
ETD Archive

2009

Regulation of Monocyte Nadph Oxidase;Role of Pattern Recognition Receptors

Deena H. Elsori
Cleveland State University

Follow this and additional works at: <https://engagedscholarship.csuohio.edu/etdarchive>

 Part of the [Biology Commons](#)

[How does access to this work benefit you? Let us know!](#)

Recommended Citation

Elsori, Deena H., "Regulation of Monocyte Nadph Oxidase;Role of Pattern Recognition Receptors" (2009).
ETD Archive. 89.
<https://engagedscholarship.csuohio.edu/etdarchive/89>

This Dissertation is brought to you for free and open access by EngagedScholarship@CSU. It has been accepted for inclusion in ETD Archive by an authorized administrator of EngagedScholarship@CSU. For more information, please contact library.es@csuohio.edu.

**REGULATION OF MONOCYTE NADPH OXIDASE: ROLE OF PATTERN
RECOGNITION RECEPTORS**

DEENA H. ELSORI

Bachelor of Science in Molecular Biology

Kuwait University

Kuwait

July, 2003

Submitted in partial fulfillment of requirements for the degree of

DOCTOR OF PHILOSOPHY IN REGULATORY BIOLOGY

at the

CLEVELAND STATE UNIVERSITY

August, 2009

© Copyright 2009 by Deena H. Elson

This dissertation has been approved for
the Department of Biological, Geological,
and Environmental Sciences and for the
College of Graduate Studies of
Cleveland State University

by

Date:
Martha Cathcart, Ph.D., Cell Biology/CC
Major Advisor

Date:
Roy Silverstein, Ph.D., Cell Biology/CC
Advisory Committee Member

Date:
Xiaoxia Li, Ph.D., Immunology/CC
Advisory Committee Member

Date:
Crystal Weyman, Ph.D., BGES/CSU
Advisory Committee Member

Date:
Christine Moravec, Ph.D., Molecular Cardiology/CC
Internal Examiner

Date:
Gary Koski, Ph.D., Immunology/CC
External Examiner

DEDICATION

To my loving husband and best friend,

Ghassan, and my wonderful son, Khalid

And

To my parents, siblings and grandparents

ACKNOWLEDGEMENTS

I would like to thank every one who made this possible and supported me throughout this long journey. I am very grateful for the constructive criticism and continuous guidance of my major advisor Dr. Martha Cathcart and the committee members, Drs. Roy Silverstein, Crystal Weyman, Xia Xiao Li and Joseph Fontes.

I would also like to thank my parents and siblings back home for their unlimited support, and their unfailing love and faith in me. I am extremely thankful to my outstanding husband for his love and support and being there for me through all these years. And last but not least I would like to thank my son, who at the end of this journey was my major encouragement and thank you for providing a different meaning for this life.

REGULATION OF MONOCYTE NADPH OXIDASE: ROLE OF PATTERN RECOGNITION RECEPTORS

DEENA H. ELSORI

ABSTRACT

Activation of the NADPH oxidase enzyme complex results in the production of the oxygen free radical, superoxide anion (O_2^-). Superoxide anion is critical for host defense against fungal and bacterial pathogens and efficient immune responses; however, uncontrolled monocyte-derived O_2^- may contribute to chronic inflammation and tissue injury. We have previously identified several pathways that regulate the activity of NADPH oxidase in human monocytes; however, the receptor(s) responsible for the activation of NADPH oxidase in primary human monocytes have not yet been determined. This study shows that pattern recognition receptors, namely Dectin-1 and Complement Receptor 3, are essential for regulating NADPH oxidase activity in Zymosan-activated human monocytes. We show that TLR2 and TLR4 are not required for NADPH oxidase activation by Zymosan. In addition this study focuses on Dectin-1 downstream signaling and complex formation with intracellular signaling proteins. Our findings in human monocytes are supportive of the prior recognized role of Src and Syk tyrosine kinases in regulating Dectin-1-mediated ROS production in murine macrophages. Our data also shows that Src and Syk are tyrosine phosphorylated in Zymosan- treated cells and that they both regulate each others activity. Furthermore, we focused on Dectin-1 complex formation with intracellular signaling proteins including

Syk, Src and PKC δ , protein kinases that regulates NADPH oxidase activity in human monocytes. This is the first study to show the involvement of PKC δ in Dectin-1 signaling. We found that the activity of PKC δ is required for its own complex formation with Dectin-1 as well as Syk-Dectin-1 interaction. In contrast, Src and Syk inhibitors had no effect on PKC δ association with Dectin-1. Blocking the activity of Src inhibited phospho-Syk/Dectin-1 complex formation which supports the role of Src in regulating Syk tyrosine phosphorylation/activation. Our data confirms that Dectin-1, a pattern recognition receptor, is a key player in the regulation of NADPH oxidase in Zymosan-activated human monocytes and we introduce PKC δ as a novel player in Dectin-1 signaling.

To expand on this project, the study also includes data reporting an endogenous pathophysiological protein, osteopontin, as a novel ligand for Dectin-1. We show here that osteopontin induces the oxidative burst in monocytes through Dectin-1. We also show data supporting binding of osteopontin to Dectin-1.

Taken together, our study provides new insights into Dectin-1 ligands and downstream signaling in primary human monocytes and highlights novel signaling pathways utilized in these important cells. Our findings are relevant for understanding the regulation of NADPH oxidase in the innate immune response and in chronic inflammatory diseases.

TABLE OF CONTENTS

	Page
LIST OF ABBREVIATIONS.....	x
LIST OF FIGURES.....	xii
CHAPTER I : INTRODUCTION1
1.1. White blood cells: Monocytes.....	1
1.1.1. Isolation of human monocytes from peripheral blood.....	2
1.2. Leukocyte NADPH oxidase.....	3
1.3. Role of NADPH oxidase and O_2^- in innate immunity.....	5
1.4. Chronic Granulomatous Disease.....	7
1.5. Chronic Inflammation: Atherosclerosis.....	9
1.5.1. LDL oxidation: Role of O_2^-	10
1.6. Signaling pathways regulating monocytic NADPH oxidase.....	12
1.7. Receptors of the innate immune system.....	13
1.7.1. Leukocyte β -Glucan Receptor: Dectin-1.....	16
1.8. Osteopontin : a novel ligand for Dectin-1.....	17
1.8.1. Osteopontin structure and biosynthesis.....	17
1.8.2. Different immune functions of Osteopontin.....	18
1.8.3. Osteopontin: Role in Atherogenesis.....	18
1.9. Rationale and Proposal.....	19

CHAPTER II : DECTIN-1 SIGNALING IN PRIMARY HUMAN MONOCYTES: REGULATION OF NADPH OXIDASE ACTIVITY.....	21
Abstract.....	21
Introduction.....	23
Material and Methods.....	27
Results.....	32
Discussion.....	61
CHAPTER III : OSTEOPONTIN: A NOVEL NON-MICROBIAL LIGAND FOR DECTIN-1.....	65
Abstract	65
Introduction.....	67
Materials and Methods.....	70
Results.....	72
Discussion.....	79
CHAPTER IV: GENERAL CONCLUSIONS.....	81
BIBLIOGRAPHY.....	84

LIST OF ABBREVIATIONS

AA	Arachidonic Acid
BCS	Bovine Calf Serum
CGD	Chronic Granulomatous Disease
CR3	Complement Receptor 3
cPLA₂	Cytosolic Phospholipase A ₂
EDTA	Ethylenediaminetetraacetic Acid
H₂O₂	Hydrogen Peroxide
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
NOX	NADPH oxidase
O₂^{•-}	Superoxide Anion
OPN	Osteopontin
PBS	Phosphate Buffered Saline
PGN	Peptidoglycan
PKC	Protein Kinase C
PVDF	Polyvinylidene Difluoride
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SOD	Superoxide Dismutase
TLR	Toll like Receptor
ZOP	Opsonized Zymosan
PRR	Pattern Recognition Receptors
PAMP	Pathogen-Associated Molecular Patterns
RGD Sequence	Arginine-Glycine-Aspartate
MPO	Myeloperoxidase

LIST OF FIGURES

	Page
Figure 1. Model of leukocyte NADPH oxidase under basal and activated conditions.....	4
Figure 2. Dysfunction of the NADPH oxidase enzyme complex in chronic granulomatous disease.....	8
Figure 3. Model depicting some of the biological and biochemical events that occur in a blood vessel during atherosclerotic lesion formation.....	11
Figure 4. Regulation of NADPH oxidase in human monocytes.....	13
Figure 5. Methods of pathogen recognition by pattern-recognition receptors.....	15
Figure 6. TLR4 and TLR2 are not involved in Zymosan or ZOP-induced O_2^- production.....	34
Figure 6A. O_2^- production is not regulated by TLR4 in activated monocytes.....	34
Figure 6B. TLR2 is also not involved in Zymosan-induced O_2^- production.....	34
Figure 6C. TLR2 is not required for ZOP-induced O_2^- release.....	34
Figure 7. Dectin-1 and complement receptor 3 (CR3) both regulate O_2^- production in activated human monocyte	37
Figure 7A. Zymosan and ZOP signal through Dectin-1 and CR3 for O_2^- production in human monocytes.....	37
Figure 7B. In ZOP-induced cells CR3 blocking antibody inhibited more than laminarin and the two together caused additive inhibition.....	37
Figure 8. Zymosan triggers Dectin-1 activation.....	39

Figure 9.	Zymosan induces tyrosine phosphorylation of Src kinase through Dectin-1 and Src regulates NADPH oxidase activity.....	41
Figure 9A.	Src is tyrosine phosphorylated in activated monocytes and this event is regulated by Dectin-1 and Syk.....	41
Figure 9B.	PP2 and SU6656 block Zymosan-induced superoxide anion production.....	41
Figure 10.	Syk is tyrosine phosphorylated following monocyte activation and Syk activity is required for superoxide anion production.....	44
Figure 10A.	Laminarin blocks Syk tyrosine phosphorylation in Zymosan- activated monocytes.....	44
Figure 10B.	Tyrosine phosphorylation of Syk kinase is regulated by Zymosan and Src activity.....	44
Figure 10C.	Syk inhibitor blocks Zymosan-induced superoxide anion production in a dose dependent manner.....	44
Figure 11.	PKC δ , Src and Syk associate with Dectin-1	47
Figure 12.	Zymosan induces Dectin-1, Src and Syk phosphorylation in an time point fashion.....	49
Figure 12A.	Dectin-1 tyrosine phosphorylation.....	49
Figure 12B.	Src tyrosine phosphorylation.....	49
Figure 12C.	Syk tyrosine phosphorylation.....	49
Figure 13.	PKC δ regulates Syk association with Dectin-1 but Syk and Src have no effect on PKC δ association with Dectin-1	53

Figure 13A.	PKC δ regulates the Zymosan-induced PKC δ / Dectin-1 and rottlerin inhibits this complex formation.....	53
Figure 13B.	Syk/Dectin-1 complex formation is regulated by PKC δ	53
Figure 13C.	PP2 and Oxindole had no effect on PKC δ /Dectin-1 complex Formation.....	53
Figure 14.	PKC δ does not regulate the tyrosine phosphorylation of Dectin-1, Src or Syk.....	55
Figure 14A.	Rottlerin and Dectin-1 tyrosine phosphorylation.....	55
Figure 14B.	Rottlerin and Src tyrosine phosphorylation.....	55
Figure 14C.	Rottlerin and Syk tyrosine phosphorylation.....	55
Figure 15.	Src regulates phospho-Syk association with Dectin-1.....	58
Figure 16.	Regulation of NADPH oxidase by Dectin-1 and CR3 in human monocytes.....	59
Figure 17.	Osteopontin stimulates superoxide anion production through Dectin-1.....	73
Figure 18.	Dectin-1 binds directly to OPN.....	75
Figure 18A.	A sensorgram presenting binding of Dectin-1 antibody (2nM) to immobilized Dectin-1.....	75
Figure 18B.	Representative profiles of the SPR responses for osteopontin binding (concentrations ranging from 15.6nM to 500 nM) to Dectin-1.....	76
Figure 18C.	Comparing OPN, de-glycosylated OPN and de-phosphorylated OPN Binding to Dectin-1.....	76

Figure 18D.	Upper panel shows binding of laminarin (2nM) to Dectin-1 followed by injection of CD44 and CD36.....	77
Figure 18E.	Testing OPN binding to CD44 and CD36.....	77

CHAPTER I

INTRODUCTION

1.1 White blood cells: Monocytes

Monocytes are produced in the bone marrow and this is followed by their release into the blood stream. They are phagocytic mononuclear leukocytes. They have a large oval-shaped nucleus surrounded by a cytoplasm and several organelles. Monocytes are the largest cells among white blood cells/leukocytes (15-18 μm) and make up on average 2-8% of the total leukocyte population (1, 2). In addition to monocytes, leukocytes also include neutrophils, basophils, eosinophils, B-lymphocytes and T-lymphocytes. A bipotential stem cell termed colony forming unit-granulocyte monocyte (CFU-GM) is responsible for the formation of monocytes and granulocytes (2, 3). CFU-GM is derived from a multipotential progenitor cell termed colony forming unit-granulocyte erythroid, macrophages, megakaryocyte (CFU-GEMM) that can differentiate into granulocytes, monocytes, platelets or erythrocytes. Growth factors such as IL-3 and granulocyte macrophage stimulating factor (GM-CSF) direct the differentiation of CFU-GM to monocytes or granulocytes. For the differentiation of CFU-GM to monocytes the presence of IL-3, GM-CSF and macrophage colony stimulating factor (M-CSF) is

required. However, granulocytes are developed by substituting M-CSF with granulocyte colony stimulating factor (G-CSF) giving rise to granulocytes (2).

Monocytes play a major role in host defense. They are capable of migrating to sites of infection where they can differentiate to macrophages. Macrophages can destroy invading pathogens by phagocytosis followed by releasing proinflammatory mediators. In addition, macrophages and another form of differentiated monocytes, dendritic cells, can serve as antigen presenting cells. As antigen presenting cells they can expose denatured components of an antigen by expressing it on their surface along with human leukocyte antigen (HLA) class II molecules to T-cells which then triggers an adaptive immune response (3).

1.1.1 *In vitro* isolation of human monocytes from peripheral blood

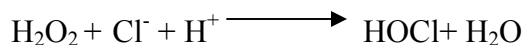
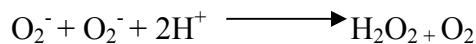
Mononuclear cells including monocytes and lymphocytes are isolated from the human peripheral blood using a ficoll-hypaque density separation method (4). One method commonly used to separate monocytes from lymphocytes is adherence to plastic or glass (5-7). Monocytes adhere very strongly to plastic and it is difficult to release them. However, studies have shown that pre-treating the plastic with calf serum before plating monocytes made it easier to release them which is usually done by incubating them with EDTA in phosphate buffer saline (6). Isolation of monocytes by adherence to serum-coated flasks produces monocytes of 90-95% purity which then can be used in *in vitro* experiments. A detailed description of the protocol used to isolate monocytes for experiments in this thesis is included in the next chapter.

1.2 Leukocyte NADPH oxidase

The nicotineamide-adenine dinucleotide phosphate (reduced form; NADPH) oxidases are a group of plasma membrane-bound enzymes. Over the past years different homologues of NADPH oxidases (NOX) have been identified, however, the leukocyte NADPH oxidase also known as NOX2 is best studied (8, 9). At first most of the studies were done on neutrophil NADPH oxidase (10, 11) but later attention has been drawn to monocyte NADPH oxidase which is the focus of our lab (12). NADPH oxidase catalyses a reaction that results in the production of superoxide anion ($O_2^{\cdot-}$) by the one-electron reduction of molecular oxygen. NADPH oxidase uses NADPH, provided by the pentose phosphate pathway as the electron donor:

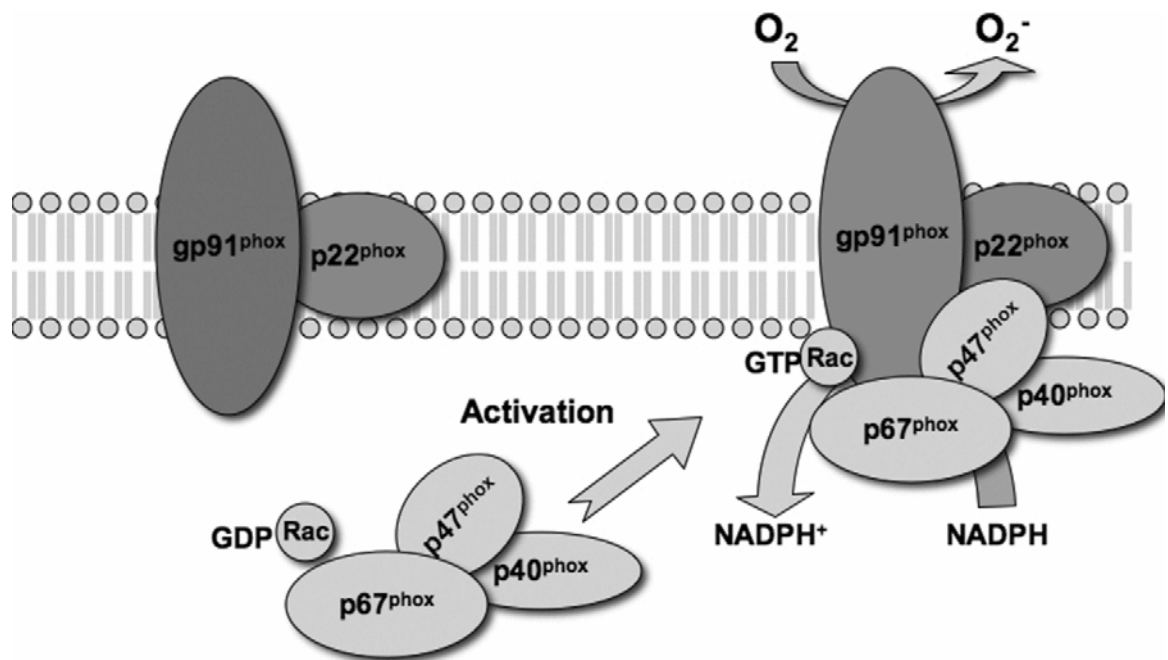


Superoxide anion produced by monocytes is readily converted to more potent reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^{\cdot}), hypochlorite (OCl^-) and singlet oxygen (1O_2):



ROS are responsible for killing bacterial and fungal pathogens. NADPH oxidase is also known as the respiratory burst oxidase because the production of $O_2^{\cdot-}$ and the more potent oxidants during phagocytosis leads to a rise in oxygen consumption. The leukocyte NADPH oxidase consists of several subunits which are divided into membrane components including flavocytochrome b558 (consisting of gp91 phox/NOX2 and p22 phox) and cytosolic components including p67phox, p47phox, p40phox and a small

guanosine 5'-triphosphate-binding protein Rac1/2 (12, 13). The components of NADPH oxidase in neutrophils and monocytes are very similar with the exception that Rac1 is present in monocytes and Rac2 in neutrophils (12, 14). The term “phox” stands for phagocytic NADPH oxidase. In leukocytes the components of this enzyme complex are unassembled, however once the cells are activated the cytosolic components translocate to the membrane and form the active enzyme complex. This assembly activates NADPH oxidase and results in the production of $O_2^{\cdot -}$, an oxygen free radical (Figure 1).



Biochemical Society Transactions (2006) 34, (960-964)

FIGURE 1. Model of leukocyte NADPH oxidase under basal and activated

conditions. Activation of the oxidase involves the stimulus-induced translocation of the cytosolic subunits p47^{phox}, p67^{phox}, and p40^{phox} and GTP-bound Rac to the cytochrome b558 which is composed of gp91^{phox} and p22^{phox}. The assembled oxidase can then use NADPH as a substrate for the reduction of molecular oxygen to $O_2^{\cdot -}$.

1.3 Role of NADPH oxidase and O_2^- in innate immunity

To trigger an efficient immune response against invading pathogens our immune system has been divided to innate and adaptive immunity which are made up of interconnected events. The first line of defense against infectious agents is the innate immune response. Innate immune receptors are responsible for the recognition of infectious non-self, pathogen-associated, molecular patterns (PAMPs). White blood cells, including monocytes, macrophages, dendritic cells, neutrophils, eosinophils, mast cells and NK cells are the major players for the innate immune system. They work by secreting cytokines and chemokines which regulate the recruitment and activation of immune cells. In addition, epithelial cells from organs such as the lung or gastric tract are also part of innate immunity (15). Reactive oxygen species (ROS) produced by NADPH oxidase play an essential role in innate immune responses against fungal and bacterial infections. In addition, granule-derived proteases and modification of the pH produce an environment that is essential for the killing of pathogens (16). Superoxide anion is readily converted to H_2O_2 which is a substrate for lactoperoxidase (LPO) to oxidize thiocyanate anions (SCN^-) to hypothiocyanite (HOSCN). HOSCN is a strong antimicrobial agent (17, 18). This is used by the epithelium to prevent colonization and infections. H_2O_2 is also a substrate for Myeloperoxidase (MPO). During neutrophil's respiratory burst, MPO catalyses a reaction that results in the production of hypochlorous acid (HOCl) from H_2O_2 and chloride anion (Cl^-). This reaction requires heme as a cofactor. MPO also uses H_2O_2 to oxidize tyrosine to tyrosyl radical (19). Neutrophils use Hypochlorous acid and tyrosyl radical to kill pathogens such as bacteria.

ROS released by the respiratory burst can be damaging to nearby tissues by interacting with other factors such as proteins, lipids and nucleic acids. In some tissues, NADPH oxidase-induced ROS are produced at concentrations that are not toxic and are usually an essential part of their function and regulation of cellular signaling. This function is also involved in innate immunity.

