minutes. Association (AI), Phagocytic (PI), Binding (BI) indexes, and Phagocytic Efficiency were determined. These panels represent data from 10 independent experiments. **e-g)** Phagocytosis assays were carried out as above, with $MnCl_2$ stimulated shCtrl and shRIAM cells which were left to phagocytose for a set amount of time and Association (AI) and Phagocytic (PI) indexes, and Phagocytic Efficiency were determined. Time points represent data from 5 independent experiments. Data are normalized with respect to AI of unstimulated C3-RBC challenged shCtrl cells and presented as mean±SD. Significance (t-test) has been calculated with respect to controls of the shCtrl cell line; * denotes $p<0.05$; **, $p<0.01$.

In control cells (shCtrl), Mn^{2+} stimulation produced an increase in phagocytosis of C-RBCs in both shCtrl and ShRIAM cells compared with Media. However, shRIAM cells had a statistically significant reduction in AI (41%) that was even more prominent in PI (75%) compared to shCtrl cells (**Figure 3.1 a-b**). The strong reduction in PI was accompanied by an increment in BI for shRIAM cells that was approximately double of shCtrl (**Figure 3.1 c**). In accordance with the observed increase in BI, analysis of Phagocytic Efficiency revealed that shRIAM cells were more than 3 times less efficient in internalizing particles than shCtrl cells (**Figure 3.1 d).** The discrepancy between the moderate reduction in association and the severely reduced efficiency suggests that RIAM plays a critical role in particle internalization that is somehow distinct from its described role in integrin activation.

To elucidate possible disturbances in phagocytic dynamics, a time-course of AI, PI and PE was performed by allowing Mn^{2+}-stimulated shCtrl and shRIAM cells to phagocytose C3-RBCs for different lengths of time (**Figure 3.1 e-g**). For the shCtrl cell line, AI reached a maximum at 20 minutes and experienced a fall after. Internalization, as determined by PI, followed the same dynamics up to saturation at around 40 minutes. Phagocytic dynamics were markedly different in the RIAM deficient cells. Whilst AI in shRIAM cells followed similar dynamics as control cells, albeit at a reduced rate, internalization was visibly disrupted. This was reflected by an altered phagocytic efficiency in shRIAM cells that showed a decreasing trend with minimal values at 20 minutes, increasing at longer time points mainly due to much reduced AI values. On the contrary, control cells presented higher efficiency values with a maximum at 10 minutes, declining afterwards to reach the lowest values at 30 and 60 minutes and presenting a small increase in phagocytic efficiency at 40 minutes suggestive of a recovering or recycling of the phagocytic machinery. The trend in PE followed by shRIAM cells is suggestive of an inefficient internalization that seems to

be independent of a deficiency in association, since this should result in reduced index values but not in a change in overall dynamics.

3.2.2. RIAM Knockdown Diminishes Downstream Signaling and F-Actin Enrichment at the Phagocytic Cup

As RIAM knockdown resulted in inefficient particle internalization, the downstream events in integrin signaling, such as ERK phosphorylation, were also expected to be consequently altered. To assess this, Mn^{2+}-stimulated shCtrl and shRIAM cells were incubated with C3-RBC for different periods of time and pERK and ERK were determined by Western blot (**Figure 3.2 a-b**). In shCtrl cells ERK phosphorylation followed a pulsed response showing a peak at 15 minutes and decreasing at longer times, as C3-RBCs were internalized. This contrasted with the kinetics observed in the shRIAM cells that had diminished ERK phosphorylation reaching an early peak at 5 minutes, slowly decreasing over time. These results are suggestive of abnormal β_2 integrin downstream signaling and concur with the observed reduced phagocytosis efficiency in shRIAM cells (**Figure 3.1 e-g**).

F-actin enrichment is a hallmark of phagocytic cup formation and depends on β_2 integrin downstream outside-in signaling. To determine if RIAM deficiency affects actin dynamics during phagocytic cup formation neutrophilic shCtrl and shRIAM cells stimulated with Mn^{2+} were challenged with C3-RBCs, fixed and stained with phalloidin-TRITC. Resulting confocal microscopy images were analyzed and fluorescence intensity of phalloidin-TRITC at RBC-cell contact sites was quantified and referred to the contact volume (**Figure 3.2 c-d)**. Control cells displayed F-actin-rich phagocytic cups, as well as a cortical F-actin staining pattern (**Figure 3.2 c)**. In contrast, shRIAM cells displayed a diffuse staining pattern with decreased F-actin at C3-RBC-cell contact sites, indicating poor actin polymerization (**Figure 3.2 c-d)**. These

results indicate that RIAM deficiency results in abatement of phagocytic cup F-actin content and concur with the observed reduced internalization, suggesting a functional connection between RIAM and the actin cytoskeleton.

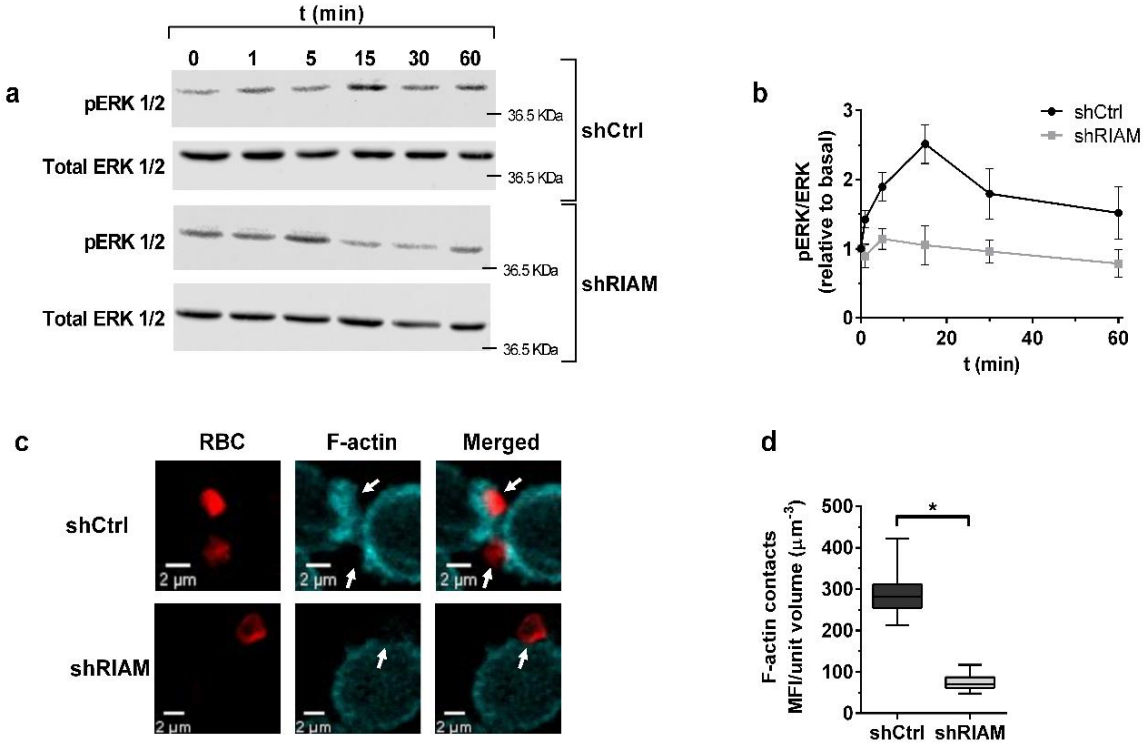


Figure 3.2. RIAM controls downstream signaling and phagocytic cup F-actin.

a) shCtrl and shRIAM HL-60 cells stimulated or not with 1 mM MnCl2 were left to phagocytose for different time points, cell lysates were prepared and pERK and total ERK were analyzed by western blot. The figure shows representative results of 5 independent experiments. **b**) Quantification of ERK activation determined as the ratio of pERK to total ERK during phagocytosis. Results are represented as relative to basal activation and are from 5 independent experiments. **c)** Mn^{2+} stimulated ShCtrl and shRIAM HL-60 cells were challenged with C3-opsonized RBCs, left to phagocytose for 20 minutes, fixed and stained for F-actin (fluorescent TRITC-phalloidin). Cells were imaged using confocal microscopy. Figure shows representative results from one confocal plane. **d**) Analysis of quantified confocal images. Phalloidin-TRITC mean fluorescence intensity (MFI) was measured at the contact zones between cells and particles. Data is presented as mean±SD. Significance (t-test) has been calculated with respect to controls of the shCtrl cell line, * denotes $p<0.05$ and **, $p<0.01$.

3.2.3. Efficient particle internalization during complement-dependent phagocytosis required VASP expression

VASP has actin polymerase activity and interacts with RIAM. Therefore, VASP could be a potential candidate link to explain the connection between RIAM and F-actin. To evaluate VASP contribution to the phagocytic process, HL-60 cells stably expressing EGFP-VASP or only EGFP were generated (**Figure 3.3 a**).

Phagocytosis assays using these cell lines stimulated with either LPS or $MnCl_2$ were carried out to determine differential effects of inside-out versus outside-in induced activation. Association (AI), Phagocytic (PI) Binding (BI) indexes were determined and represented as relative to unstimulated EGFP cells (in standard media). Phagocytic Efficiency was also represented (**Figure 3.3 b-e)**. LPS-mediated inside-out stimulation did not induce any differential effect in VASP overexpressing cells compared to their counterparts. As for outside-in stimulation with Mn^{2+}, there were no remarkable differences between control EGFP and EGFP-VASP in terms of association, yet VASP overexpression significantly impaired internalization, as evidenced by the reduced PI and correspondingly increased BI and decreased PE (2.8 fold decrease). This defect in EGFP-VASP cells paralleled the blocked internalization observed for shRIAM cells (**Figure 3.1 b-d**).

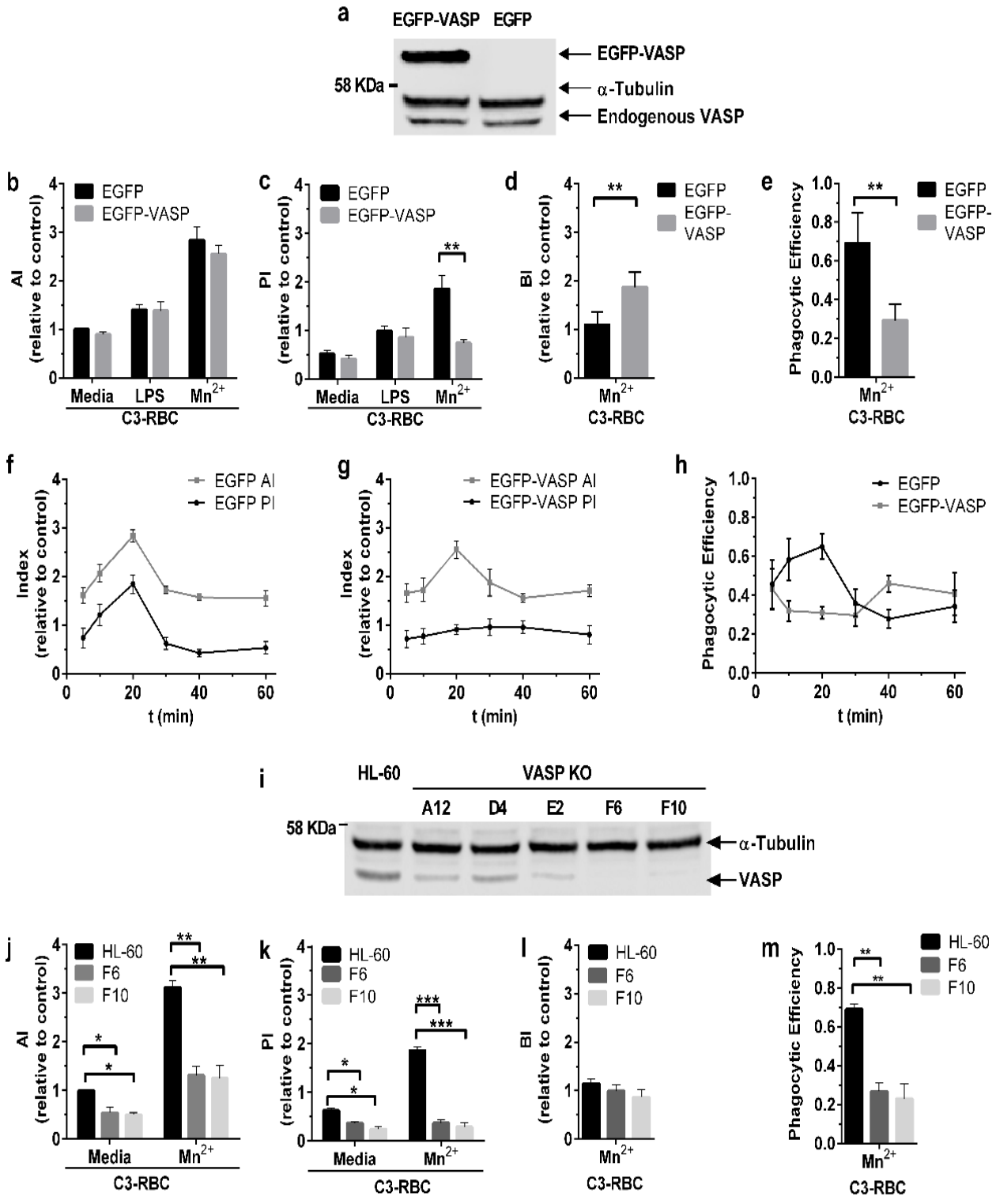


Figure 3.3. Overexpression or Knockout of VASP diminishes phagocytosis.

a) Newly generated HL-60 cell lines (EGFP and EGFP-VASP) were tested for VASP expression via western-blot. **b-e**) Phagocytic cells were challenged with C3-RBC after being

stimulated with 1 mM MnCl2, 320 nM LPS or unstimulated (Mn2+, LPS and Media, respectively) and Association (AI), Phagocytic (PI), Binding (BI) indexes and Phagocytic Efficiency were obtained. **f-h**) Phagocytosis assays were carried out as above. EGFP (**f**) and EGFP-VASP (**g**) cells stimulated with $MnCl_2$ were left to phagocytose for different length of time and the Association, Phagocytic indexes and Phagocytic Efficiency were determined. All data are normalized with respect to the AI of unstimulated C3-RBC challenged EGFP cells. Each time point represents data from 3 independent experiments. **i**) Newly generated HL-60 knockout monoclonal cell lines (VASP KO A12, D4, E2, F6 and F10 clones) were tested for VASP expression by western blot. **j-m**) Phagocytic HL-60 cells and VASP KO F6 and F10 clones were challenged with C3-RBC after being stimulated with 1mM $MnCl_2$ or left unstimulated (Mn^{2+} and Media, respectively), Association (AI), Phagocytic (PI), Binding (BI) indexes and Phagocytic Efficiency were obtained. Data are normalized with respect to the AI of unstimulated C3-RBC-challenged HL-60 cells. Data are presented as mean±SD, where the error bars denote standard deviation. Significance (t-test) has been calculated with respect to controls of the EGFP cell line, * denotes $p<0.05$; **, $p<0.01$; a *** $p<0.005$.

Since VASP is an actin polymerase, its overexpression may lead to excessive actin polymerization that might slow down and/or interfere with the phagocytic process. To test this hypothesis, the effect of VASP overexpression on phagocytic dynamics was explored. EGFP and EGFP-VASP cells were stimulated with $MnCl_2$ and challenged with C3-RBC. Cells were left to phagocytose for different periods of time and the different indexes and phagocytic efficiency were determined (**Figure 3.3 f-h**). Concurring with the previous results, no alterations were observed in association dynamics, as both cell lines displayed similar indexes. However, internalization dynamics were distinctly altered in EGFP-VASP cells, with PI oscillating around a constant value of 0.88±0.06, suggestive of ineffective phagocytosis. In parallel to the shRIAM phenotype, EGFP-VASP phagocytic efficiency followed a similar trend with an efficiency which oscillated around a value of 0.35, with reduced values at 10 and 20 minutes compared to control cells and reaching a maximum at 40 minutes, mainly due to a reduction in AI.

To further elucidate the role of VASP during phagocytosis, VASP knockout clones were generated in HL60 cells through CRISPR/Cas9 targeting VASP exon 2

(**Figure 3.3 i)**. VASP KO F6 and F10 clones were selected and stimulated with $MnCl_2$ or not (Media) and AI, PI, BI indexes and PE were determined and represented as relative to HL-60 parental cells (**Figure 3.3 j-m**). Contrasting with the overexpression phenotype, VASP KO resulted in a statistically significant reduction in AI (**Figure 3.3 j**). Particle internalization was completely abolished in VASP KO and showed no increment above basal levels after Mn^{2+} stimulation (**Figure 3.3 k)**. VASP KO BI levels were almost equal to those of parental cells (**Figure 3.3 l**), and their phagocytic efficiencies were less than half of those of parental cells (**Figure 3.3 m**), reflecting defective internalization. Taken together, these data suggest that correct VASP expression and function are critical for the effective phagocytosis of C3-opsonized targets in neutrophilic cells, highlighting the importance of this actin polymerase over other factors.

3.2.4. VASP localized to phagocytic cups in a RIAM-dependent manner

VASP has been reported to be recruited to FcγR-induced phagocytic cups [331]. To assess VASP localization during complement-dependent phagocytosis, time-lapse confocal microscopy was performed using EGFP-VASP cells stimulated with $MnCl_2$ and challenged with C3-RBCs (**Figure 3.4 a**). Prior to the addition of C3-RBCs, VASP appeared evenly distributed in the submembranal cortical cytoplasm. However, upon contact with C3-RBCs, VASP relocalized and became enriched at the RBC-cell contact sites. This enrichment was represented as fold enrichment of contact mean fluorescence intensity (MFI) (**Figure 3.4 b**). Contact MFI substantially increased in VASP-EGFP cells, around the 20 min time point whilst it remained stable in the

EGFP control cells. This peak coincided with maximal particle association observed in the previous experiments.

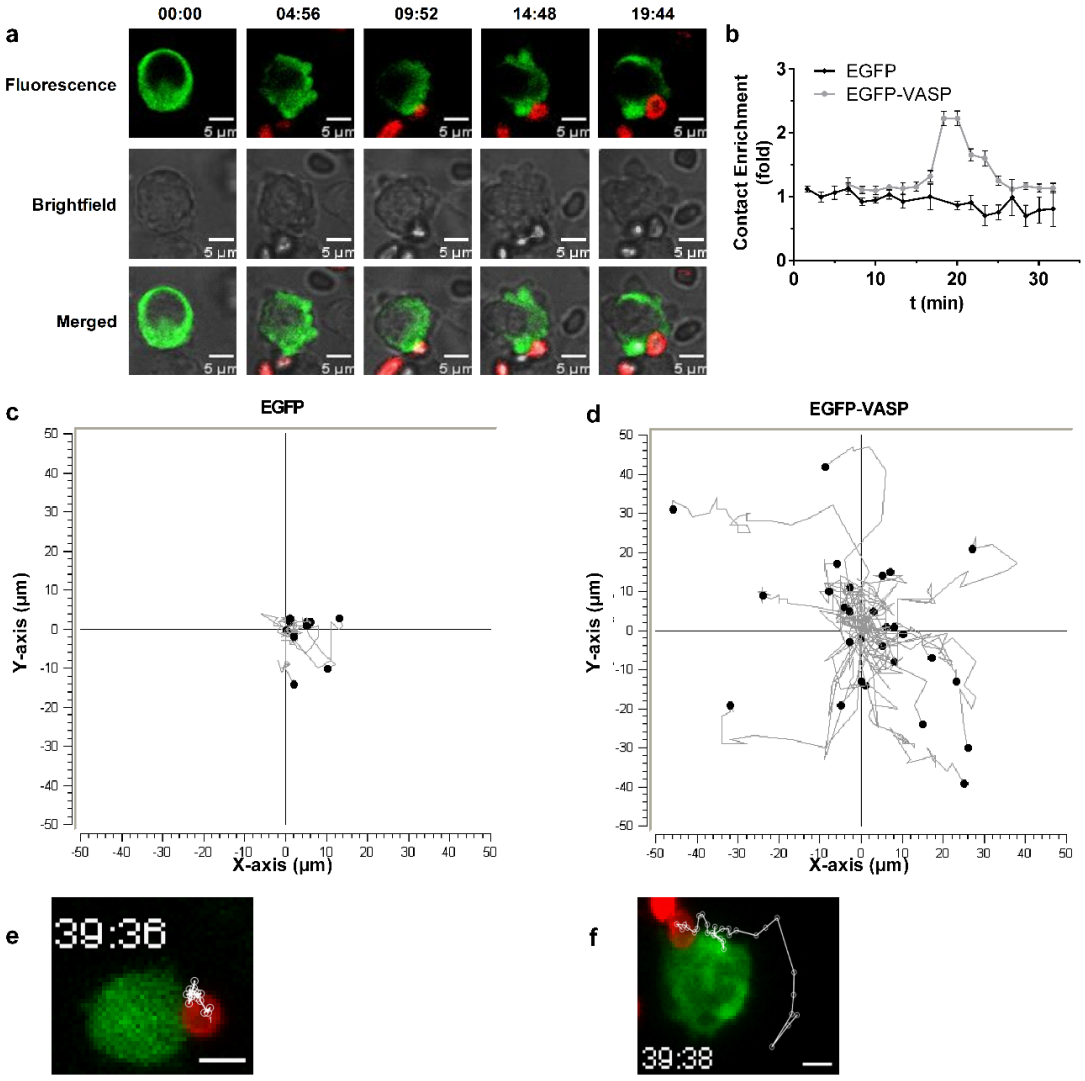


Figure 3.4. Phagocytic cup recruitment of VASP produced overactive cells.

a). Time-lapse confocal microscopy was performed on $MnCl_2$ stimulated C3-RBC-challenged EGFP-VASP HL-60 cells. RBCs are shownin red and EGFP-VASP is in green. The images show representative data from a single confocal plane. Scale bars denote represent 5 µm. **b**) Time-lapse images were analyzed and contact mean fluorescence intensity was quantified and graphed. Results are represented as fold increase with respect to total cell mean fluorescence at t=0. Represented data corresponds to 50 contacts per cell line. Data are presented as mean±SD, where the error bars denote standard deviation. Significance (t-test) has been calculated with respect to controls of the EGFP cell line. **c**) and **d**) RBC displacement was tracked on the x-y axis and represented for phagocytosing EGFP and EGFP-VASP cells (c and d, respectively) with n=50. **e**) and **f**). Representative images of either EGFP (**e**) or EGFP-VASP (**f**)

phagocytosing HL-60 cells corresponding to those analyzed in **c**) and **d**), respectively. The scale bars represent 5 μm. The images show representative data from the maximum intensity projection of all acquired confocal planes.

