CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITECNICO NACIONAL

Unidad Zacatenco

Departamento de Biomedicina Molecular

“Papel de la proteína de unión a actina HS1 en las funciones efectoras de los neutrófilos”

TESIS que presenta

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Para obtener el grado de:

Maestro en Ciencias

En la especialidad de

Biomedicina Molecular

Director de tesis

Dr. Michael Schnoor

Ciudad de México, México Agosto, 2018

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Thesis presented by

Idaira María Guerrero Fonseca

To obtain the degree

Master of Science

In the specialty

Molecular Biomedicine

Thesis Director

Dr. Michael Schnoor

Mexico City, Mexico August, 2018

Dedicatoria

A mi familia por su amor incondicional.

A mi otro yo, mi otra mitad: Idania

A mis hijos, monty y paco.

Agradecimientos

Al Dr. Michael por permitirme desarrollar mi proyecto de maestría en su laboratorio. Por sus enseñanzas y paciencia. Por su apoyo y excelente asesoría.

A la Dra. Carmen Sánchez, Dr. Leopoldo Santos y al Dr. Carlos Rosales por la asesoría brindada.

Gracias infinitas a mis padres y hermanos por apoyarme en cada paso que doy en mi vida, aunque eso implique estar lejos de mi hogar.

A mis compañeros de laboratorio y ahora amigos, Sandy, Karla, Martha, Ramon, Ivan, Alex y Eduardo, por su ayuda y retroalimentación en el laboratorio, y por la valiosa amistad que me han brindado en este corto tiempo. A los amigos que he hecho fuera del laboratorio, Eduardo, Adrián, José Luis, Gustavo, Angie, Zaida, Daniel, Joss, Felipe, por hacer más ameno y divertido este difícil camino.

A la Dra. Hilda Vargas, nuestra auxiliar de laboratorio, por su excelente desempeño y disposición para ayudar en lo que se requiera en el laboratorio. Al Dr. Fernández encargado del bioterio, por su apoyo en el manejo de ratones de laboratorio.

Al Cinvestav por proveerme las herramientas necesarias para mi formación y por el financiamiento para asistir a congresos. Al Consejo Nacional de Ciencia y Tecnología (CONACYT) por la beca recibida durante el periodo 2016-2018 que me permitió realizar mis estudios de maestría.

Al M en C. José Castaños, por este camino que iniciamos hace 6 años. Por el impulso que me has brindado en cada momento.

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List of abbreviations

ABP	Actin binding proteins
ABTS	(2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid))
AF633	Alexa Fluor 633
Arp 2/3	Actin-Related Proteins 2/3
BCR	B Cell Receptor
BM	Bone marrow
BSA	Bovine serum albumin
Cdc42	Cell Division Cycle 42
CK2	Casein kinase
CMP	Common myeloid progenitor
CR3	Complement receptor 3
DAMPs	Damage-associated molecular patterns
DC	Dendritic cells
DHR-123	Dihydrorhodamine-123
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide Triphosphates
EC	Endothelial Cell
EDTA	Ethylenediaminetetraacetic acid
ESAM	Endothelial selective adhesion molecule
E-selectin	Endothelial selectin
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FAK	Adhesion kinase
FBS	Fetal Bovine Serum

FBS	fetal bovine serum
FcγR	Fcγ Receptor
fMLP	N-Formyl-methionyl-leucyl-phenylalanine
FSC	Forward scatter
G-CSF	Granulocyte colony-stimulating factor
GEF	Guanine nucleotide exchange factors
GM-CSF	Granulocyte-Macrophage colony-stimulating factor
H_2O_2	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
Hck	Hematopoietic cell kinase
HOCl	Hypochlorous acid
HRP	Horse-radish peroxidase
HS1	Hematopoietic cell-specific lyn substarte 1 (HS1)
ICAM	Intracellular Adhesion Molecules
ICAM-1	Intracellular adhesion molecule -1
ICAM-2	Intracellular adhesion molecule - 2
IgG	immunoglobulin G
IL-17	Interleukin-17
IL-1β	Interleukin-1-beta
IL-23	Interleukin-23
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
ITAM	Immunoreceptor tyrosine-based activation motifs
JAMs	Junctional adhesion molecules
KO	Knockout
Lck	Lymphocyte-specific protein tyrosine kinase

LFA-1	Lymphocyte-function associated antigen 1
LPS	Lipopolysaccharide
LTB_4	Leukotriene B4
Mac-1	Macrophage antigen 1
MPO	Myeloperoxidase
NE	Neutrophil Elastase
NETs	Neutrophils Extracellular Traps
NFP	Nucleation-promoting factor
NLR	NOD-like receptors
NO	Nitric oxide
NPF	Nucleation promoting factor
NTA	N-terminal acidic
NTC	Non-template Control
PAD4	Protein-arginine deiminase 4
PAMPS	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline-Tween
PCR	Polymerase Chain Reaction
PECAM1	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PI (3,4) P2	Phosphatidylinositol-4,5-bisphosphate
PI (3,4,5) P3	Phosphatidylinositol- 3,4,5-trisphosphate
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein cinase C
PMA	phorbol myristate acetate
PMN	Polymorphonuclear cells

PRRs	pattern recognition receptors
P-selectin	Platelet-Selectin
PSGL1	P-selectin glycoprotein ligand-1
RLR	RIG-like receptors
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RT	Room Temperature
SCF	Stem Cell Factor
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFK	SFK
SSC	Side Scatter
Syk	Spleen tyrosine kinase
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween
TCR	T-Cell Receptor
TEM	Transedothelial Migration
TLR	Toll-like receptors
TNFα	Tumor Necrosis Factor-α
VCAM-1	Vascular cell adhesion protein 1
VE-cadherin	Vascular endothelial-cadherin
VLA-4	Very Late Antigen-4
WASp	Wiskott-Aldrich Syndrome protein
WAVE	WASP-family verprolin-homologous protein 2
WT	Wild type

RESUMEN

Los neutrófilos son reconocidos como mediadores clave de los procesos inflamatorios. Estas células emplean múltiples mecanismos para eliminar patógenos, incluyendo la captación de patógenos por fagocitosis, la producción de especies de oxígeno altamente reactivas (ROS), la liberación de contenido granular y la liberación de trampas extracelulares de neutrófilos (NET). La mayoría de las funciones son reguladas por la reorganización del citoesqueleto de actina y por proteínas moduladoras de actina. HS1 es una proteína de unión a actina que promueve la dinámica de actina a través de su interacción con el complejo Arp2/3 y con algunas GTPasas como Rap1, Rac1 y Cdc42. HS1 se expresa exclusivamente en células hematopoyéticas y está involucrado en múltiples funciones en diversas células inmunológicas. Se ha reportado que HS1 es requerida para la adhesión, quimiotaxis y extravasación de neutrófilos, pero su participación durante las funciones efectoras de estas células no se ha investigado. En este estudio, evaluamos el papel de HS1 en la respuesta efectora de los neutrófilos. El análisis de la captación de zymosan por inmunofluorescencia, mostró que la ausencia de HS1 en neutrófilos resultó en una significativa reducción en el porcentaje de fagocitosis y en el número de partículas fagocitadas por célula. Además, la localización de HS1 en las copas fagocíticas de los neutrófilos WT, sugiere que HS1 tiene un papel importante para la fagocitosis eficiente. Sin embargo, encontramos que tanto neutrófilos WT como HS1 KO tienen la misma capacidad de producir ROS en respuesta a PMA, fMLP y LPS. En conjunto, nuestros resultados sugieren que HS1 regula la efectividad del proceso de fagocitosis, pero no es requerida para la producción de ROS.

ABSTRACT

Neutrophils are recognized as key mediators of inflammatory processes. These cells exert multiple mechanisms to eliminate pathogens, including the uptake of pathogens by phagocytosis, the production of highly reactive oxygen species (ROS), release of granular content, and the release of neutrophils extracellular traps (NETs). Most functions are regulated by the reorganization of the actin cytoskeleton and by actin modulating proteins. HS1 is an actin-binding protein that promotes actin dynamics through its interaction with the Arp 2/3 complex and with some GTPases such as Rap1, Rac1 and Cdc42. HS1 is expressed exclusively in hematopoietic cells and fulfills multiple functions in various immune cells. It has been reported that HS1 is required for adhesion, chemotaxis and extravasation of neutrophils, but its participation during neutrophil effector functions has not been investigated. Thus, in this study, we evaluated the role of HS1 in the neutrophil effector response. Analysis of the uptake of zymosan by immunofluorescence showed that the absence of HS1 in neutrophils resulted in a significant reduction in the percentage of phagocytosis and in the number of phagocytosed particles per cell. In addition, HS1 localized at phagocytic cups of WT neutrophils suggesting that HS1 is important for efficient phagocytosis. By contrast, we found that both WT neutrophils and HS1 KO had similar capacities to produce ROS in response to PMA, fMLP and LPS. Taken together, our results suggest that HS1 regulates the efficiency of phagocytosis, but that it is not required for the production of ROS.

I. INTRODUCTION

1.1. Overview of the innate immune system and inflammation

Innate immunity represents the first line of defense against invading pathogenic microorganisms (1). It begins with the physical barriers formed by the internal and external epithelial layers, and molecules they produce (mucus, defensins, and antimicrobial enzymes), which must to be overcome to allow entry of pathogens into the organism. If a microorganism manages to penetrate the epithelial surface, it is usually recognized and eliminated efficiently by mechanisms of innate immunity, which starts immediately in tissues underlying the epithelia. A variety of immune cells and plasma proteins work together to prevent the dissemination of pathogens. Macrophages, dendritic cells, neutrophils, basophils, eosinophils, mast cells and natural killer cells are considered the effector leukocytes of innate immunity (1,2). All these cells are armed with a variety of pattern recognition receptors (PRRs) such as Toll-like receptors (TLR), NOD-like receptors (NLR), and RIG-like receptors (RLR) that allow the detection of pathogenic microorganisms and dead cells. These receptors identify a variety of conserved pathogenic molecules, termed pathogen-associated molecular patterns (PAMPS), and molecules released by damaged and dead cells, termed damage-associated molecular patterns (DAMPs) (3,4).

Once such molecules have been recognized by tissue-resident innate immune cells, they initiate an inflammatory response in order to eliminate the causing agent. During this process, proinflammatory cytokines such as interleukin-1-beta (IL-1β), IL-6, tumor necrosis factor-alpha (TNF-α), and a variety of chemokines are secreted. Cytokines induce changes that include increased blood flow in inflamed tissues, vasodilatation, increased adhesion of circulating leukocytes to the endothelial wall, and increased vascular permeability leading to plasma protein leakage (complement, antibodies, and acute phase reactants) to extravascular tissue (5,6). Endothelial cells get activated and express adhesion molecules that, together with the released chemokines, capture, activate and recruit leukocytes to the inflamed tissue. In this context, neutrophils are the first leukocytes to reach the sites of inflammation and mediate the onset of the effector response. At sites of inflammation, these cells have a critical role in directly eliminating pathogens through phagocytosis or secretion of reactive oxygen species (ROS) and antimicrobial peptides (7).

1.2. Neutrophils

Neutrophils, or polymorphonuclear leukocytes (PMN), are a population of short-lived myeloid cells that are characterized by their multilobed segmented nucleus and their high content of cytoplasmic granules. They represent approximately 15% of total leukocytes in the peripheral blood in mice, while in humans neutrophil abundance reaches up to 50-70% of total leukocytes in the circulation (7,8). Neutrophils are considered key players during early stages of an inflammatory process because they are the first to arrive at these sites (9,10).

1.2.1. Granulopoiesis

Neutrophil production occurs in the bone marrow (BM) during hematopoiesis. This is driven by different transcription factors and a coordinate set of cytokines such as interleukin-1 (IL-1), interleukin-3 (IL-3), SCF, G-CSF, GM-CSF, but depends mainly on granulocyte colony-stimulating factor (G-CSF), which is produced and secreted by macrophages, T cells, endothelial cells, and fibroblasts after activation by antigen recognition or cytokines such as IL-1, IL-6, and TNFα (11). During inflammation, macrophages also regulate granulopoiesis through the production and secretion of IL-23. IL-23 stimulates the production of IL-17 by T lymphocytes, which upregulates G-CSF production (11,12)

The process begins with stimulation of hematopoietic stem cell by interleukin-1 (IL-1), interleukin-3 (IL-3), SCF, G-CSF, GM-CSF that gives rise to a common myeloid progenitor (CMP). The CMP are further stimulated by IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) to form myeloblasts, the progenitors of neutrophils, macrophages and eosinophils. Then, while stimulation with GM-CSF directs the formation of monocytes, the stimulation of these cells with G-CSF leads to the differentiation, maturation and functional activation of neutrophils. Subsequently, development of neutrophils can be divided into 5 sequential stages based on nuclear morphology, cell size and granule content (Figure 1) (12,13). Myeloblasts have a round and large nucleus, small amount of cytoplasm and granules are still absent. Promyelocytes are larger than myeloblasts and contain more cytoplasm. They begin to form azurophil granules regulated by the transcription factor Gfi-1 and C/EBP-α. Myelocytes are smaller than their predecessors and have an eccentric nucleus. In this stage, the transcription factor C/EBP-ε, controls the formation of secondary granules.

Metamyelocytes have a kidney-shaped nucleus and contain tertiary granules. Neutrophils with a band-shaped nucleus represent the last stage of maturation that is maintained in the bone marrow. These cells contain all 3 types of typical neutrophil granules. Finally, the transcription factors C/EBP-β,δ,ζ and PU.1 determine the transition to segmented neutrophils, which are fully mature granulocytes with a segmented nucleus and secretory vesicles that egress from the bone marrow into the peripheral blood (11,14,15).