Toll-like receptors (TLRs) are the most studied pathogen recognition receptors. They play a key role in triggering innate immune responses. It has been recently shown that some NADPH oxidase homologues were found to signal downstream of TLRs (20). For example, after LPS activation TLR4 in neutrophils signals through NADPH oxidase 2 (NOX2) for the activation of NF- κ B-mediated gene expression in adjacent lung endothelial cells. This indicates that NOX-mediated ROS production is involved in communication between cells and this triggers an appropriate immune response through the activation of intracellular downstream signaling. Furthermore, NOX4 has been shown to be an essential player in NF- κ B-induced cytokine production after LPS binding to TLR4. In a recent study it was shown that LPS interaction with TLR4 triggers a direct interaction between NOX4 and TLR4 through the C-terminus of NOX4 and the intracellular TIR domain of TLR4. This is required for LPS-induced ROS production and NF- κ B activation (21). NADPH oxidase also signals downstream of the β -glucan receptor, Dectin-1, another innate immune receptor.

Recently, researchers are focusing on studying the role of innate immunity in antiviral immune signaling during virus infections. A study has shown that mice lacking NOX2 activity demonstrated an accelerated viral clearance of influenza infection (22). In addition, in humans inhibition of NOX2 expression in epithelial cells of the airway

interfered with innate immune responses in an NF- κ B-dependent manner following respiratory Syncytial virus and Sendai virus infections (23).

1.4 Chronic Granulomatous Disease

In some cases neutrophils and macrophages can not induce a respiratory burst after exposure to bacterial or fungal infections and this is usually due to a defect in one of the subunits of NADPH oxidase (NOX2/gp91phox, p22phox, p67phox or p47phox) which causes a rare genetic disorder known as chronic granulomatous disease (CGD) (24).

Chronic Granulomatous Disease (CGD) is a rare inherited disease that affects 1 in 250,000 people in the United States (25). It can be inherited by an X-linked mutation (X-CGD) or an autosomal recessive form (AR-CGD) (26). Typical symptoms of CGD patients include recurrent infections that start in childhood and these patients are more susceptible to bacterial and fungal infections especially when the infection is caused by a catalase positive pathogen. Fighting the infection leads to chronic inflammation and granuloma formation. The X-linked form is caused by point mutations, mainly in the β subunit of the gene that codes for the 91 kDa glycoprotein of NADPH oxidase enzyme complex (27). This is the most common form of CGD and it accounts for about 60% of the cases (Figure 2). The protein of gp91phox is not detectable in patients with X-CGD but these patients express normal amounts of mRNA with normal size transcripts.

Patients with AR-CGD have normal amounts of gp91phox expressed by their phagocytic cell; however, they lack the phosphorylated form of p47phox associated with NADPH oxidase during the activation of the respiratory burst in neutrophils (28). This indicates that the protein is either missing or a kinase responsible for its phosphorylation

is defective. Studies later showed that it was due to the absence of p47phox protein (26, 29). It was also shown that superoxide anion production by NADPH oxidase could be restored *in vitro* using cytosol fractions from the AR-CGD patients and a recombinant p47phox protein (30). Deficiency in p47phox protein accounts for ~25% of AR-CGD cases, however, mutations in p22phox and p67phox accounts for 5% of AR-CGD cases (Figure 2).

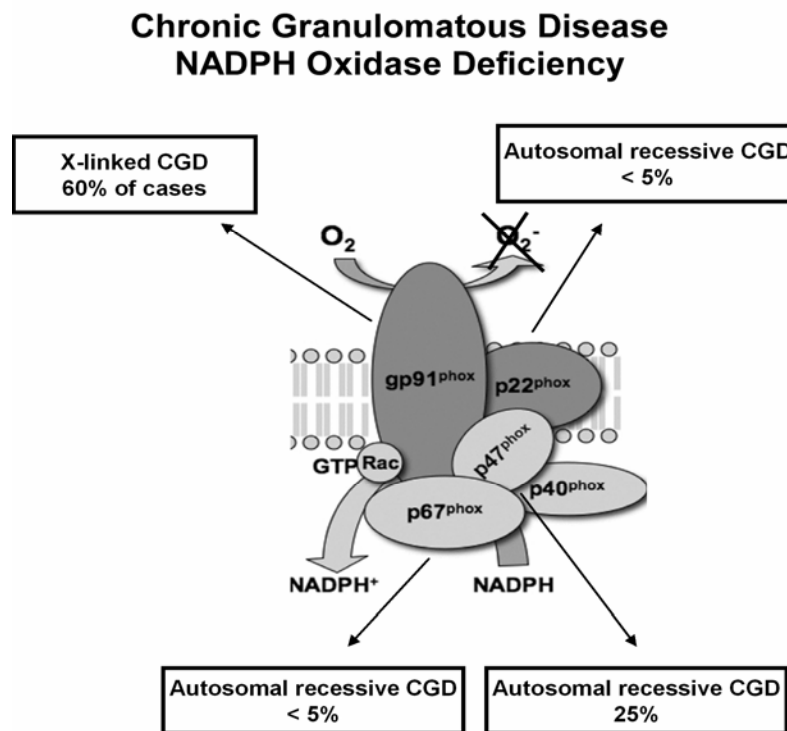


FIGURE 2. Dysfunction of the NADPH oxidase enzyme complex in chronic granulomatous disease. CGD is caused by mutations in NADPH oxidase subunits. Most of the cases are caused by a point mutation in gp91 phox (60%) which is the X-linked form of CGD. Mutations in p22 and p67 phox accounts for 5% of the autosomal recessive cases of CGD. Mutations in p47 phox accounts for 25% of autosomal recessive cases. In all of the cases the patients are more susceptible to infections due to the lack of superoxide anion production.

1.5 Chronic inflammation: Atherosclerosis

In addition to the role of NADPH oxidase- induced O_2^- in host defense and protection against invading pathogens, its uncontrolled production by monocytes can lead to chronic inflammation, specifically atherosclerosis. Atherosclerosis is a chronic inflammatory disease of the vessel wall caused by recruitment of macrophages in the vessel wall and foam cell formation. The oxidation of lipids is reported to be involved in atherogenesis and the progression of lesion formation. It was also shown that macrophages have an increased affinity for oxidized LDL (31, 32) and oxidized LDL causes injuries to cells (33-35).

Depending on the developmental level of the lesion, atherosclerotic lesions can be divided into 3 stages including: initial stage or fatty streak, intermediate lesion and the advanced fibrous plaque. The accumulation of foam cells derived from monocyte-differentiated macrophages and smooth muscle cells forms the fatty streaks (36). This is followed by monocyte and T-lymphocyte recruitment into the lesion by factors such as cytokines and chemokines (chemotactic protein-1, MCP-1) and activation of adhesion molecules present on the surface of endothelial cells (Figure 3).

Interestingly, it was recently shown that the progression of an atherosclerotic lesion does not only result from the recruitment and accumulation of monocytes in the arterial wall but it is also due to their reduced removal from the lesion. A study showed that lysophosphatidic acid and platelet-activating factor (factors that promote lesion progression) inhibited monocyte conversion to migratory cells and that promoted their accumulation in the subendothelium. In the same study, *in vivo* experiments showed that

monocytes and monocyte-derived cells demonstrated little emigration from progressive plaques but were more migratory during lesion regression (37).

1.5.1 LDL oxidation: Role of $O_2^{\cdot-}$

For many years the focus of our lab was to clarify the role of $O_2^{\cdot-}$ in monocyte-mediated low density lipoprotein (LDL) oxidation. Accumulating studies have shown that if monocytes were prevented from entering the vessel wall, lesion formation would be compromised (38-40). In addition we have shown that both monocytes and neutrophils can mediate the oxidation of LDL through $O_2^{\cdot-}$ and this was confirmed by showing inhibition by superoxide dismutase (SOD). This suggests that superoxide anion participates in LDL oxidation, which makes oxidized LDL a pro-atherogenic molecule (34). We have also shown that monocyte-mediated LDL oxidation is dependent on activation of the monocytes (34, 41) and that although monocyte-derived superoxide anion appears to be required for the oxidation of LDL, it was not entirely responsible for its oxidation (35). Monocytes in the blood are in resting state but can be exposed to different activating agents. After their activation, monocytes adhere to endothelial cells on the blood vessel wall and migrate into the adjacent tissue. While residing in the tissue, they differentiate to monocyte-derived macrophages. In the tissue, monocytes are activated by different factors and one of their immediate responses is the production of the potent oxygen free radical, superoxide anion ($O_2^{\cdot-}$), a product of a reaction catalyzed by NADPH oxidase. Monocyte NADPH oxidase is the main source of $O_2^{\cdot-}$ in atherosclerotic lesions. Superoxide anion results in the oxidation of LDL. Macrophages engulf oxidized LDL and form foam cells which can further cause cell injury, smooth

muscle proliferation and secretion of cytokines and acceleration of monocyte recruitment (Figure 3) (12). These are the initial steps for atherosclerotic lesion formation. Therefore, it is important to understand the regulation of the activity of this enzyme complex to prevent atherogenesis.

Inflammation in Atherosclerosis

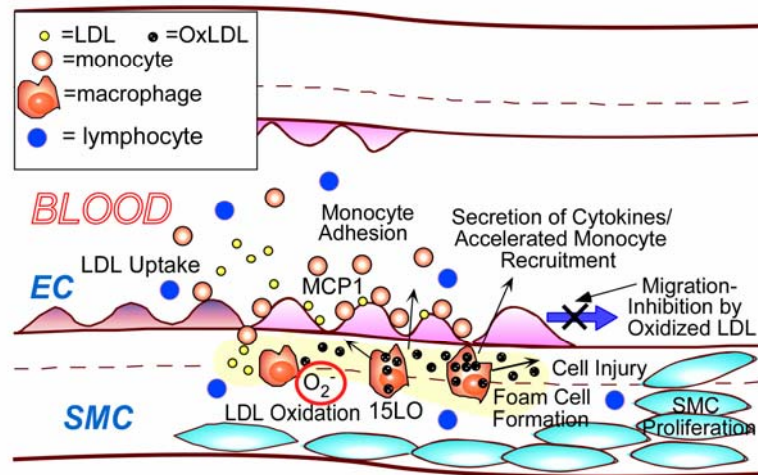


FIGURE 3. Model depicting some of the biological and biochemical events that occur in a blood vessel during atherosclerotic lesion formation. These events include monocyte migration into the vessel wall in response to chemotatic signals such as LDL and monocyte chemoattractant protein-1 (MCP-1), adhesion of monocytes to endothelial cells along the vessel wall, uptake of LDL by monocytes, migration of lipid-filled monocytes/macrophages into the intimal space, foam cell formation, cell injury and smooth muscle cell proliferation. In addition to reduced monocyte and monocyte-derived cells removal from the lesion.

1.6 Signaling pathways regulating monocytic NADPH oxidase.

The cellular mechanisms leading to the activation of NADPH oxidase and production of superoxide anion have been the focus of many investigators since the identification of the components of this enzyme complex. Activation of NADPH oxidase requires: 1) phosphorylation of some proteins in the complex to be phosphorylated, 2) translocation of cytosolic components to join membrane components to form the active enzyme complex.

Our lab has identified and studied several signaling pathways that regulate the activity and assembly of monocyte NADPH oxidase (Figure 4). In our model we use opsonized Zymosan (ZOP) to stimulate monocytes and study active NADPH oxidase. Zymosan is a mimic of a yeast pathogen; it is a cell wall preparation of *Saccharomyces cerevisiae* and has been used as a model system for activating the respiratory burst in monocytes and in other models of inflammation. ZOP is opsonized or serum-coated Zymosan. Zymosan is composed of β -glucan, mannans, mannoproteins and chitin which are components of yeast recognized by the innate immune system. Using ZOP to activate monocytes, our lab has shown that the following mechanisms regulate the enzyme's activity and assembly: 1) calcium influx and release from intracellular stores (42), 2) protein kinase C α (PKC α)-dependent phosphorylation and activation of cytosolic phospholipase A₂ (cPLA₂) (43-45), 3) cPLA₂ generation of arachidonic acid which regulate p47 and p67phox translocation, 4) PCK δ -dependent phosphorylation and translocation of p47phox and p67phox (46-48) and 5) Rac-1 dissociation from rhoGDI followed by its translocation to the membrane (14). However, the receptor(s) responsible for Zymosan and ZOP-induced O₂⁻ and for regulating NADPH oxidase activity in primary human

monocytes have not yet been determined. This will be the focus of Chapter 2 of this thesis.

Regulation of Monocyte NADPH Oxidase

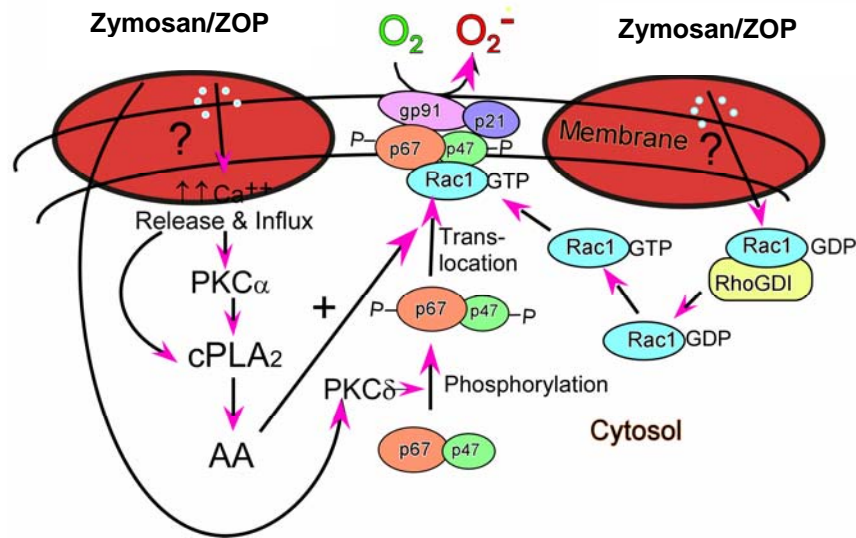


FIGURE 4. Regulation of NADPH oxidase in human monocytes. This model summarizes the signaling pathways that regulate the assembly and the activation of NADPH oxidase in human monocytes. Pathways including calcium influx, phosphorylation of cPLA_2 by $\text{PKC}\alpha$, arachidonic acid (AA) release, $\text{PKC}\delta$ -dependent phosphorylation of p47phox/p67phox and Rac-1 translocation to the membrane regulate NADPH oxidase activity in activated human monocytes.

1.7 Receptors of the innate immune system

The innate immune system detects pathogens using a group of receptors known as the pattern recognition receptors (PRR). PRRs recognize pathogen-associated molecular patterns (PAMPs). Different PRRs are distinguished by their expression profile, localization (cell surface, cytosolic, secreted into serum and tissue fluids) and function

(49). They are divided into three major categories including signaling PRRs, phagocytic PRRs and Secreted PRRs. Signaling PRRs are expressed either on the cell surface or intracellularly. Activation of pro-inflammatory signaling pathways including NF κ B, Jun N-terminal kinase (JNK) and p38 MAP kinase is common among this group. The best known family of receptors in this category is the Toll-like receptors (TLRs).

Phagocytic PRRs are mainly expressed on the surface of phagocytic cells such as monocytes, macrophages, neutrophils and dendritic cells. These receptors recognize PAMPs on pathogens and allow uptake by phagocytosis. Once phagocytosed, these pathogens are placed in intracellular lysosomal compartments and further destroyed by different mechanisms. In the case of macrophages and dendritic cells, proteins from degraded microorganisms are presented as antigens on their surface along with MHC molecules which can be recognized by T-cells and stimulates an adaptive immune response. Receptors in this category include: Scavenger Receptors, Macrophage Mannose Receptor, and the β -Glucan Receptor, Dectin-1 (50). Secreted PRRs include receptors such as the mannan-binding lectin (MBL) and peptidoglycan- recognition proteins (PGRPs). They can activate complement, opsonize microbial cells and act as accessory protein to aid recognition of pathogens by surface PRRs e.g. TLRs.

PRRs recognize PAMPs by different methods (Figure 5). Receptors such as Dectin-1 and the mannose receptor bind and recognize PAMPs through direct non-opsonic recognition. However, other PRRs such as complement receptors recognize PAMPs through non-direct opsonic recognitions. Opsonization is a term used for coating pathogens or molecules with proteins or opsonins such as complement, pentraxins and surfactant proteins A and D to allow for easier recognition by receptors. And in some

cases receptors recognize a pathogen through both opsonic and non-opsonic recognition. Pathogen recognition can also occur intracellularly through some members of the TLRs. Recognition of PAMPs by different mechanisms ends up in microbial uptake and killing and initiation of an efficient immune response (49).

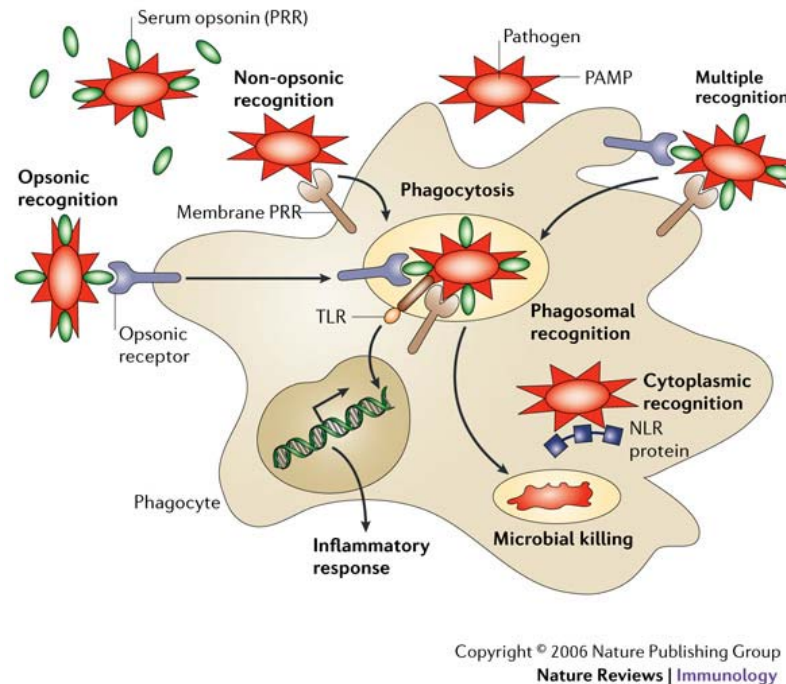


FIGURE 5. Methods of pathogen recognition by pattern-recognition receptors.

Pathogens such as fungi and bacteria can be recognized directly by PRRs through non-opsonic recognition or indirectly through opsonic recognition. In some cases recognition requires both opsonic and non-opsonic recognition. Some recognition also occurs intracellularly within vacuoles.

1.7.1 Leukocyte β -Glucan Receptor: Dectin-1

The β -glucan receptor Dectin-1 was first identified by Brown and Gordon through an expression cloning protocol (51). It is expressed on monocytes, macrophages, neutrophils and on subsets of dendritic cells and T-cells (52). Dectin-1 is a type II transmembrane receptor that contains one carbohydrate recognition domain (CRD) at the C-terminal portion of the protein and an ITAM-like motif in the N-terminal cytoplasmic region (53, 54). The CRD of Dectin-1 belongs to the C-type lectin-like subfamily. Dectin-1 is considered a non-classical C-type lectin for 2 reasons. First, it lacks a site for calcium binding in its lectin-like domain which indicates that it doesn't require calcium for ligand binding and this is not the case for other C-type lectins. The second reason is that the ITAM motif of Dectin-1 is not a typical ITAM in that the distal tyrosine residue (Tyr-3) in the membrane is not in the YXXL/I sequence, only the membrane proximal tyrosine (Tyr15) is located in this sequence (55, 56). Dectin-1 was first identified on dendritic cells but was later found to be expressed on monocytes and macrophages as well (51). Dectin-1 is specific for β -1,3,-linked and β -1,6-linked glucans, which are PAMPs found in fungal and other microbial cell walls. ITAMs were originally characterized in T-cell receptors, Fc receptors and NK receptors. Activation of these receptors involves ITAM tyrosine phosphorylation by Src kinase and docking of Syk kinase on phosphotyrosine residues followed by its activation and finally leading to activation of pro-inflammatory signaling pathways. The presence of the ITAM-like motif in Dectin-1 suggests that this receptor signals through Src/Syk tyrosine kinases. The role of Dectin-1 in regulating NADPH oxidase and its downstream signaling is the focus of Chapter 2 of this thesis.

1.8 Osteopontin: a novel ligand for Dectin-1

1.8.1 Osteopontin structure and biosynthesis

Osteopontin (OPN) is an acidic secreted glycoprotein with an approximate molecular weight of 32 kDa (57). Osteopontin is post translational modified through glycosylation (5-6 O-linked and one N-linked oligosaccharides) and variable phosphorylation (12 phospho-Ser and one phospho-Thr) and it is also sulphated. Interestingly, in rat kidney cells (58) and calcitrol-stimulated mouse JB6 epidermal cells (59) OPN is secreted in a non-phosphorylated form. OPN has a calcium, hydroxyapatite, RGD and a thrombin/MMP cleavage site (57). Cleaved fragments of OPN expose new domains and may induce different functions. The amino acid sequence of OPN is conserved throughout different species including human, rat, mouse and pig. At first, OPN was thought to be biosynthesized by cells of the osteoblastic lineage and deposited on mineralized matrix; however, it was later shown that several bone marrow-derived cells including dendritic cells, macrophages, smooth muscle cells and endothelial cells also produce OPN.

Studies have shown that osteoclasts bind to OPN *in vitro* through an RGD- $\alpha\text{v}\beta3$ integrin binding (60). OPN in the bone accumulates in areas where osteoclasts are attached to the underlying mineral surface (61). Osteopontin also plays a role in bone turnover (62). Furthermore, OPN has been shown to bind to a non-integrin receptor, namely the hyaluronan-receptor CD44 (63). Binding of OPN to CD44 was shown to be essential for bone resorption (64).