During imaging recording, EGFP-VASP cells were observed to be very motile and bound erythrocytes actively moved on the cells surface during phagocytosis, whilst C3-RBCs on EGFP control cells remained much more stationary. Erythrocyte trajectories were mapped for both cell lines using the MTrackJ Image J plugin (**Figure 3.4 c-f**). On control EGFP cells, C3-RBCs remained constrained and barely moved from their initial contact site, whilst on EGFP-VASP cells bound C3-RBC underwent drastic changes in position and velocity. These long-range changes in RBC location on the surface of phagocytic cells correlated with the observed deficient internalization and suggest that VASP overexpression leads to an overactive cortical actin cytoskeleton.

RIAM translocates from cytoplasm to the membrane during integrin activation [332]. As RIAM binds VASP, we hypothesized that this adaptor protein may be instrumental in recruiting VASP to the phagocytic cup. To establish if VASP localization was dependent on RIAM expression, EGFP-VASP cells were transfected with either a RIAM-specific or a negative control siRNA, stimulated with $MnCl_2$ and challenged with C3-RBCs. EGFP-VASP and F-actin recruitment to RBC-contact sites were quantified by confocal microscopy (**Figure 3.5 a-b**). RIAM silencing resulted in a statistically significant reduction of 58.8% in phalloidin-TRITC and 62.5% in EGFP-VASP mean fluorescence intensity at the RBC contact site. This result indicated that RIAM controlled VASP localization and may be responsible for its recruitment to phagocytic cups.

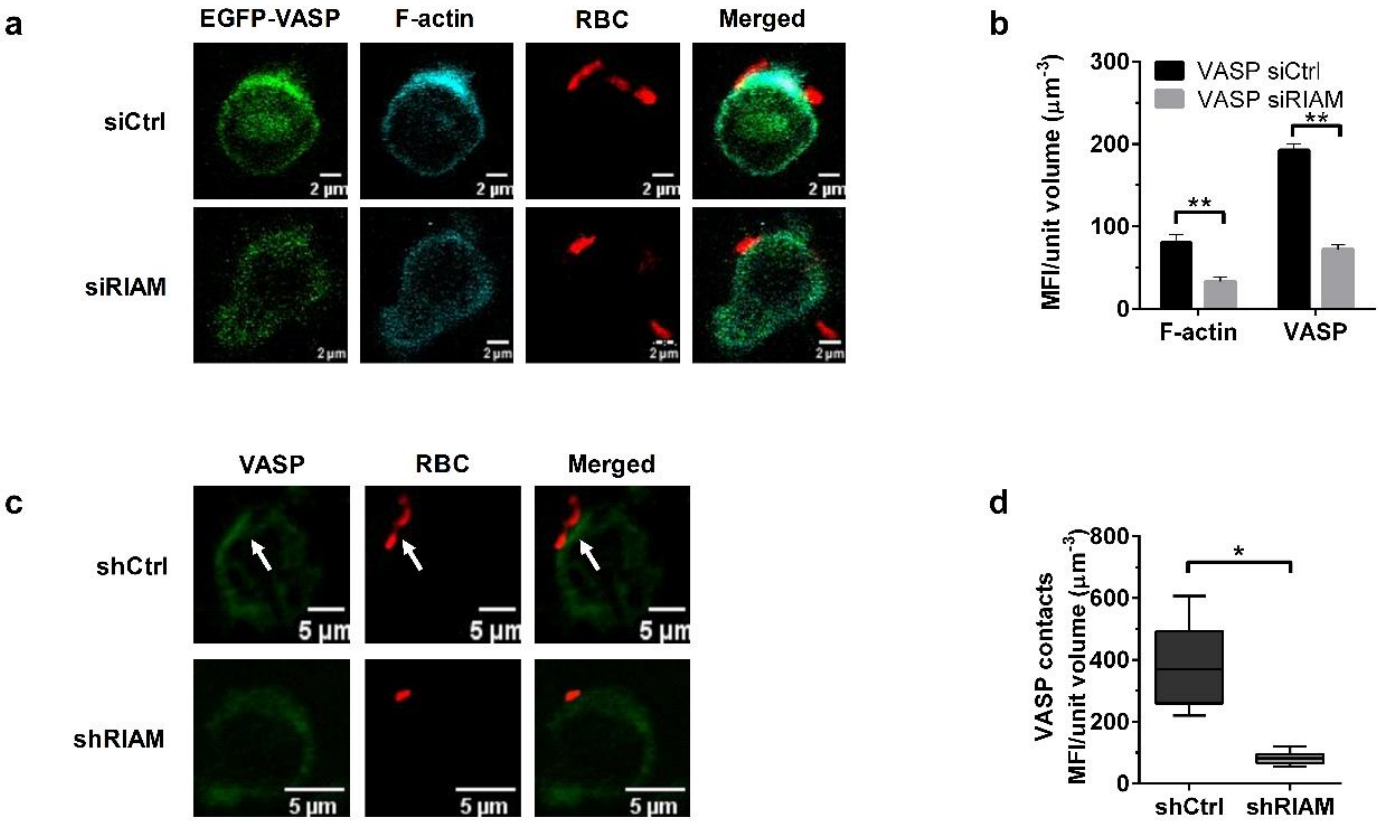


Figure 3.5. RIAM expression determined VASP membrane localization.

a) EGFP-VASP HL-60 cells were transfected with either control (siCtrl) or RIAM-specific siRNA (siRIAM) stimulated with 1 mM $MnCl_2$ and challenged with C3-RBCs. Figure shows representative images from a single confocal plane. **b)** Mean Fluorescence Intensity (MFI) of both EGFP-VASP and phalloidin-TRITC (F-actin content) was quantified for the contacts between cell and RBC and graphed. Data are presented as mean±SD, where the error bars denote standard deviation. Significance (t-test) has been calculated with respect to the siRNA controls of the VASP cell line. Double asterisk denotes a significance of $p<0.01$. **c)** $MnCl_2$ stimulated shRIAM and shCtrl cells were challenged with C3-opsonized-RBCs left to phagocytose for 20 minutes, and then fixed and stained for endogenous VASP. RBCs were stained by using an anti-C3b antibody for this assay. Cells were imaged through confocal microscopy. The figure shows representative results from a single confocal plane. **d)** Analysis of endogenous VASP staining intensity (MFI) at the RBC-Cell contact sites quantified from the confocal images shown in **c)**. Data are presented as mean±SD, where the error bars denote standard deviation. Significance (t-test) has been calculated with respect to controls of the shCtrl cell line, * denotes $p<0.05$.

Endogenous VASP distribution was also analyzed during phagocytosis in shCtrl and shRIAM cells (**Figure 3.5 c**). In control cells, VASP was located at the submembranal cortical cytoplasm accumulating at the RBC-contact site, whereas in RIAM knockdown cells VASP presented a more diffuse pattern with a reduced MFI at the RBC contact site (**Figure 3.5 d**), mirroring the pattern observed for F-actin.

3.2.5. RIAM expression is necessary for VASP pSer^{157} phosphorylation

VASP membrane localization is dependent on Ser^{157} phosphorylation, and *in vivo* integrin dependent adhesion and migration assays suggest a relationship between Ser^{157} phosphorylation and actin polymerase activity [326, 329, 330]. Since VASP and F-actin were reduced at C3-RBC-contact sites in shRIAM cells, the relationship between pSer^{157}-VASP and RIAM expression was explored during phagocytosis. Control (shCtrl) and shRIAM cells stimulated with Mn^{2+} were challenged with C3-RBC for different periods of time and VASP and pSer^{157}-VASP levels determined by western blot (**Figure 3.6 a**).

In control cells, VASP phosphorylation followed saturation kinetics and reached a peak at around the 30 minute time point. However, in shRIAM cells VASP showed lower phosphorylation and altered kinetics (**Figure 3.6 b**). Using confocal microscopy pSer^{157}-VASP localization was assessed for shRIAM and shCtrl cells during phagocytosis **Figure 3.6 c-d**). In control cells pSer^{157}-VASP was markedly localized in proximity to the RBC contact site, whereas in shRIAM cells pSer^{157}-VASP displayed a diffuse pattern with significant reduction in MFI at the RBC-cell contact site.

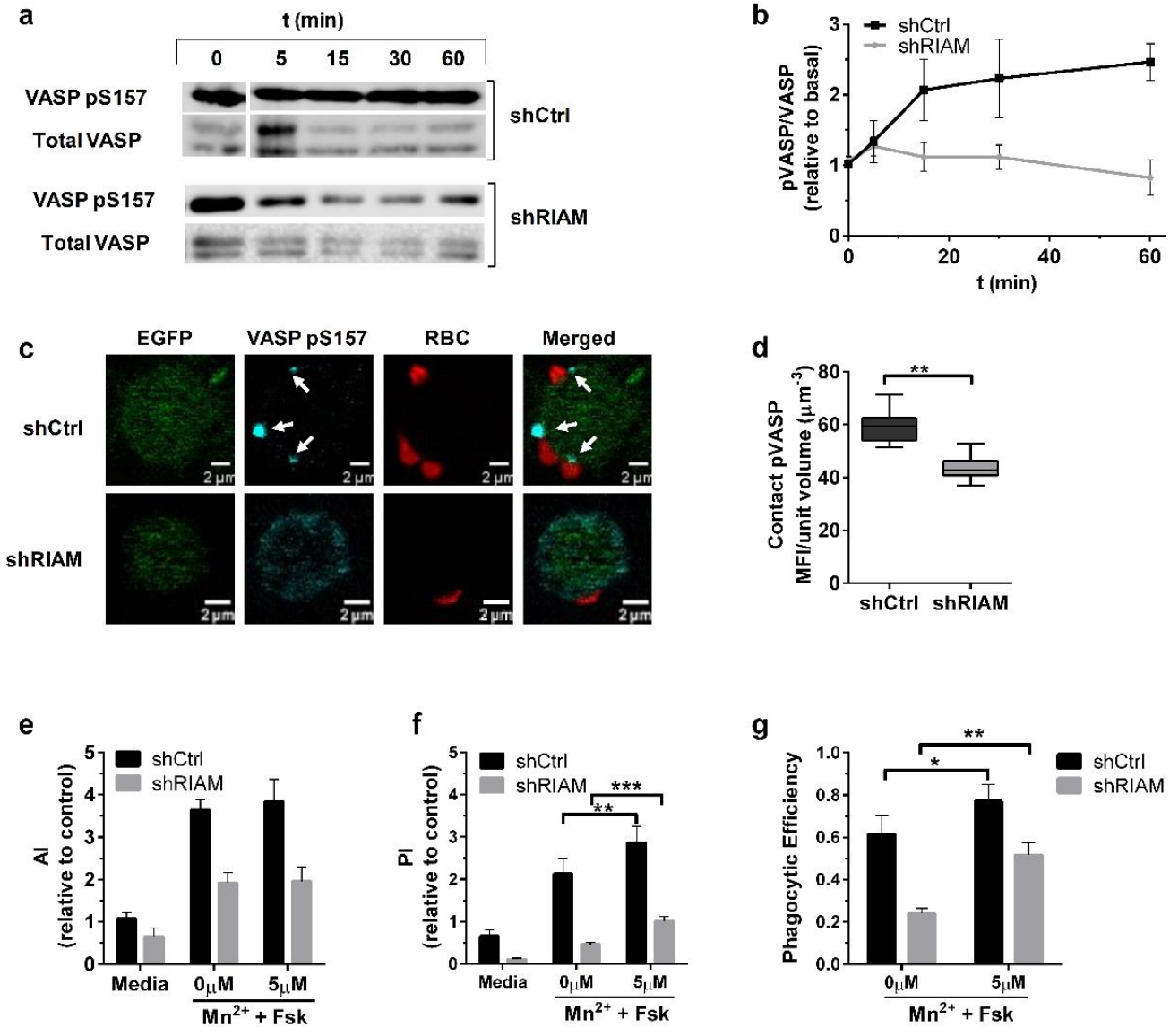


Figure 3.6. RIAM was required for pSer^{157} VASP phosphorylation.

a) Phagocytosis assays were carried with $MnCl_2$ stimulated shCtrl and shRIAM HL-60 cells for different periods of time. Cells were lysed and pSer^{157}-VASP (pVASP) and total VASP content determined by western blot. A. representative experiment out of four is shown. **b)** Quantification of pSer^{157}-VASP determined as the ratio of pVASP and total VASP signal during phagocytosis at different time points in shCtrl and ShRIAM cells. Results are represented as values relative to basal activation and are derived from five independent experiments. Data are presented as mean±SD error bars denote standard deviation. **c)** pSer^{157}-VASP localization was assessed through confocal microscopy in phagocytosing shCtrl and shRIAM cells. Arrows indicate pVASP enrichment at contacts with RBCs. The figure shows representative results from a single confocal plane. **d**) pSer^{157}-VASP mean fluorescence intensity at the RBC contact site was quantified from cells in (c). The figure shows results

from 50 cells. **e**), **f**), **g**), and **h**) AI, PI, BI and Phagocytic Efficiency were obtained from phagocytosis assays using unstimulated (Media) or $MnCl_2$-stimulated ShCtrl and shRIAM cells that were either untreated (0μM) or treated with 5μM Forskolin (Fsk). The figure represents data from 5 independent experiments. Data are normalized with respect to the AI of unstimulated C3-RBC challenged shCtrl cells. Significance (t-test) is calculated with respect to controls of shCtrl cells, a * denotes significance of $p<0.05$; **, $p<0.01$; a ***, $p<0.005$.

Considering that shRIAM cells have reduced $pSer^{157}$-VASP we proposed that an increment in VASP Ser^{157} phosphorylation might alleviate the internalization deficiency observed in shRIAM cells. To test this hypothesis cells were treated with forskolin (Fsk), which activates PKA and increases $pSer^{157}$-VASP levels [330]. Whilst Fsk treatment after $MnCl_2$ stimulation did not provoke significant changes in AI (**Figure 3.6 e**), a statistically significant two fold increase in PI was observed for both cell lines (**Figure 3.6 f**). This result suggested that forskolin treatment was capable of partially reverting the RIAM knockdown phenotype in terms of particle internalization as indicated by the increase in phagocytic efficiency induced by Fsk treatment (**Figure 3.6 h**).

3.3. Discussion

Complement-dependent phagocytosis requires receptor activation, cytoskeletal reorganization with membrane extension and particle internalization. This process requires strict coordination between different pathways, making signaling platform molecules, such as RIAM, essential. The data presented here suggest a critical role for RIAM in the coordination of β_2 integrin-induced actin cytoskeletal dynamics via its interaction with VASP. While the role of RIAM in CR3 activation after inside-out stimulation is already established [128, 152], it is uncertain if RIAM is also involved in integrin outside-in signaling.

We demonstrate that RIAM knockdown reduced complement-mediated phagocytosis in cells treated with Mn^{2+}, a stimulus that promotes full integrin

activation and high affinity for their ligands. Under these stimulation conditions shRIAM cells showed a 42% reduction in association (AI) of C3-opsonized particles. This defect did not result in altered association dynamics, as shRIAM cells followed the same trend as control cells. By contrast, RIAM knockdown cells showed impaired internalization with altered dynamics. Control cells showed a pulsed response, whereas shRIAM cells exhibited a delayed and flat response. This emphasizes the importance of RIAM in particle engulfment, as reduced expression of this adaptor molecule may slow effector recruitment acting as a bottleneck for the process. In line with this observation, macrophages from RIAM null mice showed defective adhesion to ICAM-1 that was partially rescued by Mn^{2+} treatment. Based on these results, Klapproth et al. concluded that RIAM may be involved in outside-in signaling [166].

The defective complement-phagocytosis coincided with overall reduced pERK levels and distorted ERK phosphorylation kinetics in shRIAM cells. A reduction in this signaling pathway has been described in RIAM knockdown melanoma and breast carcinoma cell lines to cause impaired adhesion turnover that correlates with an inhibition of the MEK-ERK pathway and deficient RhoA activation [333]. The results reported herein suggest that RIAM expression determines ERK phosphorylation and its kinetics during complement-mediated phagocytosis in a manner comparable to adhesion turnover regulation for migrating cells.

Scaffolding proteins coordinate signal transmission but require specific expression levels to do so. Suboptimal concentration of Ste5 (yeast) and of its mammalian equivalent KSR1/2, both controlling the MAPK pathway, result in delayed cell responses, due to unbalance between concentrations of these scaffolds and their effectors [334-337]. Both phagocytic dynamics and ERK phosphorylation kinetics of shRIAM cells are very reminiscent of these responses, further emphasizing the importance of RIAM as a signaling platform.

In addition to presenting altered ERK phosphorylation kinetics, RIAM deficient cells exhibited reduced levels of F-actin at phagocytic cups. This may explain the diminished particle internalization observed for these cells and concurs with the defects in neutrophil migration and extravasation observed for *RIAM*$^{-/-}$ mice [166], as both processes require actin cytoskeleton remodeling. Similarly, these mice show defective bone marrow and lymph node homing of B and T cells [338], further associating motile processes with RIAM expression.

Our results suggest that VASP plays a crucial role in particle internalization after Mn^{2+} stimulation. VASP overexpression significantly reduced the Phagocytic Index over time, suggesting altered internalization and ineffective phagocytosis. By using time-lapse fluorescence microscopy, we observed that, whereas in the EGFP control cell line bound C3-RBCs remained rather stationary, C3-RBCs bound to EGFP-VASP cells moved haphazardly (a process reminiscent of membrane ruffling) and this might explain the poor phagocytosis. In accordance with these results, exogenous VASP expression induces ruffle formation in endothelial cells [339], and Mena and VASP overexpression inhibits fibroblast cell motility [340], which the authors attribute to membrane ruffling. This phenotype is dependent on Ena/VASP protein localization. Fibroblasts in which Ena/VASP proteins are recruited to the plasma membrane (by co-expression of a construct containing an EVH1 binding motif and a plasma membrane targeting domain) exhibit rapid withdrawing of lamellipodial protrusions or membrane ruffles [341].

These observations concur with our data, which showed that EGFP-VASP was enriched at the phagocytic cup and was related to F-actin content, all of which were dependent on RIAM expression. The same holds true for endogenous VASP, as its localization at phagocytic cups was diminished as a result of RIAM knockdown. Our results demonstrate that VASP localization is dependent on RIAM expression

pointing to a link between these two proteins and their role in regulating complement-mediated phagocytosis.

VASP knockout cell clones presented strong defects in particle internalization and this contrasts with prior reports stating that fibroblast migration is enhanced in the absence of all Ena/VASP proteins [340]. Another study suggests that VASP expression controls tensile strength, contractility and cytoskeletal rigidity [342]. VASP-deficient fibroblasts show thickened stress fibers and a delayed but more stable adhesions. The discrepancy between our observations in complement-mediated phagocytosis and adhesion assays could be explained because phagocytosis is a more dynamic process and a delay in establishing a stable adhesion may halt the process. Furthermore, fibroblast adhesions are typically characterized by branched (Arp2/3-mediated) F-actin and lamellipodial protrusions and not by lineal filopodial extensions like those mediated by VASP or formins. These two groups of actin assembly factors may compete for a pool of profilin-bound G-actin and negatively regulate each other [343].

The fact that in our model phagocytosis was practically abolished in VASP KO cells highlights its importance over other factors that could compensate the defect. Of special note is mDia, a formin that is recruited specifically to CR3 phagocytic cups by binding to microtubule-binding CLIP-170. At phagocytic cups, mDia1 interacts with active RhoA which promotes local actin polymerization. In accordance, mDia1-deficient macrophages display inefficient particle internalization [202, 203]. VASP and mDia are unbranched actin filament polymerases with potentially overlapping functions. However, studies in *Drosophila melanogaster* cells have revealed that both proteins drive formation of filopodia with distinct morphology and dynamics, suggesting their roles are not redundant. Long, stable filopodia require mDia whereas, Ena (VASP homologue) promotes more dynamic

elongations. Ena inhibits mDia activity allowing cells to switch from long, persistent protrusions to a dynamic mixes of lamellipodia and filopodia [344]. The differences in polymerase activity suggest that these factors may have non overlapping functions, explaining the defects in phagocytosis observed in mDia or VASP deficient cells.

In addition to the previous,the actin nucleator Arp2/3 was described to participate in CR3 phagocytosis whilst being dispensable for FcγR phagocytosis in primary bone marrow-derived mouse macrophages [343] Arp2/3 activity was shown to drive the leading edge of protrusions and phagocytic cup formation during complement phagocytosis, whereas mDia1 activity coupled advancing protrusions to the particle surface. Association between Talin and Vinculin was also demonstrated in phagocytic cup containing β_2 integrins, organized in structures similar to focal adhesion complexes [53].

In migrating epithelial cells, actin assembly factors act sequentially starting with mDia1, followed by Arp2/3-mediated formation of cortical actin, and elongation mediated by mDia2 or VASP [345]. A hierarchy is also proposed during complement-dependent phagocytosis [53], where Arp2/3 may provide the cortical actin platform for mDia activity. We propose that in complement-mediated phagocytosis VASP in coordination with Arp2/3 and mDia1 may work sequentially to promote balanced F-actin dynamics during phagosome formation.