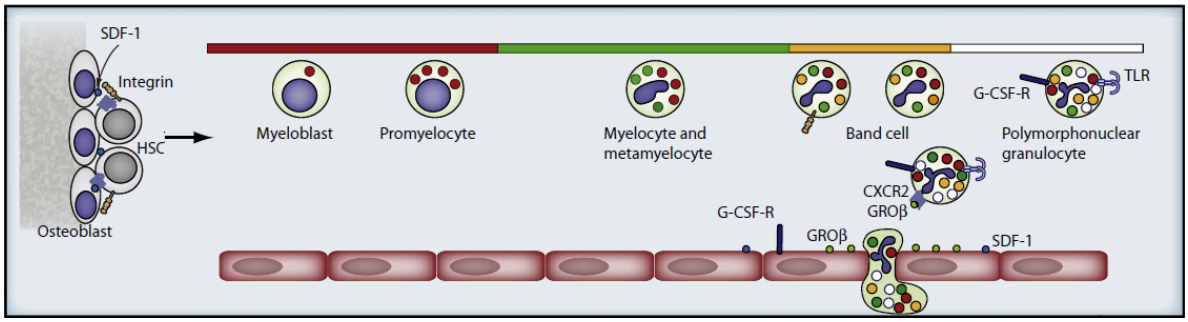

Figure 1. Granulopoiesis and granular content. Stem cells are located in the stroma of the bone marrow formed by osteoblasts and endothelial cells that produce CXCL12 to keep them in this niche. G-CSF and GM-CSF produced by macrophages, T cells, endothelial cells, and fibroblasts, act together to stimulate the generation of myeloblasts that mature into neutrophils with a segmented nucleus. During the maturation process, the granular content is formed sequentially, starting with azurophilic granules (red), specific granules (green), gelatinase granules (yellow) and secretory vesicles (white). The release of mature neutrophils from the bone marrow is regulated by increased expression of CXCR2 in neutrophils and their ligands CXCL1 and CXCL2 in endothelial cells (11).

Neutrophil trafficking from the BM to the blood circulation is controlled by retention and release signals. The maintenance of neutrophil precursors in the BM is mediated by the production of CXCL12 by BM stromal cells, which stimulates signaling via CXCR4 that is highly expressed in immature neutrophils (16). By contrast, the release of mature neutrophils is determined by signaling via the CXCR2 receptor and its ligands CXCL1 and CXCL2. G-CSF can act on immature neutrophils by inducing their complete maturation and increasing the expression of CXCR2, and on stromal cells by downregulating the expression of CXCL12, and upregulating the expression of CXCL1 and CXCL2 in BM endothelial cells (11,14,17) (Figure 1). Therefore, high levels of G-CSF induce intravasation of mature neutrophils from the BM into the systemic circulation.

Neutrophils have a half-life of 6-8 hours in the circulation. During this time, these cells await the signals to be recruited to sites of inflammation;but it has also been observed that neutrophils can also capture pathogens in the blood by intravascular NET release during endotoxemia (18). Once the neutrophils are recruited into the tissues and accomplish their function, they undergo apoptosis and are finally eliminated by phagocytosis by resident macrophages and dendritic cells. Neutrophil apoptosis also regulates the generation of neutrophils in the bone marrow. After being phagocytosed, macrophages reduce their production of IL-23 and as a consequence, the production of G-CSF is reduced. This results in the reduction of neutrophil generation when they have eliminated the pathogens and are no longer required in the tissues (11).

Blood neutrophils are guided by the vascular endothelium to the site of inflammation through a gradient of chemokines. When neutrophils are recruited into the extravascular tissue, they are further activated by several stimuli including cytokines such as TNF-α, IFN-γ, and IL-1β; chemoattractants such as CXCL1/2, C5a, LTB4; adhesion molecules; and pathogen molecules such as lipopolysaccharide (LPS) or N-Formyl-methionyl-leucyl-phenylalanine (fMLP). Following activation, these cells exploit various mechanisms to fight pathogens. They can phagocytose a large number of pathogens, release their granular content to the extracellular environment, rapidly produce large amounts of reactive oxygen species (ROS), and form neutrophil extracellular traps (NETs) (8,10).

1.2.2. Phagocytosis

Phagocytosis is a process in which cells engulf large particles (>0.5 μm) including pathogenic microorganisms in order to eliminate them (19,20). This process depends on actin polymerization and requires the recognition of ligands on the surface of target particles by specialized receptors on immune cells. Neutrophils are recognized as professional phagocytes, since they express a wide range of phagocytic receptors allowing them to recognize several targets (21). Some of these receptors such as the mannose and dectin-1 receptors recognize polysaccharides, while others recognize particles opsonized with immunoglobulin G (IgG) molecules, and complement products. Neutrophils express the type 3 complement receptor (CR3, known as Mac-1 or CD11b/CD18) and CR4 (CD11c/CD18) that recognize fragments of C3 degradation such as iC3b (inactive C3b complement); and Fcγ-receptors (RI, RIIA,

RIIIB) that recognize the Fc portion of IgG (22,23). After interaction of receptors with their ligands, receptor-bound signaling molecules induce complex signaling cascades leading to the polymerization of actin and remodeling of the plasma membrane to engulf the target and form the phagosome, a specialized and dynamic subcellular structure that allows to compartmentalize pathogens to allow their elimination by various mechanisms (24,25).

Phagocytosis mediated by FcγR occurs through pseudopods that are extended around the target particle coated with IgG (Figure 2) (26). The process begins with the recognition of the particle that induces the phosphorylation of ITAMs (immunoreceptor tyrosine-based activation motifs) in the cytoplasmic tail of FcγR by Src family kinases (mainly Lyn and Hck). Next, Syk kinase binds to ITAM and phosphorylates phosphatidylinositol 3-kinase (PI3K) to activate it, thus inducing the conversion of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) into phosphatidylinositol- 3,4,5-trisphosphate (PI(3,4,5)P3), which plays a central role for signal transduction during phagocytosis. Syk also has an important role in the activation of the GTPase Rac by activating its GEF Vav-1. Several molecules that promote the polymerization of actin also require a rapid recruitment to membrane regions that are in contact with the target. These include small GTPases Rac1 and Cdc42 that activate the actin nucleators WASP and WAVE (24,27), which in turn activate the Arp2/3 complex to mediate local generation of branched F-actin networks (28,29). This assembly of actin filaments induces the prolongation of pseudopods around the target particle forming the phagocytic cup. Proteins that regulate the contraction of actin filaments, such as myosins, support the complete extension of the membrane and the fusion of the edges of the cup to close the phagosome (30,31).

After internalization, the phagosome initiates a maturation process. Unlike macrophages, where the phagosome follows the route of endosomal maturation, neutrophils do not have a classic endosomal pathway. In neutrophils, the intracellular preformed granules are recruited by microtubules to fuse with the phagosome. Additionally, the NADPH oxidase complex is assembled in the phagosome membrane to initiate the respiratory burst within the phagosome (30). The wide variety of effector enzymes contained in the granules and the release of ROS within the phagosome, allows the rapid elimination of the pathogen.

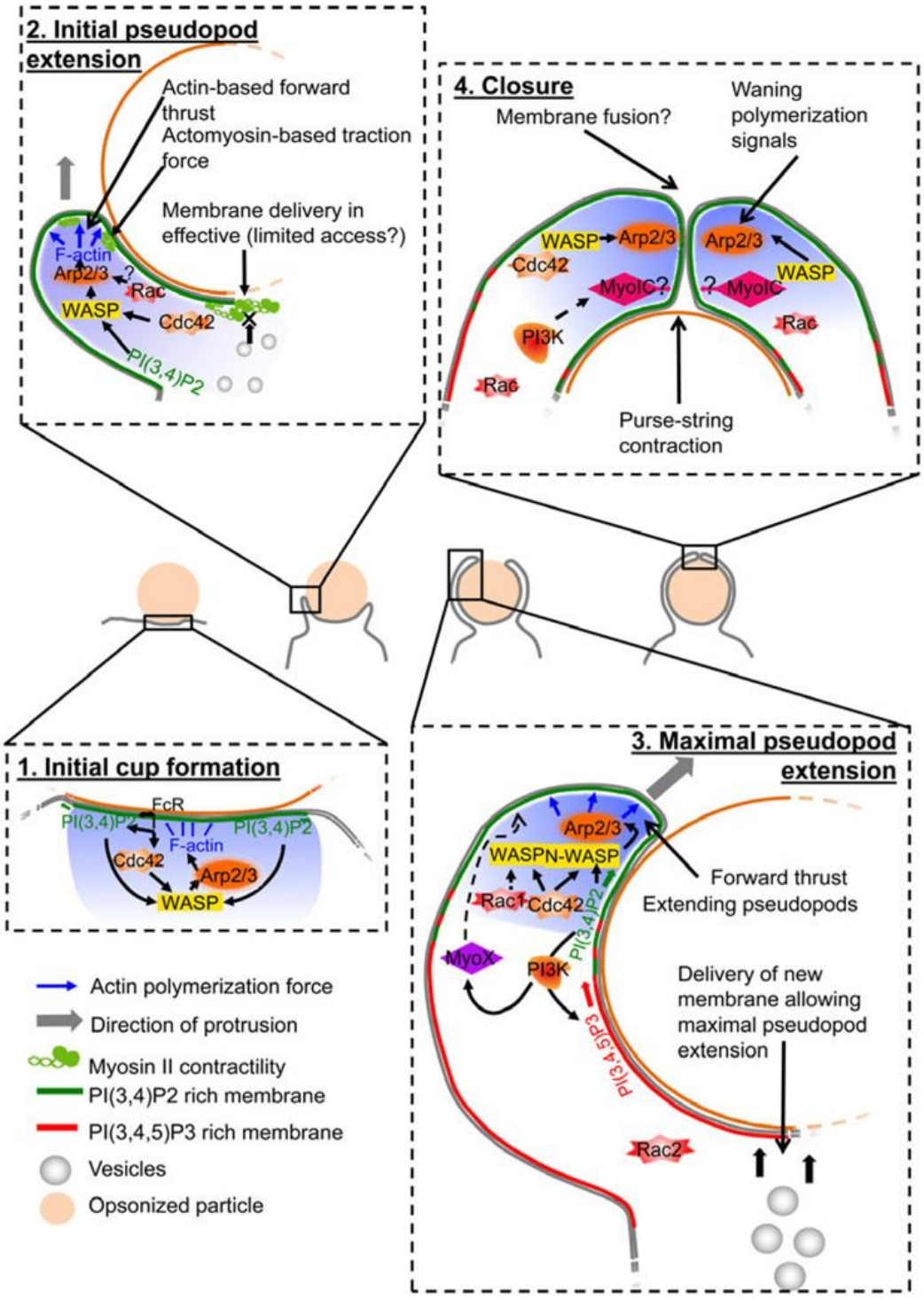

Figure 2. Effector moleculess during phagocytosis. (1) During the onset of phagocytosis, Cdc42 triggers the assembly of Arp2/3-dependent branched actin filaments at contact areas. In these regions, PI(3,4)P2 is present for signal transduction during phagocytic cup formation. (2) Polymerization of actin, and actomyosin contractility promote engulfment of the pathogen by pseudopods that form a phagocytic cup. (3) Once the cup has formed, actin dynamics are enhanced until the pseudopod completely covers the particle. (4) When the pseudopod envelops the particle completely, myosins support the fusion of the membrane edges to close the phagosome (23).

1.2.3. Degranulation

Cytoplasmic granules are a distinctive feature of neutrophils and represent a crucial microbicidal weapon during their effector response. This is due to the variety of cytolytic enzymes, antimicrobial peptides and matrix proteins that are stored within these granules. These are divided into three types based on the proteins they store: primary granules or azurophils contain mainly nyeloperoxidase (MPO), elastase, defensins, cathepsin G and inducible nitric oxide synthase (iNOS); secondary or specific granules contain lactoferrin and lysozyme; and tertiary granules contain mainly gelatinase and metalloproteinases. All of these proteins are derived from the trans-Golgi pathway during the maturation of neutrophils (9,11). They also contain secretory vesicles that rapidly mobilize proteins that need to be transported to the cell surface. The granular content can be fused with phagosomes to induce its maturation or can be release into the extracellular medium by exocytosis. The release of granular contents can be induced by chemokine and cytokine stimulation, adhesion, or integrin activation. But, unlike phagocytosis where actin polymerization is fundamental, degranulation requires depolymerization of actin fibers because the cortical actin network blocks the access of the granules to the plasma membrane and therefore its fusion (32).

Phagosomes fuse with azurophil granules that release cationic peptides such as α-defensins and cathelicidins that can form pores in the bacteria's negatively charged membrane and inhibit the synthesis of DNA and RNA to induce pathogen death (33). Proteolytic enzymes are also released into the phagosome. Lysozyme destroys the bacterial wall, whereas neutrophil elastase (NE) and cathepsin G (CG) can cleave proteins from the plasma membrane. Other microbicidal proteins such as lactoferrin and calprotectin can chelate important metals for bacterial growth thus preventing their survival within the phagosome (34,35).

The release of granules into the extracellular space can also occur during the transmigration of neutrophils. The proteases contained in the granules facilitate the degradation of the basement membrane that underlies the endothelial wall, and therefore the elimination of a barrier that limits its extravasation. This favors the rapid exit of the neutrophil to the extravascular tissue. Likewise, the granules can be released directly into the inflamed tissue where they can cause tissue damage if this process is not well controlled (8,34).

1.2.4. Oxidative burst: generation of reactive oxygen species

The respiratory burst is one of the most important cytotoxic mechanisms used by neutrophils. This is characterized by the dramatic increase in the consumption of molecular oxygen (O_2) that is used by NADPH-oxidase to generate reactive oxygen species (ROS). The NADPH-oxidase complex can be assembled at the plasma membrane and at the phagosomal membrane, where it then catalyzes the reduction of O_2 to generate superoxide ions (O_2-) and hydrogen peroxide (H_2O_2) that are released into the extracellular space or inside the phagosome (10,30). MPO is an enzyme contained in primary granules that catalyzes the production of hypochlorous acid (HOCl) from hydrogen peroxide and chloride. MPO can also convert molecular oxygen into singlet oxygen. In addition, primary granules contain the enzyme iNOS that produces reactive nitrogen species (RNS) such as nitric oxide (NO), which also exerts bactericidal effects (36).

NOX2 is the NADPH-oxidase found in neutrophils, and it is the most potent to produce ROS. A functional NADPH-oxidase requires the efficient assembly of its 6 subunits (Figure 3): the transmembrane component flavocytochrome b558, which consist of the subunits gp91phox (or NOX2) and p22phox, the three regulatory cytosolic components, p47phox, p40phox and p67phox, and the small GTPase Rac1 or Rac2 (37,38). After stimulation by inflammatory mediators or capture of pathogens, the cytosolic components are translocated to the membrane through binding to the membrane-bound subunits gp91phox and p22phox. The association of the cytoplasmic complex is regulated by the interaction of p47phox with p22phox. This interaction requires p47phox to become phosphorylated by PKC. Thus, p47phox serves as an adaptor protein allowing the assembly of the cytosolic complex and the membrane complex. In addition, a key step during complex assembly is the interaction of p67phox with active Rac1 or Rac2 (39,40).