1.8.2 Different immune functions of Osteopontin

Osteopontin (OPN) is expressed by different immune cells including monocytes/macrophages, neutrophils and lymphocytes. It has different functions in different cells. OPN regulates immune responses by acting as a chemokine and promoting cell recruitment to inflammatory sites (65). It triggers cell attachment through its integrin binding domain (RGD) (66) and wound healing (67). In addition, OPN mediates cytokine production and cell activation (68). Finally, this protein was shown to play an important role in cell survival by regulating apoptosis (68, 69).

1.8.3 Osteopontin: Role in Atherogenesis

As mentioned above OPN is a multi-functional molecule expressed by different immune cells. It is highly expressed in chronic inflammatory including atherosclerosis and it is also over-expressed in autoimmune diseases. During chronic inflammation, OPN accumulates in and around inflammatory cells. OPN has been shown to regulate atherogenesis by acting as a chemokine and recruiting monocytes/macrophages to inflammatory site and through its adhesive properties (RGD sequence) which promotes cell attachment through integrin binding. In addition, it modulates cytokine production in dendritic cells, monocytes/macrophages and T-cells during chronic inflammation. OPN plasma levels have been used clinically to diagnose different inflammatory diseases including cardiovascular disease. In addition to its role as a proinflammatory molecule, OPN is an inhibitor of mineralization and vascular calcification (70, 71).

1.9 Rationale and Proposal

The activation of NADPH oxidase in monocytes results in the production of large quantities of the highly reactive oxygen species, $O_2^{\cdot-}$. ROS are known to play a major role in the pathogenesis of atherosclerosis and studying mechanisms that regulate the activity of NADPH oxidase and ROS production provides useful information for designing new tools and therapies to control the progression of this chronic inflammatory disease. Our lab had identified several pathways that regulate the function and assembly of this enzyme complex but the receptors responsible for regulating the Zymosan-induced NADPH oxidase activity in human monocyte had not been identified.

In chapter 2 of this study we tested whether pattern recognition receptors can regulate the function of NADPH oxidase and $O_2^{\cdot-}$ produced by Zymosan and ZOP-activated human monocytes. We looked at the family of Toll-like receptors, TLR-2 and TLR-4, and then studied the role of the β -glucan receptor, Dectin-1, in regulating NADPH oxidase in human monocytes. In addition, we tested the involvement of complement receptors namely Complement Receptor 3 (CR3) in ZOP-activated monocytes. Finally, we studied Dectin-1 downstream signaling and complex formation.

In chapter 3, we address the role of a secreted glycoprotein known as Osteopontin as a novel non-microbial ligand for Dectin-1. So far all of the ligands that are used to study Dectin-1 function and signaling are of microbial nature. While trying to look for novel non-microbial ligands for Dectin-1, OPN was one of the proteins that we wanted to test and the reason for that is mainly due to its highly repetitive structural characteristics (featuring a pattern of numerous phosphates and sugars). In addition, the fact that this

protein is expressed by different immune cells including monocytes and that it regulates chronic inflammation, and specifically atherogenesis, made it more interesting for us since this is the focus of studies in our lab. Therefore, chapter 3 of this thesis focuses on the role of OPN as a ligand for Dectin-1 and activation of NADPH oxidase in human monocytes.

CHAPTER II

DECTIN-1 SIGNALING IN PRIMARY HUMAN MONOCYTES: REGULATION OF NADPH OXIDASE ACTIVITY

Deena H. ElSORI and Martha K. Cathcart

ABSTRACT

Dectin-1 belongs to the family of pattern recognition receptors and has recently been identified as the leukocyte β -glucan receptor. This study shows that both Dectin-1 and Complement Receptor 3 are essential for regulating NADPH oxidase activity in Zymosan-activated human monocytes. In addition, we show that TLR2 and TLR4 are not required for NADPH oxidase activation by Zymosan. Activation of NADPH oxidase results in the production of the oxygen free radical, superoxide anion ($O_2^{\cdot -}$). Superoxide anion is critical for host defense against fungal and bacterial pathogens and efficient immune responses; however, uncontrolled monocyte-derived $O_2^{\cdot -}$ may contribute to chronic inflammation and tissue injury. We have previously identified several proteins that regulate the activity of NADPH oxidase in primary human monocytes. In this study we focus on Dectin-1 downstream signaling and complex formation with intracellular signaling proteins. Our findings in human monocytes are supportive of the prior

recognized role of Src and Syk tyrosine kinases in regulating Dectin-1-mediated ROS production in murine macrophages. Our data also shows that Src and Syk are tyrosine phosphorylated in Zymosan- treated cells and that they both regulate each others activity. However, we show for the first time a requirement for a novel PKC family member, PKC δ , in Dectin-1 signaling cascade. Endogenous Dectin-1 forms a complex with PKC δ , Syk and Src tyrosine kinase. The activity of PKC δ is required for its complex formation with Dectin-1 and it also acts upstream of Syk in Dectin-1 complex formation. In contrast, Src and Syk pharmacological inhibitors have no effect on PKC δ /Dectin-1 complex formation.

Taken together, our study provides new insights into Dectin-1 downstream signaling in primary human monocytes and highlights distinct pathways utilized in these important cells in innate immunity.

INTRODUCTION

Blood leukocytes, including monocytes, play a major role in host defense against bacterial and fungal pathogens. One of the critical responses of monocytes to a variety of stimulating agents is the production of superoxide anion ($O_2^{\cdot -}$) through the activation of the NADPH oxidase enzyme complex. $O_2^{\cdot -}$ and other reactive oxygen species (ROS) are critical for pathogen killing and maintaining efficient host defense. Individuals that inherit a defect in NADPH oxidase activity develop a disorder known as chronic granulomatous disease (CGD) and these patients are characterized by life-threatening and recurrent infections that usually start in childhood. $O_2^{\cdot -}$ produced by activated monocytes has also been shown to oxidize lipids, specifically low density lipoproteins (LDL)(35, 44, 48, 72). The role of oxidized LDL in the development of atherosclerotic lesions and progression of atherosclerosis has been well studied. Therefore it is important to keep a balance in the production of superoxide anion to maintain efficient host defense but at the same time avoid contributing to chronic inflammatory processes.

Monocytic NADPH oxidase has several subunits which are divided into membrane components including flavocytochrome b558 (consisting of gp91 phox and p22 phox) and cytosolic components including p67phox, p47phox, p40phox and a small guanosine 5'-triphosphate-binding protein Rac-1 (12, 13). When monocytes are activated the cytosolic components translocate to the membrane to form the active complex of NADPH oxidase which will result in $O_2^{\cdot -}$ production (12). Our lab has identified and studied several signaling pathways that regulate the function of monocyte NADPH oxidase. These mechanisms include calcium release and influx (42), protein kinase C α (PKC α)-dependent phosphorylation and activation of cytosolic phospholipase A_2

(cPLA₂) (43-45), PCK δ - dependent phosphorylation and translocation of p47phox and p67phox (46-48) and Rac-1 dissociation from rhoGDI followed by its translocation to the membrane (14). In all of these studies opsonized Zymosan (ZOP) was used to activate monocytes and identify these signaling pathways; however, the receptor(s) responsible for recognizing Zymosan or ZOP and stimulating the above pathways has not been identified in primary human monocytes.

Zymosan is a mimic of a yeast pathogen, it is a cell wall preparation of *Saccharomyces cerevisiae* and has been used as a model system for activating the respiratory burst in monocytes and in other models of inflammation (73). ZOP is opsonized or serum-coated Zymosan. Zymosan is composed of β - glucan, mannans, mannoproteins and chitin which are components of yeast recognized by the innate immune system. A group of receptors of the innate immune system known as pattern recognition receptors (PRR) have been shown to bind to Zymosan particles and trigger pro-inflammatory immune responses in mouse macrophages. These receptors include the Toll like receptors (TLRs) and the more recently identified β -glucan receptor, Dectin-1. In addition, it was shown that complement receptors, namely Complement Receptor 3 (CR3, Mac-1, $\alpha_M\beta_2$), mediate non-opsonic phagocytosis of Zymosan and *Mycobacterium kansasii* and opsonic phagocytosis of ZOP in Chinese hamster ovary cells (74, 75).

Studies have shown that Zymosan binding to TLR2 and TLR6 triggers the activation of NF- κ B and production of TNF- α in rat alveolar macrophages and in RAW 264.7 cells (76-78). In addition, it was shown that phagosomes containing Zymosan recruit TLR1, TLR2, and TLR6 (77, 79). Accumulating evidence suggested that microbial-induced pro-inflammatory events are dependent on TLRs (80). More recently

studies have shown that the C-type lectin receptor, Dectin-1, collaborates with TLRs in Zymosan recognition and in initiating immune responses in mouse macrophages (81). To date very limited studies have been conducted in primary human monocytes.

The β -glucan receptor Dectin-1 was first identified by Brown and Gordon (51). It is expressed on monocytes, macrophages, neutrophils and on subsets of dendritic cells and T-cells (52). Dectin-1 recognizes intact yeast such as *Candida albicans* and β -glucan containing particles such as Zymosan. It is a type II transmembrane receptor with a C-type lectin domain at the C-terminus and an immunoreceptor tyrosine-based activation motif (ITAM)-like signaling motif in the intracellular N-terminus domain (54). ITAMs were originally characterized in T-cell receptors, Fc receptors and NK receptors. Activation of these receptors involves ITAM tyrosine phosphorylation by Src kinase and docking of Syk kinase on phosphotyrosine residues followed by its activation and finally leading to activation of pro-inflammatory signaling pathways.

It is apparent from these studies that TLRs and Dectin-1 are involved in the recognition of microbial pathogens and regulation of different immune responses. The purpose of this study was to determine whether innate immune receptors such as TLRs or Dectin-1 are involved in triggering the activity of NADPH oxidase in primary human monocytes. In particular we were interested in investigating the role of the above receptors in stimulating superoxide production in human monocytes activated by Zymosan or ZOP. Our results indicate that TLR2 and TLR4 are not involved in inducing O_2^- in Zymosan or ZOP-activated monocytes; however, we show that Dectin-1 and CR3 regulate Zymosan and ZOP-induced O_2^- release. We further show that Zymosan induces tyrosine phosphorylation of Dectin-1, Syk and Src kinase. Using laminarin, a soluble β -

glucan and a Dectin-1 inhibitor, we show that Dectin-1 regulates Src and Syk activity in Zymosan-induced monocytes. In additional studies using Src and Syk-selective inhibitors we show that both of these tyrosine kinases regulate NADPH oxidase in human monocytes. Finally, we show that PKC δ , Src and Syk form a complex with Dectin-1 and this complex formation is regulated by Zymosan and PKC δ activity. Taken together, our data indicate that pattern recognition receptors are required for monocyte NADPH oxidase activity in primary human monocytes.

MATERIALS AND METHODS

Reagents

Zymosan was purchased from MP Biomedicals (Solon, OH). Abs specific for Src phosphorylated on Tyr⁴¹⁶ (polyclonal Ab) and for Syk phosphorylated on Tyr^{525/526} (both polyclonal and monoclonal Abs) and Phospho-(Ser/Thr) Phe antibody were obtained from Cell Signaling Technology (Danvers, MA). Abs to c-Src (SRC 2) and Syk (4D10) were obtained from Santa Cruz Biotechnology (Santa Cruz, California) and PKC δ polyclonal antibody. Pharmacological inhibitors such as PP2, PP3, SU6656, oxindole, Piceatannol and Rottlerin were purchased from Calbiochem (San Diego, California). The inhibitors were dissolved in DMSO and stored at -20°C as stock solutions. Blocking antibody to human Toll-like receptor 2 (Mouse monoclonal Ab, clone TL2.1) was purchased from HyCult Biotechnology (Uden, The Netherlands). Mouse anti-human Complement Receptor 3 (CR3) blocking antibody was obtained from USBiological (Swampscott, MA). TLR4 agonist (Ultra pure *E.coli* 0111:B4 LPS), TLR4 antagonist (*E.coli* K12 *msbB* LPS) and TLR2 agonist (*E.coli* 0111:B4 peptidoglycan) were all purchased from InvivoGen (San Diego, California). Laminarin was purchased from Sigma (St. Louis, MO). Anti-phosphotyrosine antibody (PY99) was purchased from Santa Cruz Biotechnology. Human Dectin-1 monoclonal (clone 259931) and goat polyclonal antibodies were purchased from R&D Systems (Minneapolis, MN). The ExactaCruz Kit was purchased from Santa Cruz Biotechnology (Santa Cruz, California).

Preparation of opsonized Zymosan (ZOP)

To obtain serum, blood from a healthy donor was collected and allowed to clot at 37°C for 30 minutes. This was followed by spinning the blood at 524x g for 20 minutes and collecting the supernatant serum. Zymosan was reconstituted in PBS and boiled for 1 hour. Zymosan was pelleted at 931 x g and then washed two times with PBS. This was followed by incubation of 1 volume of the Zymosan pellet with 3 volumes of fresh serum for 20 minutes at 37°C with frequent mixing. The ZOP was then pelleted by centrifugation and washed two times with PBS. ZOP was resuspended in PBS to a final concentration of 40 mg/ml and aliquots were stored at -20°C (72).

Isolation of human monocytes and cell culture

Human monocytes were isolated and purified from whole blood as described previously (48, 82). PBS-diluted whole blood was layered over a Ficoll-Paque density solution and centrifuged. The mononuclear cell layer was collected and washed twice with PBS, and contaminating platelets were removed by centrifugation (280 xg) through bovine calf serum (BCS) after overlaying the serum with the mononuclear cells. This serum spin was repeated twice. Monocytes were isolated from the platelet-free mononuclear cells by adherence to flasks precoated with BCS and containing DMEM and 10% BCS (BCS/DMEM). The flasks were incubated for 2 h at 37°C in 10% CO₂. Non-adherent cells were removed by washing the flasks with BCS/DMEM. Adherent cells were detached with PBS containing 5 mM EDTA. The monocytes were collected, washed three times with BCS/DMEM, resuspended in BCS/DMEM, and incubated at 37°C in 10% CO₂ for at least 2 h before their use in experiments. In some of the experiments

monocytes were isolated from human peripheral blood using a countercurrent centrifugal elutriation method (83, 84). Monocyte preparations purified by both methods were very similar in response to Zymosan or opsonized Zymosan (ZOP) and are consistently >90% CD14⁺. ZOP or Zymosan were used to activate the monocytes by protocols previously described (72).

Treatment of cells with pharmacological inhibitors

Monocytes were plated at a concentration of $5.0 \times 10^6/2$ ml/well in six-well plates. The cells were treated for 30 minutes with or without one of the following inhibitors: PP2, a Src activity inhibitor, PP3 (an inactive analog to PP2), Syk inhibitor (oxindole), Piceatannol or Rottlerin at the indicated concentrations. After inhibitor treatment, monocytes were left unactivated or were activated with Zymosan or ZOP. Cells were collected and lysed as described below.

Western blots

Monocytes were scraped from the dish and lysed. Post-nuclear lysates were prepared using a lysis buffer containing 1% Triton X 100, NaCl 150 mM, NaF 50 mM, β glycerophosphate 30 mM, phosphoserine 0.5 mM, phosphotyrosine 0.5 mM, phosphothreonine 1.0 mM, *p*-nitrophenylphosphate 1.5 mM, Tris pH 7.4 50mM, sodium orthovanadate 1 mM, phenylmethylsulfonyl fluoride 500 μ M and protease inhibitor cocktail (1:50, Sigma Chemical Co.). After 30 min incubation on ice, extracts were centrifuged at 9300 x g for 10 min at 4 °C, the supernatant was collected and the protein concentration was determined. Post-nuclear lysates were resolved by 8% or 10% SDS-

PAGE and transferred to a PVDF membrane (0.2 μ m; Bio-Rad, Richmond, CA).

Nonspecific binding sites were blocked with 5% BSA in PBST (1X Phosphate-Buffered Saline/Tween-20 (0.1%)) at room temperature for 1 h. Human phospho-Src and phospho-Syk were detected with rabbit anti-human polyclonal Abs (Diluted 1/1000 in PBST with 3% BSA), followed by incubation with an HRP-conjugated goat anti-rabbit IgG (1/500 in PBST with 3% BSA; from KPL, Gaithersburg, Maryland). In the immunoprecipitation studies the membrane was incubated with an anti-human PKC δ polyclonal Ab, Syk monoclonal Ab or a goat polyclonal Dectin-1 Ab (Diluted 1/1000 in PBST with 3% BSA). The membrane was developed using Enhanced Chemiluminescence Detection reagents (Pierce).

Immunoprecipitation

Monocytes were pre-treated with the indicated inhibitors for 30 minutes prior to Zymosan stimulation (2 mg/ml) for 15 minutes. Post nuclear lysates were prepared as described above. The ExactaCruz Kit was used in immunoprecipitation experiments to prevent detection of the Heavy and Light chains of the IP antibody, Dectin-1 mouse monoclonal Ab. The IP was carried out by following the instructions provided by the Exactacruz Kit. Immunoblots were stripped and reprobed with Dectin-1 antibody to evaluate immunoprecipitation and assess equal loading.

Superoxide anion assay

This assay measures superoxide anion by quantifying superoxide dismutase (SOD)-inhibitable cytochrome C reduction. Human monocytes were plated in 24-well tissue

culture plates pretreated with Hank's BSS containing 10% BCS for 1 h at 37°C (500 µl respectively, 1x10⁶/ml in Hank's BSS 1X) and allowed to adhere for at least two hours. This was followed by treatment with the different inhibitors using several concentrations at indicated incubation times. Cytochrome C (160 U/ml, Sigma), with or without superoxide dismutase (300 U/ml, Sigma), was added to the wells. This was followed by Zymosan or ZOP stimulation. The cells were incubated for 1 h at 37°C. The media was collected in tubes and the absorbance of the supernatant was read in cuvettes at 550nm. The SOD-inhibitable O₂⁻ produced by monocytes was calculated by subtracting the +SOD values from the -SOD values. These values were then multiplied by the extinction coefficient of 47.6 (read in a cuvette) and expressed as nanomoles/hr/10⁶ cells. This method is a modification (46, 47, 72) of an assay previously published by Pick and Mizel (85). The following equation was used to determine the nmols of O₂⁻ produced:

$$O_2^- \text{ nmols/ml} = (A_{550} \text{ (in the absence of SOD)} \times 47.6) - (A_{550} \text{ (in the presence of SOD)} \times 47.6)$$

RESULTS

NADPH oxidase activity in Zymosan or ZOP-stimulated human monocytes is independent of TLR4 and TLR2

Previous studies have shown that Zymosan induces inflammatory signals through binding to members of the Toll-like receptor family in mouse macrophages (78). Therefore in this study we decided to determine whether TLRs might be involved in regulating Zymosan and ZOP-induced O_2^- production in primary human monocytes. We first tested the role of TLR4. Figure 6A shows that a TLR4 antagonist did not block Zymosan or ZOP-induced O_2^- production. We also induced O_2^- with LPS, a known TLR4 ligand, in the presence or absence of the TLR4 antagonist. Monocyte O_2^- production was increased after LPS stimulation and this increase was significantly blocked in the presence of this TLR4 antagonist ($p < 0.01$). These results indicate that TLR4 signaling is not required for Zymosan or ZOP-induced O_2^- production in monocytes.

Studies have shown that TLR2 binds directly to Zymosan and this triggers NF- κ B activation and TNF- α and IL-12 secretion in mouse macrophages and dendritic cells (76, 81). It was also shown that Zymosan, but not the TLR4 ligand LPS, inhibits IFN-gamma-induced killing of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) in bone marrow-derived mouse macrophages and in a mouse cell line (86). Therefore, we investigated whether a monoclonal antibody recognizing a TLR2-associated epitope (TL2.1) would block NADPH oxidase activity in Zymosan or ZOP-induced cells. The effect of the function blocking TLR2 antibody on Zymosan-stimulated cells is shown in

Fig. 6B. Cells were pretreated with TLR2 blocking antibody for 1 hour followed by stimulation with Zymosan for 1 hour, which is the time frame we use when measuring O_2^- release in human monocytes. Our lab has previously reported that the peak of O_2^- production in monocytes is 1 hour after activation (35). Peptidoglycan (PGN) from gram positive bacteria is a known TLR2 ligand (87) and a potent activator of NF- κ B and TNF- α (88). We tested the ability of PGN to induce O_2^- production in the presence and absence of the TLR2 blocking Ab. In Fig. 6C the same experiment was carried out but the cells were activated with ZOP for 1 hour. Data from these experiments are shown in Fig. 6B and 6C. The results demonstrate that treatment with the TLR2 blocking antibody did not affect O_2^- production in Zymosan or ZOP activated cells. We show that PGN is a potent stimulator of NADPH oxidase in human monocytes and this was used as a control for the TLR2 blocking antibody. In contrast to ZOP and Zymosan, we show that the PGN-induced O_2^- production in human monocytes is inhibited by the TLR2 blocking antibody ($p < 0.05$). Taken together the data in Figure 6 suggest that Zymosan or ZOP do not signal through TLR4 for O_2^- production in primary human monocytes. In addition, TLR2 does not regulate O_2^- production in Zymosan or ZOP activated cells. As expected, LPS and PGN trigger O_2^- production through TLR4 and TLR2 in human monocytes.