VASP phosphorylation at Ser^{157} correlates with membrane localization [326-328, 346]. We have demonstrated that RIAM knockdown produced a notable reduction in VASP and F-actin content at the phagocytic cup, with altered VASP $pSer^{157}$ phosphorylation kinetics and overall levels. We hypothesize that RIAM may recruit VASP to active integrins, where it may be phosphorylated. Further

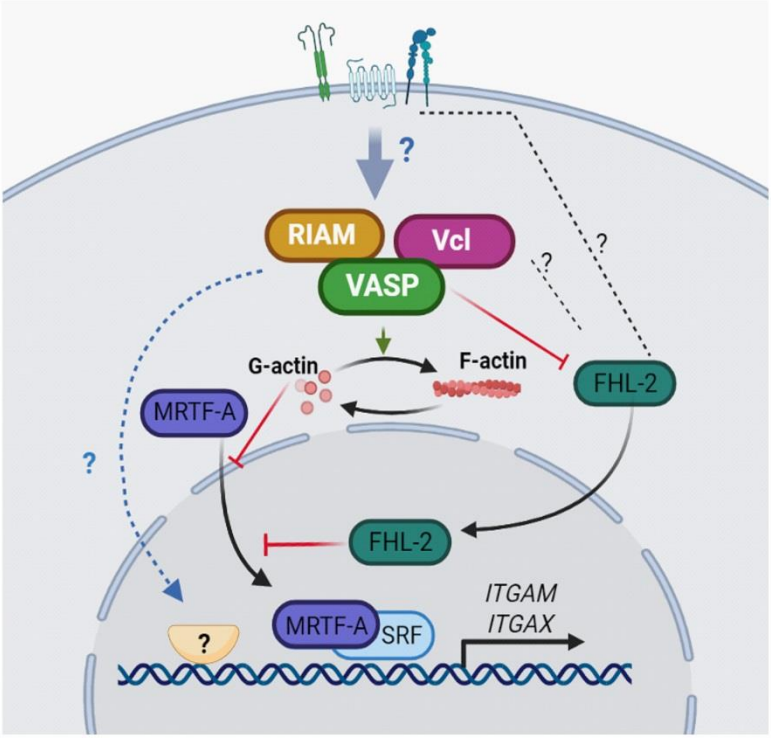
experiments using either non-phosphorylatable or phosphomimetic mutants of VASP could further corroborate this hypothesis. Indeed, in mice cells, RIAM co-immunoprecipitates preferentially with non-phosphorylatable VASP (S153A) [347] while Vinculin, another VASP binding partner, is unable to co-immunoprecipitate with it. On the contrary Vinculin-VASP interaction is induced in cells treated with acetylcholine, which promotes both Ser^{157} VASP and Vinculin Tyr^{1065} phosphorylation [330]. All this suggests that VASP phosphorylation state determines VASP preference for its binding partners.

Phosphorylation of VASP on Ser^{157} correlates with actin polymerization in vivo [326, 329, 330]. Our results using forskolin to increase VASP phosphorylation, showed an increase in phagocytosis and a partial reversion of the RIAM knockdown phenotype, suggesting increased actin polymerization. In vitro studies of recombinant VASP $pSer^{157}$ phosphomimetics have not shown an increase in polymerase activity [328]. However, other in vitro studies using lamellipodin a member of the MRL family protein involved in VASP recruitment to the lamella [348], demonstrate that this protein increases VASP processivity and therefore increase polymerization rates [349]. As RIAM is part of the MRL family, we hypothesized that a similar process occurs during complement-mediated phagocytosis, which would concur with our results.

The present work describes the role of both RIAM and VASP in complement-mediated phagocytosis. Our results demonstrate that RIAM is required for outside-in integrin signaling. We proved that RIAM acted as a relay, linking integrin activation and cytoskeletal dynamics through VASP, controlling its localization and phosphorylation status, which may in turn regulate its activity through recruitment of different binding partners.

CHAPTER 4

RESULTS II: PROXIMAL COMPONENTS OF THE INTEGRIN ADHESION COMPLEX CONTROL COMPLEMENT RECEPTOR 3 AND 4 EXPRESSION



Abstract: Activation of the integrin phagocytic receptors CR3 ($\alpha_M\beta_2$, CD11b/CD18) and CR4 ($\alpha_X\beta_2$, CD11c/CD18) requires Rap1 activation and RIAM function. RIAM controls integrin activation by recruiting Talin to β_2 subunits, enabling the Talin-Vinculin interaction, which in term bridges integrins to the actin-cytoskeleton. RIAM also recruits VASP to phagocytic cups and facilitates VASP phosphorylation and function promoting particle internalization. Using a CRISPR-Cas9 knockout approach, we have analyzed the requirement for RIAM, VASP and Vinculin expression in neutrophilic-HL-60 cells. All knockout cells displayed abolished phagocytosis that was accompanied by a significant and specific reduction in *ITGAM* (α_M), *ITGAX* (α_X) and *ITGB2* (β_2) mRNA, as revealed by RT-qPCR. RIAM, VASP and Vinculin KOs presented reduced cellular F-actin content that correlated with α_M expression, as treatment with the actin filament polymerizing and stabilizing drug jasplakinolide, partially restored α_M expression. In general, the expression of α_X was less responsive to jasplakinolide treatment than α_M, indicating that regulatory mechanisms independent of F-actin content may be involved. The Serum Response Factor (SRF) was investigated as the potential transcription factor controlling $\alpha_M\beta_2$ expression, since its coactivator MRTF-A requires actin polymerization to induce transcription. Immunofluorescent MRTF-A localization in parental cells was primarily nuclear, while in knockouts it exhibited a diffuse cytoplasmic pattern. Localization of FHL-2 (SRF corepressor) was mainly sub-membranous in parental HL-60 cells, but in knockouts the

localization was disperse in the cytoplasm and the nucleus, suggesting RIAM, VASP and Vinculin are required to maintain FHL-2 close to cytoplasmic membranes, reducing its nuclear localization and inhibiting its corepressor activity. Finally, reexpression of VASP in the VASP knockout resulted in a complete reversion of the phenotype, as knock-ins restored α_M expression. Taken together, these results suggest that expression of RIAM, VASP and Vinculin, known to be involved in integrin signaling and actin cytoskeleton modulation are necessary for the correct expression of $\alpha_M\beta_2$ and $\alpha_X\beta_2$ expression, which are key during myeloid cell differentiation and the acquisition of a phagocytic phenotype.

4.1. Background and Aims

We have previously reported that the adaptor molecule RIAM and its interacting partner VASP participate in this outside-in signaling pathway during complement-mediated phagocytosis [350]. Knocking out VASP in HL-60 cells using CRISPR/Cas9 technology also resulted in a drastic decrease in phagocytic capacity with significant decrease in particle association, which suggested integrin function or expression was altered.

Similar results had been reported for RIAM knockout mice, where these displayed a phenotype reminiscent of Leukocyte Adhesion Deficiency (LAD) syndromes, with a pronounced neutrophilic (4-fold increase) and monocytic leukocytosis. This effect was more pronounced on Talin1$^{-/-}$ mice (>30-fold increase) [166]. Leukocyte adhesion deficiency can be caused by direct loss-of-function mutations in *ITGB2* (β_2 integrin gene) [351], or mutations in components regulating integrin activation and signaling, such as Kindlin-3 [149] and CalDAG-GEFI, a Rap1 GEF [130]. This set of immunodeficiencies causes recurrent infections due to ineffective neutrophil and macrophage function, and disrupts myeloid cell differentiation, causing extreme neutrophilia (~5 to 10-fold increases) due to egress of immature precursors [219, 220, 352]. In the case of LAD-I patients, leukocyte expression of integrin β_2 is greatly diminished, or absent, with a concomitantly

reduced or null expression of its binding partner subunits α_L, α_M and α_X [353]. Mature neutrophils and macrophages require the expression of $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins in order to be functional [354]. The expression of these integrins is regulated during myelopoiesis in hematopoietic stem cell niches and is critical for their egress from the bone marrow [264].

Since our VASP knockout exhibited a phenotype compatible with the LAD-like defect observed in RIAM knockouts, we decided to characterize this deficiency in terms of integrin expression and function, and study whether this also happened for Vinculin, another component of the integrin adhesion complex (IAC). We also aimed to ascertain the cause of the observed defect and if possible use genetic reconstitution or pharmacological treatments in order to try to revert the observed phenotype.

4.2. Results

4.2.1. RIAM, VASP and Vinculin knockouts abolished phagocytosis

We previously determined that correct VASP expression is necessary for engulfment, since VASP knockout resulted in an abolished phagocytosis and VASP overexpression significantly reduced phagocytic efficiency [311]. Here, we further analyse the requirement for expression of VASP and VASP-interacting proteins, namely RIAM and Vinculin, all components of the integrin adhesome, in the process of complement-mediated phagocytosis.

Knockout of RIAM and Vinculin was performed using a double nick strategy and a transitory transfection using a CRISPR-Cas9 system. Western blot confirmed that all-*trans* retinoic acid differentiated HL-60 knockouts presented no detectable levels of the knocked-out proteins, but retained normal levels of the other assessed

proteins (**Figure 4.1 a-c**), confirming that a knockout of RIAM does not affect Vinculin (Vcl) or VASP expression levels, and vice-versa. When Talin expression was assessed, no significant change was observed, suggesting that genetic deletion of one of the genes from IAC does not affect the expression of the rest of components assessed.

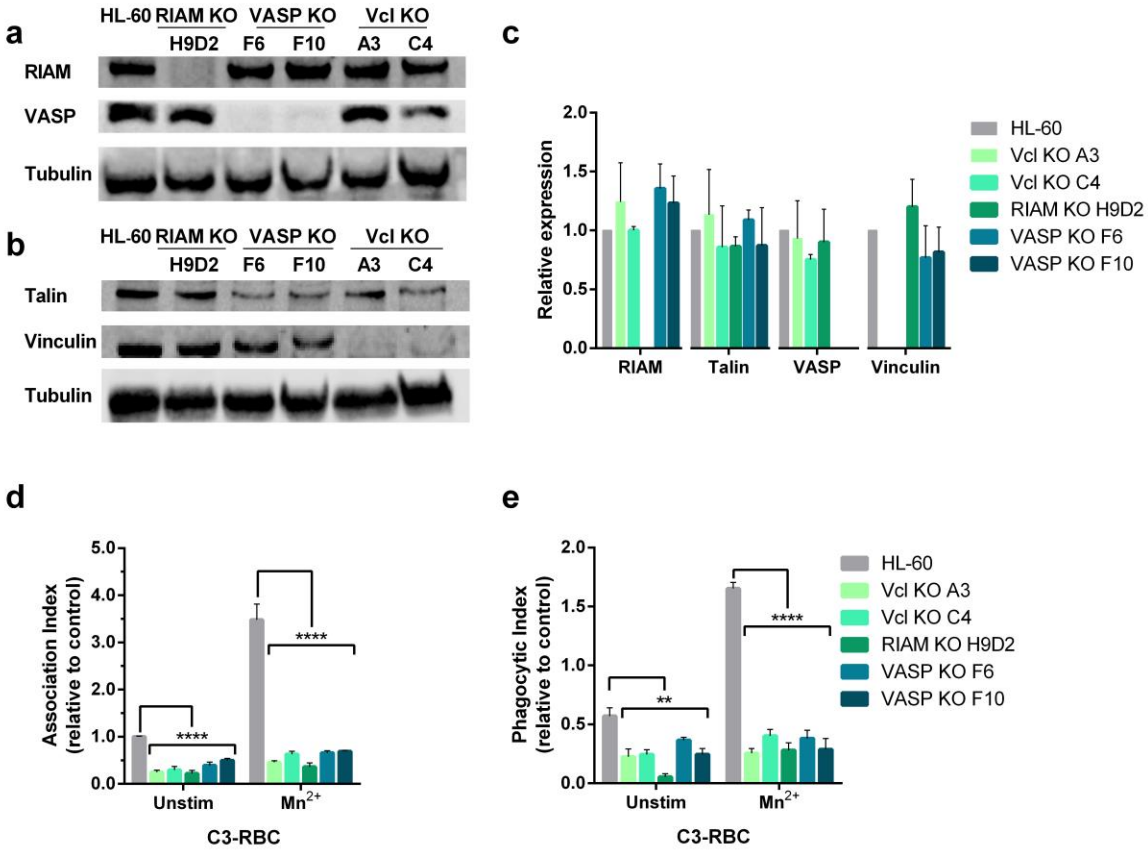


Figure 4.1. Knockout of either RIAM, VASP or Vinculin, abolishes phagocytosis.

a-b) Newly generated HL-60 RIAM, VASP and Vinculin knockout monoclonal cell lines were tested for integrin related protein expression by western blot. **c)** Quantification of protein expression in neutrophil-like HL-60 cells and derived knockouts. Results are represented as relative to HL-60 levels and are from 5 independent experiments. **d-e)** Phagocytic cells were challenged with C3-RBC after being stimulated with 1 mM $MnCl_2$ or left unstimulated, and Association (AI) and Phagocytic (PI), indexes were obtained. Data are normalized with respect to the AI of unstimulated C3-RBC-challenged HL-60 cells. Data are presented as mean±SD, where the error bars denote standard deviation. Significance (ANOVA) has been calculated with respect to HL-60 controls, ** denotes $p<0.01$; and ****, $p<0.0001$.

Since our prior reports indicated that VASP knockout abolishes particle internalization [311], RIAM and Vinculin (Vcl) knockout clones were subject to a phagocytosis assay (**Figure 4.1 d-e**). Here we observe that in a manner similar to results obtained for VASP deficient cells, RIAM and Vinculin knockout clones present a drastically diminished Association Index (AI) that was detected even at basal state, demonstrating a reduction around 70-75% for Vcl KOs, and a much more pronounced reduction (80%) for RIAM KOs (**Figure 4.1 d**). This diminished binding capacity in the absence of stimulation is suggestive of defects in the adhesive properties of these cells. When integrins were activated *via* outside-in using $MnCl_2$ (Mn^{2+}), all knockouts showed defective particle association, as stimulation was barely able to increase AI values to that of unstimulated control HL-60 cells. This defect in activation seemed to be more pronounced in RIAM KOs, a result which is in line with previous reports using RIAM-specific shRNA in HL-60 cell lines [44, 128, 311].

With regards to particle internalization (**Figure 4.1 e**), all knockouts showed severely affected PI values, where again RIAM KOs stood out as the most affected, as phagocytosis was barely detectable in these conditions. Contrasting with parental HL-60 cells, most knockouts were incapable of responding to Mn^{2+}, as no significant change was observed when comparing unstimulated to stimulated cells (1.7-fold increase versus the 3.5-fold increase observed in HL-60 parental cells). Here although RIAM KOs seemed to respond to the stimulus, it is to be noted that this could be due to a limitation in determining PI in unstimulated conditions.

Overall, the effects observed on phagocytosis seem to indicate a profound alteration in integrin activation and in the cytoskeletal rearrangements necessary for particle internalization. Beyond these effects, we speculate that integrin expression could be also affected in knockout cells. RIAM and VASP are reported to have an impact on F-actin content [128, 152] and potentially modulate transcriptional activity.

In addition, the absence of IAC components could have an effect in integrin stability and integrin recycling.

4.2.2. Expression of α_M and α_X integrin subunits is reduced in RIAM, VASP and Vinculin knockouts

To further characterize the phenotype observed in all knockouts we analyzed the surface expression of integrin subunits α_M, α_X and α_L, which pair with the β_2 subunit to form complement receptors CR3 and CR4 and integrin LFA1, respectively, as well as β_1 and β_2, and other membrane integral proteins CD59, CD9, and CD81 as controls (**Figure 4.2 a, Supplemental Figure 1 a-b**). We observed that expression (measured as the geometric mean fluorescence intensity or GMFI) of the α_M subunit was significantly reduced (74-50%), with similar values between knockout clones. A similar result was obtained for α_X, which was barely detectable in all KOs. This reduction in α_M and α_X expression was accompanied by a proportional reduction in β_2 subunit expression (40-50%) in all KOs (**Figure 4.2 a**).

No statistically significant diferences were observed for β_1 integrin or tetraspanins CD9, and CD81 (all integrin related molecules) or CD59, a molecule unrelated to integrins (**Supplemental Figure 1 b**). We confirmed that this reduction was also maintained when total cellular integrin expression was analyzed in permeabilized cells (**Supplemental Figure 1 c**), thus ruling out a defect in protein transport to the plasma membrane.

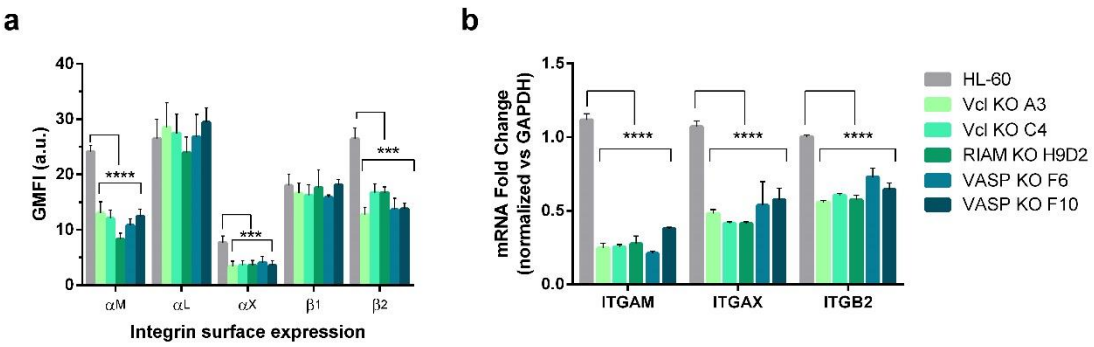


Figure 4.2. α_M and α_X subunit expression is controlled by integrin adhesion complex proteins.

a) Vinculin (Vcl), RIAM and VASP knockout cell lines and HL-60 parental cells were differentiated into neutrophilic-like cells with 1 mM all-*trans* retinoic acid (RA) and stained with monoclonal antibodies specific for α_M, α_L, α_X, β_1, β_2 integrin subunits. The geometric mean fluorescence intensity (GMFI) was obtained by flow cytometry and data represented as relative to HL-60 levels and are from 24 independent experiments done in duplicate. **b)** Expression of *ITGAM* (α_M), *ITGAX* (α_X), and *ITGB2* (β_2) mRNA levels was determined by RT-qPCR in neutrophil-like RIAM, VASP and Vcl HL-60 knockouts. Results are relative to *GAPDH* mRNA and are from 3 independent experiments done in triplicate. Data are presented as mean±SD, where the error bars denote standard deviation. Significance (ANOVA) has been calculated with respect to HL-60 controls, *** denotes $p<0.005$, and ****, $p<0.0001$.

Since the observed reductions were similar to those obtained for α_M, this suggests that surface expression of both complement receptors CR3/$\alpha_M\beta_2$ and CR4/$\alpha_X\beta_2$ require expression of RIAM, VASP and Vinculin. Furthermore, this mechanism appears to be highly specific for these integrin subunits, as it only affects the alpha subunits α_M and α_X, whilst not affecting the closely related α_L.

Next, we determined whether the decreased expression of these subunits was also detectable at mRNA level through RT-qPCR (**Figure 4.2 b**). After neutrophilic differentiation, all HL-60 knockouts cell lines showed a statistically significant 60-80% reduction in *ITGAM* (α_M gene), and 40-50% for *ITGAX* (α_X gene) mRNA expression with respect to HL-60 cells, confirming a downregulated transcription. We also observed a decrease in *ITGB2* (β_2 gene) mRNA levels that was

comparable to the reduction observed for β_2 surface expression. This downregulation was not observed for *ITGAL* (α_L gene) or *APBB1IP* (RIAM) in the VASP knockout (**Supplemental Figure 2 a**). This indicates that the expression of β_2 in knockouts was sufficient to yield normal level of $\alpha_L\beta_2$.

4.2.3. Defective upregulation of α_M and α_X during differentiation correlates with reduced F-actin

Since expression of α_M is upregulated during all-*trans* retinoic acid induced differentiation in HL-60 cells, we studied whether the low levels of α_M we observed in differentiated cells were caused by a failure to induce its expression during differentiation. In HL-60 cells, treatment with retinoic acid induced a two-fold increase in α_M and a five-fold increase in α_X expression (

Figure 4.3 a-b), as well as an increase in RIAM and VASP expression (**Supplemental Figure 2 b**).

In undifferentiated knockout clones, α_M expression was in general reduced compared to HL-60 control cells. However, whilst retinoic acid induced a two-fold increase in α_M expression for HL-60 cells, knockout clones remained insensitive to this treatment, and showed α_M expression levels comparable to undifferentiated wild-type cells, and a modest 2.5-fold increase in α_M expression (

Figure 4.3 a-b). This suggests that the differentiation program induced by retinoic acid treatment involving increased α_M and α_X transcription depends on the correct expression of the IAC components, and is more acute in the case of α_M.