ROS production is induced in neutrophils after capture of pathogens or stimulation by inflammatory mediators. (8,11). These highly reactive molecules can attack a variety of organic molecules, act as cytotoxic agents, and create a hostile environment for pathogens within the phagosome. ROS can damage DNA directly, either by producing mutations or breaking the DNA chain. It can also interact with proteins inducing a series of oxidative modifications, and trigger peroxidation of polyunsaturated fatty acids in the membrane. In addition, O_2^- can also boost the flow of potassium into the phagosome,

thus promoting the release of granule proteases into the phagosome. On the other hand, RNS can inhibit respiration and interfere with DNA replication leading to cell death (41–43). Both ROS and RNS are unspecific for their targets, and therefore they can damage both pathogens and host cells. When they are produced in excess, oxidative stress occurs, which can eventually cause serious tissue injury (36,43).

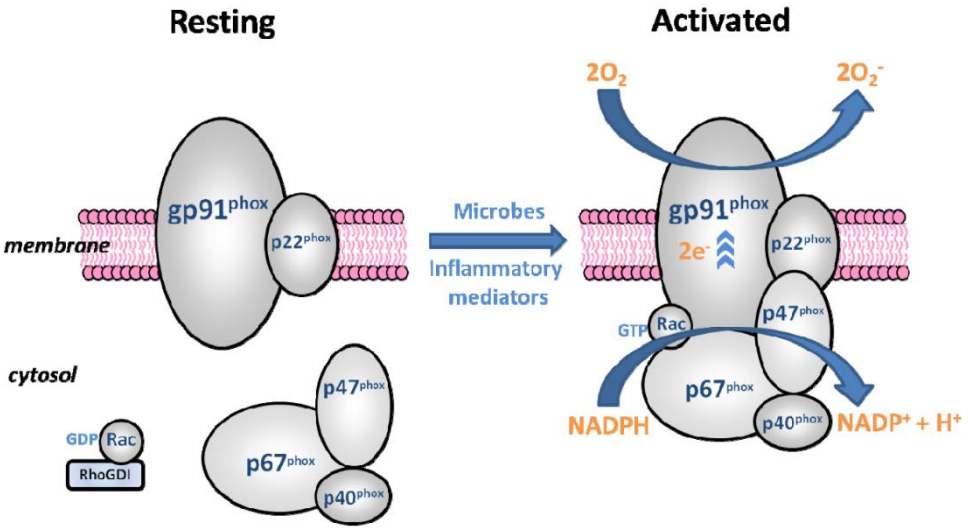

Figure 3. NADHP-oxidase assembly. The resting form of NADPH-oxidase consists of the 2 catalytic subunits gp911phox and p22phox associated to the membrane that form the flavocytochrome b558; the three cytosolic components, 47phox, p67phox, and p40phox; and the GTPase Rac. After stimulation by proinflammatory mediators or pathogen recognition, Rac gets activated and triggers translocation of the cytosolic complex to the membrane where it binds to flavocytochrome b558. Once assembled, the complex catalyzes the generation of superoxide (O_2^-) by transferring one electron from NADPH to oxygen (39).

1.2.5. Neutrophils extracelular traps (NETs)

In 2004, a novel cytotoxic mechanism was described by the group of Dr. Zychlinsky, which consists of the expulsion of net-like structures formed by decondensed chromatin fibers, histones, and granular enzymes (44,45). Analysis by immunofluorescence revealed the presence of proteases and antimicrobial proteins such as lactoferrin, cathepsin G, defensins, elastase, proteinase 3, gelatinase, and ROS-producing enzymes, such as MPO within NETs (44). The main function of these structures is to immobilize pathogens to block tissue dissemination and facilitate phagocytosis (9,46).

The exact mechanism that induces the formation and liberation of these structures is not completely understood. However, various stimuli known to activate neutrophils,

such as LPS, activated platelets, pro-inflammatory cytokines (TNF-α), PMA (phorbol myristate acetate) and stimulation of Fc receptors can trigger the rapid process of cell death in neutrophils, called NETosis, during which NETs are formed (47–50)(48,49). Interestingly, it has been shown that in some cases the release of NETs does not lead to cell death, a process called "vital NETosis" (51,52). In addition, it has also been seen that some noninfectious stimuli such as autoantibodies, platelet activation, coagulation, and cholesterol crystals can induce the formation of NETs in sterile inflammation such as systemic lupus erythematosus, rheumatoid arthritis, vasculitis, thrombosis and other autoimmune diseases. However, their role during these conditions is not well understood (49).

After neutrophil activation, NETosis begins with the activation of PKC that leads to the assembly and activation of the NADPH-oxidase complex and ROS production, which is fundamental for the formation of NETs (47). However, it is not yet clear how ROS induce NETosis. In addition, translocation of elastase and MPO to the nucleus occurs before the release of DNA into the cytoplasm (53). Both enzymes induce morphological changes within the nucleus such as decondensation of chromatin and disassembly of the nuclear envelope and granule membranes. Moreover, citrullination of histones by the enzyme PAD4 (protein-arginine deiminase 4) is necessary to decondens chromatin by reducing the positive charges of histones (53,54). Next, chromatin is released into the cytoplasm where it interacts with granular proteins. Finally, these intracellularly-formed complexes are released as NETs causing rupture of the plasma membrane and cell death (45,53,55). In the case of vital NETosis, assembly occurs similarly, however, assembled NETs are released through vesicles, thus keeping the neutrophil alive to fulfill other functions such as phagocytosis (49,56).

Although NETs have been recognized as a strategy that neutrophils apply to immobilize pathogens and prevent their dissemination, they have also been described to cause tissue damage during inflammatory responses. This is because these structures can also be released into the circulation, thus trapping erythrocytes, platelets and proteins such as fibrinogen and fibronectin, eventually leading to the formation of thrombi that can occlude the microvasculature and cause tissue hypoxia (57,58). In addition, the DAMPs released to the extracellular medium during the expulsion of the NETs amplify the immunological response, thus prolonging the inflammatory reaction that, if not controlled properly, can also cause tissue damage.

1.2.6. Recruitment and extravasation of neutrophils

Transmigration of neutrophils through the vascular endothelium is a key event during the initiation of the inflammatory response (9). This is a complex multi-stage process that is mediated by cytokines, chemokines and a range of cell adhesion molecules such as integrins and selectins. The cascade of events includes the initial binding of the neutrophil to the endothelium, rolling on the endothelial surface, firm adhesion, crawling towards the exit sites, followed by diapedesis into extravascular tissue (59,60).

The process begins with the capture of the neutrophil to the endothelial wall, which requires endothelial cell activation by inflammatory mediators (IL-1β, TNF-α, IL-17, histamine and thrombin) secreted by resident immune cells and tissue cells at the inflamed site (61,62). Endothelial activation involves an increase in the expression of adhesion molecules such as ICAM-1, ICAM-2 (intracellular adhesion molecule -1 and -2), VCAM-1 (Vascular cell adhesion protein 1), E-selectin and P-selectin. Initially, selectins interact with their ligands PSGL1 (P-selectin glycoprotein ligand-1) and L-selectin present in the microvilli of neutrophils. This union establishes a weak and transient anchor that allows tethering of the neutrophil to the surface of the endothelium, and its subsequent rolling along the vessel wall in the direction of the blood flow (61,63,64). Chemokines produced in inflamed tissues such as IL-8 (in human), CXCL1 (in mouse), C5a, and LTB_4, are exposed on the endothelial surface where they are recognized by their receptors expressed on neutrophils during rolling. The stimuli generated by the engagement of selectins and chemokines induce the activation of the β2-integrins LFA-1 (αLβ2 or CD11a / CD18) and Mac-1 (αMβ2 or CD11b / CD18), and the β1-integrin VLA-4. Integrin activation is mediated by a conformational change in its structure that results in increased affinity for its ligands ICAM-1, ICAM-2 and VCAM-1, respectively (64). The interaction between ICAM-1 and LFA-1 increases adhesion strength and decreases the speed of rolling to allow firm arrest of neutrophils. Next, the neutrophils rearrange their cytoskeleton to spread on the endothelial surface and begin crawling towards the exit site, a process mediated by interactions between Mac-1 and ICAM-1 (Figure 4) (59,65).

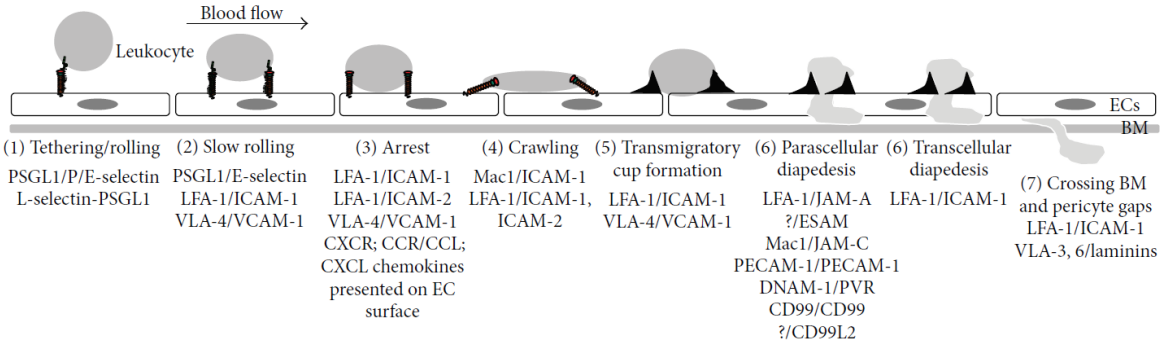

Figure 4. Trans-endothelial migration (TEM) of neutrophils: The diagram shows the sequential steps of neutrophil recruitment, as well as the interactions between the main adhesion molecules of the endothelium and the neutrophil that define each step of the adhesion cascade (60).

Finally, the actual transendothelial migration step, termed diapedesis, can occur via two distinct ways: preferentially, through the junctions between endothelial cells, called the paracellular pathway; or through the body of endothelial cells, called the transcellular route. The paracellular route is preferred in vivo by 90% of neutrophils (66), and it requires the transient and reversible opening of interendothelial junctions, and adhesive interactions between the neutrophil receptors and receptors present at the lateral region of the endothelial cells such as JAMs, PECAM1, CD99, ICAM-2, and ESAM (endothelial cell-selective adhesion molecule). During extravasation, these molecules can interact heterophilically with LFA-1 and Mac-1, and homophilically with JAM-A, PECAM-1 and CD99, located on the surface of the neutrophil to facilitate its passage through the endothelium (61,67). At cell contact areas, a large amount of ICAM-1 accumulates to form so called transmigratory cups that guide neutrophils to the spot of transmigration. The signals induced by the ICAM-1 clusters activate the tyrosine kinases Src and Pyk-2, which phosphorylate the intracellular region of VE-cadherin to promote its internalization. In addition, RhoA gets activated to induce the formation of contractile actomyosin stress fibers. Both mechanisms result in the disassembly of intercellular junctions to facilitate neutrophil passage (11,61).

After crossing the endothelium, neutrophils must pass through the basement membrane and the pericyte layer that surrounds the venules to reach the extravascular space where neutrophils can perform the effector functions described above. All of the described neutrophil functions require different forms of actin remodeling that is mainly regulated by actin-binding proteins (ABP).

1.3. Hematopoietic cell-specific lyn substrate1 (HS1)

HS1 is an ABP that is homologous to cortactin. It is specifically expressed in hematopoietic cells and has been shown to participate in several immune cell functions as described below (68–70).

HS1 has a multi-domain structure that allows it to interact with several proteins regulating actin cytoskeleton dynamics (Figure 6). It has an N-terminal acidic (NTA) domain that interacts directly with the Arp2/3 complex, followed by three and a half repeats of amino acids tandem region and a coiled-coil region that together mediate actin filament binding. In this way, HS1 has the ability to activate actin polymerization by simultaneous binding of F-actin and the Arp2/3 complex and, therefore, is considered a nucleation-promoting factor (NPF) (71–73). The central region contains a proline-rich domain, and many targets for phosphorylations. The C-terminal end contains an SH3 domain that can interact with actin-regulatory proteins such as WASP, Lck, Vav1, and dynamin 2 (Figure 6) (69,74–78). Due to this ability to interact with multiple molecules, HS1 also functions as a scaffold protein.

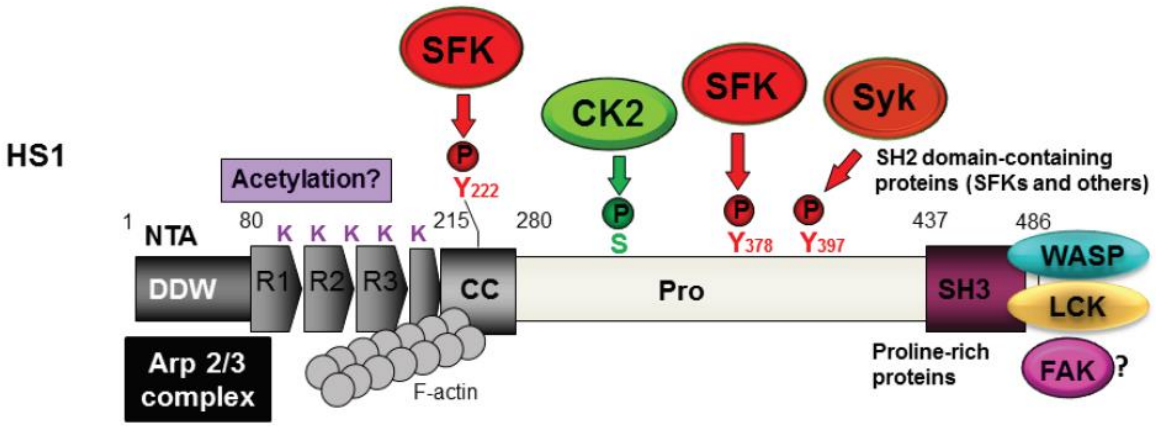

Figure 6. Structure of HS1 and interacting molecules. HS1 has an acidic domain at the amino end, 3 and a half repeats of amino acid and coiled-coild which both interact with F-actin, a proline-rich region and a SH3 domain at carboxyl end. CC, coiled-coil region; CK2, casein kinase 2; FAK, focal adhesion kinase; K, lisina; Lck, lymphocyte-specific protein tyrosine kinase; NTA, acidic domain; WASP, Wiskott–Aldrich protein. Pro, proline-rich region; R, amino acid repeat; SFKs, Src family kinases; SH3, Src homology domain; Syk, spleen tyrosine kinase (77).