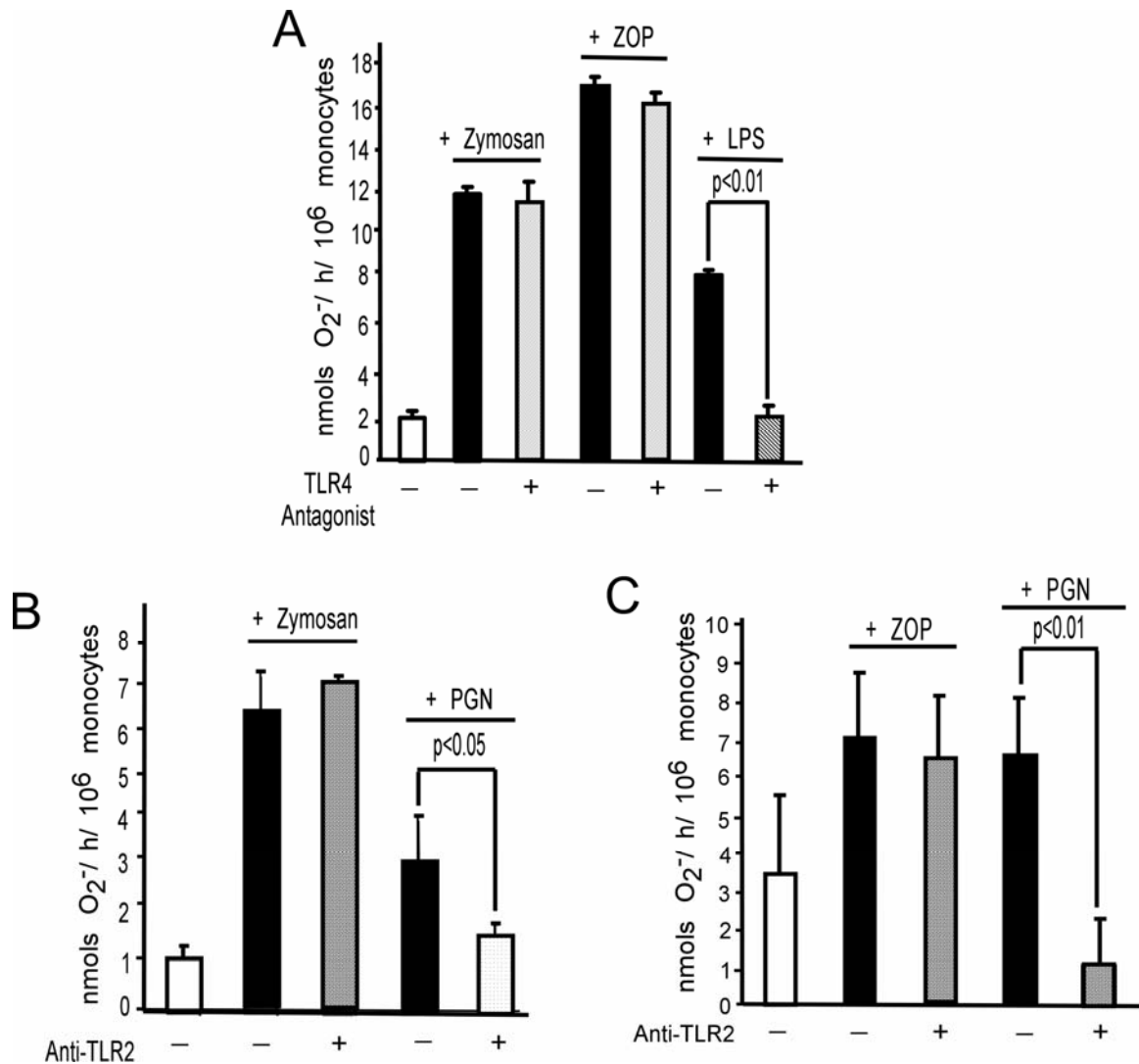


FIGURE 6. TLR4 and TLR2 are not involved in Zymosan or ZOP-induced O_2^- production. A. O_2^- production is not regulated by TLR4 in activated monocytes

Primary human monocytes were plated in 24-well plates (500 μ l; 1×10^6 /ml). Monocytes were activated with Zymosan (100 μ g/ml), ZOP (100 μ g/ml) or LPS (1 μ g/ml) in the presence or absence of the TLR4 antagonist (5 μ g/ml). Cells were pretreated with the antagonist for 30 min. Superoxide anion production was measured during the first hour of

activation. Data represent the mean \pm SD (n=3) from a representative experiment of four independent experiments that were performed. Results were similar in all experiments.

B. TLR2 is also not involved in Zymosan-induced $O_2^{\cdot-}$ production. Primary monocytes were plated as in Fig 6A. Monocytes were left untreated or were treated with a TLR2 blocking antibody (10 μ g/ml) for 1 h, followed by stimulation with Zymosan (100 μ g/ml) or *E.coli* 0111:B4 peptidoglycan (PGN) (5 μ g/ml). $O_2^{\cdot-}$ production was measured as described in Materials and Methods. **C. TLR2 is not required for ZOP-induced $O_2^{\cdot-}$ release.** Monocytes (500 μ l; 1x10⁶/ml) were treated or left untreated with a TLR2 blocking antibody (clone TL2.1) for 1 h and then activated with ZOP or PGN. Following activation for 1 h $O_2^{\cdot-}$ production was measured. Data in A and B represent the mean \pm SD (n=3) from a representative experiment of three repeat experiments that gave similar results.

NADPH activity and superoxide anion production is regulated by the leukocyte β -glucan receptor, Dectin-1, and CR3

Since neither TLR2 nor TLR4 mediated Zymosan or ZOP stimulation of $O_2^{\cdot-}$ production, we hypothesized that Dectin-1 and/or CR3 might regulate the $O_2^{\cdot-}$ production in Zymosan and ZOP-activated monocytes. We therefore proceeded to test the potential involvement of the above receptors in NADPH oxidase activity and we were also interested in the comparison of receptor utilization between Zymosan and ZOP.

Laminarin, a soluble β -glucan and a Dectin-1 inhibitor, and a CR3 function blocking monoclonal antibody were used to study the roles of Dectin-1 and CR3 in Zymosan and ZOP activation of monocyte NADPH oxidase. Both laminarin and anti-

CR3 blocking antibody were used at maximal inhibitory doses (data not shown). Laminarin caused significant inhibition (100% inhibition) of Zymosan-induced $O_2^{\cdot-}$ production ($p<0.01$). The CR3 blocking antibody also inhibited $O_2^{\cdot-}$ production ($p<0.05$); however it was not nearly as effective as the Dectin-1 inhibitor in Zymosan-activated monocytes (30% inhibition) (Fig. 7A). In contrast, in ZOP-activated cells the CR3 blocking antibody caused more inhibition (50% inhibition, $p<0.01$) than laminarin (35% inhibition, $p<0.05$). Finally, we show that a combination of laminarin and anti-CR3 monoclonal antibody is required to completely block (100% inhibition) $O_2^{\cdot-}$ production in monocytes stimulated with ZOP and the two together caused additive inhibition ($p<0.01$) (Fig. 7B).

Taken together, these results suggest that both CR3 and Dectin-1 regulate $O_2^{\cdot-}$ production in activated primary human monocytes and that Dectin-1 is the predominant receptor mediating Zymosan-induced $O_2^{\cdot-}$ production whereas CR3 is the predominant receptor mediating ZOP-induced $O_2^{\cdot-}$ production.

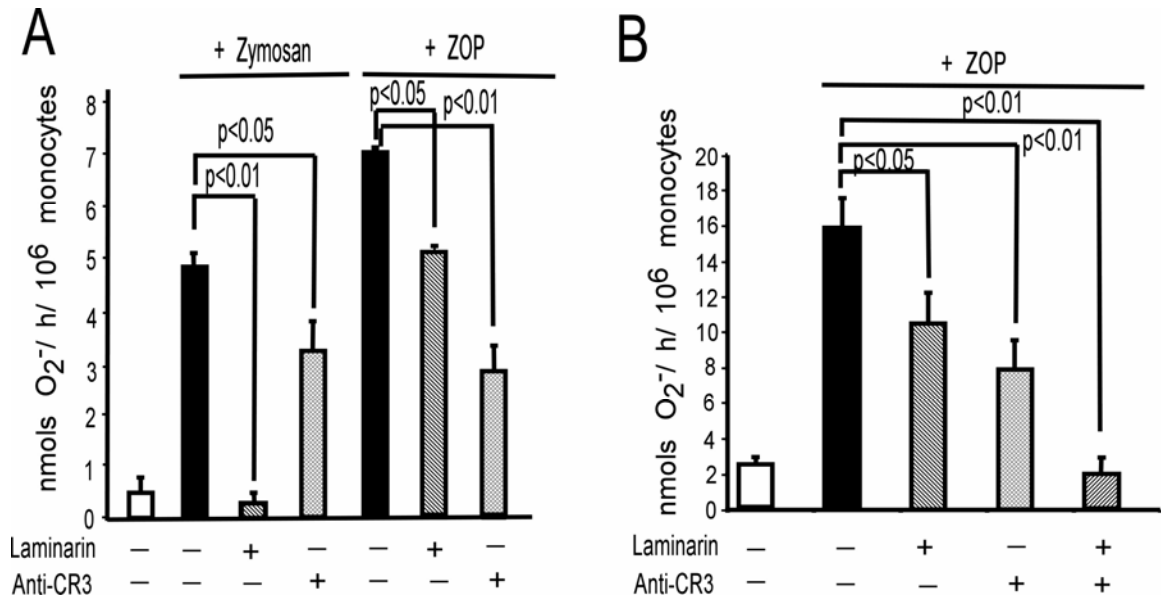


FIGURE 7. Dectin-1 and complement receptor 3 (CR3) both regulate O_2^- production in activated human monocytes. **A.** Zymosan and ZOP signal through Dectin-1 and CR3 for O_2^- production in human monocytes. Monocytes were plated as in Figure 6A. Monocytes were left untreated or were treated with laminarin (500 μ g/ml) or CR3 (10 μ g/ml) blocking antibody for 1 h. Monocytes were then activated with ZOP or Zymosan (100 μ g/ml) and O_2^- was measured during the first hour of activation. **B.** In ZOP-induced cells CR3 blocking antibody inhibited more than laminarin and the two together caused additive inhibition. Cells were activated with ZOP in the presence or absence of laminarin or CR3 blocking antibody or a combination of both. Superoxide was measured as described previously. Data in A and B show the mean \pm SD (n=3) from a representative experiment of three that were performed.

Zymosan triggers Dectin-1 tyrosine phosphorylation

Receptors such as T-cell receptors, B-cell receptors and Fc receptors have an ITAM motif in their cytoplasmic tail and tyrosine phosphorylation of this motif is required for receptor activation and initiation of downstream signaling events. Typically ITAMs are tyrosine phosphorylated by Src kinase and this is followed by Syk recruitment and autophosphorylation. In addition to the above receptors, it was also shown that Dectin-1 has a non-classical ITAM-like motif in its cytoplasmic tail (81, 89). Tyrosine phosphorylation of the ITAM-like motif of Dectin-1 is required for Syk recruitment (90). Therefore before moving on to study Dectin-1 downstream signaling in monocytes, we examined whether Dectin-1 is tyrosine phosphorylated upon stimulation with zymosan in primary human monocytes. In Figure 8 monocytes were stimulated with Zymosan, lysates were prepared and immunoprecipitated with anti-Dectin-1. Tyrosine phosphorylated Dectin-1 was detected by immunoblotting lysates with an anti-phosphotyrosine antibody, PY99.

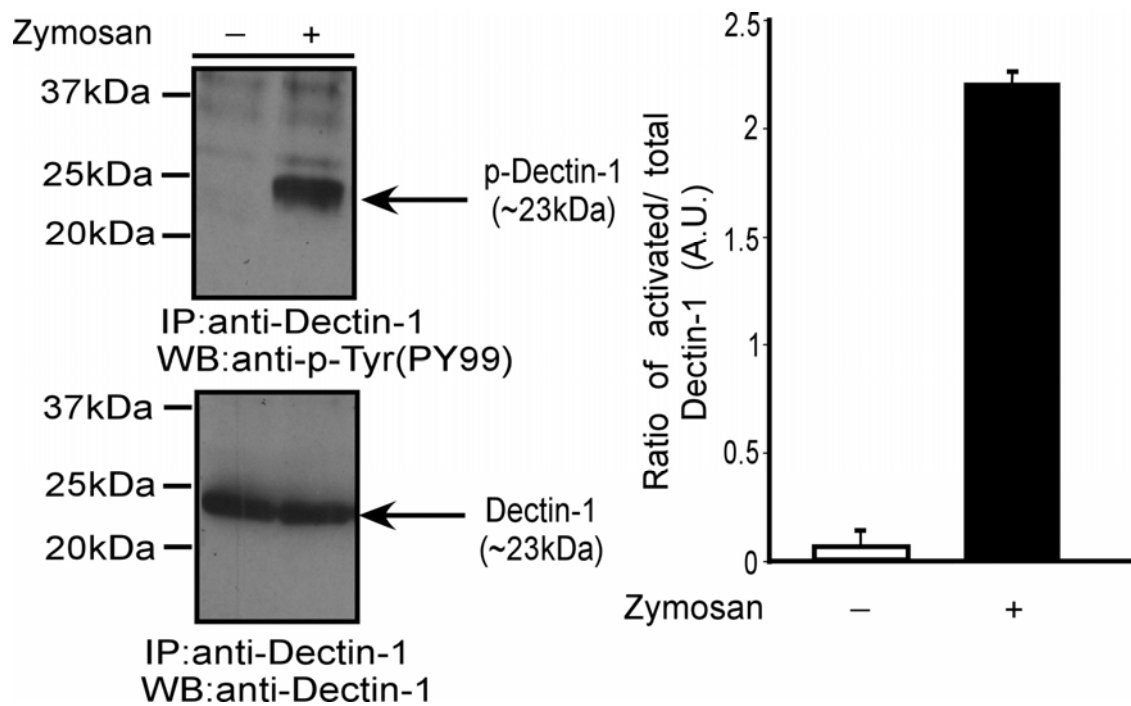


FIGURE 8. Zymosan triggers Dectin-1 activation. Human monocytes were plated in six-well plates at a concentration of 5×10^6 cells/2ml/well. Monocytes were activated using 2 mg/ml Zymosan for 15 min or left untreated. Postnuclear cell lysates were prepared, and Dectin-1 was immunoprecipitated using a mouse monoclonal antibody against Dectin-1. Immune complexes were subjected to 10% SDS-PAGE and then transferred to PVDF membranes. Dectin-1 immune complexes were first immunoblotted with an anti-phosphotyrosine antibody (PY99) and then stripped and reprobed with goat polyclonal antibody against human Dectin-1 to check immunoprecipitation and equal loading. Data were collected from three independent experiments and shown as the mean \pm data range.

Zymosan induces tyrosine phosphorylation of Src through Dectin-1 and PP2, SU6656 inhibit superoxide production in activated monocytes

We showed earlier that Zymosan requires Dectin-1 for NADPH oxidase activity in monocytes and that Dectin-1 is tyrosine phosphorylated in Zymosan-activated monocytes. In this study we were interested in looking at Src and Syk activation in Zymosan-induced human monocytes. To explore this we conducted studies to test whether Zymosan induces Src activation by assessing the auto-phosphorylation of Src after Zymosan treatment using a specific antibody that detects Src pTyr416. We activated monocytes with Zymosan for 30 minutes and representative results of this treatment on Src Tyr-416 phosphorylation are shown in Fig. 9A (upper panel). Zymosan induced Src Tyr-416 phosphorylation as compared to inactivated cells. We also tested the effect of laminarin and two Syk inhibitors, piceatannol and oxindole, on Src activity. Data shown in Fig. 9A (upper panel) demonstrate that blocking Dectin-1 signaling by laminarin caused 72% inhibition of Src tyrosine phosphorylation. In addition, Src phosphorylation was inhibited 99% by oxindole and 72% by piceatannol. Lane 6 shows cells treated with DMSO which was used as a vehicle control and showed no effect on Src activation. We then normalized for sample loading as determined by the Src immunoblot (Fig 9A lower panel) and graphed the data.

In Figure 9A we show that Zymosan induces the activation of Src kinase through Dectin-1. Therefore, we tested the role of Src in regulating NADPH oxidase activity and $O_2^{\cdot -}$ production in Zymosan-activated human monocytes. Fig. 9B shows that PP2 and SU6656, two specific inhibitors for Src family kinases, dose-dependently inhibited Zymosan-triggered $O_2^{\cdot -}$ production ($p < 0.001$); however, PP3, the inactive analogue of

PP2, had no inhibitory effect. These data suggest an essential role for Src tyrosine kinase in controlling NADPH oxidase activity in human monocytes.

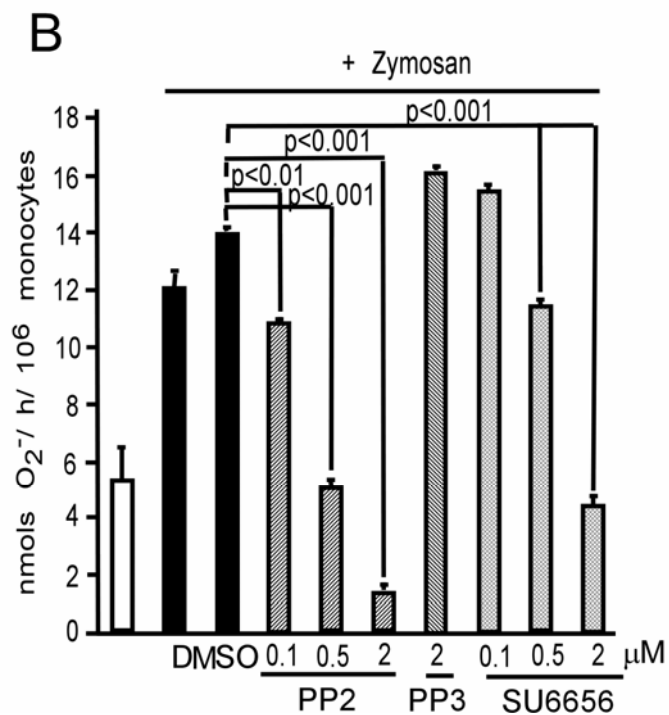
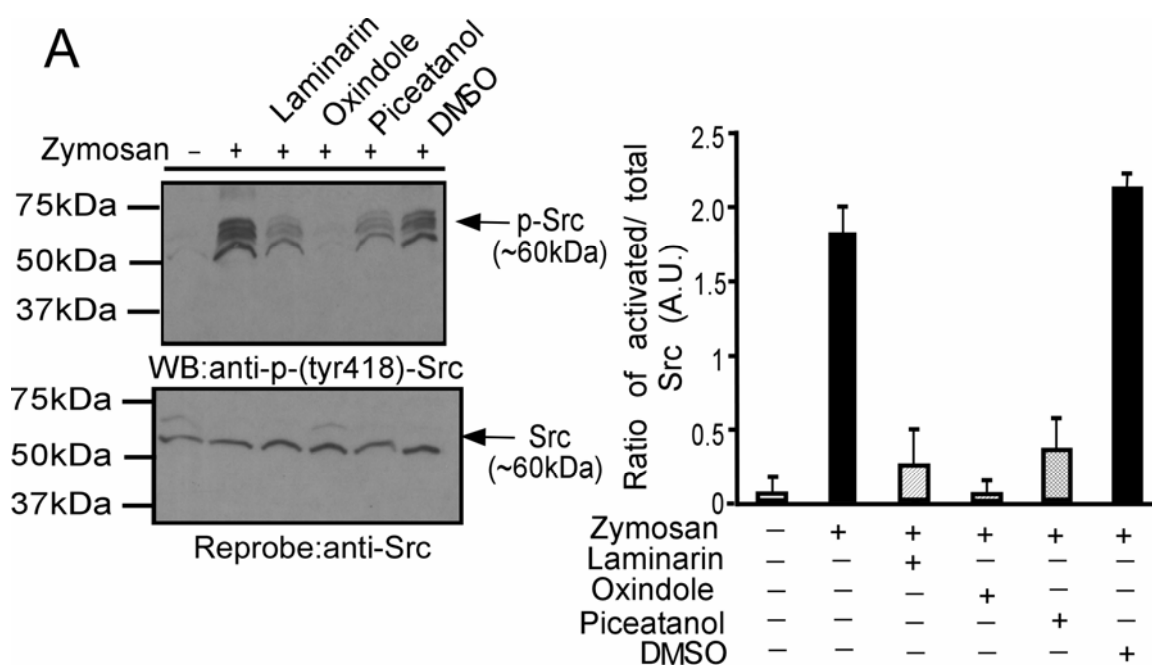


FIGURE 9. Zymosan induces tyrosine phosphorylation of Src kinase through Dectin-1 and Src regulates NADPH oxidase activity. **A. Src is tyrosine phosphorylated in activated monocytes and this event is regulated by Dectin-1 and Syk.** Human monocytes were plated as in Figure 8. The cells were then left unactivated or were activated with Zymosan (2mg/ml) for 30 minutes. The phospho-Src protein was detected by Western-blotting using a rabbit anti-human polyclonal phospho-Tyr⁴¹⁶-Src antibody. In lane 3 the cells were pretreated for 30 min with laminarin (500µg/ml) followed by Zymosan activation. In lane 4 and 5 cells were pretreated with two selective Syk inhibitors. Lane 6 represents cells pretreated with DMSO alone as vehicle control. After activation, cells were lysed and proteins were resolved by SDS-PAGE and electrophoretically transferred to a PVDF membrane. The membrane was then subjected to Western blot analysis with a p-Src rabbit polyclonal antibody. The membrane was then stripped and Src protein was detected using a rabbit anti-human polyclonal antibody (lower panel). Data represent results from two independent experiments and are shown as the mean \pm data range.

B. PP2 and SU6656 block Zymosan-induced superoxide anion production. Human monocytes plated as described in Fig 6A were activated with Zymosan. In some groups cells were pretreated with Src inhibitors PP2, SU6656 or PP3, an inactive analogue of PP2 at dose dependent manner prior to Zymosan activation. Pre-treatment with the inhibitors or vehicle solution (2% DMSO final concentration) was done for 1 hour before Zymosan activation. Superoxide anion production was measured in the first hour of activation as described in Materials and Methods. Data are from a representative experiment of four that were performed showing mean \pm SD, n=3.