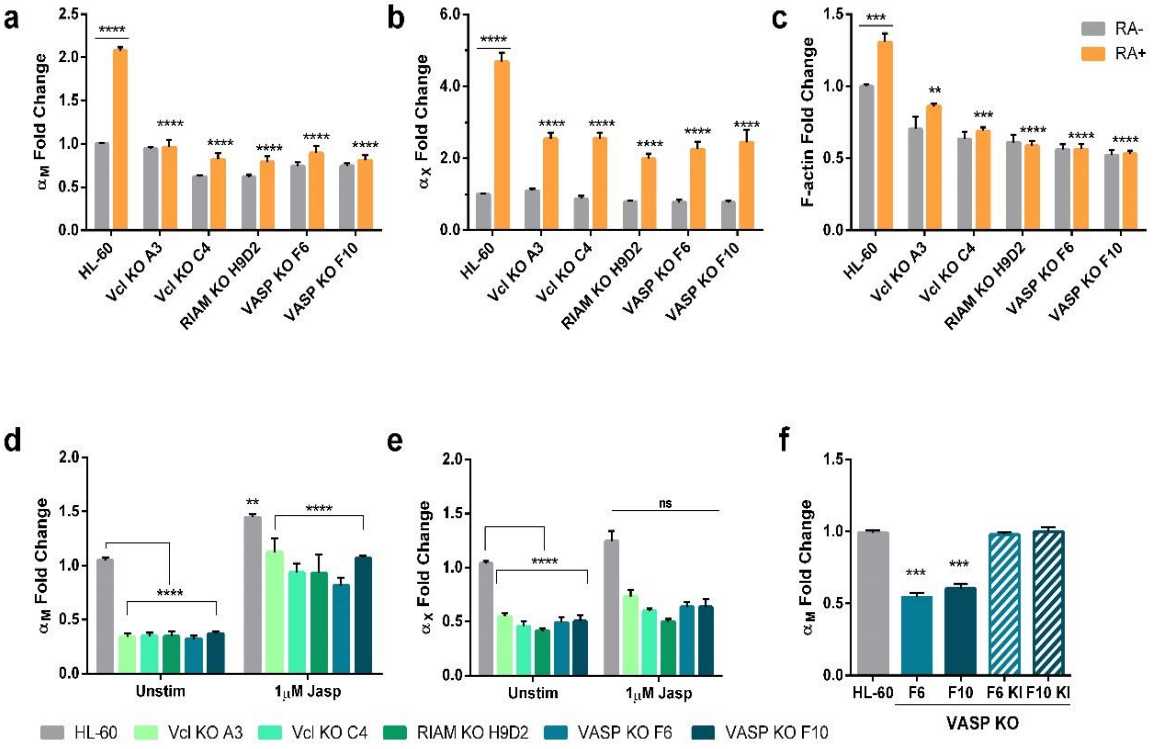


Figure 4.3. RIAM, VASP and Vinculin knockouts show an altered phenotype when undergoing neutrophilic differentiation.

a-b) Vinculin (Vcl), RIAM and VASP knockout cell lines and HL-60 parental cells were differentiated into neutrophilic-like cells with 1 mM all-*trans* retinoic acid treatment (RA+) or maintained undifferentiated (RA-), and expression of α_M and α_X integrins was analyzed by flow cytometry. **c)** Cellular F-actin content was analyzed using fluorescently labeled phalloidin in HL-60 knockout cell lines and parental cells, in undifferentiated or differentiated cells. **d-e)** Vinculin, RIAM and VASP knockout cell lines and HL-60 parental cells were treated with a 2 h 1 μM jasplakinolide stimulation, followed by a 24 h resting period during neutrophilic differentiation. Then, expression of α_M and α_X integrins was analyzed by flow cytometry. **f)** Expression of α_M integrin was analyzed in VASP F6 and F10 knockout clones and in VASP knock-in polyclonal cell lines F6 KI and F10 KI. Results are represented as GMFI relative to HL-60 wild type levels and are from at least 3 independent experiments done in triplicate. Data are presented as mean±SD, where the error bars denote standard deviation. Significance (ANOVA) has been calculated with respect to HL-60 controls, ** denotes $p<0.01$; ***, $p<0.005$, ****, $p<0.0001$; and n.s., for non-statistically significant.

Prior studies revealed that RIAM silencing resulted in a reduction in F-actin content [152]. It is also well known that the G:F-actin ratio determines the activation of transcriptional regulation programs. Hence, we decided to analyze in parallel with

the previous experiment total cellular F-actin content for all knockout HL-60 cell lines
(

Figure 4.3 c). For all conditions, F-actin content was diminished, with RIAM and VASP knockout clones showing a strong reduction (40-50%). This is in agreement with the previously observed defects in phagocytic capacity and prior reports which link these proteins in the control of the cytoskeletal rearrangements necessary for particle engulfment [128, 311]. A more moderate reduction (30-37%) in F-actin content for Vinculin knockouts was also observed. This suggests that Vinculin expression might be indirectly controlling F-actin levels, as opposed to RIAM and VASP [152, 311, 325, 330, 342, 347]. Whilst differentiation induced a modest yet significant increase (31%) in total F-actin content for wild type HL-60 cells, RIAM, VASP and Vinculin knockouts were mainly unresponsive to all-*trans* retinoic acid. Overall, this reduction in total F-actin content, correlated with the observed defect in α_M expression.

Since F-actin content is capable of controlling gene expression, and it seemed to be the common thread which tied all three knockouts, we tried to reverse the phenotype through the use of the actin stabilizer jasplakinolide (Jasp), which induces actin polymerization. Indeed, this treatment induced a significant 3-fold increase in α_M levels for all knockouts causing them to reach levels comparable to those of

unstimulated parental cells, whilst having a modest effect (1.4-fold increase) on controls (

Figure 4.3 d). However, the effect of jasplakinolide in reverting α_M expression was partial, as knockouts could not reach the expression levels induced by this drug in parental cells. Nonetheless, this served as a proof-of-concept that the reduced F-actin levels were capable of controlling integrin expression. For α_X, jasplakinolide treatment only induced a marginal 1.2-fold increase in expression, indicating a less relevant involvement of cellular F-actin content in the control of α_X expression (

Figure 4.3 e).

Finally, to ensure that the observed effect was due to the genetic deletion of the studied proteins, VASP was knocked-in in the two knockout clones, yielding the

polyclonal lines VASP F6 KI and F10 KI (**Supplemental Figure 2**). When αM levels were assessed (

Figure 4.3 f), these cells were indistinguishable from parental HL-60 cells. This served as a demonstration that genetic reconstitution of VASP was able to revert the observed phenotype and that therefore the observed effect was not due to the experimental system used.

4.2.4. Distribution of the SRF co-regulators MRTF-A and FHL-2 is controlled by RIAM, VASP and Vinculin expression

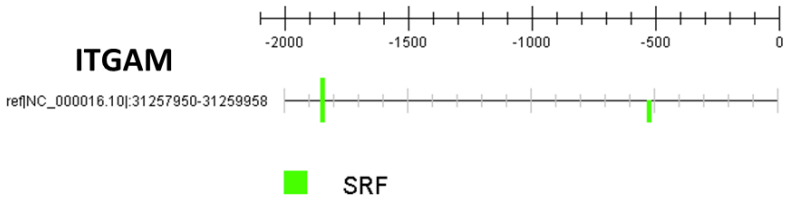
The Serum Response Factor (SRF) transcription factor along with its co-activator MRTF-A are described as the main targets of actin dynamics [292-294]. MRTF-A is sequestered in an inactive state in the cytoplasm by binding monomeric G-actin [295]. Actin filament elongation reduces total cellular G-actin and leads to the dissociation of MRTF-A-G-actin complexes, and therefore allows MRTF-A nuclear import and subsequent activation of SRF-mediated transcription.

In order to test the hypothesis that the SRF pathway could be involved in regulating αM integrin expression, we analyzed a 2kb region upstream from *ITGAM* and *ITGAX* using the freeware tool Ciiider. This program was capable of predicting

with a 0.3 cutoff value, three SRF binding sites in *ITGAM* and one in *ITGAX* (**Figure 4.4 a-b**, respectively).

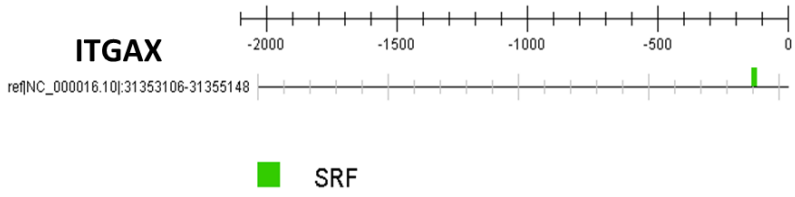
This indicates that both *ITGAM* and *ITGAX* expression could be induced through SRF activation and is in line with our previous results demonstrating that poor integrin α_M and α_X expression in knockouts occurs due to diminished F-actin content, and that this can be alleviated through the use of jasplakinolide.

a



Start Position	End Position	Strand	Core Match Score	Matrix Match Score	Sequence
-1861	-1846	+	0,776	0,709	GTTCCATGTATGTGTG
-535	-520	-	0,809	0,723	GGGCAACATAGGGAGA
-1861	-1846	-	0,785	0,712	CACACATACATGGAAC

b



Start Position	End Position	Strand	Core Match Score	Matrix Match Score	Sequence
-144	-129	+	0,776	0,705	CATCCATCTAAGCAAA

Figure 4.4. SRF promoter analysis of the alpha subunits of CR3 and CR4.
a) The figure shows the location of the putative SRF binding sites, as well as their sequence, location, strand direction and prediction parameters on the 2kb region upstream from the *ITGAM* initiation codon. **b**) A similar analysis performed on the 2kb region upstream from *ITGAX*.

This result gives credence to the hypothesis that through controlling cytoskeletal dynamics, RIAM, VASP and Vinculin induce integrin α_M and α_X expression through the SRF-MRTF-A pathway.

To test the hypothesis that the SRF pathway could be involved in regulating α_M integrin expression, we studied MRTF-A subcellular localization in wild type and knockout lines using confocal fluorescence microcopy (

Figure 4.5a).

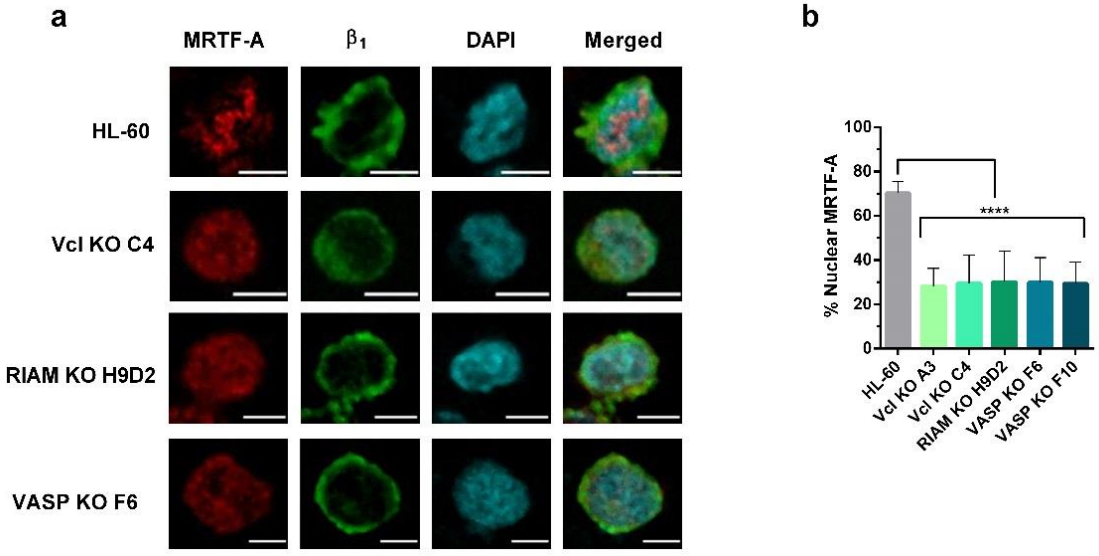


Figure 4.5. MRTF-A distribution is altered in RIAM, VASP and Vinculin knockouts.

a) HL-60 parental cells and the knockouts for Vinculin (Vcl), RIAM and VASP were differentiated into neutrophil-like cells, fixed, permeabilized and fluorescently labelled with an anti-MRTF-A mAb, DAPI for nuclear staining and anti-β_1 integrin to delimit the plasma membrane. Images show representative results from 3 independent experiments analyzed by confocal fluorescence microscopy. Bars indicate 5 μm. **b)** Quantification of MRFT-A nuclear distribution in these images is represented. Results are represented as relative to the wild-type nuclear ratio and are from 3 independent experiments with at least 50 cells. Data are presented as mean ± SD, where the error bars denote standard deviation. Significance (ANOVA) has been calculated with respect to HL-60 controls, **** denotes $p<0.0001$.

For wild type cells, MRTF-A showed a punctate pattern stain, which co-localized with DAPI. This pattern implies a nuclear translocation and is suggestive

of an active transcription of SRF controlled genes. Meanwhile, knockouts presented a diffuse staining pattern, which more closely resembles a cytoplasmic stain.

The extent of MRTF-A nuclear translocation was determined by fluorescent signal quantification (**Figure 4.5 b**). For each cell we defined the total fluorescence (or integrated density in ImageJ) in the MRTF-A channel for regions delimited by the cortical β_1 integrin stain, as total cellular MRTF-A, and the fluorescence which co-localized with the nuclear DAPI stain, as the nuclear MRTF-A. While HL-60 cells showed a primarily nuclear localization of MRTF-A (~74%) the three HL-60 knockouts showed little nuclear translocation (<30%), indicating that knockout of these proteins results in a statistically significant and drastic reduction in MRTF-A translocation. These results are in agreement with our previous observations that knockouts present reduced levels of F-actin and suggest that defective SRF activity plays a role in the loss of α_M expression observed for the knockouts.

Similarly, we studied the subcellular localization of the SRF corepressor FHL-2 (**Figure 4.6a**). FHL-2 competes with MRTF-A for binding [296]. In parental cells, FHL-2 showed a sub-membranous localization as judged by its close proximity to β_1 integrin. However, for knockouts FHL-2 staining followed a cytoplasmic and nuclear distribution. The FHL-2 fluorescence was quantified (**Figure 4.6 b-c**). Signals co-localizing with DAPI were assigned as nuclear FHL-2 and sub-membranous when localizing with integrin β_1. Total cellular FHL-2 was also determined. Our data confirmed that for RIAM, VASP and Vinculin knockouts FHL-2 localization was mainly nuclear (~60%) with a minor proportion at the sub-membrane (~20%), while in HL-60 parental cells, FHL-2 was more abundant at the membrane (~50%) and only a 35% was nuclear. This result suggests that the expression of RIAM, VASP, and

Vinculin may be required to retain FHL-2 close to the cytoplasmic membrane, preventing its corepressor activity and allowing proper integrin expression.

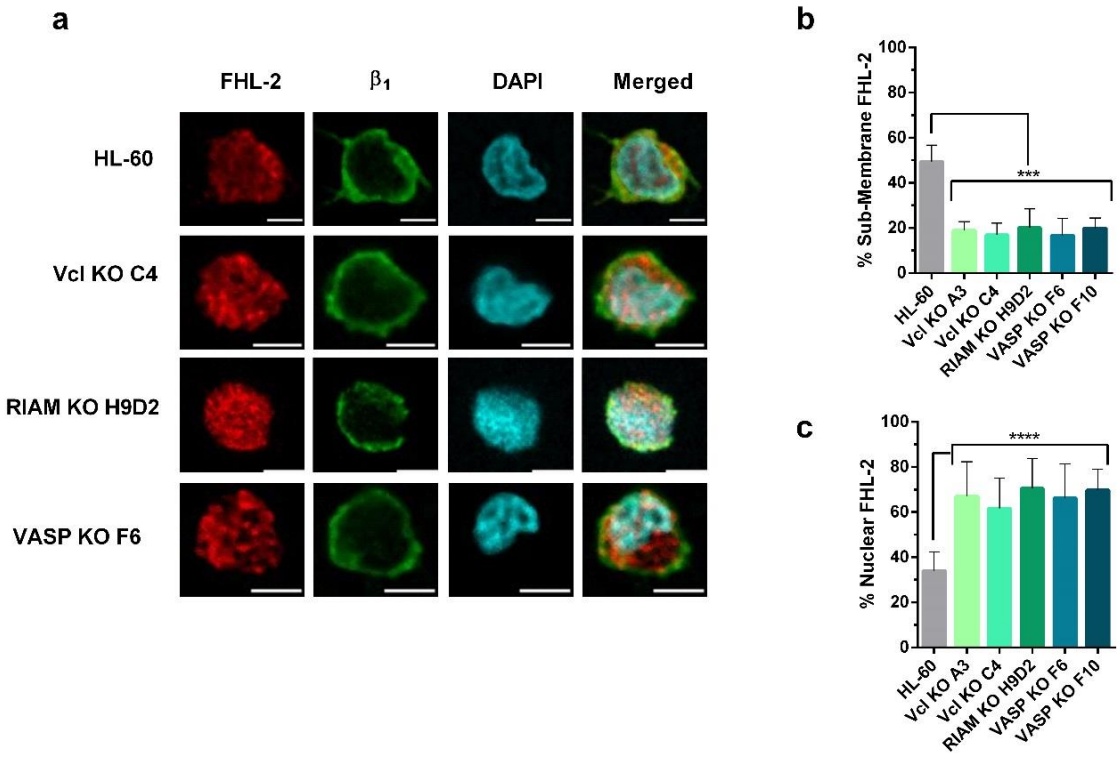


Figure 4.6. FHL-2 nuclear translocation is enhanced in RIAM, VASP and Vinculin knockouts.

a) HL-60 parental cells and the knockouts for Vcl, RIAM, VASP HL-60 cells were differentiated into neutrophilic-like cells with 1 mM all-trans retinoic acid for 48 h, fixed, permeabilized and fluorescently labelled using anti-FHL-2 and anti-b1 integrin antibodies and DAPI. Confocal microscopy images were analyzed using the ImageJ software package and are representative results from 3 independent experiments. Since there were no differences between clones, only one clone from each knockout is presented. Bars indicate 5μm. **b-c)** Subcellular distribution of FHL-2. The graphs represent the quantification of images. Results are represented as relative to the wild-type nuclear ratio and are from 3 independent experiments with at least 50 cells. Data are presented as mean ± SD, where the error bars denote standard deviation. Significance (ANOVA) has been calculated with respect to HL-60 controls, *** denotes $p<0.005$, and ****, $p<0.0001$.

4.2.5. Jasplakinolide treatment renormalizes SRF co-regulators subcellular localization

Since jasplakinolide treatment was shown to be capable of increasing α_M expression, we assessed whether this treatment could also revert the subcellular localization of MRTF-A to more closely resemble wild type cells (**Figure 4.7**).

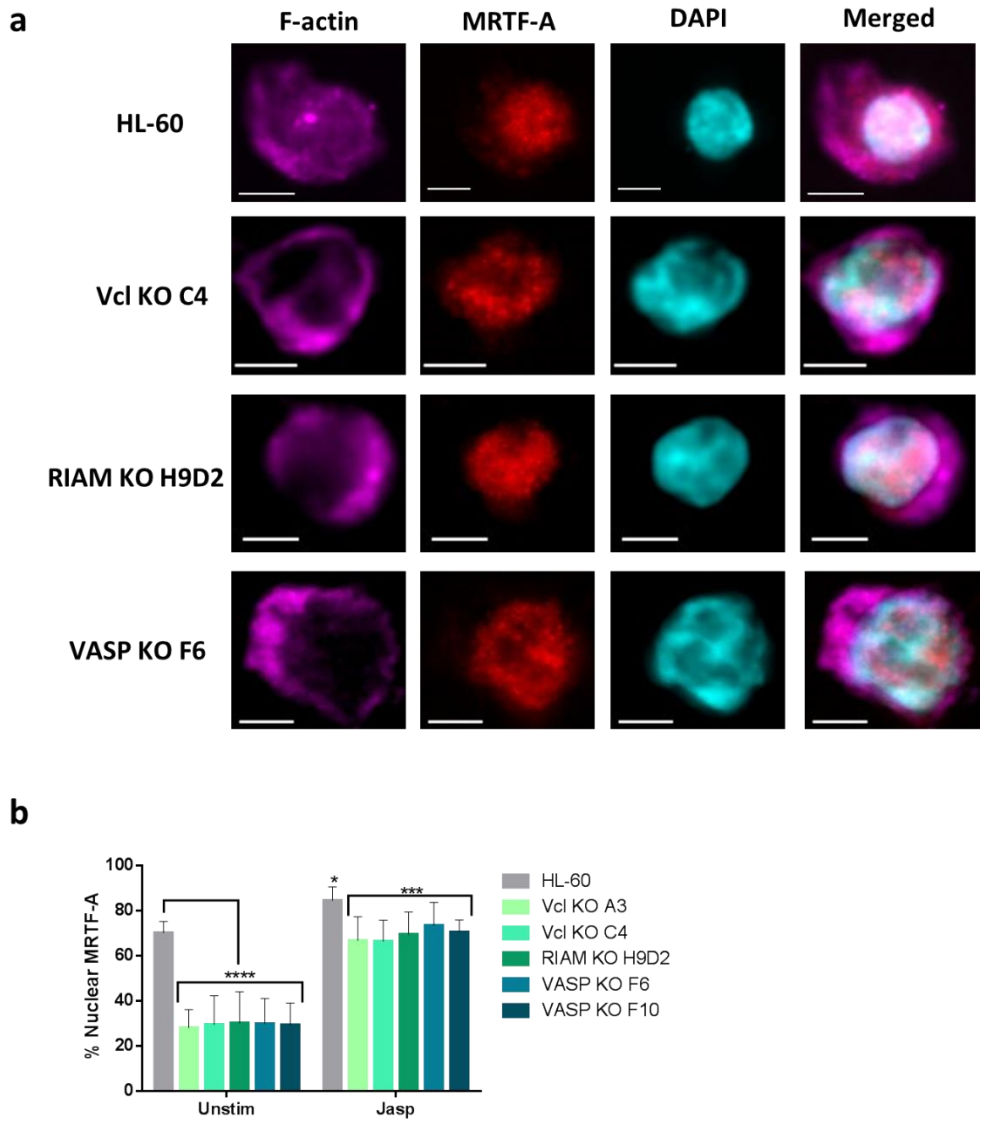


Figure 4.7. Jasplakinolide induces MRTF-A nuclear translocation in RIAM, VASP and Vinculin knockouts.

a) Differentiated HL-60 parental cells and HL-60 knockouts for Vcl, RIAM and VASP were adhered to slides, then subjected to a 1 μM 2 h jasplakinolide stimulation, fixed, permeabilized and fluorescently stained to determine MRTF-A localization. β_1 integrin was used to delimit the plasma membrane and DAPI was used as a nuclear stain. Confocal microscopy images were analyzed using

the ImageJ package and are representative results from 3 independent experiments. Bars indicate 5 μm. **b)** Quantification images from the untreated cells shown on **Figure 4.5a** and and jasplakinolide-treated cells on **Figure 4.7a**. Results are represented as relative to the wild type nuclear ratio and are from 3 independent experiments with at least 50 cells. Data are presented as mean±SD, where the error bars denote standard deviation. Significance (ANOVA) has been calculated with respect to HL-60 controls, *** denotes $p<0.005$, and ****, $p<0.0001$.