Several studies reported multiple post-translational modifications in HS1 in response to inflammatory stimuli. The central region is phosphorylated at different tyrosine residues by several kinases of the Src family (SFK) including Syk, Yes, Fyn, Fgr, Lyn, Hck, Lck and Abl, and serine residues by CK2 (75,79–81). These phosphorylations create anchoring sites for proteins containing SH2 domains such as the kinases Abl, PI3K and PLCγ, or allow interaction with signaling molecules and adaptors such as the Rac-GEF Vav1 (69,81). This network of interactions emphasizes the importance of HS1 as adapter protein.

HS1 was first described in T and B cells as substrate of the kinases Lck and Lyn in response to BCR and TCR signaling (82). Other studies showed that HS1 regulated actin assembly during the formation of the immunologic synapse in T cells (75,82), as well as in the lytic synapse in NK cells (80). Moreover, HS1 controlled chemotaxis, cytoxicity and transendothelial migration of NK cells (80,81). In dendritic cells, HS1 interacted with WASP to induce the formation of podosomes during chemotaxis (74), and with dynamin to regulate receptor-mediated endocytosis (79). To date, little is known about the role of HS1 in neutrophils. In human neutrophils, HS1 colocalized with F-actin at the leading edge during chemotactic migration in response to fMLP. In addition, HS1 depletion in the cell line PLB-985 resulted in impaired chemotaxis towards fMLP (Figure 7) (79). Intravital microscopy of CXCL1-stimulated cremaster venules revealed that HS1-deficient neutrophils had increased rolling velocity and that the number of adherent and transmigrated neutrophils were reduced (Figure 8). Additionally, *in vivo* arrest assays after CXCL1 injection showed less adhesion of leukocytes to cremaster venules in the absence of HS1 suggesting that HS1 regulates the activation of LFA-1. Of note, this effect was due to reduced activation of the GTPases Rac1 and Rap1 in the absence of HS1 (Figure 8) (83). Taken together, these findings suggest that HS1 regulates the migration and extravasation of neutrophils by controlling the activation of Rac1, Rap1 and LFA-1. However, it is currently unknown whether HS1 also contributes to neutrophil effector functions. Given the importance of actin remodeling during these functions, and the capacity of HS1 to regulate actin dynamics, we speculate that HS1 is also involved in the regulation of phagocytosis, oxidative burst, degranulation, and NETosis.

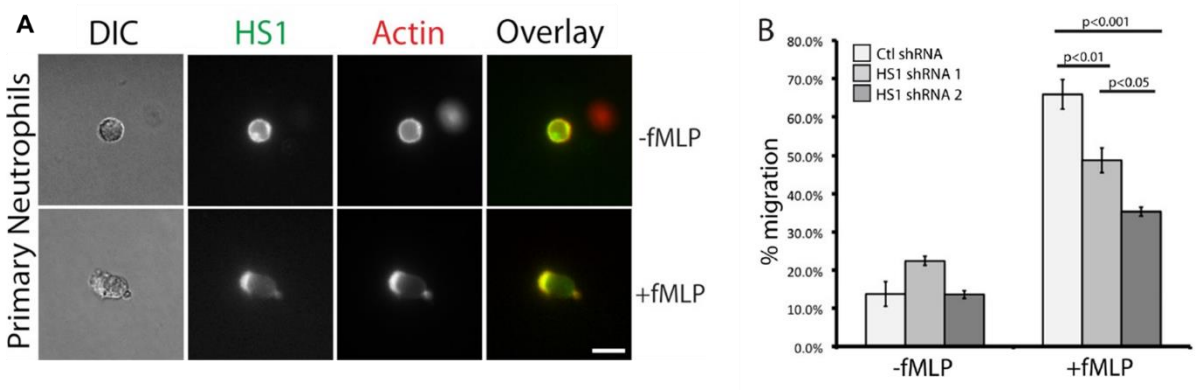

Figure 7. HS1 is required for efficient chemotaxis. (A) Immunofluorescence staining of HS1 (green) and F-actin (red) in neutrophils isolated from peripheral human blood before and after stimulation with fMLP (1µM). (B) HS1-depleted PLB-985 cells were generated using HS1-specific shRNA. Transwell chemotaxis assays were performed using transwell filters (3 µm pores) coated with 10µg/ml fibrinogen. PLB-985 cells were plated in the top chamber and cell migration towards fMLP (10nM) (bottom) was quantified by flow cytometry and depicted as percentage of the initial total cells (86).

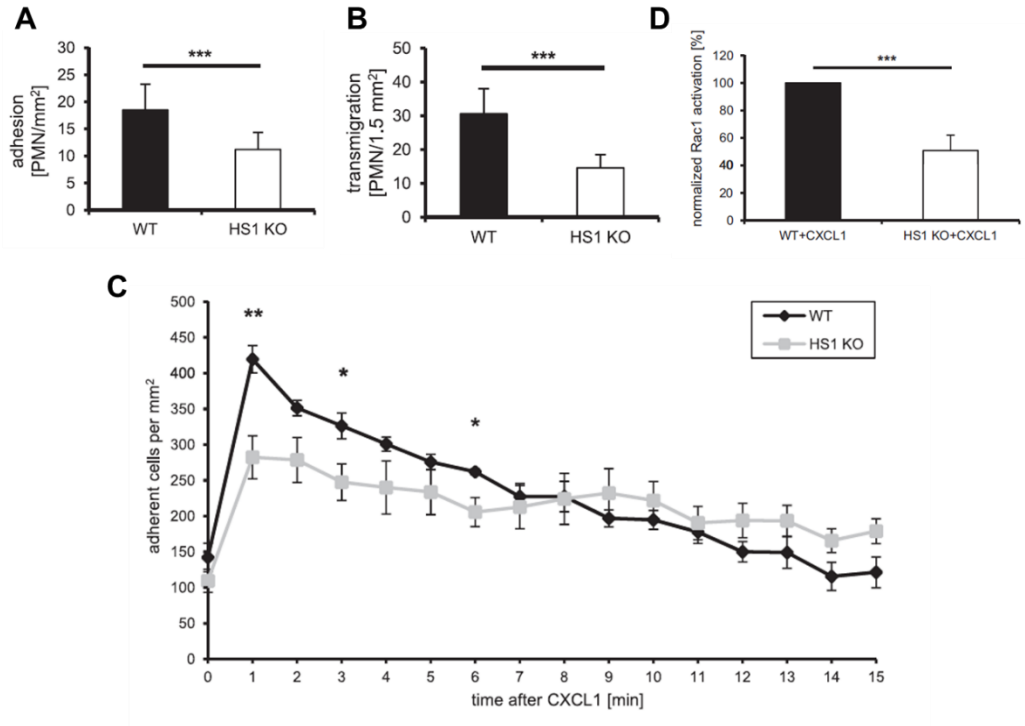

Figure 8. TEM of HS1-deficient neutrophils is reduced. Intravital microscopy of cremaster muscle venules of HS1 KO and WT mice stimulated by CXCL1 superfusion for 1 h. The number of firmly adherent cells (A), and the number of transmigrated cells (B) were evaluated. (C) Neutrophil arrest assays showed that the numbers of adherent leukocytes in cremaster venules after intra-arterial injection of CXCL1 analyzed each min was reduced. (D) Pull-down assays showed that Rac1 activation in HS1 KO neutrophils was reduced 30 s after CXCL1 stimulation (90).

II. JUSTIFICATION

Neutrophils are recognized as key mediators of the innate immune response because they are the first to arrive at sites of inflammation and rapidly eliminate pathogens using a variety of cytotoxic mechanisms. HS1 is a protein that regulates actin dynamics through binding to the Arp 2/3 complex and activation of GTPases. Likewise, HS1 is important for leading-edge formation during chemotaxis, transendothelial-migration, podosome formation and endocytosis. However, currently it remains unknown whether HS1 plays an important role for neutrophil effector functions.

III. HYPOTHESIS

HS1 deficiency decreases the efficiency of neutrophil effector functions.

IV. GENERAL OBJECTIVE

To evaluate the role of HS1 in neutrophil effector functions.

V. PARTICULAR OBJECTIVES

1. To evaluate phagocytosis in HS1 KO neutrophils compared to WT.
2. To determine production of ROS in HS1 KO vs WT neutrophils.
3. To analyze formation of NETs in HS1 KO vs WT neutrophils.

VI. MATERIALS AND METHODS

Buffers, solutions and cell culture media

1X HBSS	8000 mg/L NaCl 400 mg/L KCl 40 mg/L Na_2HPO_4 60 mg/L KH_2PO_4 350 mg/L $NaHCO_3$ 1000 mg/L D-glucose pH 7.4
1X PBS	138 mM NaCl 3 mM KCl 8.1 mM Na_2HPO_4 1.5 mM KH_2PO_4
1X RIPA	20mM Tris-HCl pH 7.5 150mM NaCl 1mM Na2EDTA 1mM EGTA 1% NP-40 1% sodium deoxycholate
5X Laemmli buffer	0.1875 M Tris-HCl pH 6.8 45% glycerol 2.5% SDS 1.78 M β-mercaptoethanol 0.00125% bromophenol blue
Agarose	Cleaver Scientific Ltd #CSL-AG500
Blocking Buffer	5 % skim-milk or BSA (SIGMA) TBS-T
HBSS Ca^{+} Mg^{2+}	HBSS 1X 140 mg/L $CaCl_2$ 120 mg/L $MgSO_4$
Lysis buffer 1 for DNA purification	25mM NaOH 0.2mM EDTA

Lysis buffer 2 for DNA purification	40mM Tris-HCl pH 5.5
Lysis buffer for protein purification	1X RIPA 3X complete protease inhibitor cocktail (Roche) 1X PhosSTOP (Roche)
RPMI-1640 Medium	Sigma #R4130
SDS-PAGE buffer	25 mM Tris 192 mM glycine 0.1% SDS pH 8.3
TBS	150 mM NaCl 10 mM Tris base pH 8.0
TBS-T	100 ml 10x TBS 0.1% Tween 20
Transfer buffer	20% methanol 25 mM Tris 192 mM glycine 0.1% SDS

Reagents and antibodies

10mM dNTP	ThermoFisher Scientific
25 mM MgCl	PROMEGA #A351H
5X Green GoTaq Flexi Buffer	PROMEGA #M891A
ABTS	SIGMA #A1888
ABTS substrate mix	1mM ABTS 1M Sodium Citrate at pH 4.5 15 mM H_2O_2 H_2O Milli-Q
Alexa Fluor 633 anti-rabbit IgG (H+L)	ThermoFisher Scientific #A21070
Cytochalasin-B	SIGMA #C6762
Fetal bovine serum	Biowest #S1810
fMLP	SIGMA #F3506
Gelatin	CTR Scientific
Goat anti-mouse IgG-HRP	Santa Cruz Biotechnology #sc-2005
Go-Taq G2 Flexi DNA	PROMEGA #M780B
Histopaque-1077 (1.077 g/ml)	SIGMA #10771
Histopaque-1119 (1.119 g/ml)	SIGMA #11191
LSP	Invivogen #
Mouse Anti-rabbit IgG-HRP	Santa Cruz Biotechnology #sc-2357
PFA (Paraformaldehyde)	SIGMA #P6148
PMA (phorbol-12-myristate-13-acetate)	SIGMA #P8139
ProLong Gold mountant with Dapi.	ThermoFisher Scientific. # P36935
Rabbit anti-HS1 (Rodent specific)	Cell signaling Technology #4557S
Rhodamine Phalloidin	ThermoFisher Scientific #R415
SuperSignal West Pico substrates	ThermoFisher Scientific. #34087
Triton X-100	Sigma #T9284
Zymosan A (S. cerevisiae)	ThermoFisher Scientific #Z2841

Animals

Female C57BL/6J wild-type (The Jackson Laboratory, Bar Harbor, Maine, USA.) and HS1-KO mice (kindly donated by Dr. Klemens Rottner, TU Braunschweig, Germany) were used at an age of 8-12 weeks. Mice were kept in the animal barrier facility at Cinvestav under specific pathogen-free conditions. All experiments have been approved by the Institutional Animal Care and Use Committee (IACUC) of Cinvestav, Mexico. In all experiments, mice were euthanized by anesthesia over-dose followed by cervical dislocation.

Genotyping of mice

To genotype the mice, genomic DNA was extracted from a segment of the tail. Tails were incubated in 75 µl of buffer 1 (25mM NaOH and 0.2mM EDTA) for 1h at 98°C in a Thermomixer (Eppendorf, Hamburg, Germany) without agitation. Then, the samples were allowed to cool and 75 µl of buffer 2 (40mM Tris-HCl pH 5.5) was added. Subsequently, all samples were centrifuged for 5 minutes at 13,000 rpm and the supernatants containing genomic DNA were transferred to fresh Eppendorf 1.5 ml tubes. PCRs were performed using the 2 µl of the supernatant, 5.5 µl of DPEC H_2O, 0.1 µl GoTaq G2 Flexi DNA polymerase, (PROMEGA, Madison, WI, USA), 0.2 µl 10Mm dNTP (Thermo Scientific, Massachusetts, USA), 2 µl Green GoTaq Flexi Buffer (PROMEGA), 1 µl 25 Mm $MgCl_2$ (PROMEGA) and 0.5 of the following primers: forward 5'-GGCATGGATGGCTGCTGGAC-3'; reverse 5'-CCTTCGTCACATGGAATATG-3'; lacZ 5'-CATGCTTGGAACAACGAGCGC-3' in a final volume of 10 µl. The PCR conditions were the following: activation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 30 s and extension at 72°C for 45 s, followed by a final extension at 72°C for 3 min. After PCR, the amplicons were analyzed by 2% agarose gel electrophoresis for 70 min at 100 mV.