Syk is activated in Zymosan-treated cells and Oxindole blocks superoxide production in activated monocytes

After exploring Src activation we performed more experiments to explore whether Syk is activated in Zymosan treated cells. Monocytes were incubated with Zymosan for 30 minutes or they were left untreated. The level of Syk activation was detected by western blotting using an antibody that only detects human Syk when phosphorylated at Tyr525/526 (Figure 10A and 10B, upper panel). Data presented in Figure 10A (upper panel) demonstrate the effect of laminarin on Syk activation. As shown in the figure Syk tyrosine phosphorylation was inhibited 97% by laminarin. Furthermore, the selective inhibitor for Src activity, PP2, reduced Syk tyrosine phosphorylation in Zymosan activated monocytes, whereas the inactive analogue of PP2, PP3, had no effect (Figure 10B). To check for protein loading both membranes were stripped and reprobed with an antibody against Syk. These studies indicate that Syk tyrosine kinase is phosphorylated at its activation site by Zymosan and that both Dectin-1 and Src regulate Syk phosphorylation.

A recent study published by Underhill *et al.* showed that Dectin-1 activates Syk in Zymosan-stimulated IFN- γ primed-mouse macrophages and this is important for the production of reactive oxygen species but not for phagocytosis (55). Therefore we tested the role of Syk in regulating NADPH oxidase activity and O₂⁻ production in Zymosan-activated human monocytes. Fig. 10C shows that oxindole dose-dependently inhibited Zymosan-induced O₂⁻ ($p < 0.001$). Taken together, our results from Figures 9 and 10 indicate that Zymosan induces Src and Syk tyrosine phosphorylation, two proteins believed to be activated downstream of Dectin-1. We show here that Dectin-1 is indeed

upstream of Src and Syk and blocking this receptor will block Zymosan-induced activation of both kinases. Using specific pharmacological inhibitors, we also show that PP2 regulates Syk activity and Oxindoles regulate Src activity. The above data suggest an essential role for both Src and Syk tyrosine kinases in controlling NADPH oxidase activity in human monocytes.

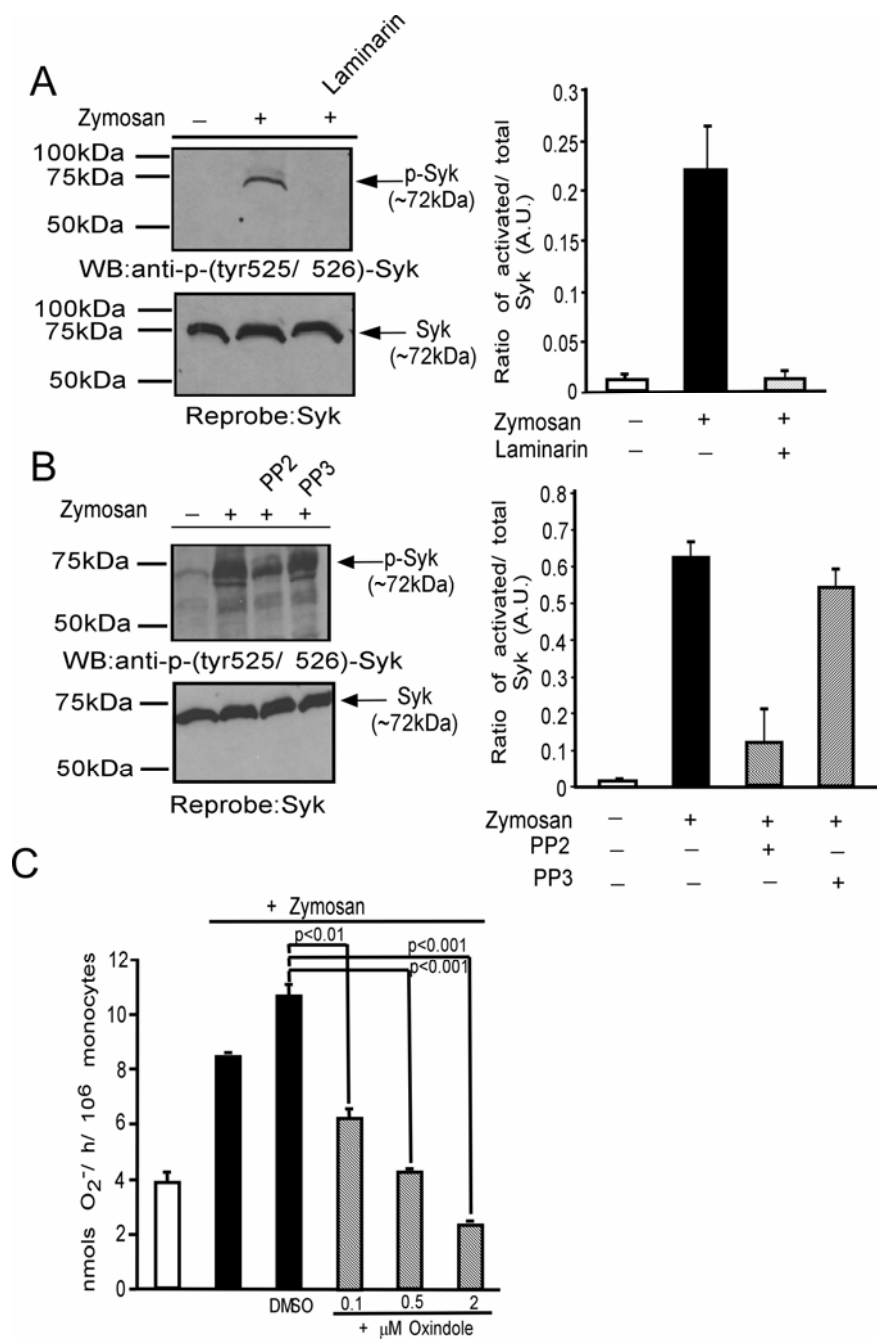


FIGURE 10. Syk is tyrosine phosphorylated following monocyte activation and Syk activity is required for superoxide anion production. A. Laminarin blocks Syk

tyrosine phosphorylation in Zymosan-activated monocytes. Phospho-Syk protein was detected using a rabbit monoclonal phospho-Syk Tyr^{525/526} antibody. In lane 3 (upper panel), monocytes were pretreated with laminarin (500µg/ml) for 30 minutes. Cells were then activated with Zymosan (2mg/ml) for 30 min. After activation cells were lysed and lysates were resolved by SDS-PAGE, electrophoretically transferred to a PVDF membrane and analyzed by Western blotting. The blot was stripped and reprobed with a Syk monoclonal antibody to check for equal protein loading (lower panel). Data were collected from two independent experiments and shown as the mean ± data range

B. Tyrosine phosphorylation of Syk kinase is regulated by Zymosan and Src activity.

Monocytes were plated as previously described and then were activated with Zymosan or were left unactivated. Cells were lysed, resolved by SDS-PAGE and analyzed by Western blot analysis. Phospho-Syk protein was detected using a rabbit polyclonal phospho-Syk Tyr^{525/526} antibody. In lane 3 and 4 (upper panel), monocytes were pretreated with PP2 (25µM) and PP3 (25µM) for 30 minutes and then activated with Zymosan (2mg/ml) for 30 min. The blot was stripped and reprobed with a Syk monoclonal antibody to check for equal protein loading (lower panel). Data were collected from two independent experiments and shown as the mean ± data range

C. Oxindole blocks Zymosan-induced superoxide anion production in a dose dependent manner. Monocytes were plated as described in Figure 6A. Monocytes were pretreated with for 30 minutes prior to Zymosan stimulation. Data are from a representative experiment of four that were performed showing mean ± SD (n=3) with similar results.

Dectin-1 complex formation

We have shown that Dectin-1 is required for the activation of Src and Syk tyrosine kinases and for the production of superoxide anion in Zymosan-activated human monocytes. We are also interested in studying Dectin-1 complex formation with intracellular proteins after monocyte activation. To evaluate possible association of Src/Syk kinases with Dectin-1, we immunoprecipitated Dectin-1 from untreated and Zymosan-treated monocyte cell lysates at different time points and analyzed the SDS-PAGE blots with antibodies to Src/Syk kinases.

Studies from our lab have shown that PKC δ is required for NADPH oxidase activation in primary human monocytes (46, 47). Our lab has also shown that PKC δ is both serine and tyrosine phosphorylated after treating monocytes with ZOP (47). From the above studies we know that PKC δ regulates NADPH oxidase activity in human monocytes. Therefore, we were interested to see whether PKC δ is also present in a complex with Dectin-1 in monocytes.

Results of these experiments are presented in Figure 11. Src, Syk and PKC δ co-immunoprecipitated with endogenous Dectin-1. The data indicate that all three proteins are endogenously associated with Dectin-1, and association is enhanced upon treatment with Zymosan. Association between the receptor and the proteins peaks at 5-10 minutes then gradually decreases and by 30 minutes it drops back to basal level of association. Reprobing the blot with Dectin-1 antibody confirmed that equal amounts of Dectin-1 protein were immunoprecipitated from the lysates (Fig.11, bottom panel). Thus Zymosan treatment enhances the association of several different proteins with Dectin-1 and this is a very rapid event taking place within 5 minutes of monocyte exposure to Zymosan. In

addition these data demonstrate for the first time that PKC δ associates with Dectin-1 and that Zymosan enhances the formation of this complex.

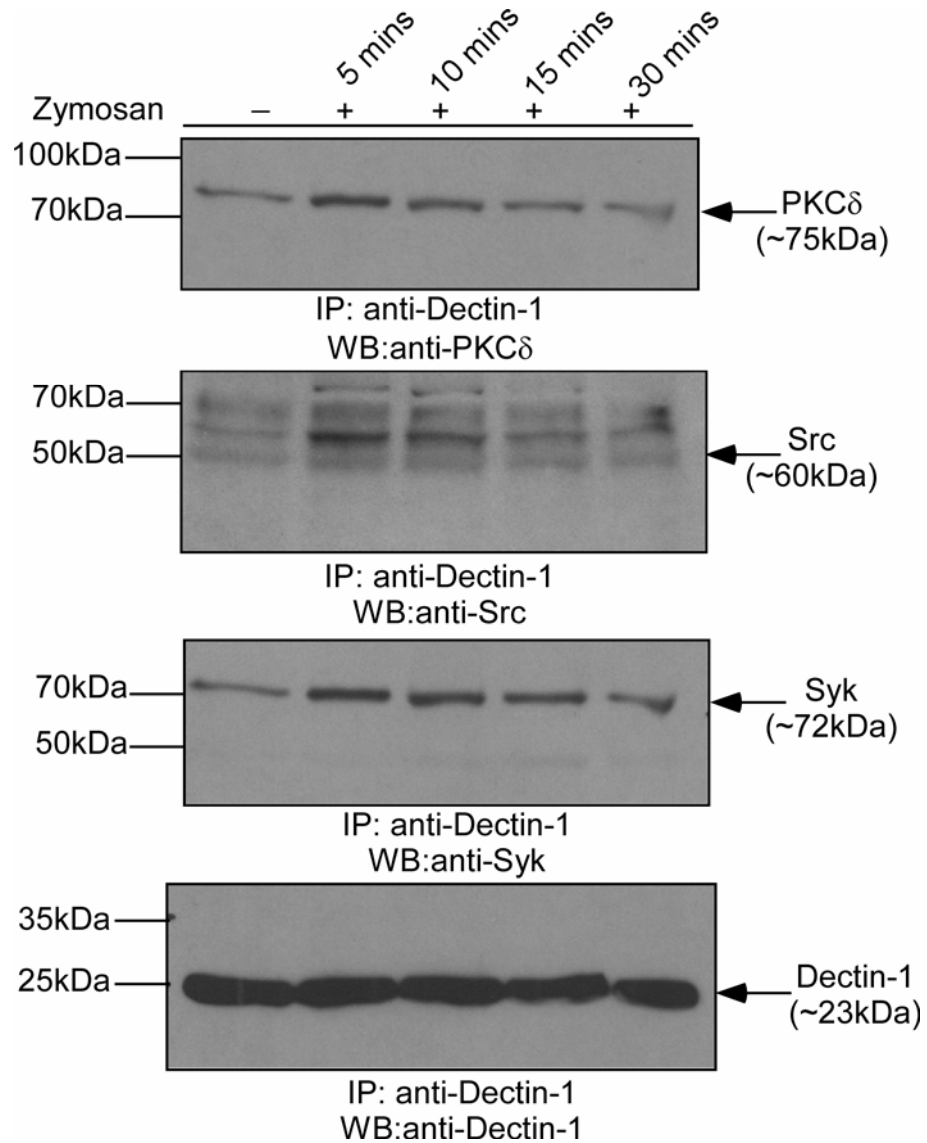


FIGURE 11. PKC δ , Src and Syk associate with Dectin-1. Human monocytes (10 million/group) were treated with Zymosan (2mg/ml) for 5, 10, 15 and 30 minutes or left untreated. All groups were lysed and immunoprecipitated with antibody specific to human Dectin-1. The immunoprecipitates were analyzed on 8% SDS-PAGE blots and

probed with antibodies to PKC δ , Src or Syk. The bottom panel displays the blot from subsequent reprobing, after stripping, using an antibody against Dectin-1. Near-equal immunoprecipitation of proteins was observed. This is a representative experiment of two performed (n=2).

Tyrosine phosphorylation of Dectin-1, Src and Syk kinase follows the same pattern as the complex formation

We have shown earlier that Dectin-1, Src and Syk are tyrosine phosphorylated after Zymosan activation. Here we carried out time course experiments, looking at tyrosine phosphorylation of the above proteins at different time points. The purpose of these experiments was to know the peak time for tyrosine phosphorylation and compare the pattern of activation with that of complex formation (shown in Fig 11). The tyrosine phosphorylation status of Dectin-1 was examined by its immunoprecipitation from untreated and Zymosan-treated monocyte lysates and then probing with an anti-phosphotyrosine antibody (PY99) to detect Zymosan-induced changes in tyrosine phosphorylation of the immunoprecipitated proteins. We observed that Zymosan markedly induced tyrosine phosphorylation of Dectin-1 (as shown earlier), however, the peak of phosphorylation was seen between 5-10 minutes although it decreased after that it was still detectable at 30 minutes (Fig 12A, upper panel). The blot was then stripped and reprobed with Dectin-1 antibody to ensure adequate immunoprecipitation (Fig 12A, lower panel).

The tyrosine phosphorylation status of Src and Syk were examined using antiphospho-Src or Syk antibodies. In these experiments 50 μ g monocyte post-nuclear

lysates from untreated and Zymosan treated (5, 10, 15 and 30 mins) monocytes were run on SDS-PAGE, transferred onto a PVDF membrane and blotted with either phospho-Src or phospho-Syk antibody. Phosphorylated Src and Syk were only detectable in Zymosan treated cells (Fig 12B and Fig 12C, upper panels). The peak phosphorylation for Src and Syk was detectable at 5-10 minutes of Zymosan activation and we observe a decrease at later time points. This observation is consistent with the pattern of tyrosine phosphorylation of Dectin-1. The lower two panels of Figure 12B and Figure 12C are a result of a strip and a reprobe with total Src or Syk antibody, showing almost equal loading.

Thus it appears from these results that Dectin-1, Src and Syk are all activated after 5 minutes of Zymosan treatment and this activation decreases with time. This is consistent with the pattern of Dectin-1 association with Syk and Src.

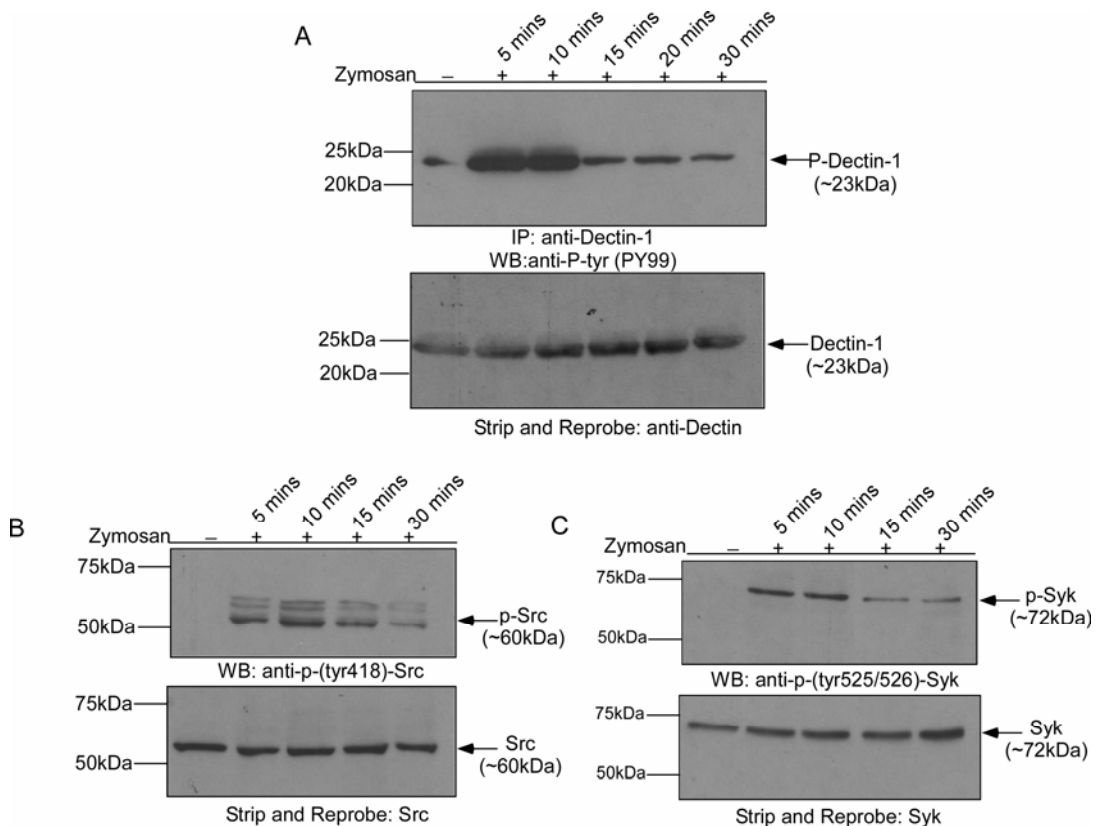


FIGURE 12. Zymosan induces Dectin-1, Src and Syk phosphorylation in a time point fashion. **A. Dectin-1 tyrosine phosphorylation.** Monocytes (10 million/group) were untreated or treated with Zymosan (2mg/ml) for 5-30 minutes, lysed, and immunoprecipitated with an antibody specific for Dectin-1. The immunoprecipitated proteins were collected on beads and separated on a 10% SDS-PAGE, transferred onto a PVDF membrane, and probed with PY-99. The bottom panel shows the stripped and reprobed blot with Dectin-1 antibody. **B. Src tyrosine phosphorylation.** Monocyte lysates were prepared from untreated or Zymosan-treated cells at the indicated time points, and protein concentrations were determined. Lysate protein (50 µg) from treated or untreated monocytes was loaded in adjacent lanes and probed with anti-phospho-Src antibody. The lower panel shows the stripped and reprobed blot with Src antibody. **C. Syk tyrosine phosphorylation.** Monocyte lysates were prepared from untreated or Zymosan-treated cells at the indicated time points. Lysate protein (50 µg) from treated or untreated monocytes was loaded in adjacent lanes and probed with anti-phospho-Syk antibody. The lower panel shows the stripped and reprobed blot with Syk antibody. This a representative experiment of two performed (n=2).

PKC δ and Dectin-1 signaling

PKC δ is required for NADPH oxidase activation in primary human monocytes (46, 47). From the above studies we have shown that PKC δ is associated with Dectin-1 and this association is enhanced by Zymosan activation in monocytes. To expand this observation we tested the effect of rottlerin, a PKC δ activity inhibitor, on Dectin-1 complex formation. In addition, we investigated the role of PP2, a Src inhibitor, and Oxindole on PKC δ association with Dectin-1. To test this we performed immunoprecipitation and Western-blotting experiments. The upper panel of Figure 13A confirms our earlier observation that there is already a weak basal level of association between Dectin1 and PKC δ which is induced by the addition of Zymosan. In the presence of rottlerin, this interaction becomes weaker which leads to the conclusion that PKC δ activity is required for this molecular complex formation. The middle panel of Figure 13A shows that when PKC δ and Dectin-1 form this molecular complex, PKC δ remains in its Ser/Thr phosphorylated form (activated form of PKC δ) and again rottlerin down-regulates the activity of PKC δ and inhibits the association between the two molecules. The bottom panel shows almost equal levels of Dectin1 in the immunoprecipitates.

Our studies in Figure 10 suggest the involvement of Syk kinase in Dectin-1 signaling and NADPH oxidase activation in human monocytes. In Figure 11 we show Syk association with Dectin-1. We next wanted to determine whether Syk association with endogenous Dectin-1 in monocytes is regulated by PKC δ . Monocytes were treated with Zymosan then lysed, and post-nuclear cell lysates were immunoprecipitated with anti-Dectin-1 antibody followed by Western blot analysis. As shown in Figure 13B Zymosan induced Syk/Dectin-1 complex formation. Pre-treating lysates with rottlerin

followed by Zymosan activation markedly reduced Syk/Dectin-1 complex formation, suggesting that PKC δ is acting upstream of Syk in this complex formation. We checked protein loading by reprobing the blot with Dectin-1 antibody (Fig 13 B, lower panel). In Figure 13C we tested the effect of PP2 and Oxindole on PKC δ association with Dectin-1. Cells were pre-treated with PP2/PP3 and Oxindole for 30 minutes before exposure to Zymosan for 10 minutes and the immunoprecipitation/Western blotting was carried out. Our results from Fig 13C show that both inhibitors had no effect on PKC δ /Dectin-1 complex formation. This indicates that Syk and Src do not regulate the interaction of PKC δ with Dectin-1. Further experiments are required to find out whether PKC δ regulates the Src and Dectin-1 complex formation.