As expected, we observed that jasplakinolide treatment increases MRTF-A signal at the nucleus for HL-60 controls, since the MRTF-A closely co-localized with the nuclear DAPI stain (**Figure 4.7 a**). MRTF-A also displayed a clear nuclear distribution in all knockout cells indicating that jasplakinolide treatment positively affected MRTF-A nuclear translocation, bypassing the lack of expression of the knocked-out proteins. Furthermore, for all jasplakinolide-treated cells, barely any cytoplasmic MRTF-A was observable.

Next we analyzed the extent of MRFT-A translocation by fluorescent signal quantification in jasplakinolide treated cells (**Figure 4.7 b**). We defined for each cell the total fluorescence (or integrated density in ImageJ) in the MRTF-A channel for regions delimited by the cortical actin stain of the Phalloidin (F-actin) channel, as total cellular MRTF-A, and the fluorescence which co-localized with the nuclear DAPI stain, as the nuclear MRTF-A signal. Using this approach, we calculate a percentage nuclear localization for MRTF-A for both unstimulated and jasplakinolide treated cells.

When analyzing jasplakinolide-treated cells, we observed a statistically significant increase in nuclear localization for MRTF-A for all cells, compared to untreated cells. Whilst the effect of the jasplakinolide treatment for HL-60 cells was minor (1.2 fold), as most MRTF-A was nuclear prior to the jasplakinolide treatment, the increase observed for knockouts oscillated between 2.3 and 2.5 fold, highlighting the severe defect in this signaling pathway. This fold change correlated with the increase in α_M expression for differentiated HL-60 cells observed in

Figure 4.5 a, and taken together with those shown in in

Figure 4.3 c, suggest that this pathway is critically involved in the expression of α_M.

FHL-2 subcellular localization was also assessed in jasplakinolide treated cells (**Figure 4.8 a**). In parental cells, FHL-2 showed a sub-membranous localization as judged by co-localization with the β_1 integrins. FHL-2 was also present in the nucleus for jasplakinolide treated HL-60 cells, although to a lesser extent, coinciding with reports which show that MRTF-A signaling induces FHL-2 expression acting as a negative feedback loop [296]. Although some nuclear distribution was also retained, all knockouts displayed an observable FHL-2 enrichment at the cytoplasm and at the sub-membranous zone induced by jasplakinolide treatment (**Figure 4.8 a**).

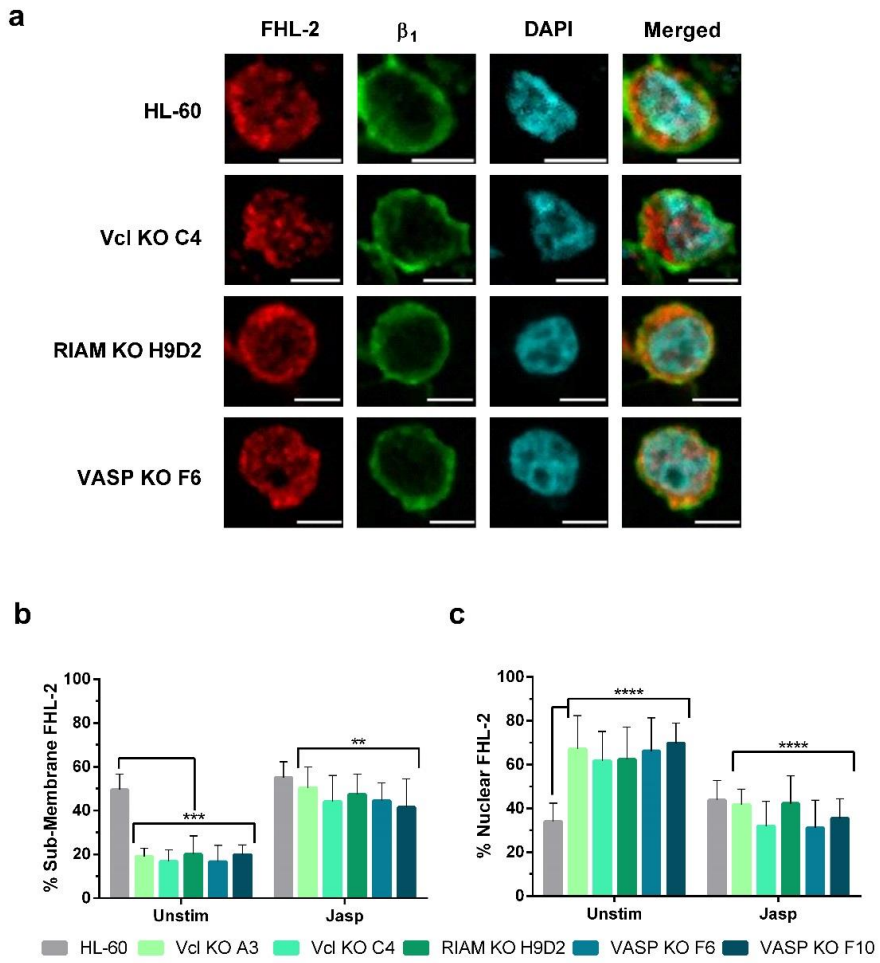


Figure 4.8. Jasplakinolide reduces nuclear localization of FHL-2 in RIAM, VASP and Vinculin knockouts.

a) Differentiated HL-60 parental cells and HL-60 knockouts for Vcl, RIAM and VASP were adhered to slides, then subjected to a 1 μM 2 h jasplakinolide stimulation, fixed, permeabilized and fluorescently stained to determine FHL-2 localization. β_1 integrin was used to delimit the plasma membrane and DAPI was used as a nuclear stain. Confocal microscopy images were analyzed using the ImageJ package and are representative results from 3 independent experiments. Bars indicate 5 μm. **b-c**) Subcellular distribution of FHL-2. The graphs represent the quantification of images from untreated cells shown on **Figure 4.6a** and and jasplakinolide-treated cells shown on **Figure 4.8a**. Results are represented as relative to the parental HL-60 nuclear ratio and are from 3 independent experiments with at least 50 cells. Data are presented as mean±SD, where the error bars denote standard deviation. Significance (ANOVA) has been calculated with respect to HL-60 controls, ** denotes $p<0.01$; *** $p<0.005$, and **** $p<0.0001$.

We analyzed the extent of FHL-2 translocation by fluorescent signal quantification in jasplakinolide treated cells (**Figure 4.8 b-c**). Jasplakinolide treatment induced an increase in FHL-2 membrane distribution (55%) on HL-60 parental cells compared to untreated cells, leading to the equal distribution of this protein between the membrane and nuclear compartments. For all knockouts jasplakinolide also increased FHL-2 localization at the membrane (from 20% to ~50%) and decreased nuclear FHL-2, compared to untreated cells. This renormalization of FHL-2 subcellular distribution correlates with our previous results showing a similar correction of MRTF-A nuclear translocation (**Figure 4.8**) and integrin α_M expression (

Figure 4.3 c) after jasplakinolide treatment. This indicates that this pathway requires the coordinated participation of both SRF co-regulators in order for promyelocytic cells to differentiate normally, express complement receptors and therefore perform their biological functions.

4.3. Discussion

Myeloid cell function is critically dependent on the correct expression of cell adhesion molecules as well as downstream effectors controlling their activation. Disruption of the expression of integrins or proteins involved in their activation have been shown to cause immunodeficiencies such as Leukocyte Adhesion Deficiencies (LAD), in the case of β_2 integrin and Kindlin-3 expression [219, 220, 352]. The results

presented herein outline that the upregulation of α_M and α_X observed during myeloid cell differentiation depends on the correct expression of RIAM, VASP and Vinculin, which are proteins involved in actin cytoskeletal dynamics and integrin signaling.

We demonstrate that deletion of either RIAM, VASP or Vinculin has a profound effect on the expression of CR3 and CR4 receptors without affecting the expression of other integrin adhesion complex components. Previously observed data indicated that RIAM knockout does not affect Talin-1, Kindlin-3, Rap1 or Cal-DAG expression in mice platelets, macrophages, or PMNs [166]. Similarly, altered expression of another MRL family member, lamellipodin (Lpd) in mouse fibroblasts does not impact consensus integrin adhesion complex molecules [355, 356], as shown in a knockout which had normal expression of RIAM, Mena, EVL and VASP [357] or in an Lpd siRNA knockdown model, where expression of integrin adhesion downstream signaling proteins FAK and CAS was unaltered [358]. To further support this, Vinculin deletion in mouse embryonic fibroblasts had no detectable effect on the expression of the core focal adhesion components Talin, Paxillin, FAK, Zyxin, and VASP [359].

Talin deficient lines should also show unperturbed expression of integrin adhesome complex proteins. Indeed, Talin-2 deficient mouse cardiomyocytes showed normal levels of Vinculin, Paxillin, Kindlin-2 and FAK [360]. Further exploring genetic alterations relevant for integrin activation and function, we find that knockout of Kindlin-3, a mediator of integrin clustering [136, 176] and critical for initiating outside-in signaling [209, 361, 362], had no impact on Talin expression in mouse platelets [363].

All the previously mentioned alterations in IAC component expression display a similar defect in integrin-mediated adhesion and/or migration [357-360,

363], which mirrors our results showing that knockout of RIAM, VASP and Vinculin abolishes phagocytosis. In addition, when integrin expression was analyzed in IAC components-deficient cells, statistically significant and specific changes in expression of integrin subunits could be detected, confirming our results where a specific reduction in α_M and α_X integrin subunits could be detected at both the protein and mRNA levels.

Talin-1 deletion was found to affect β_3 expression, but not β_1 in osteoclasts [364], causing a significant osteopetrosis, whilst Talin-1 and Talin-2 double knockout in fibroblasts exhibited increased β_3, α_5 and a slight increase in α_V, but retained normal levels of β_1 [173]. In B-cells, Talin-1 deletion caused altered B-cell differentiation, and homing to peripheral lymph nodes and defective adoptive transfer, but had normal levels of α_4 and α_L integrins. Kindlin-3 deficient platelets were observed to have reduced $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ expression [363]. Similarly, RIAM deletion in B-cells had no impact on α_4 and α_L integrins, but showed defective peripheral lymph node homing and adhesion [338], whereas T-cell specific deletion of RIAM resulted in defective α_E expression and increased α_4 [365], but showed no changes in surface expression of integrins β_1, β_2, β_7 [366], which is consistent with our results, which show subunit specificity. These results highlight that for cells to perform integrin dependent functions, a correct expression of integrin subunits is required, and that this is dependent on integrin adhesion complex proteins in a cell-type specific manner.

Deletion of EVL-VASP family proteins had similar effects on integrin subunit expression, where Ena and VASP double knockout showed a specific decrease in T-cell α_4 and its partners β_1 and β_7, and a more moderate and possibly compensatory increase in α_L [367]. hMENA silencing in lung cancer as well as breast cancer cell lines

caused a significant reduction in surface α_3, α_6 and β_1, the latter of which was also observed to have deficient *ITGB1* (β_1 gene) mRNA levels [368]. This more closely follows our results which show that RIAM, VASP and Vinculin knockouts all caused a specific reduction of *ITGAM* (α_M gene) and *ITGAX* (α_M gene) mRNA levels. Moreover, the authors determined that hMENA expression was required to maintain normal cytoskeletal organization and G:F-actin ratios [368]. This result closely resembles our own, where VASP knockouts had deficient F-actin content.

Alterations in F-actin content and aberrant cytoskeletal morphologies have been described for RIAM [128, 152, 157] and Vinculin deficient cells [369, 370]. Previous results from our group, show that RIAM deficient cells show decreased F-actin content at the phagocytic cup and that this effect correlates with deficient VASP pSer^{157} phosphorylation and phagocytic cup recruitment, thereby explaining the deficiency in phagocytosis [311]. Much like the results presented here, Ena-VASP-hMena triple knockout (mouse fibroblasts and melanoma cell lines) had aberrant lamellipodial morphology, as well as abolished motility and integrin-dependent adhesion, which was concordant with a loss of lamellipodial F-actin network organization and decreased F-actin content [371]. Our results provide a link between the lack of expression of RIAM, VASP and Vinculin and a reduction in F-actin content, leading to a loss in α_M expression which persists under all-trans retinoic acid treatment, which is known to induce their expression during neutrophilic differentiation in HL-60 cells. This is supported by the fact that the defect in α_M was partially reversed by the actin stabilizing drug jasplakinolide inducing a 2.5-fold increase in expression, indicating that α_M expression is heavily regulated by an F-actin dependent mechanism but does not exclude other additional F-actin-independent mechanisms downstream of RIAM, VASP and Vinculin.

In contrast with the results obtained for α_M, jasplakinolide treatment had a minor effect on α_X, inducing a non-significant 1.3-fold increase in expression for all cells, suggesting that α_X expression is less dependent of F-actin, and that other pathways may be more critical. Interestingly, during neutrophilic differentiation granulocyte colony-stimulating factor (G-CSF) signaling to the MAPK/ERK pathway is required to activate AP-1 [372], which in turn regulates α_X/CD11c expression [373]. We have previously reported that RIAM has impact on ERK1/2 phosphorylation dynamics [350, 374], which may result in activation of transcription factors like the aforementioned AP-1. Expression and activity of IAC proteins could have an impact on the nuclear translocation of transcription factors downstream of the MAPK/ERK pathway independently of the SRF/MRTF-A pathway, thereby explaining our results.

We also demonstrate that knockout of RIAM, VASP or Vinculin results in a change in subcellular localization of the SRF coactivator MRTF-A, which is consistent with an increase in inactive cytoplasmic G-actin-bound MRTF-A. hMENA knockouts presented a reduction in SRF activity, and were comparable to the effect observed using the SRF inhibitor CCG1423 [368]. Similarly, VASP has been shown to control SRF activity and co-immunoprecipitate with the formin mDIA1 in mouse fibroblasts [292]. Mouse hematopoietic stem cells deficient in mDIA2 presented defective engraftment and migration, reduced F-actin content. In addition, these cells had inhibited transcription of SRF target genes, which included *FHL-2*, *SRF*, *ITGA2*, *ITGAL*, *ITGAM* and *ITGB2* [291]. The authors then demonstrate that the defects observed for mDIA2 knockouts were rescued by overexpression of SRF, and subsequently identify SRF consensus binding sites on *ITGAM* and *ITGB2* [291]. However, these sites are located downstream of their promoters, and there are no

experimental results which confirm their significance in human lines, but this suggests that SRF might have a conserved role in regulating integrin expression.

Here, we identify consensus sequences for SRF-binding in *ITGAM* and *ITGAX* upstream of the promoter and altered translocation of MRFT-A in RIAM, VASP or Vinculin KO cells. In addition, there are numerous reports of integrin-mediated functions being inhibited in SRF or MRTF-A deficient cells [294, 295] and that MRTF-A loss-of-function mutations lead to severe immunodeficiency [375].

Disruption of Kindlin function was also shown to negatively impact actin dynamics and MRTF-A nuclear shuttling. This effect was linked to MRTF-A function and expression, since MRTF-A inhibition recapitulated the effect observed for Kindlin-3 knockout. Interestingly, approximately one third of the differentially expressed genes in MRTF-A knockouts were shared by β_2 deletion [293]. These genes mainly comprised components of the integrin adhesome, such as the genes for the Src Family Kinases (SFK), Fgr and Hck. A non-significant decrease in α_X expression is also described for MRTF-A inhibition. Genetic deletion of MRTF-A also caused significant defects in migration and traction force generation similar to those observed for β_2 knock-in mutants that lost the Kindlin-3 binding site [293]. The authors highlight the existence of a β_2-Kindlin3-SRF-MRTF-A pathway that regulates dendritic cell function and our results expand upon this discovery suggesting that disruption of integrin proximal components such as RIAM, VASP or Vinculin can cause severe defects in integrin expression through a dysregulation of the MRTF-A pathway. We also demonstrate that modulation of the G:F-actin ratio using jasplakinolide partially renormalizes MRTF-A localization in all knockout lines, and that this effect is in parallel with an increase in α_M expression.

Our results suggest that integrin expression is regulated through the control of SRF transcriptional activity. We demonstrate that the Four-and-a-Half LIM domain protein FHL-2, which has been described as competing with the coactivator MRTF-A for SRF binding [296], shifts localization in the absence of RIAM, VASP or Vinculin expression. Whereas FHL-2 is enriched at the sub-membranous zone in control cells, knockouts show an increased nuclear FHL-2 signal, which can be partially reverted through the use of jasplakinolide. Hence, FHL-2 localization is consistent with a described auto-regulatory feedback loop that orchestrates the expression of lineage-specific SRF target genes [296]. SRF was also shown to induce the expression of FHL-2 dependent on differentiation and RhoA signaling, which would be consistent with our findings.

In addition, RIAM silencing has been shown to cause defective Vav2-RhoA-ROCK-MLC pathway activation and expression of constitutively active Vav2 and RhoA partially rescued the invasive capacity of RIAM-deficient melanoma cells [376]. Similarly, other reports connect VASP expression with RhoA signaling and SRF activity [292, 377]. These results predict that since RhoA signaling is diminished in the absence of correct RIAM and VASP expression, SRF signaling and its negative feedback loop, which induces FHL-2 expression to stop MRTF-A/SRF-mediated gene expression, would be abolished [296]. This is in line with our results since we observe that the effects on integrin expression and SRF cofactor subcelullar localization can be reversed through activating MRTF-A nuclear translocation using jasplakinolide.

Since our results show that FHL-2 is detectable in the nucleus of both HL-60 cells and the knockouts, this raises the question of the role of FHL-2 during neutrophil differentiation. This is backed by the fact that FHL-2 has been observed to be overexpressed in acute myeloid leukemia, and overexpression of FHL-2 in a mouse model enhances proliferation of myeloid progenitors and granulopoiesis

[378]. The authors show that FHL-2 is expressed in all subsets of hematopoietic progenitors, and is downregulated during normal myelopoiesis. Therefore, FHL-2 might play a critical role in driving myelopoiesis. Since FHL-2 has no intrinsic transcriptional activity, this effect can be explained through its interaction with other transcription factors. In fact, FHL-2 has been described to act as a cofactor for CREB, RXR, and β-catenin, among others [379]. Wnt/β-catenin signaling significantly upregulate VASP protein expression, and VASP promotes the nuclear translocation of β-catenin, acting as a positive feedback loop driving cell proliferation and migration in breast cancer [380]. In addition, CREB1 is capable of directly binding to the promoter of VASP, and activate its expression, forming a signaling pathway which plays a role in breast cancer migration and proliferation [381].

Our results suggest that FHL-2 can be sequestered at the membrane and is dependent on RIAM, VASP and Vinculin expression, since their deletion favors nuclear translocation and abolishes co-localization with β_1. FHL-2 has been described to directly bind several integrin subunits, including β_2 through its N-terminal LIM domains [379, 382] and is capable of binding α-actin mainly through its C-terminal LIM domains (3 and 4) [383]. FHL-2, much like Vinculin and RIAM [160, 384], is described to form part of a mechanosensitive pathway, regulating protein expression with substrate rigidity and the strength of the adhesion [385]. Other Lim-domain containing proteins have been noted to directly bind VASP and Vinculin, such as Zyxin [386-390] and Paxillin [388, 390-393], both of which can localize to focal adhesion and form part of the consensus adhesome [355, 356]. Due to the fact that this interaction occurs through LIM domain containing regions, it is not unreasonable to assume that FHL-2 might also be able to directly bind VASP and Vinculin. The specifics of LIM domain-mediated interactions have not yet been defined precisely and seem to depend on the specific amino acid sequence of the Zn^{2+} finger domains,

linker sequence and length, as well as on the higher-order structures and the number of fingers [390, 394-396]. Furthermore, FHL-2 has been shown to directly bind tensed F-actin in a force-dependent manner, thereby preventing its nuclear translocation in fibroblasts[397]. This could also explain the observed results. FHL-2 has also been shown to directly bind the SH2 domain adaptor protein Grb7 in a tyrosine phosphorylation dependent manner *via* its RA and PH domains [398]. This interaction seemed to be critical for breast cancer invasion and survival [399]. Since RIAM shares a similar molecular architecture, a RA-PH module and Proline-rich regions [44, 128, 152, 156, 376], and has been compared to the Grb7 protein family [152, 153, 156], it is also possible that this protein directly interacts with FHL-2, which is supported by our findings. Alternatively, this interaction could also be indirect, through RIAM-mediated recruitment of VASP [311] or Vinculin [139, 160].

Finally, we show that the decrease in $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrin expression observed for knockouts is concomitant with a reduction in F-actin, and that these occur during all-*trans* retinoic acid induced neutrophilic differentiation of HL-60 cells. This result suggests that knockout of RIAM, VASP or Vinculin causes a failure to upregulate these integrin subunits, and this might be a critical step in the differentiation program of myeloid cells.

Myelopoiesis requires the hierarchical and sequential activation of transcriptional programs controlled mainly by the transcription factors PU.1 and CEBPα [400]. PU.1 upregulates α_M expression in promyelocytic cell lines in response to all-*trans* retinoic acid [273, 282-284] and this effect has been shown to be in response to G-CSF-Stat3 signaling during myelopoiesis [286]. Vav1 activation through tyrosine phosphorylation helps to drive PU.1 mediated upregulation of α_M, although the authors found the formation of PU.1 complexes on the *ITGAM* promoter in Vav1 knockouts suggesting that this may be a feedforward loop [401]. Vav1 is upregulated

after all-*trans* retinoic acid treatment [402, 403] and its tyrosine phosphorylation can be performed by Src family [402-404] and Syk kinases [53, 193, 195]. Since these kinases can be activated downstream of integrin activation [44, 177, 178, 183, 184, 186], the notion that the expression of proteins necessary for integrin activation and downstream signaling (such as RIAM, VASP and Vinculin) might help to further drive this purported feedforward loop is given credence.