Western blot

To confirm HS1 deficiency in mice, HS1 protein was detected by western blot in lung lysate. Lungs were perfused via the right hearts ventricle with 20 ml of PBS. Afterwards, lungs were dissected and homogenized on ice in 500 µl of lysis buffer (1X RIPA, 3X complete and 1X PhosSTOP) using a Polytron Tissue Master (OMNI International, Georgia, USA). The lysates were centrifuged at 12,000 rpm for 15 min, and supernatants transferred to fresh Eppendorf tubes. Proteins were denaturalized by adding 5X Laemmli Buffer (250mM Tris-HCl pH 6.8, 10% SDS, 30% glycerol, 5% β-mercapitalethanol and 0.02% bromophenol blue) and boiling at 95°C for 5 min. Cell lysates were then stored at -20°C until further use.

Equal amounts of proteins were loaded in each lane and separated by 8% SDS-PAGE for 1.5 h at 110 volts. Subsequently, proteins were transferred to nitrocellulose membranes (Bio-Rad California, USA.) for 1.5 h at 220 mA. Membranes were blocked with TBS-Tween 0.1% containing 5% skim milk for 1 h at room temperature to prevent non-specific binding of primary antibodies. Membranes were then incubated with a 1:1,000 dilution of rabbit anti-mouse HS1 (Cell Signalling Technology, Massachusetts, USA) and 1:4,000 of mouse anti-mouse tubulin (Thermo Fisher Scientific) as loading control, diluted in blocking solution overnight at 4°C with gentle agitation. Blots were subsequently washed 3 times with TBS-Tween 0.1 % for 10 minutes before incubation with anti-rabbit IgG and anti-mouse IgG secondary antibodies coupled to horse-radish peroxidase (HRP) (Santa Cruz Biotechnology, Texas, USA.) for 1 h at room temperature. Following incubation, blots were washed three times with TBS-Tween as before and then the bands were visualized using SuperSignal West Pico substrates (Thermo Fisher) and a ChemiDoc device (Bio-Rad).

Isolation of neutrophils

For functional assays, bone marrow neutrophils from WT and HS1 KO mice were isolated from bone marrow of the femurs and tibias. Bones were aseptically removed and placed in a petri dish with RPMI-1640 1X (SIGMA) medium. Bone ends were cut and then, the bone marrow cells were flushed out by injecting 5mL of cold PBS (phosphate-buffered saline) + 3% of FBS (fetal bovine serum) through the epiphysis and filtered through a 70µm nylon mesh in a 50mL falcon tube to remove bone particles. The cell suspension was centrifuged at 1500 rpm for 5 minutes at 4°C, and

then resuspended in 1mL of PBS. Cells were separated by Histopaque 1119 and 1077 density gradient centrifugation (density, 1.119 g/ml and 1.077 g/ml respectively, SIGMA, Missouri, USA) at 700xg for 30 minutes at room temperature without brakes. Neutrophils were collected at the interface of the Histopaque 1119 and Histopaque 1077 layers and washed twice with 10 ml of PBS + 3% FBS. Finally, the neutrophil pellet was resuspended in 5ml of PBS + 3% FBS and counted in a neubauer chamber with trypan blue solution to determine the number of live cells.

Blood collection

Peripheral blood was obtained by puncture of the right heart ventricle of anaesthetized mice and collected in sterile Eppendorf tubes. To obtain serum, the blood was centrifuged at 1,000xg for 10 minutes and the supernatant was transferred into a fresh Eppendorf tube. The serum was used to opsonize zymosan particles.

Phagocytosis assay

$10x10^7$ zymosan-fluorescein particles (Thermo Fisher) were incubated with 1.5 ml of fresh serum for 1 hour to allow for opsonization. $2x10^5$ neutrophils isolated from bone marrow were adhered on a cover-slip coated with gelatin in a 24-well plate (Corning Life Sciences, USA) for 30 minutes. Then, the attached neutrophils were incubated with $2x10^6$ opsonized or non-opsonized zymosan particles in 500 µL of RMPI-1640 for 60 minutes at 37°C and 5% CO_2 to allow phagocytosis. After incubation, cells were washed twice with cold PBS to stop the phagocytosis process. Subsequently, cells were fixed with 3% PFA (SIGMA) for 20 minutes at RT and then washed 3 times with 1X PBS.

Afterwards, cells were permeabilized using 500 µL 0.3% Triton X-100 in 1X PBS for 10 minutes at room temperature and then stained with rhodamine-phalloidin (Thermo Fisher) at a 1:300 dilution in 1X PBS for 1 h at room temperature to stain the actin cytoskeleton. To visualize HS1, neutrophils were blocked with 50 µL PBS + BSA 3% for 1 h after permeabilization, and then incubated with the primary antibody rabbit anti-mouse HS1 (Cell Signaling Technology) in PBS + BSA 3% at 4°C overnight. Next, cells were washed 3 times with 1X PBS and incubated with an anti-rabbit-AF633-labelled secondary antibody and rhodamine-phalloidin at room temperature for 1 h. Finally, the

cover-slips were mounted on slides with special ProLong Gold mounting medium with Dapi (Thermo Fisher) and visualized using a confocal laser microscope (SP8, Leica). Acquired images were analyzed using ImageJ software. The percentage of phagocytosis was determined by the proportion of cells that internalized particles with respect to the total number of cells in at least 100 randomly chosen fields.

Oxidative burst assay

Quantification of ROS production in WT and HS1-deficient neutrophils was measured using dihydrorhodamine-123 (DHR123) (SIGMA), which upon oxidization by ROS exhibits green fluorescence that is easily detectable by flow cytometry (84). Neutrophils ($1x10^6$ cells/ml) were pre-warmed at 37°C for 10 minutes and then incubated with 1µM DHR123 in a water bath at 37°C for 20 minutes to allow the permeable DHR-123 to enter the cells. Next, neutrophils were activated with 100nM PMA (SIGMA), 1µg/ml LPS (Invivogen, San Diego, USA), or 1µM fMLP (SIGMA) to stimulate ROS production. After 5, 10, 15, 20, 25 and 30 minutes of stimulation, the reaction was stopped by putting the cells on ice for 10 minutes. Oxidation of DHR123 to fluorescent rhodamine was measured using a Cytoflex flow cytometer (Beckman Coulter, USA). The analysis was performed only with cells found in the granulocyte gate identified in the forward-side scatter dot-plot using the FlowJo Treestar V10 software.

NET formation assay

The ability of WT neutrophils of mice and human neutrophils to form NETs was evaluated by fluorescence microscopy. $2x10^5$ neutrophils in 500 µL RPMI medium without supplements were added to 24-well plates for 30 min at 37°C and 5% CO_2. Subsequently, neutrophils were incubated with 100 nM PMA or $2x10^6$ zymosan particles in RMPI to stimulate formation of NETs for 4 h. In all experiments, unstimulated neutrophils were used as control. Following stimulation, cells were fixed with 2% PFA (SIGMA) for 30 min at 37°C. Afterwards, DNA released as NETs was stained with 150 nM DAPI + Triton X-100 0.1% in PBS for 30 min at room temperature in the dark. Finally, NETs were visualized using an inverted fluorescence microscope (Olympus IX50).

Statistics analysis

Statistical analysis was performed using Student's t-test for comparison of two groups, or one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons in the case of kinetics (GraphPad software). Data of each experiment are presented as means ± standard deviation (SD). $P \leq 0.05$ was considered statistically significant.

VII. RESULTS:

Before isolation of neutrophils, mice were genotyped using genomic DNA and end-point PCR. The PCR-amplified amplicon of HS1-deficient mice corresponds to a single band of 250 bp, whereas the WT band has a size of 350 bp (Figure 9). In the case of heterozygous mice, both bands can be observed, although heterozygous mice were not used in any experiment. In some cases, when we were unable to get a clear band pattern, mice were characterized by western blot using lung lysates. In agreement to the reported size of the protein band (74,85), we also detected the HS1 band with a weight of approximately 75kDa. This band was visible only in WT mice, but absent in HS1-deficient mice (Figure 10).

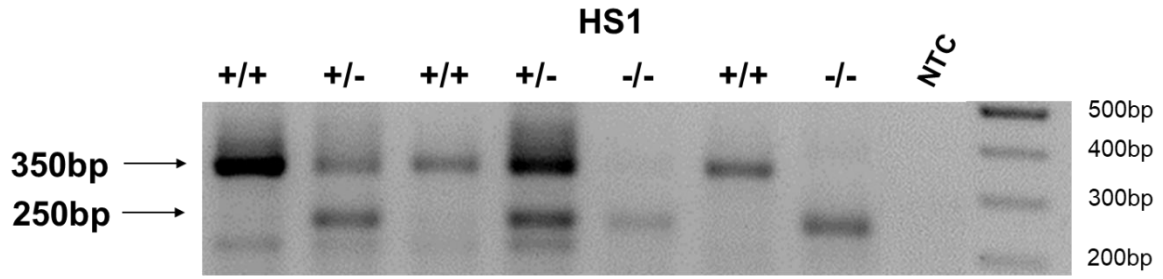

Figure 9. 2% agarose gel showing the amplicons for the indicated genotypes: WT (+/+), heterozygous (+/-) and KO (-/-) mice. NTC: no-template control.

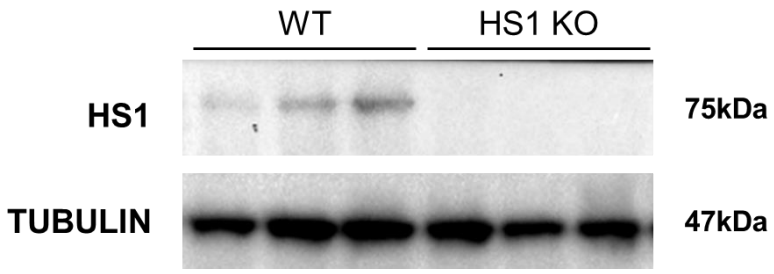

Figure 10. Representative blot from lung lysates show a band corresponding to HS1 at 75 kDa only in WT mice. Tubulin served as loading control.

HS1 regulates the efficiency of phagocytosis

To assess the role of HS1 during phagocytosis, bone marrow neutrophils from WT and HS1 KO mice were co-incubated with opsonized (Op) and non-opsonized (NOp) zymosan particles for 1 h and analyzed by immunofluorescence microscopy. To confirm particle internalization, z-stack images were captured using a Leica SP8 confocal microscope. After 1h, both WT and HS1 KO neutrophils were able to phagocytose on average 2 +/- 0.74 and 2 +/- 0.69 NOp-zymosan particles respectively and there was no statistically significant difference in the numbers of phagocytosed particles per cell (Figures 11A and B). However, the proportion of cells that phagocytosed NOp-Zym was lower in HS1 KO with an average of 29.95 +/- 10.62%, whereas 44.75 +/- 6.98 of WT neutrophils were able to phagocytose (Figure 11C). Interestingly, the frequency of phagocytosis with Op-Zym increased two-fold in both WT and HS1-KO neutrophils (Figures 12A and C). However, as with NOp-zym, HS1 KO neutrophils showed significantly lower phagocytosis capacity. While 86.84 +/- 3.68% of all WT neutrophils phagocytosed Op-Zym, only 72.08% +/- 9.11% of HS1 KO PMN phagocytosed Op-Zym (Figures 12C). Similarly, the amount of particles internalized by HS1 KO PMN was significantly reduced with only 3 +/- 0.68 phagocytosed particles per cell, whereas WT PMN phagocytosed on average 5 +/- 0.6 particles (Figure 12C). These data suggest that HS1 deficiency diminishes the efficiency of phagocytosis.

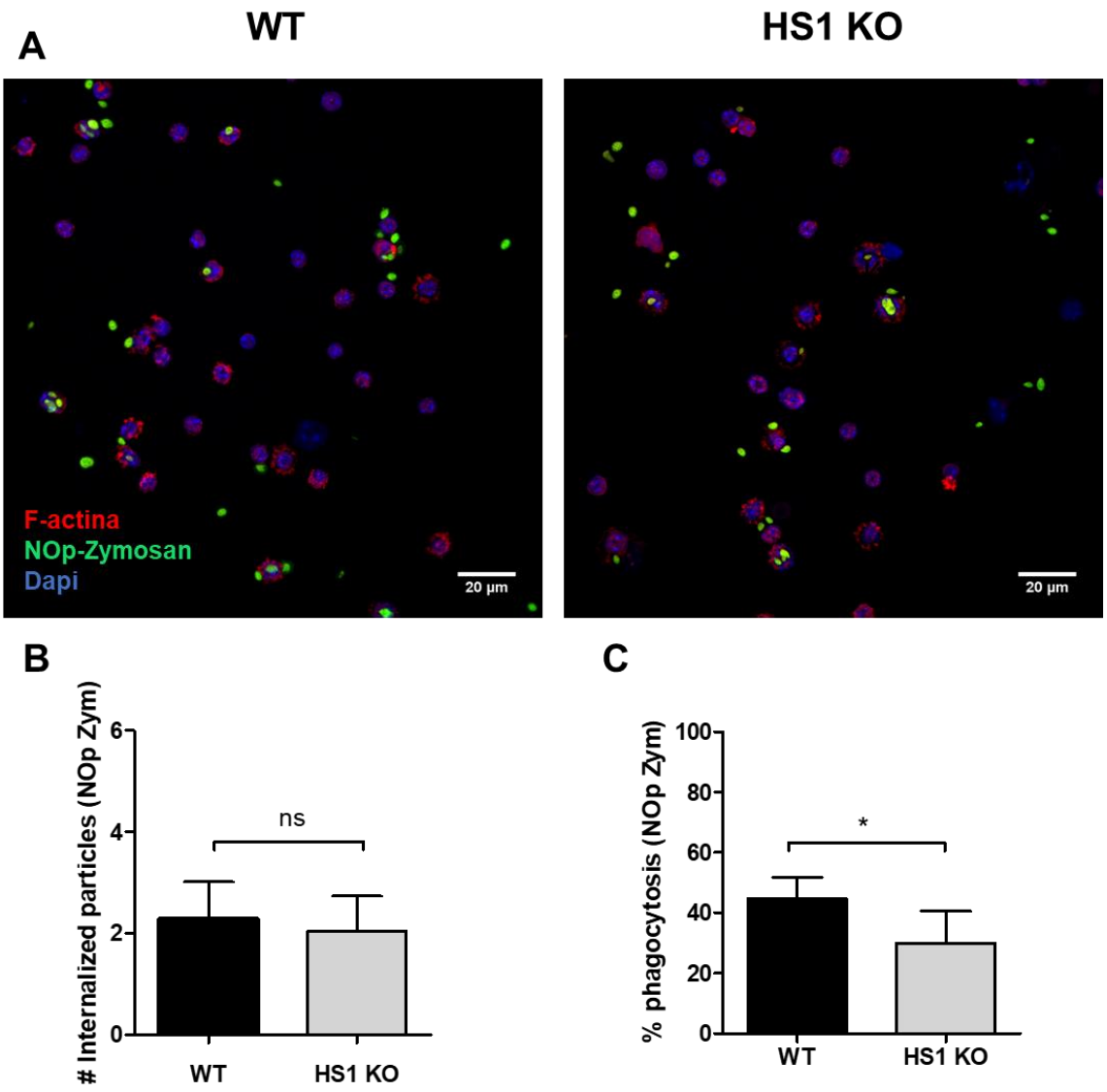

Figure 11. Phagocytosis of Non-opsonized zymosan particles. (A) WT and HS1 KO PMN were incubated with 10 non-opsonized zymosan-fluorescein particles (green) per cell at 37°C for 1 h to allow phagocytosis. Staining with DAPI (blue) and rhodamine-phalloidin (red) was performed to visualize nuclei and F-actin, respectively. (B) Quantification of the numbers of particles phagocytosed by each WT or HS1-KO PMN. (C) Percentage of WT and HS1-KO PMN that have phagocytosed at least one particle after 1 h. *p<0.05. ns, not significant. Data are represented as mean ± SD (n=4).