Bijli *et al* showed that PKC δ mediates the activation of Syk tyrosine kinase and that inhibition of PKC δ activity using pharmacological and genetic approaches blocked thrombin-induced Syk activation in human endothelial cells (91). In Fig 13B we showed that PKC δ regulates Syk association with Dectin-1. We therefore asked the question whether PKC δ can regulate Syk activation. To test this we pre-treated monocytes with rottlerin for 30 minutes followed by Zymosan activation for 10 minutes and observed the level of tyrosine phosphorylation of Syk in untreated, Zymosan-treated and rottlerin pre-treated cells. In addition, we carried out similar experiments to test the effect of rottlerin on Dectin-1 and Src tyrosine phosphorylation. We show in Figure 14 that rottlerin had no effect on the Zymosan-induced tyrosine phosphorylation of Syk, Src or Dectin-1. Taken together, our data shows that PKC δ regulates Dectin-1 complex formation with Syk and Src while having no effect on their tyrosine phosphorylation.

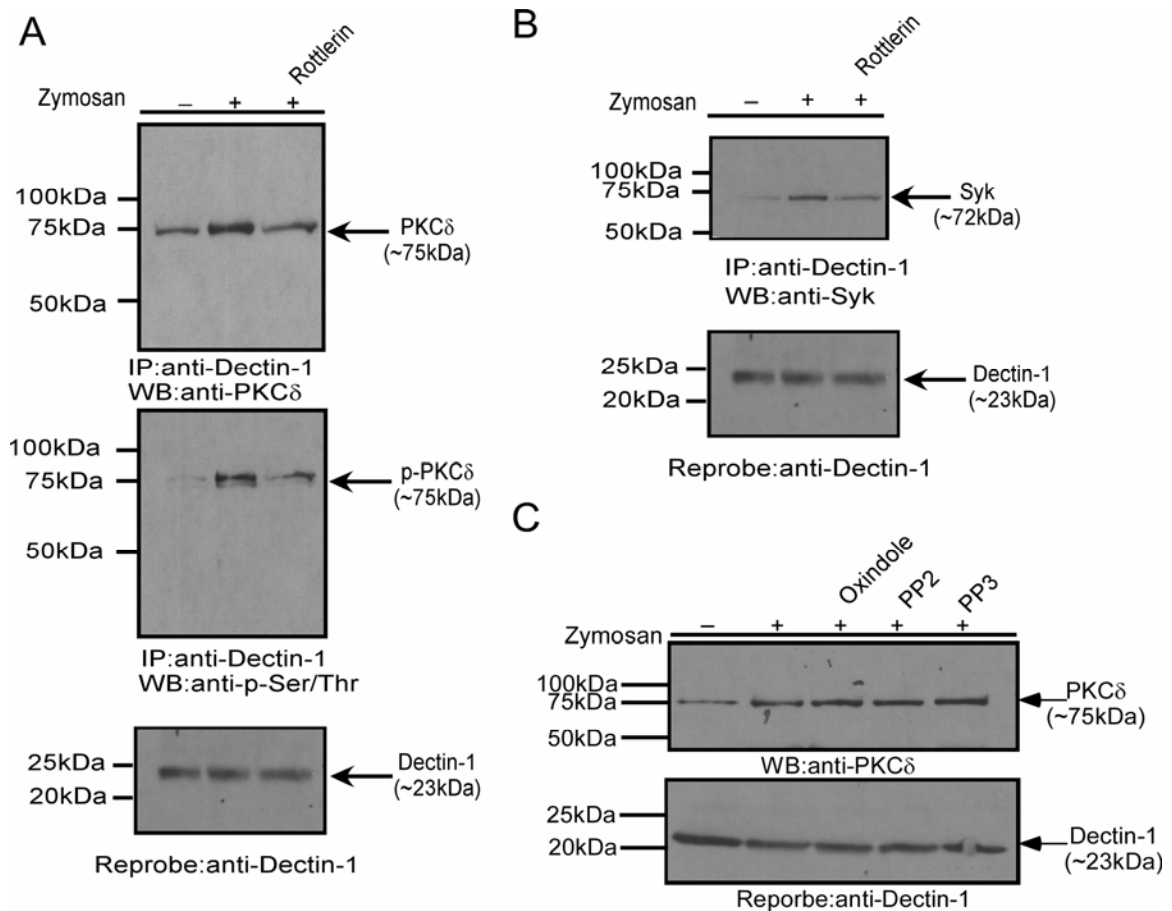


FIGURE 13. PKC δ regulates Syk association with Dectin-1 but Syk and Src have no effect on PKC δ association with Dectin-1. A. PKC δ regulates the Zymosan-induced PKC δ / Dectin-1 complex formation. Monocytes (10×10^6 /group) were left untreated or treated with rottlerin (5 μ M) for 30 minutes prior to Zymosan (2mg/ml) stimulation for an additional 10 minutes. Monocyte lysates from untreated or those exposed to Zymosan were immunoprecipitated (IP) with a mouse monoclonal antibody against human Dectin-1. Immune complexes were subjected to 10% SDS-PAGE and then transferred to PVDF membranes. Dectin-1 immune complexes were first immunoblotted with rabbit polyclonal antibody against p-Ser/Thr and then stripped and reprobed with anti-PKC δ Ab. Finally the blot was reprobed with a goat polyclonal antibody against

human Dectin-1. **B. Syk/Dectin-1 complex formation is regulated by PKC δ .** Monocytes were plated as in Fig 13A and were left untreated or treated with Rottlerin (5 μ M) for 30 minutes prior to Zymosan stimulation for an additional 10 minutes. Cells were lysed and protein was immunoprecipitated with anti-Dectin-1 Ab and blotted with Syk Ab. The blots were reprobed with anti-Dectin-1 polyclonal Ab to assess loading (lower panels).

C. PP2 and Oxindole had no effect on PKC δ /Dectin-1 complex formation. Monocytes were plated as in Fig 13A and were left untreated or treated with PP2, PP3 or oxindole (20 μ M) for 30 minutes prior to Zymosan stimulation for an additional 10 minutes. Cells were lysed and immunoprecipitated with anti-Dectin-1 Ab and blotted with Syk Ab. The blots were reprobed with anti-Dectin-1 polyclonal Ab to assess loading (lower panels).

This is a representative experiment of two performed (n=2).

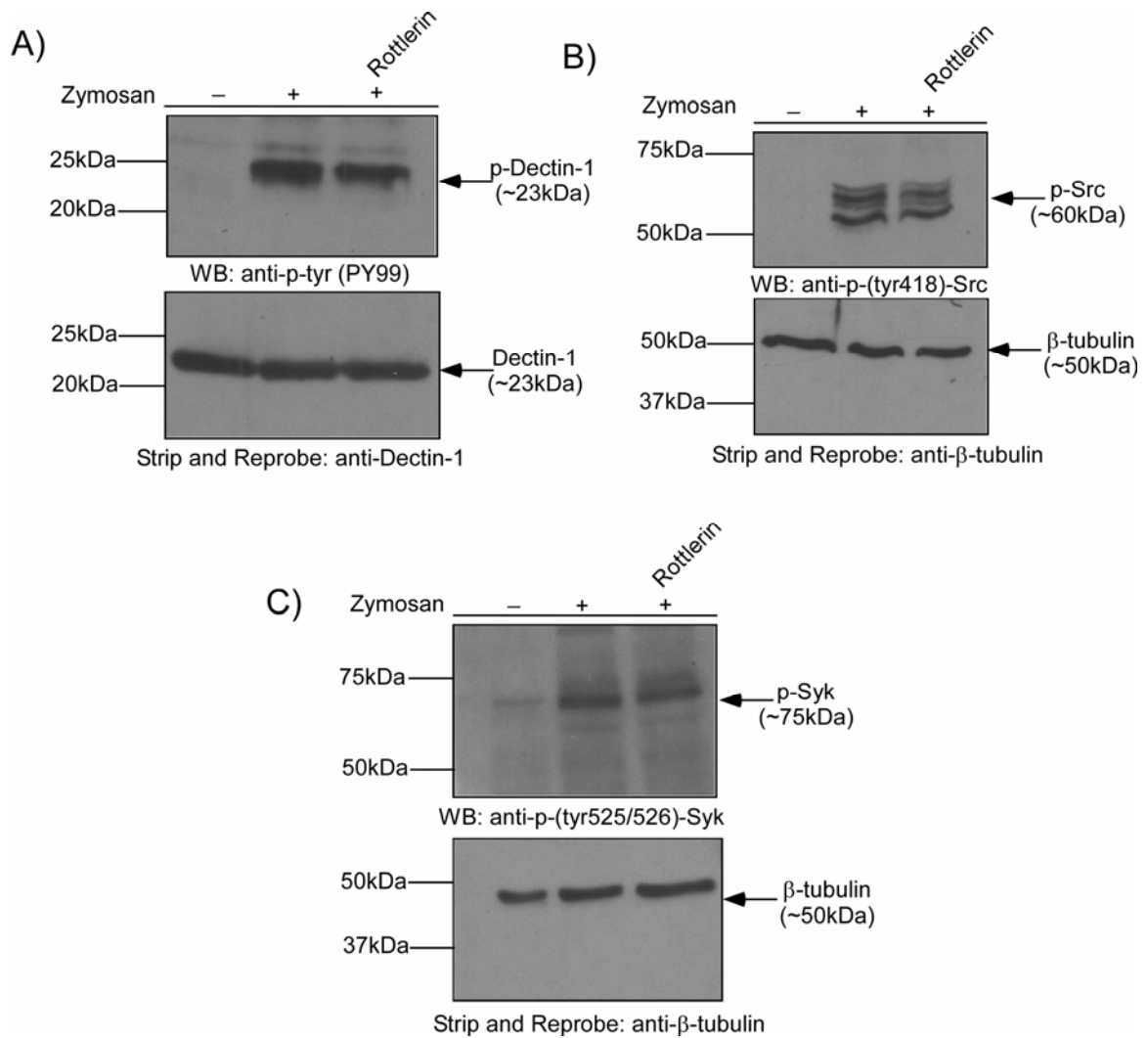


FIGURE 14. PKC δ does not regulate the tyrosine phosphorylation of Dectin-1, Src or Syk. **A. Rottlerin and Dectin-1 tyrosine phosphorylation.** Monocytes (10×10^6 /group) were untreated, treated with Zymosan (2mg/ml) for 10 minutes with or without rottlerin (5 μ M) pre-treatment (30 minutes), lysed, and immunoprecipitated with an antibody specific for Dectin-1. The immunoprecipitated proteins were collected on beads and separated on a 10% SDS-PAGE, transferred onto a PVDF membrane, and probed with PY-99. The bottom panel shows the stripped and reprobed blot with the Dectin-1 antibody. This is a representative experiment of three performed (n=3).

B. Rottlerin and Src tyrosine phosphorylation. Monocyte lysates were prepared from untreated or Zymosan-treated cells with or without rottlerin pre-treatment, and protein concentrations were determined. Lysate protein (50 µg) from treated or untreated monocytes was loaded in adjacent lanes and probed with anti-phospho-Src antibody. The lower panel shows the stripped and reprobed blot with Src antibody. **C. Rottlerin and Syk tyrosine phosphorylation.** Monocyte lysates were prepared from untreated or Zymosan-treated cells with or without rottlerin pre-treatment and protein concentrations were determined. Lysate protein (50 µg) from treated or untreated monocytes was loaded in adjacent lanes and probed with anti-phospho-Syk antibody. The lower panel shows the stripped and reprobed blot with Syk antibody.

Phospho-Syk forms a complex with Dectin-1 and this event is regulated by PP2 and Oxindole

We have shown in Figure 10B that PP2, a Src kinase activity inhibitor, but not PP3 blocks tyrosine phosphorylation of Syk. This indicates that Src acts upstream of Syk and that it regulate the activity of Syk kinase. We were interested to know whether phospho-Syk can form a complex with Dectin-1 and if Src would regulate this interaction.

To test this we performed Immunoprecipitation/Western-blotting experiments. The upper panel of Figure 15 shows that Zymosan induces phospho-Syk complex formation with Dectin-1. In the presence of PP2, this interaction is markedly inhibited. PP3, the structural negative analog for PP2, had no effect. In addition, pre-treating the cells with Oxindole also significantly reduced this complex formation. The middle panel

shows that there is already a weak level of association between Dectin-1 and total Syk, which is induced by Zymosan-stimulation. However, PP2 and Oxindole had no inhibitory affect on Syk-Dectin-1 complex formation. The bottom panel shows equal level of Dectin-1 in the immunoprecipitates.

Our results are supportive of Figure 10B which demonstrate that Src regulates tyrosine phosphorylation of Syk. Here we show that it regulates phospho-Syk complex formation with Dectin-1. In addition, we show that Oxindole reduces this complex formation. This could be explained by 1) direct inhibition of Syk kinase activity by the inhibitor 2) we have shown in the previous results that Src kinase activity is required for Zymosan-induced Syk Tyr 525 phosphorylation/activation and Oxindole regulates Src kinase activity therefore it would makes sense that Oxindole could regulate the complex by inhibiting Src kinase activity. Total Syk complex formation with Dectin-1 was induced by Zymosan (as shown before) but was not inhibited by either Oxindole or PP2.

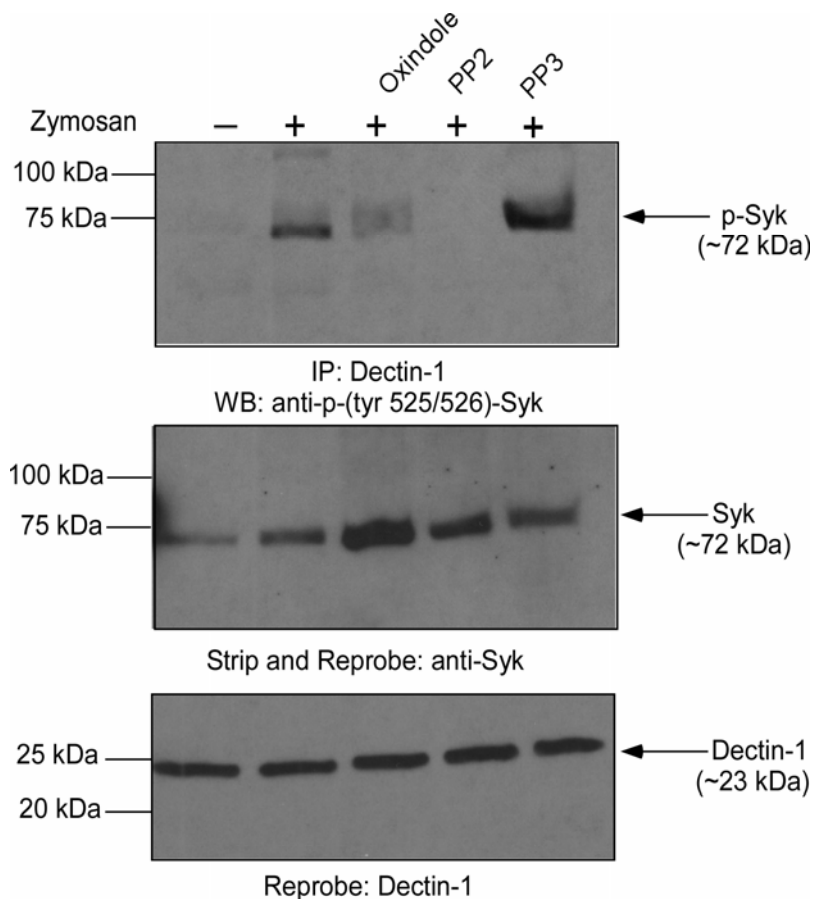


FIGURE 15. Src regulates phospho-Syk association with Dectin-1. Zymosan induces phospho-Syk/ Dectin-1 complex formation and PP2 and Oxindole inhibits this event. Monocytes ($5 \times 10^6/2$ ml) were left untreated or treated with Oxindole, PP2 or PP3 (20 μ M) for 30 minutes prior to Zymosan (2mg/ml) stimulation for an additional 15 minutes. Monocyte lysates from untreated or those exposed to Zymosan were immunoprecipitated (IP) with a mouse monoclonal antibody against human Dectin-1. Immune complexes were subjected to 10% SDS-PAGE and then transferred to PVDF membranes. Dectin-1 immune complexes were first immunoblotted with rabbit monoclonal antibody against p-Syk and then stripped and reprobred with anti-Syk Ab. Finally the blot was reprobred with a goat polyclonal antibody against human Dectin-1.

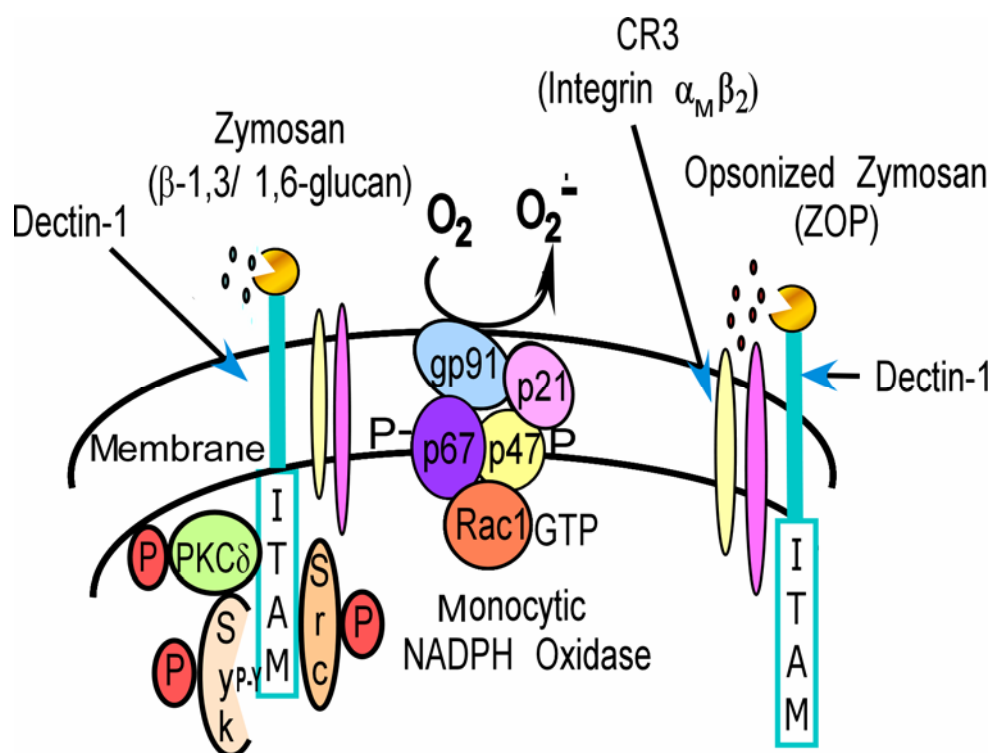


FIGURE 16. Regulation of NADPH oxidase by pattern recognition receptors in

human monocytes. This model summarizes the receptors and signaling pathways that regulate the assembly and the activation of NADPH oxidase in human monocytes.

Previously we have shown that several pathways, including calcium influx, phosphorylation of cPLA₂ by PKCα, arachidonic acid (AA) release, PKCδ-dependent phosphorylation of p47phox/p67phox and Rac-1 translocation to the membrane regulate NADPH oxidase activity in activated human monocytes (12, 14, 42, 45-48). In this study we show that Dectin-1 and CR3 serve as receptors for ZOP and Zymosan and they both regulate the activity of this enzyme complex *in vitro*. In addition, we have shown that Zymosan induces the activation of Syk and Src kinase, two proteins that signal downstream of Dectin-1. We have identified a role for Src and Syk kinase in regulating the production of superoxide anion and activity of monocyte NADPH oxidase. Finally,

we show that Zymosan induces Dectin-1 complex formation with PKC δ , Syk and Src.

PKC δ is upstream of Syk in the complex.

DISCUSSION

The activation of NADPH oxidase and reactive oxygen species production through pattern recognition receptors have been studied in various cell lines and bone marrow-derived mouse macrophages (55, 74, 81, 92). A key element of ROS production in response to stimuli containing β -glucans is the activation of Dectin-1. Activation of mouse macrophages and dendritic cells by Zymosan and other yeast particles containing β -glucans results in Dectin-1 activation and Src/Syk phosphorylation (55, 81, 93). Dectin-1 has also been reported to activate cPLA₂ and COX2 expression in mouse macrophages (94, 95) and Syk-dependent ERK activation regulates IL-2 and IL-10 production by dendritic cells stimulated with Zymosan (96).

To understand the relevance of innate immune receptors in controlling NADPH oxidase activity in primary human monocytes, we investigated the oxidative burst in the presence and absence of different receptor inhibitors. Using selective inhibitors and blocking antibodies against TLR2, TLR4, CR3 and Dectin-1, we found that Zymosan and ZOP signal primarily through Dectin-1 and CR3 for the activation of NADPH oxidase and O₂⁻ production in human monocytes. We show that TLR2 and TLR4 are not required for Zymosan or ZOP-induced O₂⁻ production. These results are consistent with reports showing that Zymosan-induced ROS production in mouse macrophages is independent of TLR2 and MyD88 (81).

In addition to our data showing a Dectin-1 requirement for ROS production through Zymosan activation, we report that CR3 can contribute to O₂⁻ release in activated human monocytes. CR3 was initially characterized for its binding to opsonized particles. More recently it has been shown to play a major role in non-opsonic recognition of

Zymosan and *Mycobacterium kansasii* through its lectin binding domains (74) . Using a maximal inhibitory dose of a blocking antibody against human CR3, we show in this study that blocking CR3 significantly reduced Zymosan and ZOP-induced $O_2^{\cdot-}$ production. More CR3 involvement was seen with ZOP-treated cells likely because ZOP is coated with serum proteins including C3bi, a ligand for CR3. Laminarin did not inhibit as much as the CR3 blocking antibody in ZOP-induced cells because we expect that a significant amount of $O_2^{\cdot-}$ production was triggered through the C3bi binding site (I-domain) on CR3 which was less engaged in cells activated with unopsonized Zymosan. This further supports the fact that CR3 can act as both an opsonic and non-opsonic receptor, binding to β -glucan elements of Zymosan and ZOP through the lectin domain and to iC3b on ZOP through the I-domain.