We propose a model where initial transcription of integrin subunits occurs via myelopoiesis-specific transcription factor networks like PU.1, C/EBPα, C/EBPε and Gfi-1 [400], which are also involved in the expression of the G-CSF receptor. This would drive the expression of FHL-2, which has been shown to be upregulated in progenitors and is downregulated thereafter [378]. Concomitantly, VASP, RIAM and Vinculin could be upregulated, which concurs with our observations noting RIAM and VASP upregulation after all-*trans* retinoic acid treatment and the appearance of an upper band consistent with the phosphorylated form of VASP (**Supplementary Figure 2b-c**)[128]. An initial increase in FHL-2 could serve as a mechanism to retain non-mature precursors in the bone marrow by inhibiting α_M and α_X expression, since reports indicate that β_2 integrin expression facilitates the release of mature neutrophils from the bone marrow during myelopoiesis [230].

RIAM/VASP/Vinculin activity downstream of different receptors would increase F-actin content allowing MRTF-A/SRF mediated upregulation of α_M and α_X, with concomitant FHL-2 sequestration at the membrane. Additionally, activation of other transcription factors induced by RIAM/VASP/Vinculin activity may contribute to regulate α_M and α_X expression. Deficiency in $\alpha_M\beta_2$ and $\alpha_X\beta_2$ expression has not been described in RIAM, VASP or Vinculin knockout mice [166, 173, 370, 405, 406]. This discrepancy could be explained by the existence of compensatory mechanisms operating during myelopoiesis, involving homologous proteins and/or alternative

signaling pathways, including those that promote F-actin polymerization. Our results therefore highlight the existence of a pathway that occurs during all-*trans* retinoic acid-induced differentiation of HL-60 cells that works to ensure the upregulation of α_M and α_X integrins, and subsequently the acquisition of a phagocytic phenotype.

CHAPTER 6

OVERALL DISCUSSION AND FUTURE WORK

The present work dissects the contribution of several proteins involved in the downstream signaling of Complement Receptor 3 and 4 (integrin $\alpha_M\beta_2$ /$\alpha_x\beta_2$) and discusses the implications of their expression or lack thereof, over the phagocytic function of myeloid cells, specifically neutrophils.

The results outlined in **Chapter 3** showed that the adaptor molecule RIAM had two independent and distinct roles; it determined integrin activation and also particle engulfment during complement-dependent phagocytosis. In accordance, RIAM knockdown diminished and distorted ERK signaling, and resulted in impaired actin polymerization at phagocytic cups. We demonstrate that RIAM controlled VASP recruitment to phagocytic cups and determined VASP phosphorylation levels. VASP had a critical and non-redundant role in phagocytosis as VASP knockout completely abolished particle internalization, whereas overexpression resulted in inefficient engulfment, highlighting its importance in phagocytic actin dynamics. We proposed that RIAM relays conformational information to actin cytoskeleton through VASP. This pathway is likely shared by other integrins and may also be relevant for cell migration, as RIAM is located at the tip of probing protrusions or "sticky fingers" [332].

Our results show that RIAM is required for outside-in signaling [311]. Recently, RIAM has been shown to require Src Family kinase-mediated phosphorylation at Tyr^{264} and Tyr^{427} [407]. Phoshorylation in these residues unmask the pleckstrin homology domain, allowing for membrane localization of RIAM. Since Src Family kinase activation is one of the earliest steps in the outside-in pathway [44],

the requirement for this phosphorylation explains the poor phagocytosis observed for these cells [311]. However, little is known about the signaling leading to RIAM phosphorylation and the phosphorylation state of RIAM during the phagocytic process. This may be approached by the use of phosphomimetic or phophorylation-inhibited RIAM mutants. It would also be interesting to test if these phosphorylations trigger additional signaling driving exclusion of RIAM from phagocytic cups or triggering its dephosphorylation for the recycling of the phagocytic machinery.

RIAM has another FAK-mediated phosphorylation at Tyr^{45} [408], which is purported to release an autoinhibitory interaction between the N-terminus an the Ras association domain, thereby preventing interaction with Rap1. Since activation of FAK and Pyk2 are considered to be later signals [44], it is possible that initial engagement between the phagocytic target and CR3 is mediated by a faster direct Rap1-Talin interaction [173, 174], triggering integrin downstream signaling, which in turn lead to a more profound and RIAM mediated activation of the CR3 pool. Indeed, the direct interaction between Rap1 and Talin has been demonstrated to be the main pathway for integrin activation in platelets[409], where RIAM deletion has no effect [166], and plays a role in neutrophil adhesion, extravasation, whilst not having an impact on macrophage adhesion and migration [410]. Recently, the relative contribution of the RIAM-Talin and Rap1-Talin pathway have been studied for β_2 integrins in mouse neutrophils [405]. RIAM was shown to play a central role in β_2 activation, yet the loss of integrin associated functions in RIAM knockouts could be exacerbated by the loss of the Rap1-Talin interaction, and the double mutant presented a phenotype similar to Talin knockouts[405]. The authors conclude that "the Rap1-Talin pathway only contributes to Talin activation by recruiting it to the plasma membrane, while the Rap1-RIAM-Talin pathway plays a dual role". The results presented in **Chapter 3**, cement this notion.

The Rap1-Talin direct interaction was shown to be evolutionarily conserved, and requires Rap1 cooperation with $PI(4,5)P_2$ to direct Talin towards integrin β tails [173, 174]. However, the results obtained for direct activation of Rap1 through the use of 8CPT-2Me-cAMP indicates that this pathway cannot compensate for RIAM deficiency and RIAM expression is still necessary in complement-mediated phagocytosis [128].

The Rap1-Talin seems to constitute an evolutionarily older pathway. This pathway was observed to be the main mediator of integrin-mediated migration in the amoeba *Dictyostelium discoideum* [411], and is proposed to regulate the collective cell migration observed in *D. discoideum* slugs (a multicellular like formation of the amoeba) [412]. Interestingly, Amoebozoans have been shown to have functional integrin adhesion complexes, complete with α-actinin, Vinculin and VASP orthologues [413, 414]. Correct expression of these have been shown to be necessary for adhesive, migratory and phagocytic functions [414-416]. The appearance of integrins therefore seems to be have occurred earlier in eukaryote evolution than previously thought [413], and predates Amorphea (clade within the domain Eukaryota containing amoebas, animals, fungi, and choanoflagellates).

Although no RIAM does not exist beyond mammals [411, 417], MRL family proteins can be found in several species, including *Drosophila melanogaster* (Pico) [418], *Caenorhabditis elegans* (Mig-10), and *Dictyostelium discoideum* (DydA) [419]. All of these are Ras GTPase family binding proteins and may act as signaling platforms. For *D. discoideum*, adhesion is mainly regulated through the direct interaction of TalinB and Rap1,and this interaction is essential for its multicellular development [411]. In *D. discoideum*, MRL proteins seem not to play a direct role in integrin activation, since DydA has been shown to bind exclusively Ras and not Rap1 [419].

When scrutinizing other MRL proteins for their ability to bind Rap1 orthologues, which appears to be a common feature of integrin activation [411, 420], one finds that *D. melanogaster* Pico can bind both Ras and Rap [418], mammalian Lamellipodin has been shown to bind Ras isoforms but not Rap1[348, 421-423] and mammalian RIAM mainly binds Rap1, but shows a very weak interaction with Ras [152]. This suggests that the evolution of MRL proteins may have been due to the co-opting of a Ras mediator in order to ensure a more robust integrin activation than that observed for direct Rap-Talin [173, 174]. For mammals, a genetic duplication may have occurred, resulting in two specialized MRL proteins with distinct functions. RIAM appears to provide a more specialized signaling platform for β_2 integrins [166], allowing for the formation of filopoidal architectures mediated by proteins such as VASP [311]. Indeed, Lamellipodin and RIAM seem to have non-redundant roles, since the affinity for phosphoinositides of RIAM is greater than that of Lpd [424], and knockout of Lamellipodin does not significantly alter VASP membrane localization [425]. Lamellipodin seems to regulate the cytoskeleton through activating Scar/WAVE complex and Arp2/3-mediated actin branching typical of lamellipodia [426, 427].

Our results may shed insight into the evolutionary process of MRL proteins and the role that these might play in the appearance of the first multicellular organisms. A more in-depth comparative study of the biochemical properties MRL family proteins could uncover how mammalian RIAM and Lpd may have evolved and how their roles as integrators of integrin activation and cytoskeletal dynamics have specialized in order to construct and fine-tune different actin cytoskeleton structures.

In a manner reminiscent to RIAM, Lamellipodin has been shown to coordinate with VASP in order to induce membrane protrusion and actin

polymerization needed for the engulfment of IgG-coated sheep RBCs [428]. The authors identify VASP as a key mediator of phagocytosis which is in agreement with our results. These findings highlight that Lpd, contrary to RIAM, is necessary for Fc-mediated phagocytosis and further prove that these MRL proteins play non-redundant roles and further serve to differentiate the mechanisms involved in complement versus Fc-mediated phagocytosis. Furthermore, the authors link the Lpd-VASP with phosphoinositide metabolism, which are reported to delimit microdomains in the phagocytic cup, serving as a signal for effector recruitment [67]. The authors note that Lpd dissociates from the maturing phagosome prior to the disappearance of the $PI(3,4)P_2$. This suggests that a similar mechanism for RIAM could take place in complement-mediated phagocytosis and would therefore serve as a basis to study the localization of RIAM in the phagocytic cup, with relation to its phosphorylations and PIP metabolism.

Our results also highlight the importance of VASP in phagocytosis and suggest that $pSer^{157}$ VASP phosphorylation, which correlated with its recruitment to phagocytic cups and was dependent on RIAM expression, may be critical in regulating its polymerase activity [311]. Previous *in vitro* experiments with isolated VASP phosphomimetics indicated that $pSer^{157}$ phosphorylation did not increase F-actin elongation rates [340], however, this phosphorylation may facilitate the appearance of VASP-Vinculin complexes reducing the pool of VASP-RIAM, in a manner similar to the dynamics observed for Talin-RIAM and Talin-Vinculin [53, 139, 160, 370]. Since Vinculin favors adhesion maturation [139], Vinculin may restrict VASP elongation rates or lead to VASP displacement from adhesions. This change in binding partners may explain why *in vitro* experiments to assess the role of $pSer^{157}$ conflict with results obtained *in vivo* [311, 326, 329, 330].

In addition to the classical role of phagocytosis in pathogen clearance, RIAM and VASP seem to play a role in microglial phagocytosis in order to ensure tisular homeostasis through the removal of apoptotic neurons, debris, protein aggregates, synapses ('synaptic pruning') and axons [429-431]. Altered synaptic pruning may underlie neurodevelopmental disorders as autism, schizophrenia and epilepsy [432] , and RIAM has been identified through GWAS as a risk factor for prepulse inhibition in mice and schizophrenia in humans [433]. Furthermore, Lpd knockout mice also develop behavioral problems, showing hyperactivity and increased anxiety [434] pointing to the critical role of MRL proteins in phagocytic synapse pruning, although the regulation of these pathways remains to be fully explored.

A genetic linkage analysis identified RIAM polymorphisms, along with other genes localized on chromosome 10, as conferring susceptibility for the late onset of Alzheimer's disease. [435]. Similarly, VASP was shown to be significantly upregulated in microglia in Alzheimer's disease [436]. An in-depth understanding of the mechanisms and control points of this pathway may inform new strategies to target diseases which rely upon $\alpha_M\beta_2$ function.

Chapter 4 outlines the critical importance of correct expression of integrin adhesion complex proteins RIAM, VASP and Vinculin in the expression of the α_M and α_X integrin subunits. These results hint at the possibility that a defect in other integrin proximal proteins might result in a similar phenotype, which would be reminiscent of Leukocyte Adhesion Deficiencies.

There is growing evidence that leukocyte adhesion defects might be closely related to a subset of primary immunodeficiencies termed actinopathies [223, 225, 227]. Actinopathies are characterized by defects affecting actin polymerization that range from mutations in actin proteins (*ACTB*), actin nucleators and their regulators

(*ARPC1B, CORO1A, WAS, WIPF1, NCKAP1L*), small GTPases and their regulators (*RAC2, DOCK2, DOCK8, STK4*), actin severing (*WDR1*), and actin-dependent transcription cofactors (*MRTF-A*) [223, 225, 375]. All of these genes have been demonstrated to be targets of SRF-mediated transcription, and MRTF-A serves as its coactivator [223]. Though hypothesized to exist, no immunodeficiency-causing mutations have been detected for Ena/VASP family proteins [225]. Our research outlines the phagocytic defects that would be observed for such a disease, and therefore might serve as a model to test new therapeutic strategies.

We identified the importance of the transcription factor SRF and its cofactors MRTF-A and FHL-2. MRTF-A mutations have been described to cause phagocytosis and migration defects, and MRTF-A knockout in HL-60 cells also cause profound defects in F-actin content [375, 442]. One of the cases of MRTF-A deficiency also reports that the patient presented LAD-like symptoms, with leukocytosis and neutrophilia [442]. Knockouts for RIAM, VASP and Vinculin presented similar defects in F-actin, which presents a mechanical link which could explain the underlying molecular mechanisms of MRTF-A deficiency.

When analyzing integrin expression in MRTF-A deficient primary neutrophils or HL-60 lines, no defects in integrin expression were found [375, 442], which suggests the existence of compensatory mechanisms which could regulate α_M or α_X expression. Further supporting this notion, SRF knockout mice present normal or a modest downregulation in *ITGAM* mRNA, yet when surface expression was analyzed, the authors found a paradoxical increase in neutrophil surface integrin α_M [294]. However, SRF knockout mice neutrophils still presented abolished migration and defective outside-in integrin signaling and functions, which might be explained by an additional defect in RIAM, VASP or Vinculin expression [294].

In line with the previous observation, MRTF-A deficiency might also cause defects in Vinculin expression. HL-60 knockouts presented a significantly reduced expression of a non-muscle myosin IIa component (*MYH9*), which resulted in abnormal uropod retraction during migration [442]. Since Vinculin expression is required for stable uropodial F-actin localization and neutrophil polarization and migration [370], the defect observed for MRTF-A knockouts might also be explained by a defect in Vinculin expression.

We also demonstrate that the defects observed for RIAM, VASP and Vinculin knockout can be partially reversed through the use of actin stabilizers like jasplakinolide, which might inform new therapies for emerging immunodeficiencies where the actin cytoskeleton is disrupted. Direct targeting of VASP, RIAM, Vinculin or the use of similar actin stabilizers might also reverse defects in neutrophil functions. Curative treatment of actinopathies still consist of bone-marrow transplants [223], and our results provide a pathway to be explored as paliative treatment whilst patients are awaiting transplant or as a therapeutic option in cases where transplantation is not advised.

Finally, these results might serve to dissect the pathways involved in myeloid cell differentiation or polarization and further study of the events leading to SRF-mediated α_M and α_X upregulation. In this regard, *VASP* mRNA expression was shown to be upregulated in M1 polarized macrophages, which also reflected in an increase in protein expression and pSer^{157} phosphorylation[406]. Conversely, M2 polarization significantly reduced *VASP* mRNA. Interestingly, VASP$^{-/-}$ macrophages show a skew towards an M1 phenotype [443] caused by increased STAT1 signaling and was related to NO production through iNOS [406, 444]. This may indicate a dual role for VASP in polarization. RIAM has also been identified as having a role in

myeloid cell fate commitment and polarization, controlling tumor progression [445]. However, the results from this work remain to be published.

CHAPTER 7

CONCLUSIONS

CHAPTER 3

1. Reduced RIAM expression in neutrophilic HL-60 cells results in deficient particle internalization during complement-mediated phagocytosis, following Mn^{2+} integrin activation.

2. Disruption of RIAM expression dampens outside-in signaling downstream of $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins and actin polymerization.

3. VASP is critical for the phagocytic engulfment of complement-opsonized particles and is recruited to phagocytic cups following outside-in signaling.

4. Through VASP recruitment, RIAM controls F-actin enrichment at phagocytic cups.

5. RIAM controls VASP $pSer^{157}$ phosphorylation, which is required for efficient phagocytosis.

CHAPTER 4

1. Knockout of RIAM, VASP or Vinculin in HL-60 cells completely abolishes complement dependent-phagocytosis.

2. RIAM, VASP and Vinculin specifically control surface expression of the integrin subunits α_M, α_X and β_2 at the mRNA level.

3. Upregulation of α_M and α_X during neutrophilic differentiation of HL-60 cells is compromised in RIAM, VASP and Vinculin knockout cell lines.

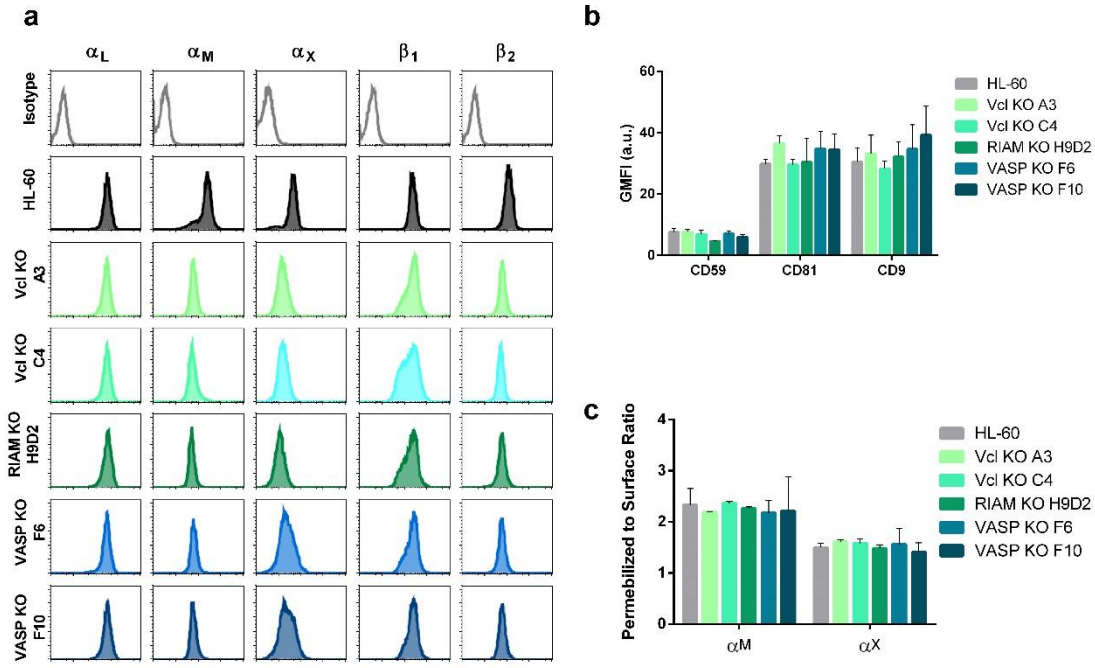
4. Expression of α_M and α_X can be induced through the use of F-actin stabilizers in RIAM, VASP and Vinculin knockout HL-60 cells. This finding connects the expression of these integrin proximal components to an increased F-actin cellular content and the control of α_M and α_X expression in HL-60 cells.

5. RIAM, VASP and Vinculin control the nuclear translocation of SRF co-activator MRTF-A in an F-actin dependent manner.

6. RIAM, VASP and Vinculin may play a role in sequestering the SRF corepressor FHL-2 at the plasma membrane, determining SRF activity.

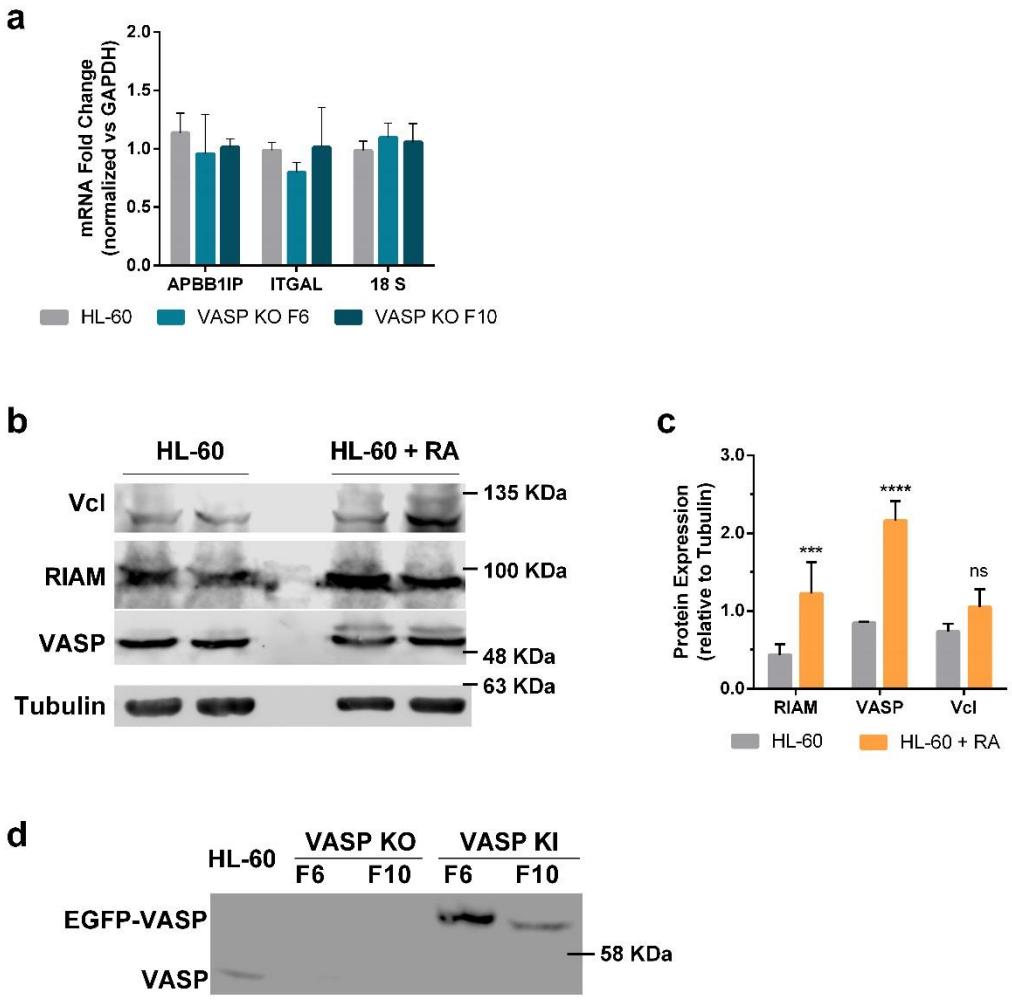
APPENDIX

7.1. Supplemental Figures



Supplemental Figure 1. Additional results for Figure 4.2a.

a) Expression of α_M, α_L, α_X, β_1, β_2 integrin subunits was determined for RA-differentiated HL-60 parental cells and the Vinculin, RIAM and VASP knockout cell lines. Flow cytometry profiles from a representative experiment corresponding to Figure 4.2a are shown. **b)** Neutrophilic-like Vinculin (Vcl), RIAM and VASP knockout cell lines and HL-60 parental cells were stained with monoclonal antibodies specific for the tetraspanins CD81 and CD9, or cell surface molecule CD59. Geometric Mean Fluorescence Intensity (GMFI) was obtained by flow cytometry. The results show data from 3 independent experiments done in duplicate and represented as mean±SD, where the error bars denote standard deviation. **c)** Neutrophilic-like Vinculin (Vcl), RIAM and VASP knockout cell lines and HL-60 parental cells were permeabilized with 0,1% Triton X-100 for 10 minutes or left untreated. Cells were stained for α_M and α_X expression and GMFI was obtained by flow cytometry and represented as the ratio of permeabilized staining to surface staining. Data is from 6 independent experiments done in duplicate. Error bars denote standard deviation. Significance (ANOVA test) has been calculated with respect to HL-60 controls, however, no significance was found between samples.