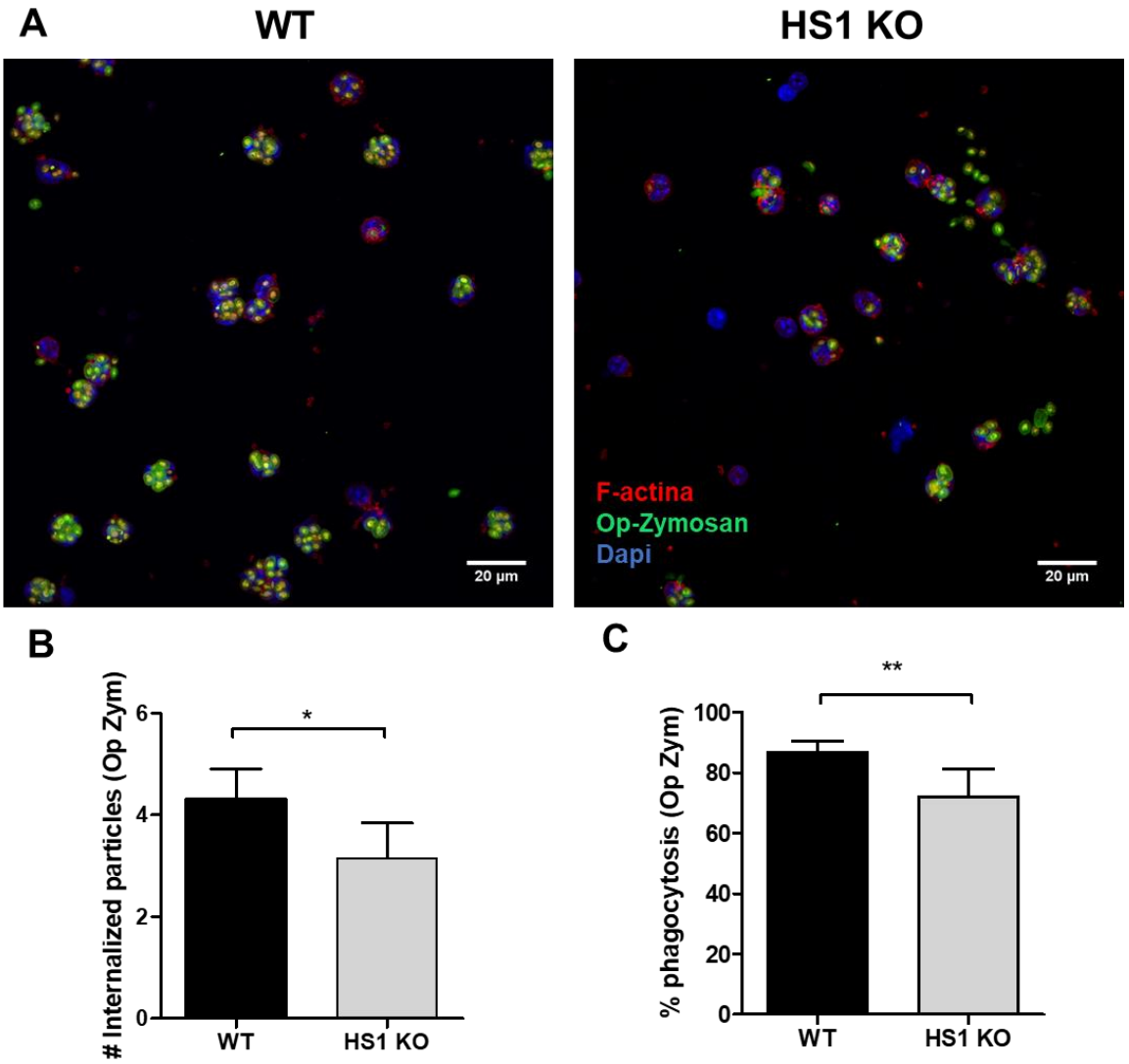

Figure 12. HS1 is required for efficient phagocytosis of opsonized zymosan particles. (A) WT and HS1 KO PMN were incubated with opsonized zymosan particles (1:10, green) at 37°C for 1 h. Staining with DAPI (blue) and rhodamine-phalloidin (red) was performed to visualize nuclei and F-actin, respectively. (B) Quantification of the numbers of particles phagocytosed by each WT or HS1-KO PMN. (C) Percentage of WT and HS1-KO PMN that have phagocytosed at least one particle after 1 h. Data are represented as mean ± SD (n=6), *p<0.05, **p<0.01.

To confirm these results, we characterized in more detail the localizations of HS1 and actin during phagocytosis. It is known that several actin regulatory proteins are recruited into the phagocytic cups to allow membrane expansion and engulfing of the particle (27–29). We identified phagocytic cup-like structures rich in actin filaments in WT PMN after 1 h of incubation at the interface where particles bound to the cell surface (Figure 13A, arrowhead), during internalization (Figure 13B, arrow) and when the phagosome had closed (Figure 13A, arrow). Interestingly, in WT PMN, we detected HS1 co-localization with F-actin in these structures, while it was dispersely located in the periphery of cells that did not phagocytose (Figure 13). Given the reported importance of HS1 for actin remodeling, we expected these structures to be disrupted in HS1 KO PMN. However, after 1 h, we found that these cells also formed phagocytic cups that were structurally similar to those observed in WT PMN (Figure 14). To further corroborate that HS1 is not required for phagocytic cup formation, it will be important to evaluate these structures in HS1 KO neutrophils at earlier time points to exclude the possibility of a delay in the formation of phagocytic cups. Taken together, these data suggest that HS1 is not per se required for phagocytosis in neutrophils, but that its presence enhances phagocytic efficiency.

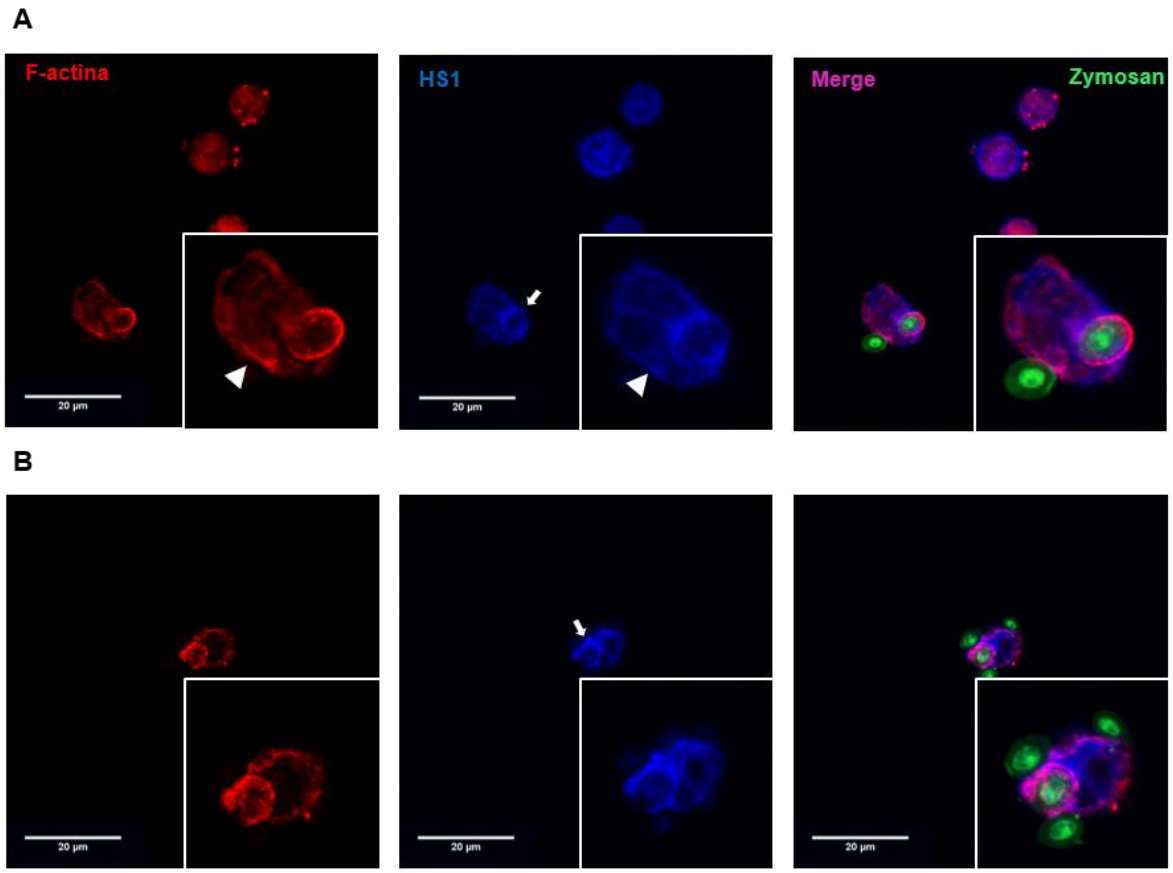

Figure 13. HS1 and actin are enriched in phagocytic cups. Staining of HS1 (blue) and F-actin (red) in WT PMN incubated with Op-Zym particles (green) for 1 h. Arrows show regions enriched in F-actin and HS1 in the phagocytic cups. Arrowheads indicate contact regions between zymosan particles and cells enriched in F-actin and HS1 as an initial cup formation. Representative images of at least 4 independent experiments are shown.

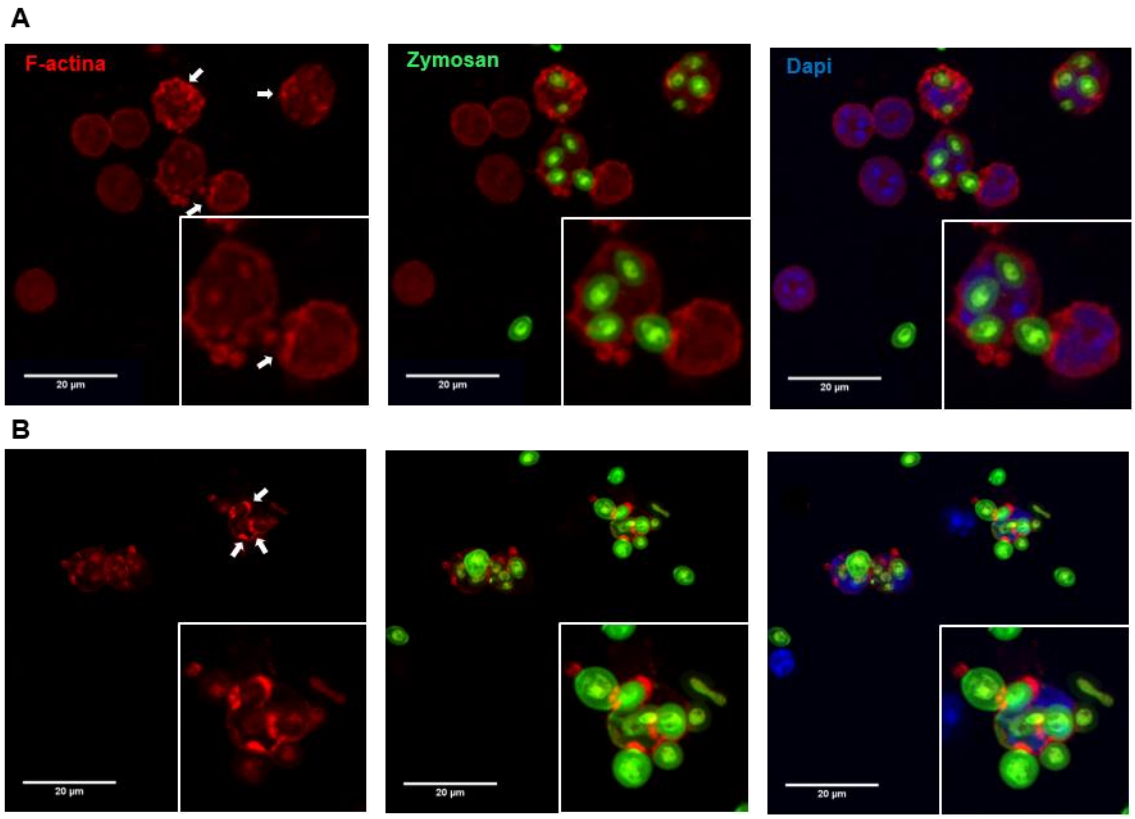

Figure 14. HS1 KO neutrophils are able of to form phagocytic cups. Staining of F-actin (red), and DAPI (blue) in HS1 KO PMN incubated with Op-zymosan particles (green) for 1 h. Two randomly selected fields are shown in A and B. Arrows show regions enriched in F-actin in the phagocytic cups. Representative images of at least 4 experiments are shown.