Signaling through ITAM-containing receptors triggers activation and tyrosine phosphorylation of their ITAM cytoplasmic domain. A typical ITAM motif has 2 motifs arranged as YXXL and phosphorylation of both tyrosine residues in the dual YXXL motif is required to activate Syk/ZAP70 and downstream signaling in ITAM receptors; however, in the case of the lectin receptors, Dectin-1 and CLEC-2, there is only a single YXXL motif in the cytoplasmic tail and tyrosine phosphorylation of this motif is sufficient to recruit Syk and initiate immune responses (90, 97). The ITAM motif of Dectin-1 is not a typical ITAM in that the distal tyrosine residue in the membrane is not in the YXXL/I sequence, only the membrane proximal tyrosine is located in this sequence (90).

Our findings in human monocytes are supportive of the prior recognized role of Src and Syk tyrosine kinases in regulating Dectin-1-mediated ROS production in murine

macrophages. Our data also show that Src and Syk are tyrosine phosphorylated in Zymosan- treated cells and that they both regulate each others activity. The regulation of Syk phosphorylation by Src is consistent with that published in another study using IFN- γ primed bone marrow-derived mouse macrophages (55). However, in this study we show novel evidence of Syk regulating Src activity in Zymosan-activated monocytes in an apparent feedback activation loop.

Previous findings from our lab showed that PKC δ is required for ZOP activation of NADPH oxidase activity in primary human monocytes (46, 47) . We also showed that PKC δ is an upstream kinase regulating p67 phosphorylation (46) and p47phox phosphorylation and translocation (47) which are required for NADPH oxidase activity. In this study we show for the first time a requirement for a novel PKC family member in Dectin-1 signaling. PKC δ forms a complex with endogenous Dectin-1 and this complex is enhanced by Zymosan. In addition PKC δ /Dectin-1 complex formation is regulated by rottlerin, a selective inhibitor of PKC δ activity. Our data also indicate that when PKC δ and Dectin1 form a molecular complex, PKC δ remains in its Ser/Thr phosphorylated form (activated form of PKC delta) and that rottlerin inhibits the association between the two molecules. Our lab has shown in an earlier report that rottlerin blocks PKC δ kinase activity using an in vitro kinase activity assay (47). These data provide evidence that PKC δ activity is required for PKC δ /Dectin-1 complex formation. Our study also shows that endogenous Dectin-1 forms a complex with Syk and Src tyrosine kinases and that PKC δ acts upstream of Syk in the complex formation. In contrast, Src and Syk pharmacological inhibitors have no effect on PKC δ /Dectin-1 association.

We additionally performed time-course experiments for Dectin-1 association with PKC δ , Syk and Src and observed rapid association of all three proteins with Dectin-1 which was detectable at 5 minutes and reduced over time. Our results from these experiments demonstrated a similar pattern for the formation of molecular complex. Finally, we confirmed that Src regulates the activity of Syk by showing that phospho-Syk and Dectin-1 interaction is significantly inhibited by PP2. Oxindole also blocks this complex formation presumably through a Src dependent pathway.

In summary, in this study we introduce for the first time PKC δ as a new player in Dectin-1 signaling cascade and show novel data on Dectin-1 complex formation in activated human monocytes. Taken together, our results suggest that non-TLR pattern recognition receptors are important regulators of NADPH oxidase function in primary human monocytes. This provides insight into mechanisms for controlling superoxide anion production in human monocytes.

CHAPTER III

OSTEOPONTIN: A NOVEL NON-MICROBIAL LIGAND FOR DECTIN-1

Deena H. Elson, Satya P. Yadav and Martha K. Cathcart

ABSTRACT

Dectin-1 is a well known receptor for β -glucan rich microbial ligands. To date non-microbial endogenous ligands for Dectin-1 have not yet been identified. This study supports the role of an endogenous glycoprotein, osteopontin (OPN), as a novel ligand for Dectin-1. OPN is an acidic phosphorylated adhesion protein. It is a multi-functional molecule expressed by different immune cells including monocytes, macrophages, neutrophils and T-lymphocytes and it is also highly expressed in chronic inflammatory diseases including atherosclerosis. In search for potential novel non-microbial Dectin-1 ligands, OPN was one of the candidates that we wanted to test due to its highly repetitive structural characteristics (featuring a pattern of numerous phosphates and sugars). In addition, the fact that osteopontin is expressed by inflammatory cells and that it promotes the pathogenesis of atherosclerosis made this protein an even more tantalizing candidate

In this study we show that osteopontin signals through Dectin-1 for the activation of NADPH oxidase and superoxide anion production in human monocytes and this activation is inhibited by laminarin, a lectin blocking reagent. Using the method of surface plasmon resonance (SPR), we show direct binding of human recombinant OPN to Dectin-1 immobilized on a sensor chip.

CD44, a cell-surface glycoprotein and hyaluronic acid receptor, is also known to bind to OPN. To show that laminarin specifically blocks OPN-induced superoxide anion through Dectin-1 and not CD44, we used SPR to exclude laminarin binding to CD44. In addition we also show that laminarin does not bind to CD36, another pattern recognition receptor.

Taken together, our results suggest that OPN is a novel ligand for Dectin-1. We show for the first time that OPN signals through Dectin-1 for the activation of NADPH oxidase in primary human monocytes and demonstrate selective binding between the two proteins.

INTRODUCTION

The β -glucan receptor Dectin-1 was first identified by Brown and Gordon (51). It is expressed on monocytes, macrophages, neutrophils and on subsets of dendritic cells and T-cells (52). It is a type II transmembrane receptor with a C-type lectin domain at the C-terminus and an immunoreceptor tyrosine-based activation motif (ITAM)-like signaling motif in the intracellular N-terminus domain (54). Dectin-1 recognizes intact yeast such as *Saccharomyces cerevisiae*, fungal pathogens including *Candida albicans* and *Pneumocystis carinii*, and β -glucan containing particles such as Zymosan (yeast cell wall preparation). Soluble β -glucans such as laminarin and glucan phosphate block the binding of Dectin-1 to Zymosan. Dectin-1 binds specifically to β -1,3-linked or both β -1,3- and β -1,6-linked glucose monomers through the lectin-like domain (53). Dectin-1 doesn't have a typical lectin domain in that it binds to glucans in a calcium independent manner (51). Dectin-1 also recognizes intact fungal and yeast pathogens through glucan binding. (51, 92, 98)

Non-microbial endogenous ligands recognized by Dectin-1 have yet been determined. All of the ligands used to study Dectin-1 function are exogenous β -glucan rich particles and intact microorganisms. It is noteworthy to mention that in addition to binding to glucans, Dectin-1 binds to a subset of T-lymphocytes and triggers their proliferation (51, 54). Soluble glucans do not inhibit Dectin-1 binding to T-cells (51) and this endogenous ligand is still not identified.

In this study we investigate the role of a non-microbial phosphorylated protein, osteopontin, as novel endogenous ligand for Dectin-1. Osteopontin (OPN) is an acidic secreted glycoprotein with an approximate molecular weight of 32 kDa (57). Osteopontin is post-translationally modified through glycosylation (5-6 O-linked and one N-linked oligosaccharides) and variable phosphorylation (12 phospho-Ser and one phospho-Thr) and it is also sulphated. Interestingly, in rat kidney cells (58) and calcitrol-stimulated mouse JB6 epidermal cells (59) OPN is secreted in a non-phosphorylated form. OPN has a calcium, hydroxyapatite, RGD and a thrombin/MMP cleavage site (57). Cleaved fragments of OPN expose new domains and may induce different functions. The amino acid sequence of OPN is conserved throughout different species including human, rat, mouse and pig. At first, OPN was thought to be synthesized by cells of the osteoblastic lineage and deposited on mineralized matrix; however, it was later shown that several bone marrow-derived cells including dendritic cells, macrophages, smooth muscle cells and endothelial cells also produce OPN.

OPN binds to different members of the integrin family, α_v and β_1 integrins, through its RGD binding domain. In addition, OPN has been shown to bind to a non-integrin receptor, namely the hyaluronan-receptor CD44 (63). Binding of OPN to CD44 was shown to be essential for bone resorption (64).

Osteopontin (OPN) is expressed by different immune cells including monocytes/macrophages, neutrophils and lymphocytes. It has different functions in different cells. OPN regulates immune responses by acting as a chemokine and promoting cell recruitment to inflammatory sites (65). It triggers cell attachment through its integrin binding domain (RGD) by binding to different members of the integrin family

(66). In addition it has been shown to be essential for wound healing (67). OPN mediates cytokine production and cell activation (68). Finally, this protein was shown to play an important role in cell survival by regulating apoptosis (68, 69). It is highly expressed in chronic inflammatory diseases including atherosclerosis (70, 99) and it is also over-expressed in autoimmune diseases (100).

During chronic inflammation, OPN accumulates in and around inflammatory cells. OPN has been shown to regulate atherogenesis by acting as a chemokine, by recruiting monocytes and macrophages to inflammatory sites, and through its adhesive properties (RGD sequence) which promotes cell attachment through integrin binding. In addition, it modulates cytokine production in dendritic cells, monocytes/macrophages and T-cells during chronic inflammation (70). OPN plasma levels have been used clinically to diagnose different inflammatory diseases including cardiovascular disease. In addition to its role as a proinflammatory molecule, OPN is an inhibitor of mineralization and vascular calcification (99).

The reason for choosing OPN as a possible ligand for Dectin-1 is mainly due to its highly repetitive structural characteristics (featuring a pattern of numerous phosphates and sugars). In addition, OPN is expressed in human monocytes and plays a role in the pathogenesis of atherosclerosis.

We investigated the role of OPN in regulating superoxide anion production in a Dectin-1 dependent manner by using the SOD-inhibitable Cytochrome C reduction assay. In addition, using SPR (Surface Plasmon Resonance) we tested the binding of recombinant osteopontin to Dectin-1 immobilized on a chip and excluded laminarin binding to CD44 and CD36, another pattern recognition receptor.

MATERIALS AND METHODS

Reagents

Zymosan was purchased from MP Biomedicals (Solon, OH). Laminarin and human CD44 monoclonal antibody were purchased from Sigma (St. Louis, MO). Human Dectin-1 monoclonal antibody (clone 259931), Dectin-1, osteopontin and CD44 recombinant proteins were purchased from R&D Systems (Minneapolis, MN). Human recombinant CD36 was a generous gift from Dr M.Febbraio and Dr R.Silverstein (Cleveland Clinic, Cleveland, OH).

Preparation of Zymosan

Zymosan was reconstituted in PBS and boiled for 1 hour. Zymosan was pelleted at 931 x g and then washed two times with PBS and resuspended in PBS to a final concentration of 40 mg/ml and aliquots were stored at -20°C (72).

Isolation of human monocytes and cell culture

Human monocytes were isolated and purified from whole blood as described previously (48, 82). PBS-diluted whole blood was layered over a Ficoll-Paque density solution and centrifuged. The mononuclear cell layer was collected and washed twice with PBS, and contaminating platelets were removed by centrifugation (280xg) through bovine calf serum (BCS) after overlaying the serum with the mononuclear cells. This serum spin was repeated twice. Monocytes were isolated from the platelet-free mononuclear cells by adherence to flasks precoated with BCS and containing DMEM and 10% BCS

(BCS/DMEM). The flasks were incubated for 2 h at 37°C in 10% CO₂. Non-adherent cells were removed by washing the flasks with BCS/DMEM. Adherent cells were detached with PBS containing 5 mM EDTA. The monocytes were collected, washed three times with BCS/DMEM, resuspended in BCS/DMEM, and incubated at 37°C in 10% CO₂ for at least 2 h before their use in experiments. In some of the experiments monocytes were isolated from human peripheral blood using a countercurrent centrifugal elutriation method (83, 84). Monocyte preparations purified by both methods were very similar in response to Zymosan or opsonized Zymosan (ZOP) and are consistently >90% CD14⁺. ZOP or Zymosan were used to activate the monocytes by protocols previously described (72).

Surface plasmon resonance studies (SPR)

The interaction between Dectin-1 and various ligands was measured using surface plasmon resonance (SPR) using a Biacore 3000 instrument (Biacore, Uppsala, Sweden). Dectin-1 was covalently coupled via primary amines, at a concentration of 5000 response units, to the dextran matrix of CM5 sensor chips. Different concentrations of recombinant human osteopontin in Hank's (HBSS) buffer containing 1 mM CaCl₂, 1 mM MgSO₄ and 1 mM MgCl₂ were flowed over flow cells on the sensor chip containing Dectin-1 or nothing (reference cell). All data were corrected for the response obtained using a blank reference flow cell that was activated with EDC/NHS and then blocked with ethanolamine. Experiments were performed by injecting the analytes at 20 µl/min for 2 minutes. The chip surface was regenerated using 2 M NaCl plus 50 mM NaOH. Data were analyzed using the BIAevaluation 3.1 program (Biacore, Uppsala, Sweden).

RESULTS

Osteopontin triggers superoxide anion production through Dectin-1

Non-microbial endogenous ligands for Dectin-1 have not yet been identified. We have already shown in chapter II that a microbial ligand, yeast Zymosan, induces NADPH oxidase activation through Dectin-1 in primary human monocytes. In this experiment we tested the role of a non-microbial pathophysiological ligand, osteopontin, in regulating the activity of monocyte NADPH oxidase through Dectin-1.

The effect of laminarin on Zymosan and OPN-stimulated cells is shown in Figure 17. Monocytes were pre-treated with laminarin for 1 hour followed by stimulation with Zymosan or OPN for 1 hour, which is the time frame we use when measuring $O_2^{\cdot -}$ release in human monocytes. Our results demonstrate that Dectin-1 regulates OPN- induced $O_2^{\cdot -}$ production in primary human monocytes. Zymosan signaling through Dectin-1 is supportive of our results from chapter II. In Figure 17 we carried out a dose response experiment using increasing levels of OPN. OPN concentrations used in this experiment are similar to OPN plasma levels found in patients with chronic inflammatory disease. Results indicate that increasing OPN concentration increases the level of $O_2^{\cdot -}$ release in monocytes and laminarin showed inhibition in all three doses.

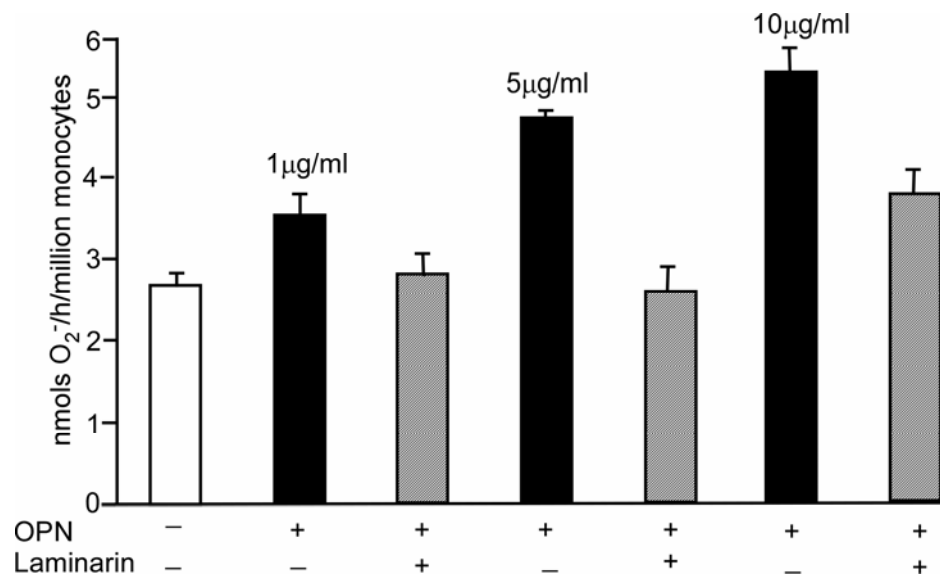
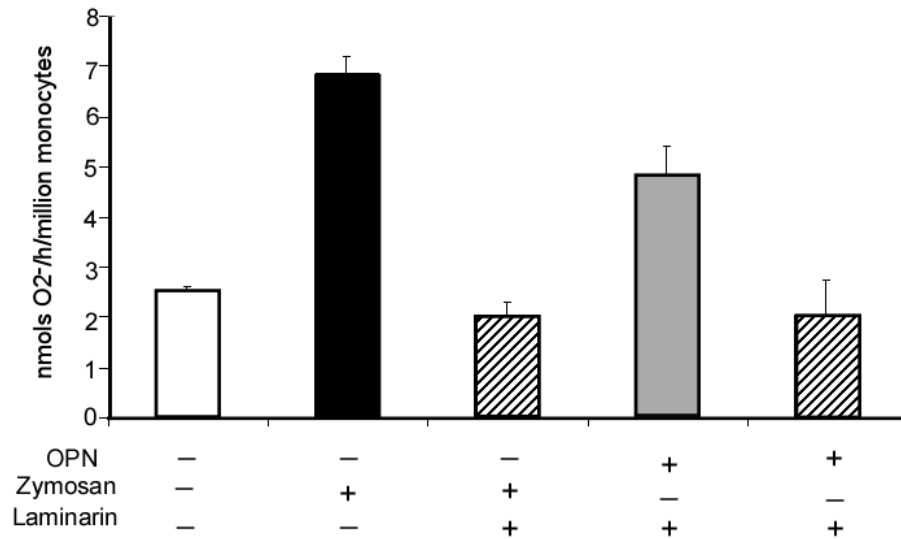


FIGURE 17. Osteopontin stimulates superoxide anion production through Dectin-1.

Primary human monocytes were plated in 24-well plates (500µl; 1x10⁶/ml). Monocytes were activated with Zymosan (100µg/ml), OPN (5µg/ml) in the presence or absence of laminarin (500µg/ml). Cells were pretreated with laminarin for 1 hour. Superoxide anion

production was measured during the first hour of activation. Data represent the mean \pm SD (n=3).

Osteopontin binds to Dectin-1

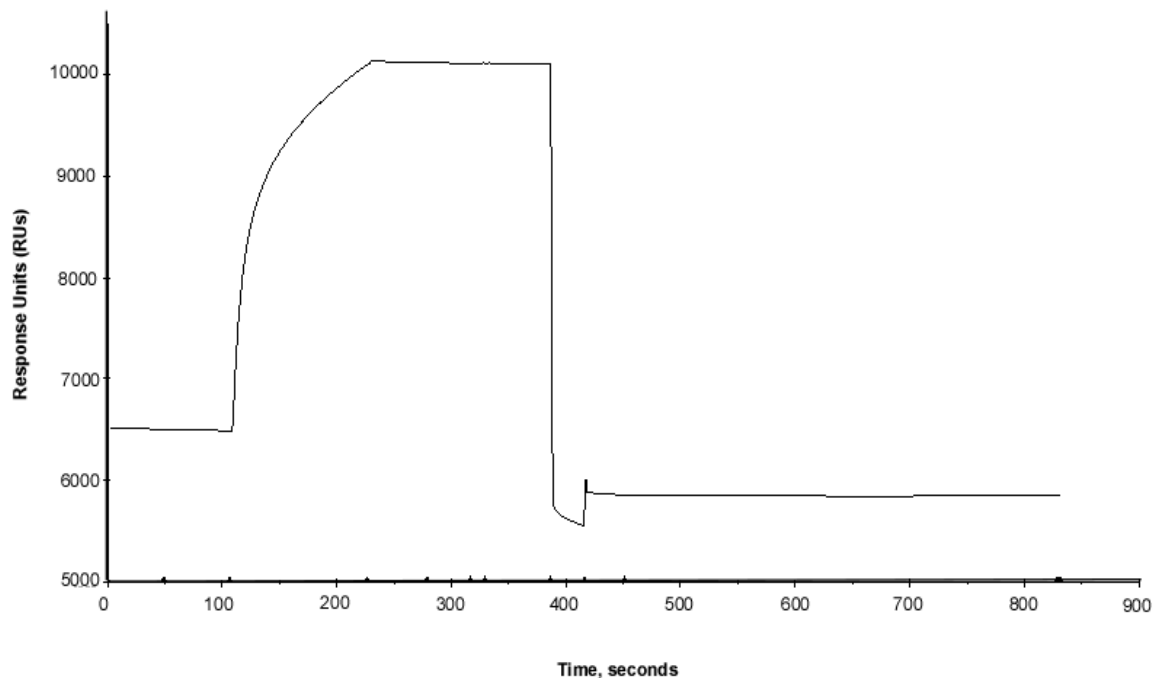
We wanted to see if Osteopontin binds to Dectin-1 directly. We examined the ability of recombinant human Osteopontin to interact with Dectin-1. A mature recombinant human Osteopontin was obtained commercially and based on the N-terminal sequencing, the protein encompasses residues Ile 17-Asn 314 and has calculated molecular mass of \sim 33 kDa. OPN is post-translationally modified and due to glycosylation it migrates at \sim 65 kDa in SDS-PAGE under reducing conditions. The DNA sequence used to generate recombinant human Dectin-1 codes for the extracellular domain of Dectin-1 (amino acid residues 66-201). Both recombinant proteins were expressed in a mouse myeloma cell line, NS0.