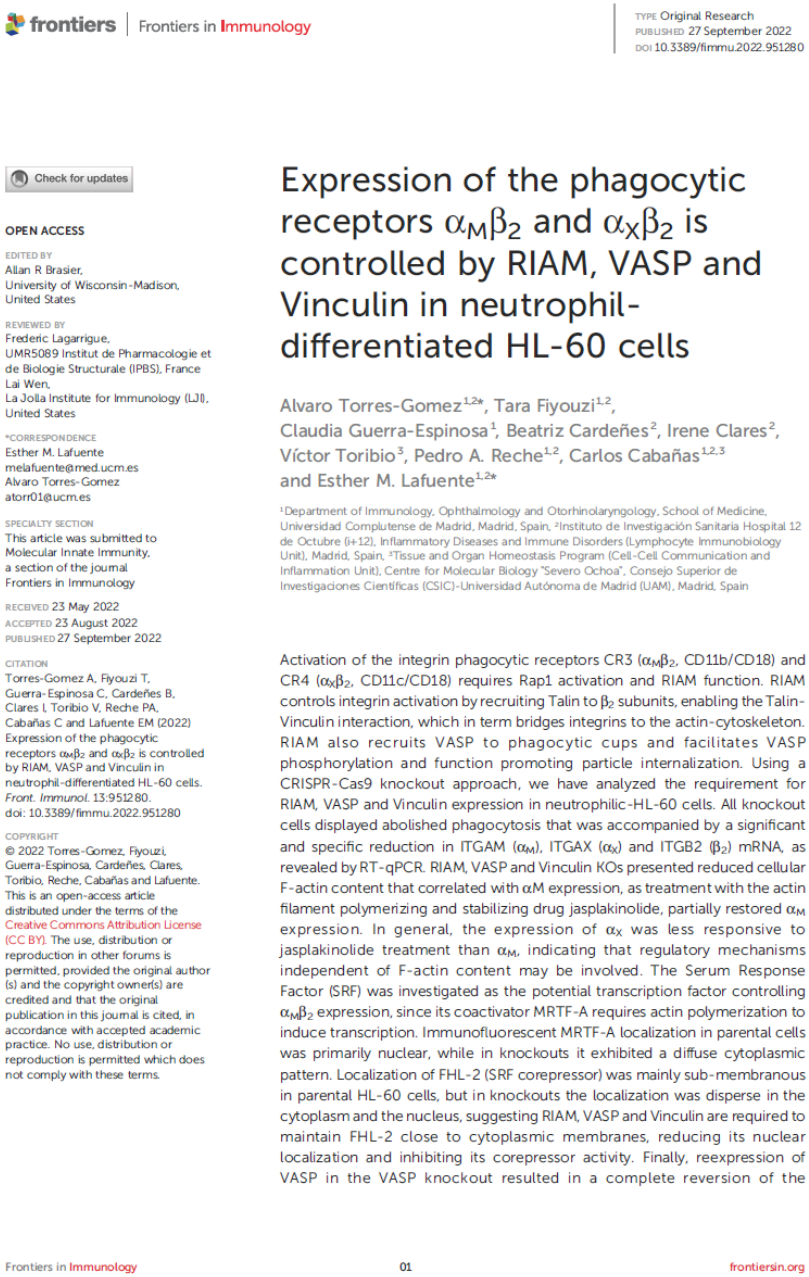
Supplemental Figure 2. Additional results for Figures 4.2b and 4.3.

a) Expression of *APBB1IP* (RIAM) and *ITGAL* (integrin α_L gene) and 18S rRNA mRNA levels were determined by RT-qPCR in neutrophil-like HL-60 cells and VASP F6 and VASP F10 knockout clones. Results are represented as relative to *GAPDH* mRNA levels and are from 3 independent experiments done in triplicate. Data are presented as mean±SD, where the error bars denote standard deviation. **b-c)** Cell lysates from undifferentiated HL-60 cells or RA differentiated HL-60 cells (HL-60+RA) were analyzed by western blot for RIAM, VASP and Vinculin expression. The images show representative results from two experiments done in triplicate. Protein band intensity is represented as relative to Tubulin. Significance (ANOVA) has been calculated with respect to HL-60 controls, *** denotes $p<0.005$, **** $p<0.0001$, and ns denotes no significance. **d)** VASP KO F6 and F10 clones were transduced with an EGFP-VASP retroviral plasmid and the corresponding polyclonal knock-in cell lines VASP KI F6 and VASP KI F10, were generated. Expression of VASP was analyzed by western blot in HL60, VASP KO F6 and F10 clones and their respective VASP KI F6 and VASP KI F10 polyclonal cell lines.

7.2. Publications and Poster Presentations

7.2.1. Thesis Related Publications

1. **Torres-Gomez, A.**; Fiyouzi, T.; Guerrera-Espinosa, C.; Cardeñes, B., Clarés, I., Toribio, V.; Reche, P.A.; Cabañas, C.; Lafuente, E.M. "Expression of the Phagocytic Receptors $\alpha_M\beta_2$ and $\alpha_X\beta_2$ is Controlled by RIAM, VASP and Vinculin in Neutrophil-Differentiated HL-60 cells", 2022, Frontiers in Immunology, 13:951280, 1-16, https:/doi.org/10.3389/fimmu.2022.951280.



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Expression of the phagocytic receptors $\alpha_M\beta_2$ and $\alpha_X\beta_2$ is controlled by RIAM, VASP and Vinculin in neutrophil-differentiated HL-60 cells

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Activation of the integrin phagocytic receptors CR3 ($\alpha_M\beta_2$, CD11b/CD18) and CR4 ($\alpha_X\beta_2$, CD11c/CD18) requires Rap1 activation and RIAM function. RIAM controls integrin activation by recruiting Talin to β_2 subunits, enabling the Talin-Vinculin interaction, which in term bridges integrins to the actin-cytoskeleton. RIAM also recruits VASP to phagocytic cups and facilitates VASP phosphorylation and function promoting particle internalization. Using a CRISPR-Cas9 knockout approach, we have analyzed the requirement for RIAM, VASP and Vinculin expression in neutrophilic-HL-60 cells. All knockout cells displayed abolished phagocytosis that was accompanied by a significant and specific reduction in ITGAM (α_M), ITGAX (α_X) and ITGB2 (β_2) mRNA, as revealed by RT-qPCR. RIAM, VASP and Vinculin KOs presented reduced cellular F-actin content that correlated with αM expression, as treatment with the actin filament polymerizing and stabilizing drug jasplakinolide, partially restored α_M expression. In general, the expression of α_X was less responsive to jasplakinolide treatment than α_M, indicating that regulatory mechanisms independent of F-actin content may be involved. The Serum Response Factor (SRF) was investigated as the potential transcription factor controlling $\alpha_M\beta_2$ expression, since its coactivator MRTF-A requires actin polymerization to induce transcription. Immunofluorescent MRTF-A localization in parental cells was primarily nuclear, while in knockouts it exhibited a diffuse cytoplasmic pattern. Localization of FHL-2 (SRF corepressor) was mainly sub-membranous in parental HL-60 cells, but in knockouts the localization was disperse in the cytoplasm and the nucleus, suggesting RIAM, VASP and Vinculin are required to maintain FHL-2 close to cytoplasmic membranes, reducing its nuclear localization and inhibiting its corepressor activity. Finally, reexpression of VASP in the VASP knockout resulted in a complete reversion of the

2. Duygu, S-A; **Torres-Gomez**, A; Yazicioglu,Y-F; Christofides, A.; Patsoukis, N.; Lafuente, E.M; Boussiotis, V.A., "Structural, biochemical, and functional properties of the Rap1-Interacting Adaptor Molecule (RIAM)", 2021, Biomedical Journal, https://doi.org/ 10.1016/j.bj.2021.09.005.

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Short review

Structural, biochemical, and functional properties of the Rap1-Interacting Adaptor Molecule (RIAM)

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ABSTRACT

Leukocytes, the leading players of immune system, are involved in innate and adaptive immune responses. Leukocyte adhesion to endothelial cells during transmigration or to antigen presenting cells during T cell activation, requires integrin activation through a process termed inside-out integrin signaling. In hematopoietic cells, Rap1 and its downstream effector RIAM (Rap1-interacting adaptor molecule) form a cornerstone for inside-out integrin activation. The Rap1/RIAM pathway is involved in signal integration for activation, actin remodeling and cytoskeletal reorganization in T cells, as well as in myeloid cell differentiation and function. RIAM is instrumental for phagocytosis, a process requiring particle recognition, cytoskeletal remodeling and membrane protrusion for engulfment and digestion. In the present review, we discuss the structural and molecular properties of RIAM and the recent discoveries regarding the functional role of the Rap1/RIAM module in hematopoietic cells.

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Article

RIAM-VASP Module Relays Integrin Complement Receptors in Outside-In Signaling Driving Particle Engulfment

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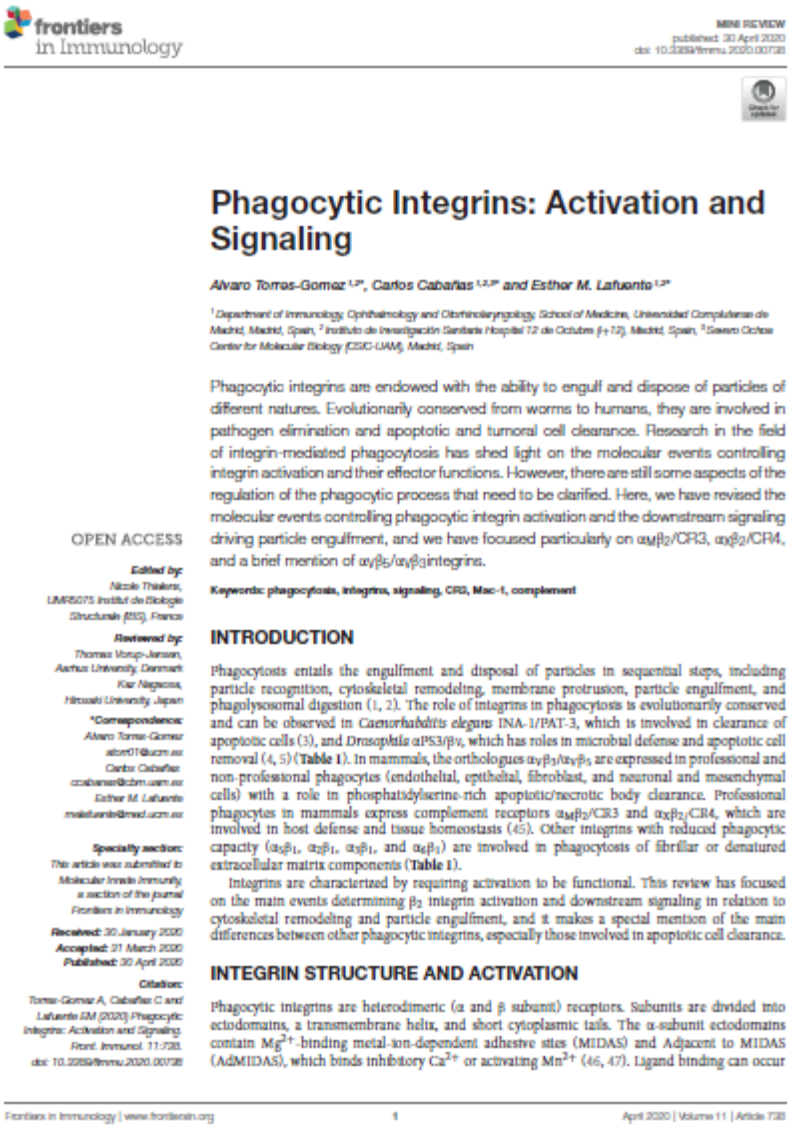
Abstract: The phagocytic integrins and complement receptors $\alpha_M\beta_2$/CR3 and $\alpha_X\beta_2$/CR4 are classically associated with the phagocytosis of iC3b-opsonized particles. The activation of this receptor is dependent on signals derived from other receptors (inside-out signaling) with the crucial involvement of the Rap1-RIAM-Talin-1 pathway. Here, we analyze the implication of RIAM and its binding partner VASP in the signaling events occurring downstream of β_2 integrins (outside-in) during complement-mediated phagocytosis. To this end, we used HL-60 promyelocytic cell lines deficient in RIAM or VASP or overexpressing EGFP-tagged VASP to determine VASP dynamics at phagocytic cups. Our results indicate that RIAM-deficient HL-60 cells presented impaired particle internalization and altered integrin downstream signaling during complement-dependent phagocytosis. Similarly, VASP deficiency completely blocked phagocytosis, while VASP overexpression increased the random movement of phagocytic particles at the cell surface, with reduced internalization. Moreover, the recruitment of VASP to particle contact sites, amount of pSer157-VASP and formation of actin-rich phagocytic cups were dependent on RIAM expression. Our results suggested that RIAM worked as a relay for integrin complement receptors in outside-in signaling, coordinating integrin activation and cytoskeletal rearrangements via its interaction with VASP.

Keywords: phagocytosis; complement; CR3; CR4; Mac-1; β_2 integrins; RIAM; VASP; outside-in

1. Introduction

Essential immunological processes like immune synapse formation, cell migration, leukocyte extravasation, neutrophil extracellular trap formation, NK cell killing and phagocytosis depend on integrin activation and the recognition of their ligands [1–4]. Of special note are the integrins $\alpha_M\beta_2$ and $\alpha_X\beta_2$ (also known as CR3, CD11b/CD18, Mac-1 and CR4, CD11c/CD18, respectively),

4. **Torres-Gomez, A.;** Cabañas, C.; Lafuente, E.M., "Phagocytic Integrins: Activation and Signaling", 2020, Frontiers in Immunology, 11, 738. https://doi.org/10.3389/fimmu.2020.00738.



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MINI REVIEW
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Phagocytic Integrins: Activation and Signaling

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Phagocytic integrins are endowed with the ability to engulf and dispose of particles of different natures. Evolutionarily conserved from worms to humans, they are involved in pathogen elimination and apoptotic and tumoral cell clearance. Research in the field of integrin-mediated phagocytosis has shed light on the molecular events controlling integrin activation and their effector functions. However, there are still some aspects of the regulation of the phagocytic process that need to be clarified. Here, we have revised the molecular events controlling phagocytic integrin activation and the downstream signaling driving particle engulfment, and we have focused particularly on $\alpha_M\beta_2$/CR3, $\alpha_X\beta_2$/CR4, and a brief mention of $\alpha_V\beta_5$/$\alpha_V\beta_3$integrins.

Keywords: phagocytosis, integrins, signaling, CR3, Mac-1, complement

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INTRODUCTION

Phagocytosis entails the engulfment and disposal of particles in sequential steps, including particle recognition, cytoskeletal remodeling, membrane protrusion, particle engulfment, and phagolysosomal digestion (1, 2). The role of integrins in phagocytosis is evolutionarily conserved and can be observed in *Caenorhabditis elegans* INA-1/PAT-3, which is involved in clearance of apoptotic cells (3), and *Drosophila* αPS3/βν, which has roles in microbial defense and apoptotic cell removal (4, 5) (**Table 1**). In mammals, the orthologues $\alpha_V\beta_3$/$\alpha_V\beta_5$ are expressed in professional and non-professional phagocytes (endothelial, epithelial, fibroblast, and neuronal and mesenchymal cells) with a role in phosphatidylserine-rich apoptotic/necrotic body clearance. Professional phagocytes in mammals express complement receptors $\alpha_M\beta_2$/CR3 and $\alpha_X\beta_2$/CR4, which are involved in host defense and tissue homeostasis (45). Other integrins with reduced phagocytic capacity ($\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_9\beta_1$, and $\alpha_6\beta_1$) are involved in phagocytosis of fibrillar or denatured extracellular matrix components (**Table 1**).

Integrins are characterized by requiring activation to be functional. This review has focused on the main events determining β_2 integrin activation and downstream signaling in relation to cytoskeletal remodeling and particle engulfment, and it makes a special mention of the main differences between other phagocytic integrins, especially those involved in apoptotic cell clearance.

INTEGRIN STRUCTURE AND ACTIVATION

Phagocytic integrins are heterodimeric (α and β subunit) receptors. Subunits are divided into ectodomains, a transmembrane helix, and short cytoplasmic tails. The α-subunit ectodomains contain Mg^{2+}-binding metal-ion-dependent adhesive sites (MIDAS) and Adjacent to MIDAS (AdMIDAS), which binds inhibitory Ca^{2+} or activating Mn^{2+} (46, 47). Ligand binding can occur

5. Patsoukis, N; Bardhan, K; Weaver, JD; Sari, D; **Torres-Gomez, A;** Li, L; Strauss, L; Lafuente E.M., Boussiotis V.A."The adaptor molecule RIAM integrates signaling events critical for integrin-mediated control of immune function and cancer progression.", 2017, Sci Signal., 10. https://doi.org/10.1126/scisignal.aam8298.

SCIENCE SIGNALING | REVIEW

CELL BIOLOGY

The adaptor molecule RIAM integrates signaling events critical for integrin-mediated control of immune function and cancer progression

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Lymphocyte activation requires adhesion to antigen-presenting cells. This is a critical event linking innate and adaptive immunity. Lymphocyte adhesion is accomplished through LFA-1, which must be activated by a process referred to as inside-out integrin signaling. Among the few signaling molecules that have been implicated in inside-out integrin activation in hematopoietic cells are the small guanosine triphosphatase (GTPase) Rap1 and its downstream effector Rap1-interacting molecule (RIAM), a multidomain protein that defined the Mig10-RIAM-lamellipodin (MRL) class of adaptor molecules. Through its various domains, RIAM is a critical node of signal integration for activation of T cells, recruits monomeric and polymerized actin to drive actin remodeling and cytoskeletal reorganization, and promotes inside-out integrin signaling in T cells. As a regulator of inside-out integrin activation, RIAM affects multiple functions of innate and adaptive immunity. The effects of RIAM on cytoskeletal reorganization and integrin activation have implications in cell migration and trafficking of cancer cells. We provide an overview of the structure and interactions of RIAM, and we discuss the implications of RIAM functions in innate and adaptive immunity and cancer.

Identification of RIAM

The human Rap1-interacting molecule (RIAM) was identified in a yeast two-hybrid screen for candidate effectors of the small guanosine triphosphatase (GTPase) Rap1 (*1*). Before its identification as a Rap1-interacting molecule, RIAM was identified as a binding partner of the amyloid β (A4) precursor protein-binding, family B, member 1 (APBB1; also known as Fe65) and was named amyloid β (A4) precursor protein–binding, family B, member 1 interacting protein (APBB1IP) accordingly (*2*). This interaction is mediated by the WW (tryptophan-tryptophan) domain of Fe65 interacting with the proline-rich regions of RIAM (*2*). In an independent study, the gene encoding RIAM was also identified as transcriptionally induced in response to all-trans retinoic acid (ATRA) in the promyelocytic HL-60 cell line, and the protein was accordingly named retinoic acid–responsive proline-rich protein 1 (RARP-1) (*3*). In that system, it was found that forced expression in various cell types suppressed transactivation of activator protein 1 (AP-1) and serum response element (SRE), leading to the conclusion that this protein was functionally involved in cell growth arrest. An independent group also identified RIAM as an interactor of Enabled/vasodilator-stimulated proteins (Ena/VASP) family, which are involved in cell motility and actin polymerization, and named it proline-rich EVH1 ligand 1 (PREL1) (*4*). This study reported that RIAM colocalized with Ena/VASP proteins at the tips of lamellipodia and at focal adhesions in response to epidermal growth factor (EGF) treatment of fibroblasts. Because this event coincided temporally with Ras activation, the authors suggested that RIAM might link Ras signaling to cytoskeleton remodeling during cell migration and spreading (*4*). However, direct evidence for such interaction was not identified.

Structure and Homologs of RIAM

The open reading frame of RIAM is 1998 base pairs and encodes a protein of 665 amino acids. Structurally, RIAM contains an RA (RalGDS/AF-6 or Ras-association) domain, a PH (pleckstrin homology) domain, and two proline-rich regions. Two putative coiled-coil regions are present at the N terminus (amino acids 62 to 89 and amino acids 149 to 181) (Fig. 1A) (*1*).