HS1 is not required for ROS production

Given that HS1 is known to play a role for Rac1 activation (79,83), which is essential for the assembly of the NADPH-oxidase complex (86,87), we evaluated whether ROS production is affected in the absence of HS1. In order to prove this, the respiratory burst was stimulated with 100 nM PMA, and the produced ROS were quantified by measuring the amount of DHR-123 oxidation to rhodamine by flow cytometry. The neutrophil population was selected from the SSC vs FSC gate (Figure 15A). After 30 minutes of stimulation, we observed ROS production in both cell types (Figure 15B). However, ROS production was not significantly different between WT and HS1 KO neutrophils (Figure 15B-C), although we observed a discrete tendency towards lower ROS production in HS1 KO neutrophils. Therefore, we decided to perform a time course with analysis 5, 10, 20, 25 and 30 min after stimulation. Again, at all times, we observed a trend towards lower ROS production in HS1-KO PMN at later time points, but the differences were not statistically significant (Figure 15D).

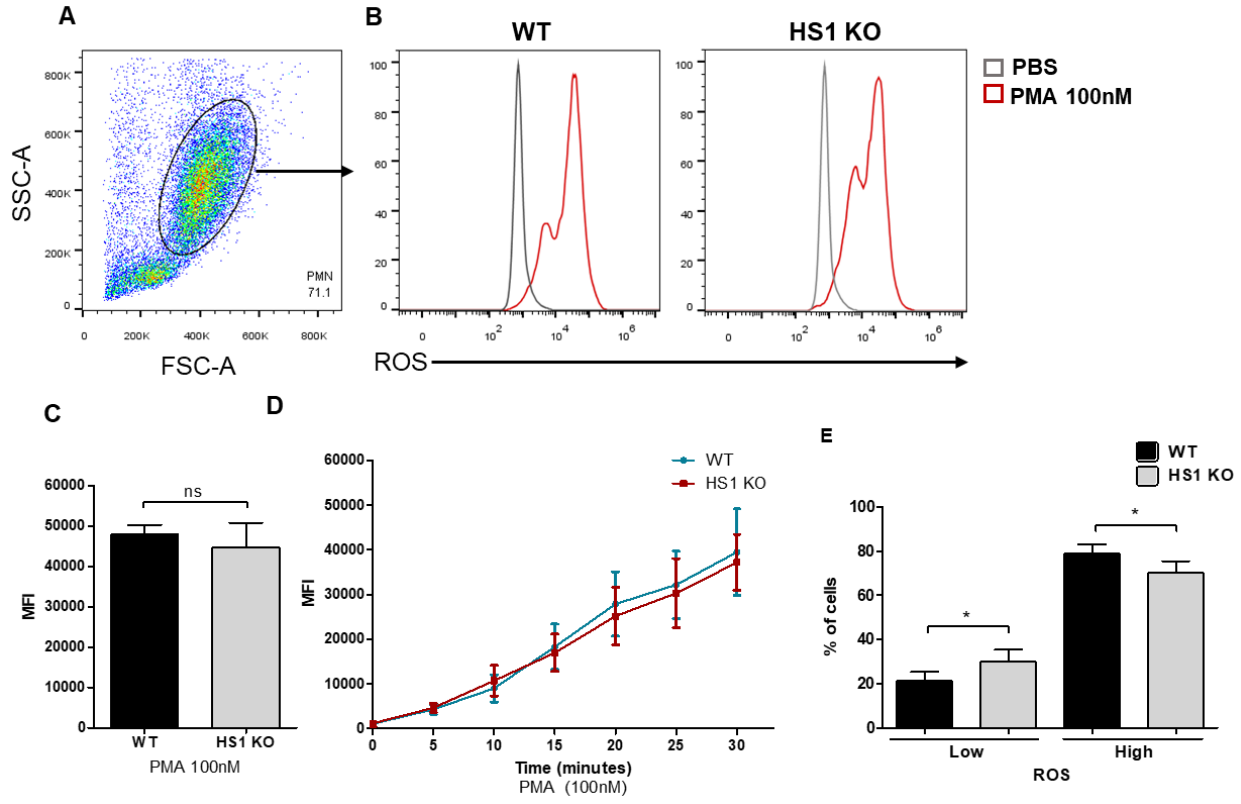

Figure 16. ROS production after stimulation with LPS or fMLP. (A) MFI of rhodamine after 30 min of stimulation with 1 µg/mL LPS. (B) WT and HS1 KO PMN were stimulated with 1 µM fMLP at the indicated times and ROS production was measured by flow cytometry. However, no significant differences were observed between WT and HS1-KO PMN. Data are represented as mean ± SD (n=4).

Of note, in both histograms, the neutrophil population after activation with PMA was heterogeneous with two very well-defined peaks (Figure 7B). One population produced large amounts of ROS (between 10^4 and 10^6 on the x-axis; termed ***High ROS***); and another one that produced fewer ROS (between 10^2 and 10^4 on the x-axis, termed ***Low ROS***). Thus, we assessed the percentage of cells in these two populations separately and compared the percentage of WT and HS1-KO PMN in these populations with different ROS production. Interestingly, we found significantly less cells that produce high amounts of ROS in HS1 KO PMN (Low ROS: 29.94+/-5.44%; High ROS: 70.06+/-5.44%) compared to WT (Low ROS: 21.13+/-4.19%; High ROS: 78.87+/-4.19%) Accordingly, a significantly higher percentage of HS1-KO PMN produced low amounts of ROS compared to WT. This finding suggests that the non-significant trend to reduced ROS production in total HS1-KO PMN might be due to the fact that there is a higher proportion of HS1-KO PMN with a decreased activation compared to WT PMN.

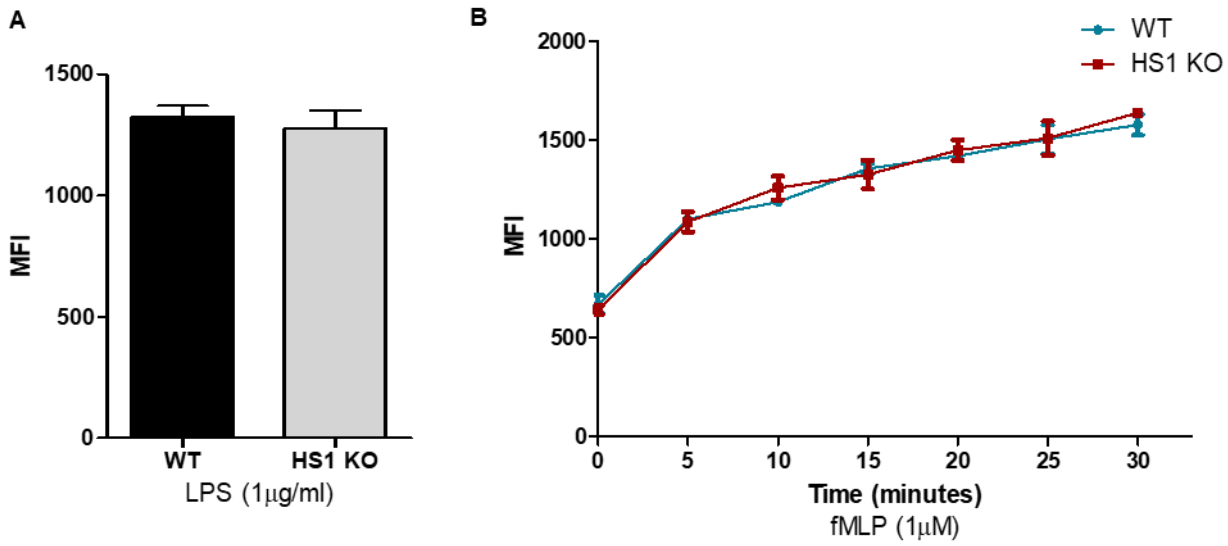

Figure 15. ROS production in WT and HS1-KO PMN. (A) Representative dot-plot of a WT granulocyte gate showing SSC vs FSC. (B) Representative histograms showing WT and HS1 KO neutrophils without stimuli in gray and with 100nM PMA for 30 min in red. (C) Mean fluorescence intensity (MFI) of rhodamine after 30 min of stimulation. (D) WT and HS1 KO PMN were stimulated for the indicated times and mean fluorescence intensity (MFI) of rhodamine was measured. No significant differences were observed between WT and HS1-KO PMN. (E) Percentages of neutrophils that produce high and low ROS after 30 min for the respective genotypes. Data are represented as mean ± SD (n=6), *p<0.05.

It is possible that the strong activation that induces PMA to produce ROS prevents the detection of a difference in ROS production in the absence of HS1. Therefore, we decided to evaluate ROS production using 1 µg/mL LPS and 1 µM fMLP as pro-inflammatory stimuli. However, even with these milder stimuli, we failed to observe significant differences in ROS production between WT and HS1-KO PMN (Figure 16).

Formation of NETs in mouse PMN

NET formation was originally analyzed by fluorescent visualization of extracellular DNA. Several studies indicate that PMA is a strong stimulus to induce NET formation, therefore, we used PMA at 100nM as a NET inducer (44,49,88). However, after 4 h of stimulation, we observed only some structures resembling NETs, and some cells with decondensed nuclei, a feature of cells before releasing DNA in NETs (Figure 17). However, only 1% of all murine PMN showed NETs. Since this procedure has mostly been done in human neutrophils, we performed experiments with human peripheral blood neutrophils to prove that the experimental strategy works. We found that by using the same PMA in murine (100 nM) or human PMN (20 nM), only human PMN were able to form significant amounts of NETs (approximately 80% of cells per field) (Figure 17) suggesting that the stimulus works in human PMN but, is probably insufficient to induce NET formation in murine PMN. Using 200 nM PMA, we also failed to observe NETs (data not shown). Thus, it will be essential to evaluate NET formation in future experiments using higher concentrations of PMA.

Due to these problems with PMA concentrations, we decided to use zymosan ($2x10^6$) to activate PMN, since zymosan has been reported to be an effective inducer of NETs in bovine neutrophils (89). However, after 4h of simulation, we only observed 12.81% of total cells that formed NETs (Figure 18). We will try using opsonized-zymosan with extended incubation times in future experiments to evaluate if the percentage of NET-producing murine PMN can be increased under these conditions. Once we established the appropriate stimulus to induce the formation of NETs in murine WT PMN, we will compare it to HS1 KO PMN.

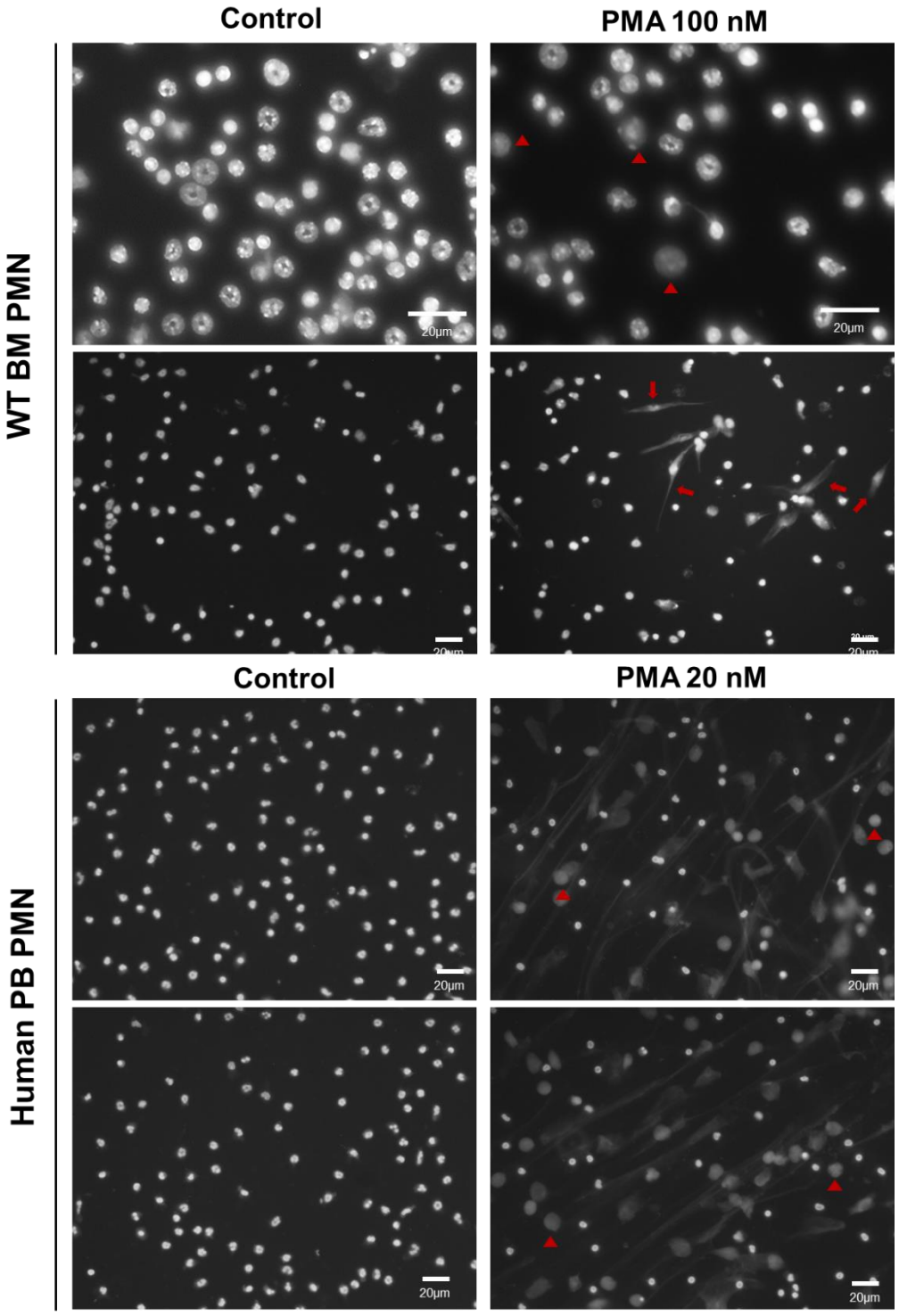

Figure 17. Microscopic analysis of neutrophil extracellular trap formation. Bone marrow neutrophils from WT mice and human peripheral blood neutrophils were adhered to a 24-well plate and stimulated with 100 nM and 20 nM PMA, respectively, for 4 hr. DNA was stained with DAPI. Arrowheads show decondensed nuclei in mouse and human PMN. Arrows show DNA released with a typical NET structure. (n=5).