Upon identification as a Rap1-interacting molecule and structural characterization of RIAM, database searches for homologous genes revealed that the proteins with highest homology to RIAM are human lamellipodin (Lpd) (also known as KIAA1681 and AY494951) and Lpd-S (a short isoform of human lamellipodin, also known as ALS2CR9 and BAB69020) (*1*, *5*). Furthermore, RIAM is related to proteins CG11940 (AAF49029) in *Drosophila melanogaster* and Mig-10 (P34400) in *Caenorhabditis elegans* (Fig. 1A. Comparison of the domain structures of these proteins indicated that RIAM, Lpd, CG11940, and Mig-10 have a proline-rich region at the C terminus and a highly conserved pattern of 27 amino acids predicted to be a coiled-coil region immediately N-terminal to the RA domain. In addition, comparison of the RA and PH domains in RIAM-related proteins showed regions of these domains to be conserved among the proteins (*1*). Collectively, these proteins define the MRL (Mig-10/RIAM/Lpd) family (*6*). Phylogenetic analysis showed that the MRL proteins are conserved during evolution but *Drosophila* and *C. elegans* each only have one gene encoding an MRL family member (*1*). *Mig-10* is the first member of the MRL family and was identified in a screen for mutations associated with neuronal cell migration defects during *C. elegans* embryogenesis. Specifically, the *mig-10* gene is required for the long-range anteroposterior migration of the two canal-associated neurons, anterior lateral microtubule cells, and hermaphrodite-specific neurons and for proper development of the excretory canals (*7*).

Subsequently, mutations in the *Drosophila MRL* ortholog *CG11940* were identified, and the gene was named *pico* due to the retarded growth phenotype resulting from *pico* knockdown or loss-of-function mutation (*8*). Reduction in *pico* expression in *Drosophila* resulted in animals with

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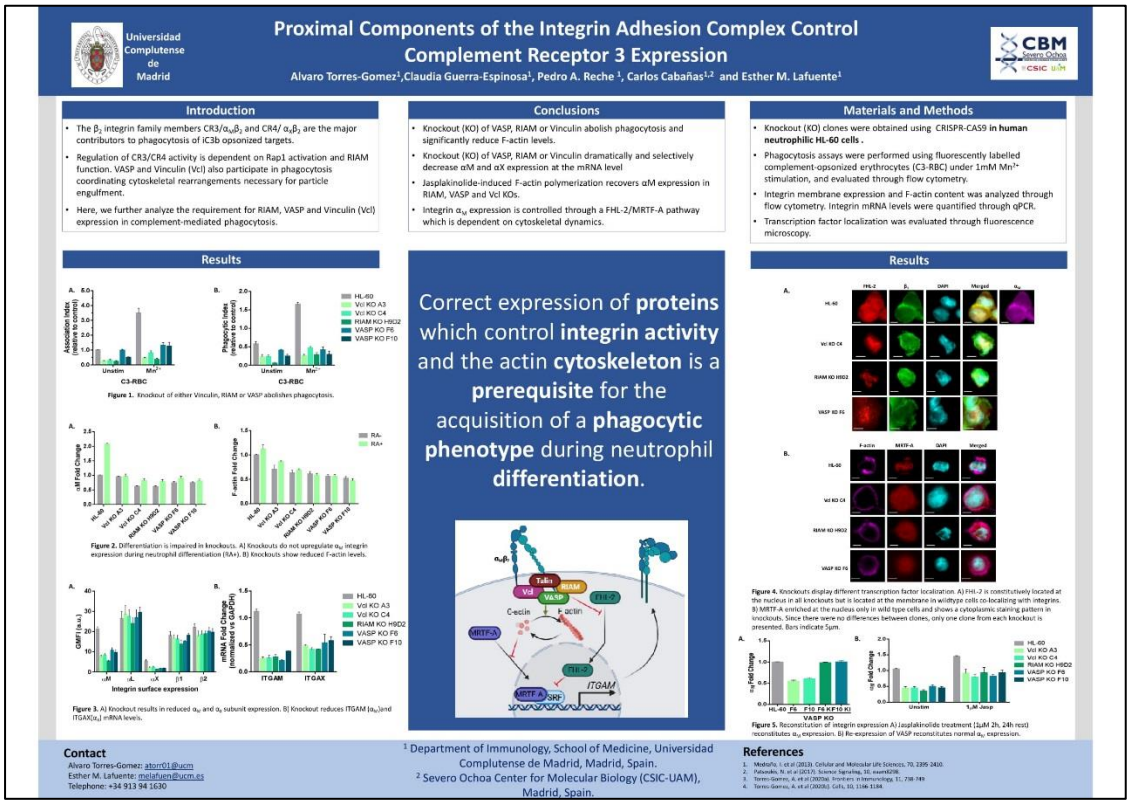
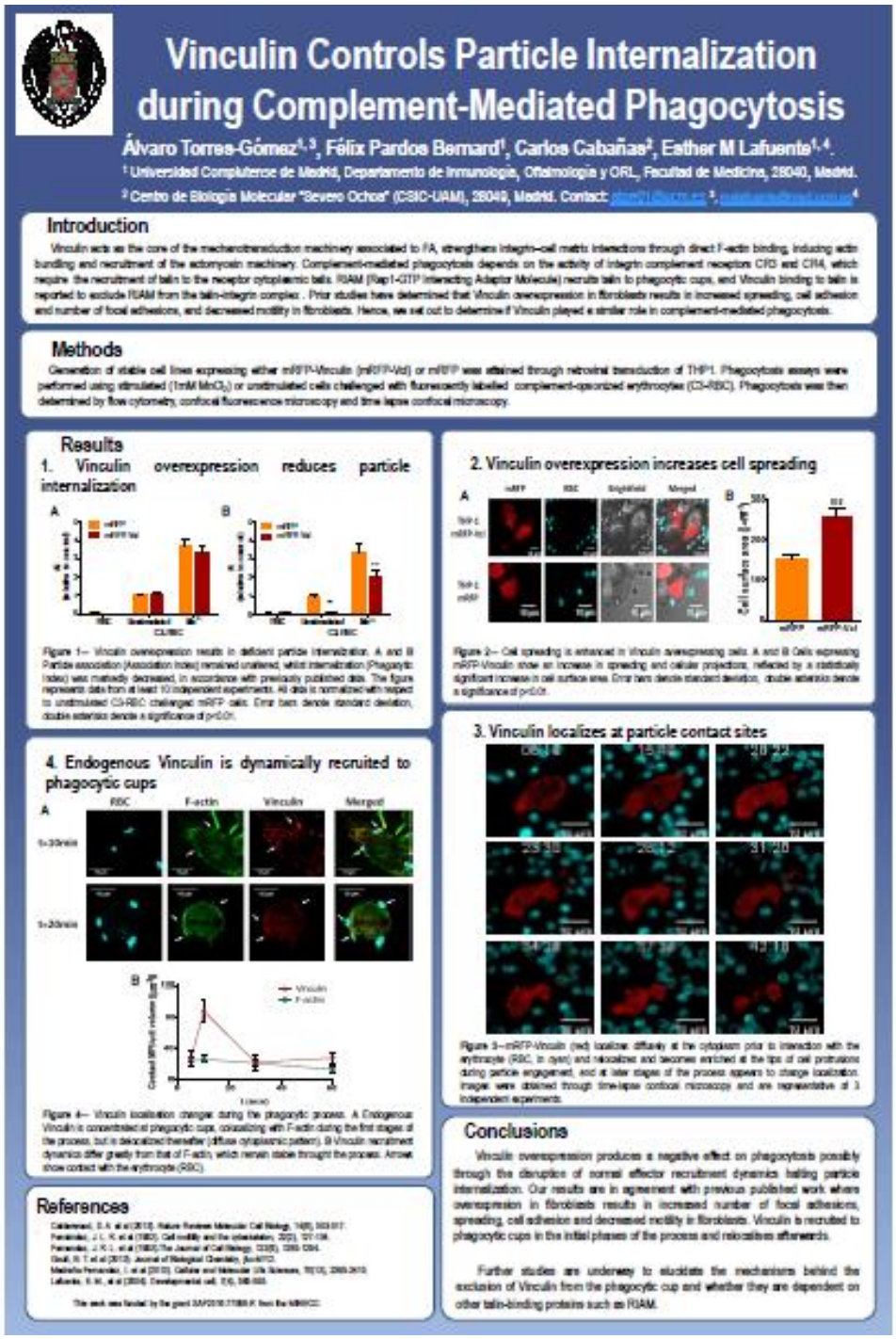
7.2.2. Posters

1. **Torres-Gomez, A**; Claudia Guerra Espinosa, C.; Reche, P.A.; Cabañas, C.; Lafuente, E.M., "Cytoskeleton Related Proteins Control Complement Receptor 3 Expression during Differentiation", 6th European Congress of Immunology, International, Turkey, 1/09/2021-4/09/2021.

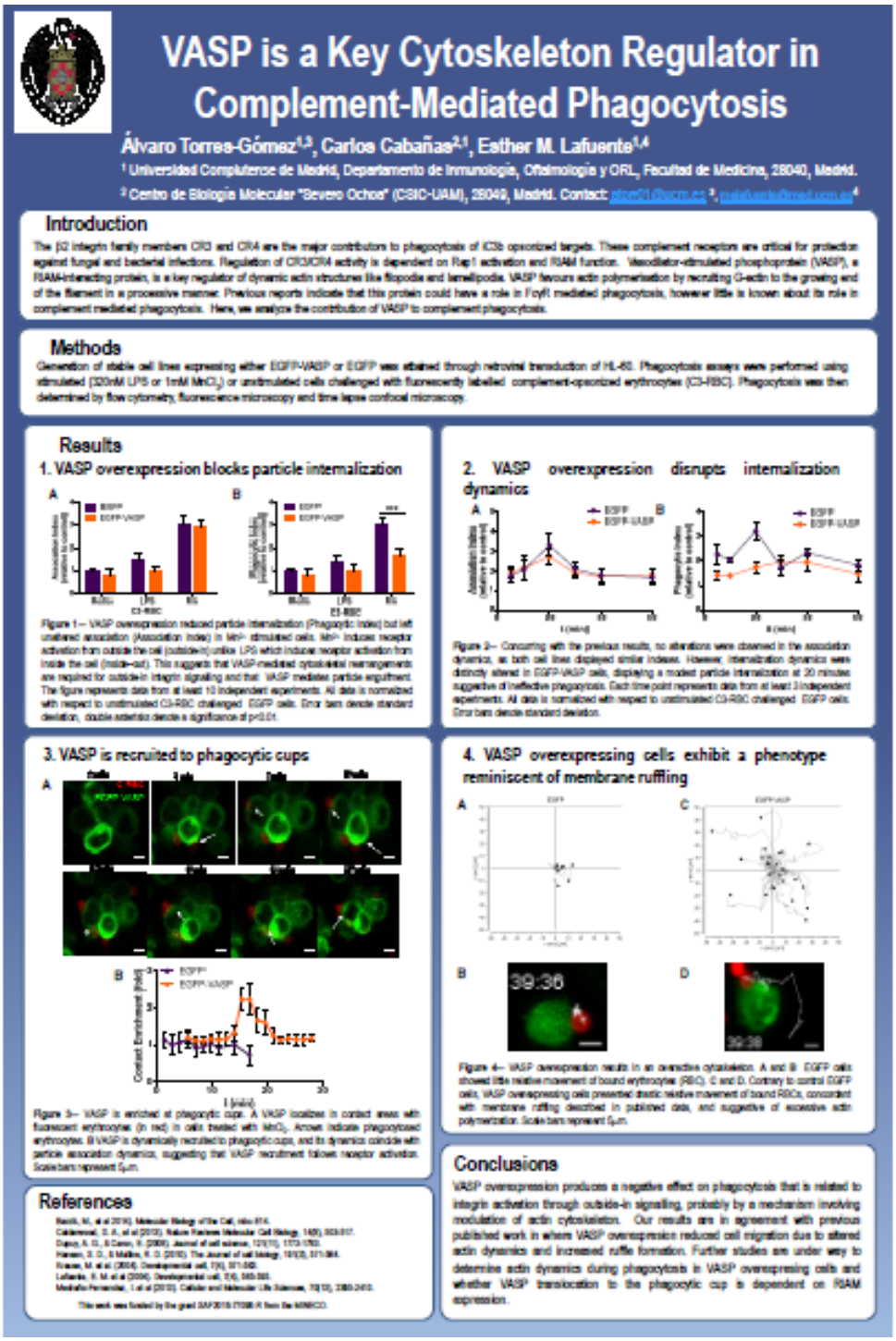
2. **Torres-Gomez, A**; Claudia Guerra Espinosa, C.; Reche, P.A.; Cabañas, C.; Lafuente, E.M., "Proximal Components of the Integrin Adhesion Complex Control Complement Receptor 3 Expression", Keystone Symposia - Innate Immunity: Mechanisms and Modulation; International, EEUU, 12/04/2021-15/04/2021.



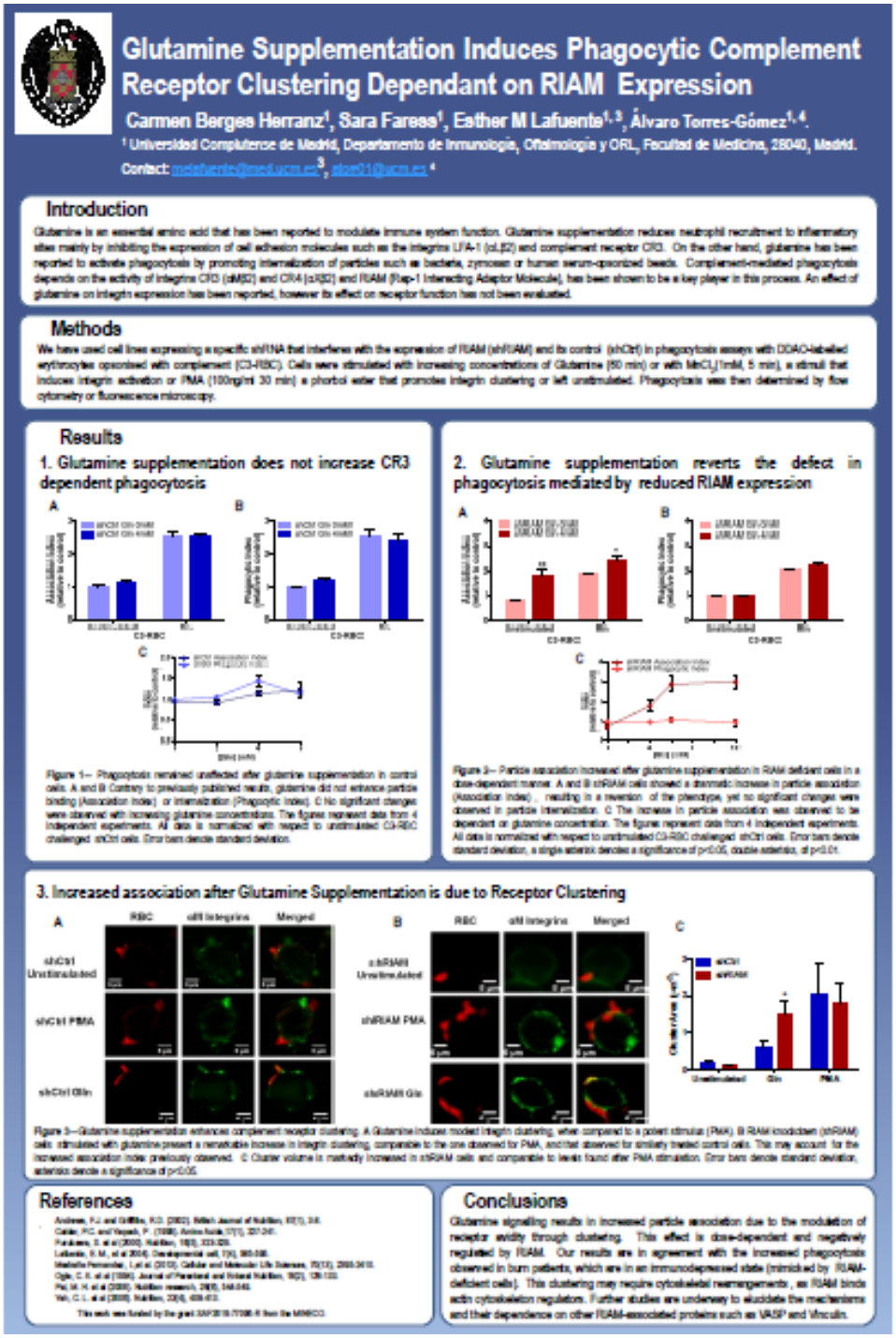
3. **Torres-Gomez, A**; Pardos Bernard, F.; Cabañas, C.; Lafuente, E.M., "Vinculin Controls Particle Internalization during Complement-Mediated Phagocytosis", IX Reunión Anual de la Sociedad de Inmunología de la Comunidad de Madrid (SICAM), National, Spain, 18/01/2019.



4. **Torres-Gomez, A**; Cabañas, C.; Lafuente, E.M., "VASP is a Key Cytoskeleton Regulator in Complement-Mediated Phagocytosis", IX Reunión Anual de la Sociedad de Inmunología de la Comunidad de Madrid (SICAM), National, Spain, 18/01/2019.



5. Berges Herranz, C.; Faress, S; Lafuente, E.M.; **Torres-Gomez, A**; "Glutamine Supplementation Induces Phagocytic Complement Receptor Clustering Dependent on RIAM Expression", IX Reunión Anual de la Sociedad de Inmunología de la Comunidad de Madrid (SICAM), National, Spain, 18/01/2019.



Glutamine Supplementation Induces Phagocytic Complement Receptor Clustering Dependant on RIAM Expression

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Introduction

Glutamine is an essential amino acid that has been reported to modulate immune system function. Glutamine supplementation reduces neutrophil recruitment to inflammatory sites mainly by inhibiting the expression of cell adhesion molecules such as the integrins LFA-1 (αLβ2) and complement receptor CR3. On the other hand, glutamine has been reported to activate phagocytosis by promoting internalization of particles such as bacteria, zymosan or human serum-opsonized beads. Complement-mediated phagocytosis depends on the activity of integrins CR3 (αMβ2) and CR4 (αXβ2) and RIAM (Rap-1 Interacting Adaptor Molecule), has been shown to be a key player in this process. An effect of glutamine on integrin expression has been reported, however its effect on receptor function has not been evaluated.

Methods

We have used cell lines expressing a specific shRNA that interferes with the expression of RIAM (shRIAM) and its control (shCtrl) in phagocytosis assays with DDAO-labelled erythrocytes opsonized with complement (C3-RBC). Cells were stimulated with increasing concentrations of Glutamine (60 min) or with MnCl$_2$1mM, 5 min), a stimuli that induces integrin activation or PMA (100ng/ml 30 min) a phorbol ester that promotes integrin clustering or left unstimulated. Phagocytosis was then determined by flow cytometry or fluorescence microscopy.

Results

1. Glutamine supplementation does not increase CR3 dependent phagocytosis

Figure 1– Phagocytosis remained unaffected after glutamine supplementation in control cells. A and B Contrary to previously published results, glutamine did not enhance particle binding (Association Index) or internalization (Phagocytic Index). C No significant changes were observed with increasing glutamine concentrations. The figures represent data from 4 independent experiments. All data is normalized with respect to unstimulated C3-RBC challenged shCtrl cells. Error bars denote standard deviation.

2. Glutamine supplementation reverts the defect in phagocytosis mediated by reduced RIAM expression

Figure 2– Particle association increased after glutamine supplementation in RIAM deficient cells in a dose-dependent manner. A and B shRIAM cells showed a dramatic increase in particle association (Association Index), resulting in a reversion of the phenotype, yet no significant changes were observed in particle internalization. C The increase in particle association was observed to be dependent on glutamine concentration. The figures represent data from 4 independent experiments. All data is normalized with respect to unstimulated C3-RBC challenged shCtrl cells. Error bars denote standard deviation, a single asterisk denotes a significance of $p \leq 0.05$, double asterisks, of $p \leq 0.01$.

3. Increased association after Glutamine Supplementation is due to Receptor Clustering

Figure 3—Glutamine supplementation enhances complement receptor clustering. A Glutamine induces modest integrin clustering, when compared to a potent stimulus (PMA). B RIAM knockdown (shRIAM) cells stimulated with glutamine present a remarkable increase in integrin clustering, comparable to the one observed for PMA, and that observed for similarly treated control cells. This may account for the increased association index previously observed. C Cluster volume is markedly increased in shRIAM cells and comparable to levels found after PMA stimulation. Error bars denote standard deviation, asterisks denote a significance of $p \leq 0.05$.

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Conclusions

Glutamine signalling results in increased particle association due to the modulation of receptor avidity through clustering. This effect is dose-dependent and negatively regulated by RIAM. Our results are in agreement with the increased phagocytosis observed in burn patients, which are in an immunodepressed state (mimicked by RIAM-deficient cells). This clustering may require cytoskeletal rearrangements, as RIAM binds actin cytoskeleton regulators. Further studies are underway to elucidate the mechanisms and their dependence on other RIAM-associated proteins such as VASP and Vinculin.

7.2.3. Other Publications

1. Cardeñes B, Clares I, Toribio V, Pascual L, López-Martín S, **Torres-Gomez A**, Sainz de la Cuesta R, Lafuente EM, López-Cabrera M, Yáñez-Mó M, Cabañas C. "Cellular Integrin α5β1 and Exosomal ADAM17 Mediate the Binding and Uptake of Exosomes Produced by Colorectal Carcinoma Cells". 2021, International Journal of Molecular Sciences,22, 9938. https://doi.org/10.3390/ijms22189938.
2. **Torres-Gómez, A.**, Cardeñes, B., Díez-Sainz, E., Lafuente, E. M., & Cabañas, C. "Functional Integrin Regulation Through Interactions with Tetraspanin CD9". 2021, In *The Integrin Interactome* (pp. 47-56). Humana, New York, NY.
3. Molero-Abraham, M., Sanchez-Trincado, J. L., Gomez-Perosanz, M., **Torres-Gomez, A.**, Subiza, J. L., Lafuente, E. M., & Reche, P. A., "Human oral epithelial cells impair bacteria-mediated maturation of dendritic cells and render T cells unresponsive to stimulation". 2019, Frontiers in immunology, 10, 1434.

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