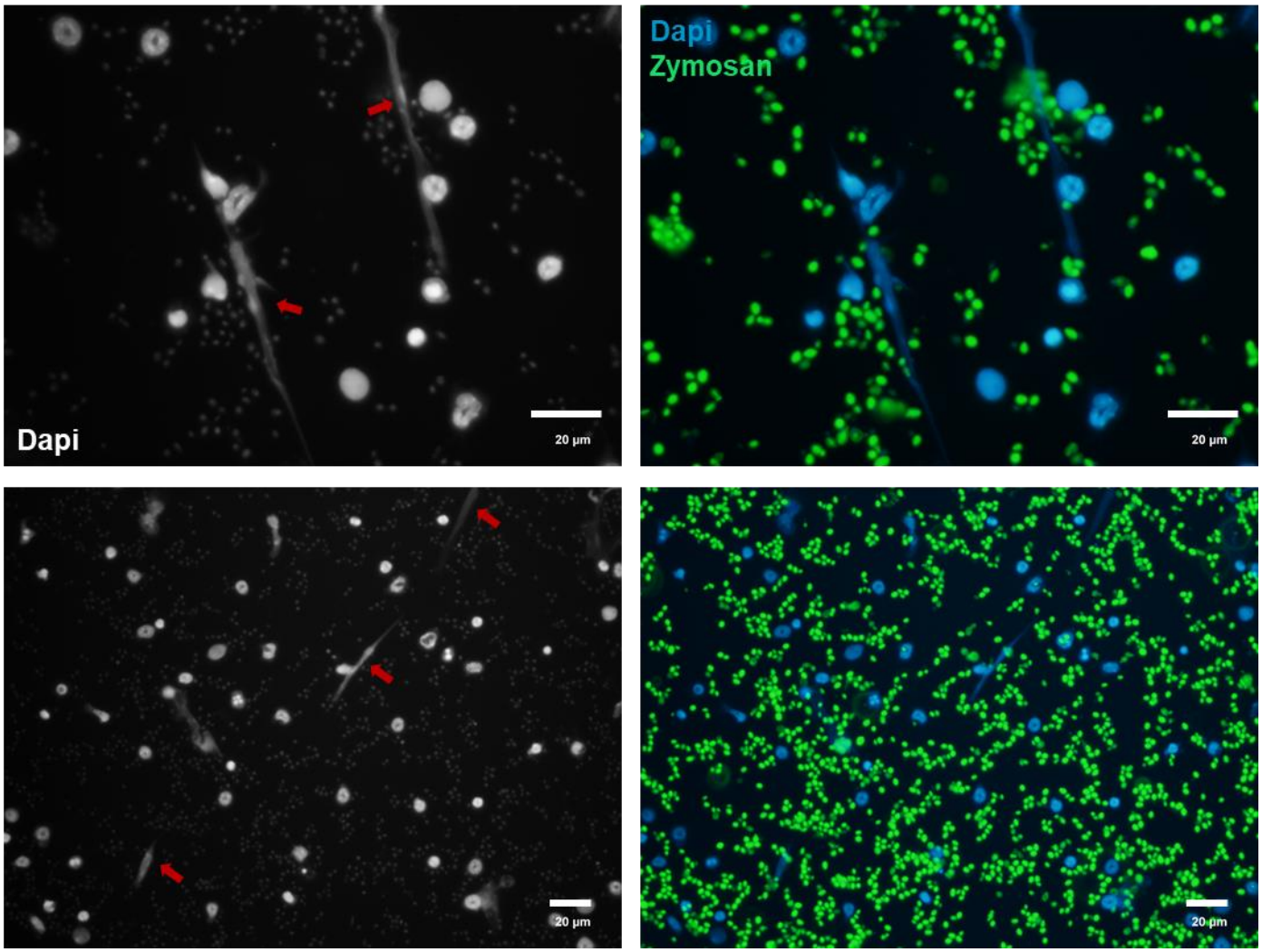

Figure 18. Zymosan induces the release of NETs in mouse PMN. Bone marrow neutrophils from WT mice were adhered to a 24-well plate and stimulated with zymosan ($2x10^6$, green) for 4 hr. DNA was stained with DAPI (blue). Arrows show DNA released with a typical NET structure. Two random fields of one experiment are depicted.

VIII. DISCUSSION

Neutrophils are key leukocytes during the first phase of the innate immune response. When they arrive at the site of inflammation, neutrophils fight pathogens by phagocytosis, production of ROS, degranulation, and the release of NETs. HS1 is an ABP exclusively expressed in hematopoietic cells that promotes actin dynamics through interaction with the Arp2/3 complex (68,70); and thus contributes to several cell functions in immune cells (compare chapter 1.3). However, the importance of HS1 in neutrophils has not been studied in detail. It has been reported that HS1 is required for efficient adhesion, chemotaxis and extravasation of neutrophils (79,83), but the role of HS1 in cytotoxic functions remains unknown. Here, we investigated the role of HS1 during the effector response of neutrophils, and found that it is not required for ROS production, that it is important for efficient phagocytosis.

Given the importance of actin polymerization for phagocytosis, we evaluated the dynamics of phagocytosis in HS1-deficient neutrophils by confocal microscopy using fluorescent zymosan particles. This strategy allowed us to analyze actin dynamics during phagocytosis in each cell. Another study of mouse bone marrow dendritic cells showed that HS1 deficiency did not cause any differences in phagocytosis of zymosan (90). However, we found that the absence of HS1 in neutrophils resulted in a reduction in the percentage of phagocytosis of non-opsonized zymosan particles (Figure 11C). Using opsonized zymosan, phagocytosis increased 2-fold in both WT and HS1-KO neutrophils, however, the efficiency of phagocytosis diminished by 20% in the absence of HS1; and the number of internalized particles per cell was significantly reduced (Figure 12 B-C), suggesting that HS1 is contributing to the effectiveness of phagocytosis in neutrophils. The discrepancy between our results and Huang the findings in dendritic cells may be due to the fact that they used non-opsonized zymosan. Maybe with opsonized zymosan they would have seen a difference. In addition, they did not analyze the number of phagocytized particles per cell where a defect could also have be seen. The differences may also be attributed to a possible redundancy effect by cortactin, since its expression has been reported in mouse spleen dendritic cells and in DC of human peripheral blood, in contrast to neutrophils where cortactin is absent (91,92). However, Dehring and colleagues showed that dendritic cells obtained from mouse bone marrow do not express cortactin (74). Whether expression of cortactin might be induced in specific populations of DC during activation has so far not been investigated. An alternative explanation could be the limited ability

of DC to phagocytose. Neutrophils are recognized as the most effective phagocytes, whose main function is to rapidly capture large number of pathogens and destroy them (93–95). By contrast, DC have been shown to downregulate their phagocytic capacity after maturation (96–98) suggesting that the mechanisms regulating phagocytosis must be different in both cell types in order to be more efficient in neutrophils. Thus, it will be important to understand how this process is regulated differently in different immune cell types. We suggest that HS1 regulates rather the efficiency of phagocytosis in neutrophils rather than induction. To further characterize this process, we plan to evaluate in future studies phagocytosis in an *in vivo* model of infectious inflammation such as polymicrobial sepsis in HS1-deficient mice.

We also characterized the localization of HS1 during phagocytosis. This strategy allowed us to visualize phagocytic cup-like structures rich in actin filaments (Figure 13). Interestingly, we observed that HS1 is localized in these structures during internalization of zymosan particles (Figure 13), but, in those cells that did not phagocytose, HS1 is found in the periphery. These findings are similar to those reported in human neutrophils, where the stimulation with the chemoattractant fMLP results in the re-localization of HS1 from the periphery to the leading edge where F-actin polarized is induced (79). In addition, in mouse bone marrow dendritic cells adhered to coverslips, HS1 colocalizes with F-actin in podosomes and lamellipodial edges (74). This means that under basal conditions, HS1 is localized in the cell periphery possibly stabilizing the cortical actin ring. However, after stimulation, neutrophils require the polymerization of actin, and HS1 is recruited in these areas such as the phagocytic cups. Thus, we expected that the phagocytic cups would be altered in HS1-deficient neutrophils, however, we were able to visualize them after 1 h of incubation. These structures were observed to be enriched in F-actin similar to WT neutrophils. A kinetic of phagocytosis as in the study of Huang and colleges in dendritic cells (90), could indicate whether the absence of HS1 rather causes a delay in the uptake of particles.

HS1 has been reported to regulate the activation of Rac1 and Rap1 during neutrophil chemotaxis induced by fMLP (79), and during adhesion mediated by LFA-1 (83). As mentioned before, this indicates that HS1 could be supporting the polymerization of actin in the phagocytic cups through the activation of Rac1. However, because HS1 is considered a weak activator of the Arp2/3 complex and alone does not have a strong actin polymerization activity (71,72), we suggest that HS1 could be regulating efficient

phagocytosis rather due to its capacity to function as an adapter protein. So, HS1 may support the recruitment of other proteins necessary for actin polymerization and stabilize the newly formed actin filaments in phagocytic cups as also reported for cortactin REF Scherer et al, JBC, 2018. On the other hand, it has been reported that, using a model where the human FcγRIII gene was transfected into the mouse macrophage P388D1 cell line, HS1 is phosphorylated at tyrosine residues after cross-linking with anti-hFcγRIII (99). This suggests that HS1 might be involved in signal transduction during phagocytosis mediated by the Fc receptor. It would be important to characterize the specific residues that are phosphorylated during Fc receptor signaling and perform phagocytosis assays using HS1 mutants where these residues are substituted.

Elimination of pathogens within the phagosomes is regulated through the rapid production of ROS and the release of granular content (22,100). Oxidative burst requires the efficient assembly of the NADPH-oxidase complex, which requires (37,40) activation of Rac2 and Rac1 (86,87). Given the importance of HS1 for the activation of Rac1, we evaluated whether the absence of HS1 has an effect on ROS production. To this end, we used dihydrorhodamine123 since it has been widely used to evaluate defects in the assembly of NADPH-oxidase. After stimulation of neutrophils with PMA, fMLP and LPS, we observed production of ROS, but no differences in ROS production was observed between WT and HS1-KO neutrophils suggesting that HS1 is not required for this process. Since it is not clear whether HS1 also regulates the activation of Rac2, it is possible that the reduced activation of Rac1 caused by the deficiency of HS1 is compensated by Rac2 during the oxidative burst.Aas Rac2 is the most predominant isoform in neutrophils suggested to play an important role in the assembly of the NADPH-oxidase complex (101–103)

Interestingly, the histograms of ROS production showed 2 distinct populations of neutrophils in both WT and HS1-KO after stimulation with PMA: one that produces more ROS (High-ROS) and one that produces less ROS (Low-ROS) (Figure 7B). We found that in HS1 KO neutrophils, the proportion of cells that produced less ROS was significantly higher. We believe that this population that produces less ROS, are more immature cells with less capacity to produce ROS. Some preliminary experiments using flow cytometry, where we performed Gr-1 staining after stimulating the cells with DHR and PMA, show that those cells that produce less ROS, have a reduced expression of Gr-1 (data not shown). However, we did not observe differences in the

expression of Gr-1 between WT and HS1 KO PMN in both population. All these data are consistent with a study showing a population of neutrophil precursors in the bone marrow with band morphology that produced less ROS (104). Interestingly, by flow cytometry they found that these precursor neutrophils had a $CD11b^{+}Ly6G^{low}$ phenotype, similar to our preliminary results suggesting that these Low-ROS cells could be immature cells. This would indicate that mature HS1-deficient neutrophils have the same capacity as WT to produce ROS, but that the tendency to reduced ROS production might be due to the fact that HS1 KO neutrophils have problems during maturation. Further research into the development and maturation of neutrophils in HS1 KO mice is needed to prove this idea.

Another inflammatory neutrophil tool are NETs. However, most studies analyzing NET formation have been performed in human neutrophils, and in murine neutrophils it is still difficult to standardize a technique due to the difficulties in obtaining mature neutrophils from peripheral blood. By contrast, bone marrow neutrophils, commonly used for functional experiments, do not respond very efficiently to the stimuli used in human neutrophils (105). Working with human neutrophils is more feasible because obtaining a good number of mature neutrophils is achieved with a small amount of peripheral blood. While in humans these represent 50-70% of all leukocytes in peripheral blood, in mice these reach only 15-20% (106,107). This is made more complicated by the fact that it is only possible to obtain around 1ml of blood from each mouse. Thus, the best option is to isolate neutrophils from bone marrow using a density gradient. However, these immature neutrophils (108,109) have a lower efficiency in producing NETs compared to human peripheral blood neutrophils (18,50,105,110), which we could confirm. We stimulated mouse bone marrow neutrophils with PMA, since it is an established inducer of NETs (44,49,88,110). However, we could barely observe NET structures in mouse BM neutrophils using 100 nM PMA, while we observed robust NET formation in human PB neutrophils using 20 nM PMA. Increasing the concentration of PMA to 200nM did not significantly increase NET formation. We are currently performing dose response experiments with a maximum concentration of 1µg / ml PMA. In addition, zymosan has also been reported as an inducer of NETs in bovine neutrophils due to its ability to activate ROS production (89). Thus, we tested this stimulus but did not observe significantly more NETs in BM PMN. The use of other stimuli, or primed neutrophils (e.g. isolated from septic mice) could solve this methodological problem.

In summary, the reduction in the efficiency of phagocytosis in the absence of HS1 and its location in phagocytic cups suggest that HS1 plays an important role for the efficiency of phagocytosis. However, HS1 is not required for the production of ROS after stimulation with PMA. The use of other stimuli is necessary to prove these findings in different contexts. It remains to be seen whether HS1 participates in the formation of NETs and in the release of cytoplasmic granules; experiments that we are currently performing. The study of neutrophil effector functions during inflammation in vivo will provide further evidence for the importance of HS1 during the innate immune response.

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