The capacity of Dectin-1 to bind osteopontin and other ligands was tested using SPR. Dectin-1 was coupled to the CM5 chip. First we wanted to test the ability of Dectin-1 binding its own antibody. Figure 18A shows a sensogram that represent strong binding of Dectin-1 to human Dectin-1 monoclonal antibody. This was done to confirm the efficiency of Dectin-1 immobilization and activation on the chip. Then we tested the binding of osteopontin to Dectin-1. A representative set of SPR profiles across a range of osteopontin concentrations flowed over Dectin-1 surface was also determined (Figure 18B). Increasing the concentration of OPN increases its binding to Dectin-1. The average dissociation constant (K_d) value of OPN binding to Dectin-1 which is the maximal responses achieved at equilibrium for each OPN concentration was 3.6 nM. In Figure 18C we show that posttranslational modification of OPN is required for its

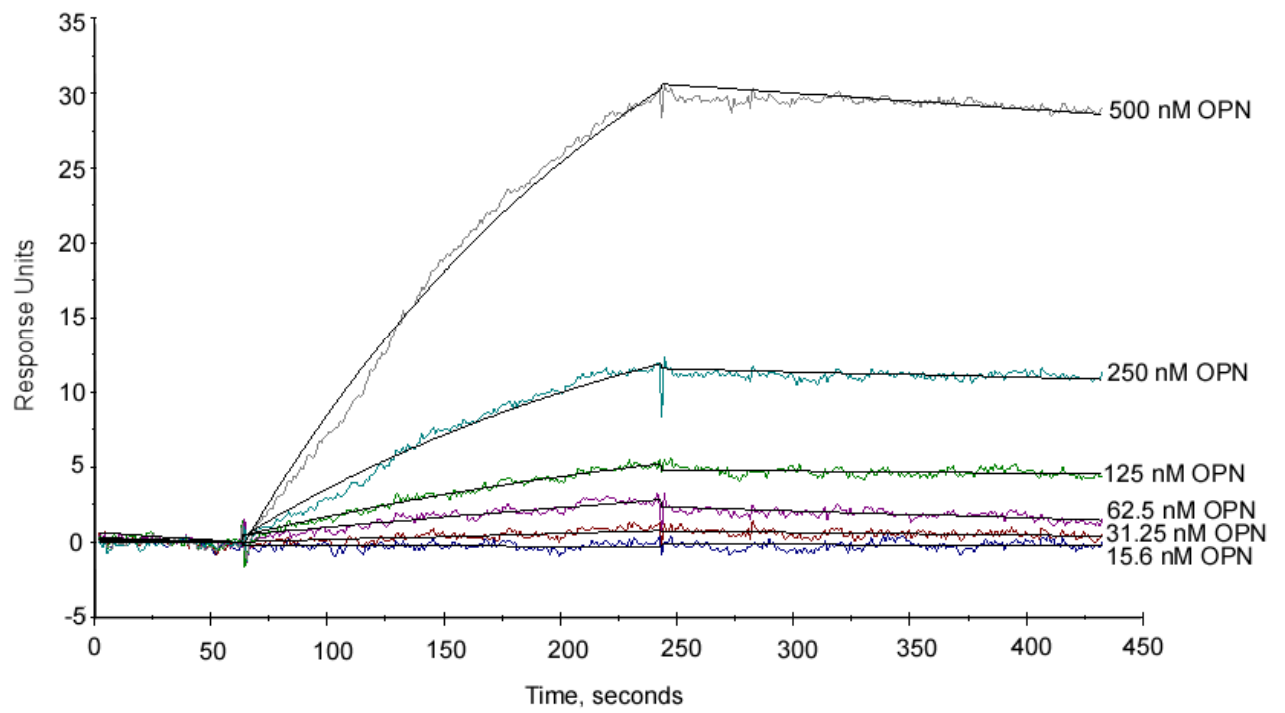
efficient binding to Dectin-1. De-glycosylated and De-phosphorylated OPN do not bind to Dectin-1 as observed in the sensogram of Figure 18C.

In Figure 18D we show laminarin binding to Dectin-1, we then injected CD44 and CD36 to test their binding to laminarin. No binding was detected between CD44 or CD36 and laminarin. This was done to confirm exclusive binding of laminarin to Dectin-1 which confirms our earlier studies that show OPN signaling through Dectin-1 for the activation of NADPH oxidase in monocytes. Finally, we tested the binding of OPN to CD44 and CD36. In Figure 18E, we show direct binding of OPN to CD44, a known receptor of OPN, but no binding to CD36 (used as a negative control).

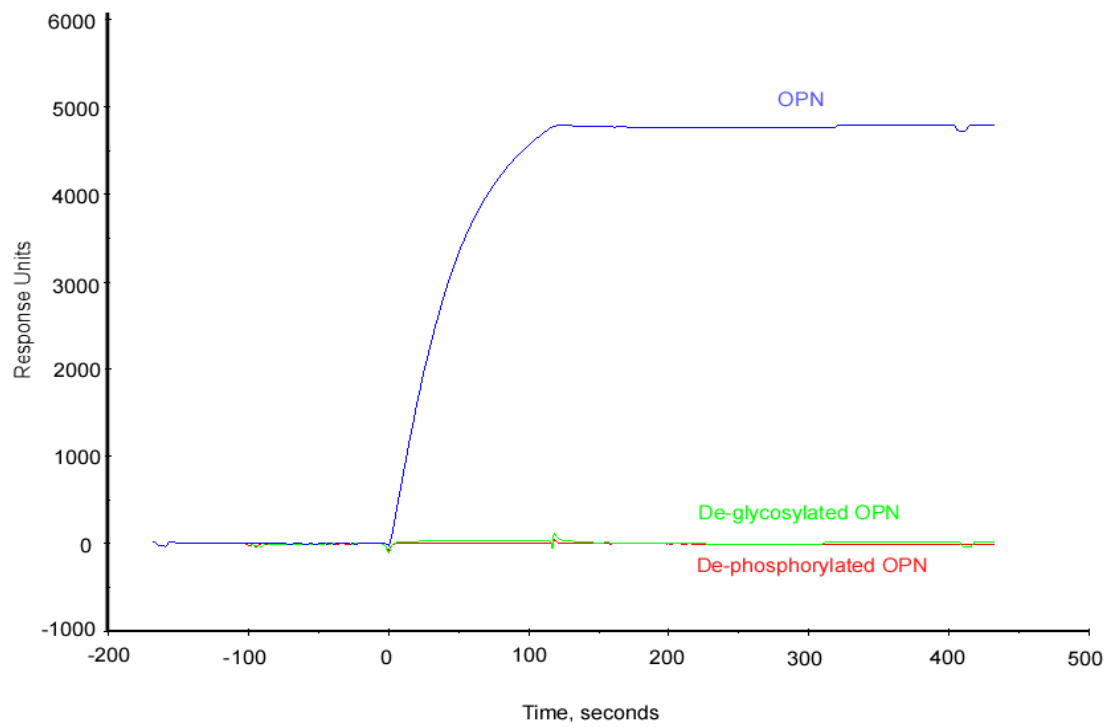
A)



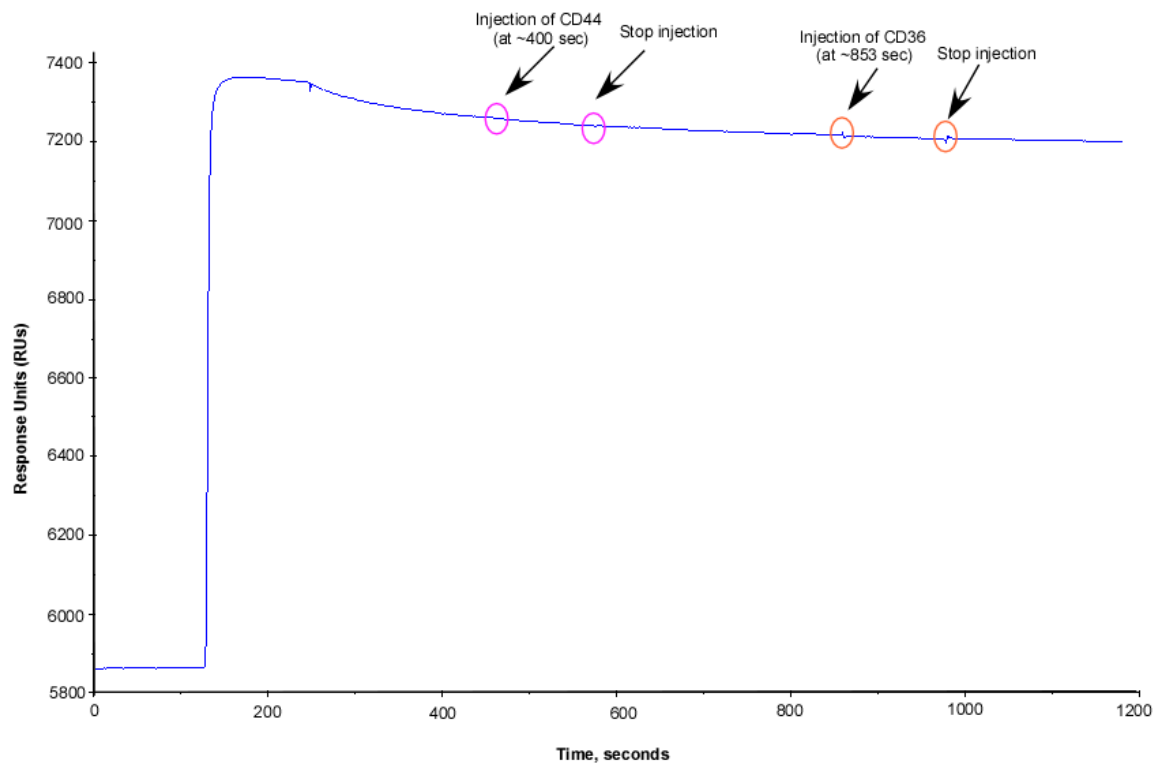
B)



C)



D)



E)

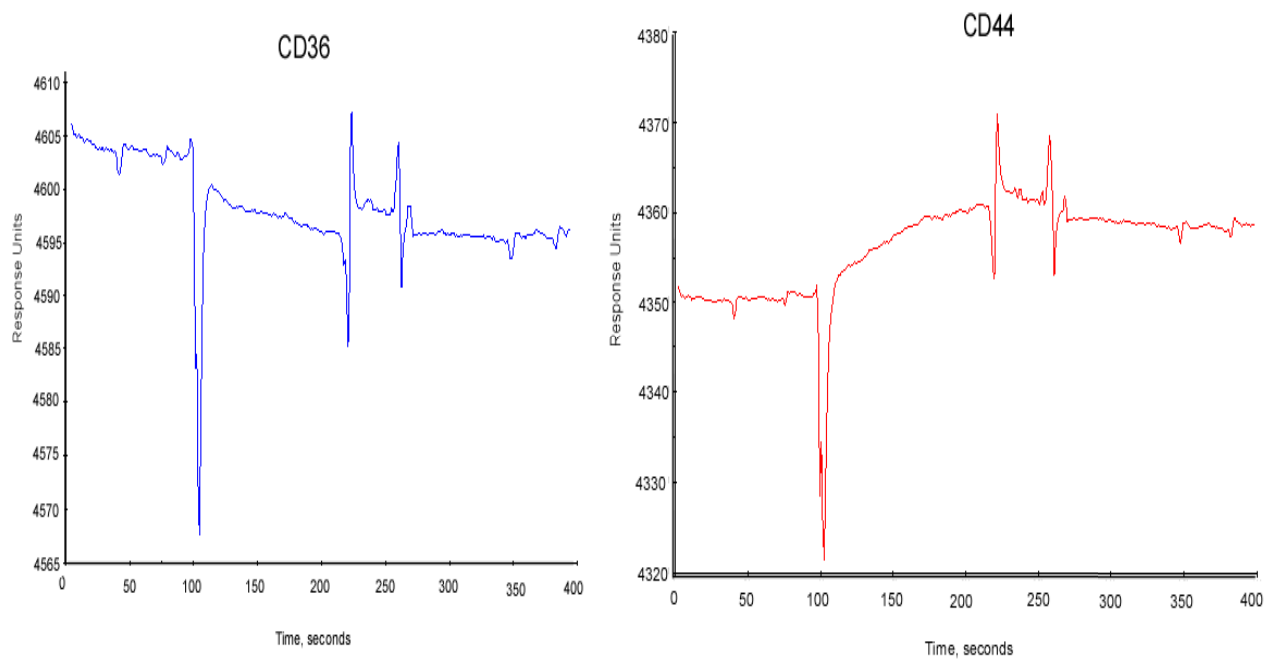


FIGURE 18. Dectin-1 binds directly to OPN. A) A sensorgram presenting binding of Dectin-1 antibody (2nM) to immobilized Dectin-1. B) Representative profiles of the SPR responses for osteopontin binding (concentrations ranging from 15.6 nM to 500 nM) to Dectin-1 in Hank's BSS media. RU indicates response/resonance units. C) Comparing OPN, de-lglycosylated OPN and de-phosphorylated OPN (all at 250 nM) binding to Dectin-1. D) Binding of laminarin (2nM) to Dectin-1 followed by injection of CD44 and CD36. E) Testing OPN binding to CD44 and CD36. Studies were done using Hank's (HBSS) buffer supplemented with 1 mM CaCl_2 , 1 mM MgSO_4 and 1 mM MgCl_2 .

DISCUSSION

Dectin-1 is a pattern recognition receptor that binds to β -glucan rich pathogens. The present experiments show that Dectin-1 can also bind other types of ligands that are non-microbial in nature. We show that osteopontin (OPN), a phosphorylated glycoprotein adhesion molecule, is a different type of ligand for Dectin-1. Our studies demonstrate that laminarin, a Dectin-1 inhibitor, blocks osteopontin-induced superoxide anion production. Osteopontin binds to Dectin-1 recombinant protein and adheres to monocytes after activation of additional receptors.

Lai and coworkers (101) have shown in a recent study that OPN regulates the production of superoxide anion production and NADPH oxidase (NOX2) subunit accumulation in aortic vascular smooth muscle cells. They show that OPN-derived superoxide anion and oxylipids promote upregulation of MMP-9 during high glucose conditions *in vitro*. In this study we show data supporting the role of OPN in activating NADPH oxidase. Our experiments were done using primary human monocytes and we were more interested to know whether Dectin-1 is involved in OPN-induced superoxide production. The fact that OPN signals through Dectin-1 for the activation of NADPH oxidase and ROS production in human monocytes is very relevant because OPN promotes the development and progression of atherosclerosis and vascular remodeling (99). It is also highly expressed in atherosclerotic lesions and found in association with monocyte-derived macrophages and foam cells. It would be interesting to do further experiments to identify downstream signaling molecules that regulate OPN signaling through Dectin-1 and NADPH oxidase activation.

Binding of osteopontin to Dectin-1 was confirmed using the SPR method. Surface Plasmon Resonance is a powerful method used to measure interactions between biomolecules in real-time without the need to label the interactants. The concept is to immobilize one of the interactants (ligand) on the sensor chip and pass the other molecule (analyte) in solution over the surface. Binding and dissociation are presented in a graph known as the sensogram and levels of association are presented as response/resonance units (RUs). In our experiments Dectin-1 was immobilized on the sensor chip and osteopontin at different concentrations was passed over the surface. Binding between the two proteins is shown by the sensograms in Figure 18. In the de-glycosylated and de-phosphorylated form, OPN does not bind to Dectin-1. We confirmed Dectin-1 immobilization and activation by showing binding to laminarin and Dectin-1 monoclonal antibody. Furthermore, OPN binds to CD44 but not to CD36. We also show that laminarin does not bind to CD44 or CD36. This information is required to confirm that laminarin specifically blocked OPN-induced superoxide through Dectin-1 and not CD44 or other pattern recognition receptors such as CD36. Additional studies are required to determine the exact binding site for OPN recognition on Dectin-1 as our data indicates that the β -glucan site is required which is demonstrated by laminarin inhibition but other sites are yet to be investigated.

Taken together, our study provides new insights into Dectin-1 ligands in primary human monocytes and introduces OPN as a novel non-microbial ligand for Dectin-1. This study highlights novel findings that are relevant for understanding this pattern recognition receptor and its role in the innate immunity and in chronic inflammatory diseases.

CHAPTER IV

GENERAL CONCLUSIONS

NADPH oxidase plays an essential role in host defense by catalyzing a reaction that results in the production of superoxide anion, an oxygen free radical. Superoxide anion readily converts to more toxic derivatives such as H_2O_2 , OH^\cdot and $^1\text{O}_2$. These toxic substances are responsible for killing pathogens and promoting host defense. A non-functional NADPH oxidase can lead to chronic infection which is a symptom of chronic granulomatous disease (CGD). CGD is a result of NADPH oxidase deficiency. On the other hand, uncontrolled production of ROS can lead to chronic inflammation specifically atherosclerosis. Therefore, the regulation of this enzyme complex is important to control the production of superoxide anion.

Various studies have developed from attempts to understand the regulation of NADPH oxidase and its product superoxide anion. Current research is focusing on the role of pattern recognition receptors in regulating NADPH oxidase and ROS production. The first goal of this research project was to address whether pattern recognition receptors can regulate Zymosan/ZOP-induced superoxide anion production in primary human monocytes. We chose to test the involvement of Toll-like receptors first.

Our results demonstrate that neither TLR4 nor TLR2 are required for superoxide anion production in Zymosan or ZOP- activated monocytes. We tested the hypothesis that the β -glucan receptor, Dectin-1, and CR3 were involved in regulating NADPH oxidase activity in Zymosan or ZOP-stimulated monocytes. We show in this study that Zymosan signal primarily through Dectin-1 for the production of superoxide anion whereas ZOP signals through both Dectin-1 and CR3. In addition, we studied Dectin-1 downstream signaling in Zymosan-activated monocytes. Our data show that Src and Syk tyrosine kinases signal downstream of Dectin-1 and that they regulate the activity of each other. Both of these tyrosine kinases are required for Zymosan-induced superoxide anion release. Furthermore, we focused on Dectin-1 complex formation with intracellular signaling proteins including Syk, Src and PKC δ , protein kinases that regulates NADPH oxidase activity in human monocytes. This is the first study to show the involvement of PKC δ in Dectin-1 signaling. We found that the activity of PKC δ is required for its own complex formation with Dectin-1 as well as Syk-Dectin-1 interaction. In contrast, Src and Syk inhibitors had no effect on PKC δ association with Dectin-1. Blocking the activity of Src inhibited phospho-Syk/Dectin-1 complex formation which supports the role of Src in regulating Syk tyrosine phosphorylation/activation. We believe our data confirms that Dectin-1, a pattern recognition receptor, is a key player in the regulation of NADPH oxidase in Zymosan-activated human monocytes. Finally, we introduce PKC δ as a novel player in Dectin-1 signaling.

The Second goal of this project was to search for novel, endogenous ligands for Dectin-1. Different microbial ligands have been used to study Dectin-1 signaling and function; however, non-microbial endogenous ligands for this receptor have not yet been

elucidated. We tested the hypothesis that osteopontin, an endogenous pathophysiological protein, is a novel ligand for Dectin-1. Our findings suggest for the first time that osteopontin triggers superoxide anion production through Dectin-1 in human monocytes. This event is inhibited by laminarin, a lectin blocking reagent. We confirm the interaction between osteopontin and Dectin-1 by showing direct binding using the technique of surface plasmon resonance. Furthermore, we show binding of OPN to CD44, a known OPN receptor, and exclude OPN binding to CD36. Finally, we show that laminarin does not bind to CD44 or CD36. Additional studies are required to explore the mechanism and downstream signaling events of osteopontin-Dectin-1 interaction.

In conclusion the data presented in this thesis suggest that pattern recognition receptors and their ligands are essential regulators of NADPH oxidase activity in primary human monocytes.

BIBLIOGRAPHY

1. Mckenzie, S. B. 1996. *Textbook of hematology*. Williams & Wilkins, Baltimore.
2. Williams, W. J. 1990. *Hematology*. McGraw-Hill Health Professions Division, New York.
3. Paul, W. E. 2003. *Fundamental Immunology*. Lippincott Williams & Wilkins.
4. Perper, R. J., T. W. Zee, and M. M. Mickelson. 1968. Purification of lymphocytes and platelets by gradient centrifugation. *J Lab Clin Med* 72:842.
5. Koller, C. A., G. W. King, P. E. Hurtubise, A. L. Sagone, and A. F. LoBuglio. 1973. Characterization of glass adherent human mononuclear cells. *J Immunol* 111:1610.
6. Kumagai, K., K. Itoh, S. Hinuma, and M. Tada. 1979. Pretreatment of plastic Petri dishes with fetal calf serum. A simple method for macrophage isolation. *J Immunol Methods* 29:17.
7. Treves, A. J., D. Yagoda, A. Haimovitz, N. Ramu, D. Rachmilewitz, and Z. Fuks. 1980. The isolation and purification of human peripheral blood monocytes in cell suspension. *J Immunol Methods* 39:71.
8. Robinson, J. M., and J. A. Badwey. 1995. The NADPH oxidase complex of phagocytic leukocytes: a biochemical and cytochemical view. *Histochem Cell Biol* 103:163.
9. Babior, B. M. 1999. NADPH oxidase: an update. *Blood* 93:1464.
10. Henderson, L. M., and J. B. Chappel. 1996. NADPH oxidase of neutrophils. *Biochim Biophys Acta* 1273:87.
11. Clark, R. A. 1999. Activation of the neutrophil respiratory burst oxidase. *J Infect Dis* 179 Suppl 2:S309.
12. Cathcart, M. K. 2004. Regulation of superoxide anion production by NADPH oxidase in monocytes/macrophages: contributions to atherosclerosis. *Arterioscler Thromb Vasc Biol* 24:23.
13. Chanock, S. J., J. el Benna, R. M. Smith, and B. M. Babior. 1994. The respiratory burst oxidase. *J Biol Chem* 269:24519.
14. Zhao, X., K. A. Carnevale, and M. K. Cathcart. 2003. Human monocytes use Rac1, not Rac2, in the NADPH oxidase complex. *J Biol Chem* 278:40788.
15. Janeway, C. A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu Rev Immunol* 20:197.
16. Ahluwalia, J., A. Tinker, L. H. Clapp, M. R. Duchon, A. Y. Abramov, S. Pope, M. Nobles, and A. W. Segal. 2004. The large-conductance Ca²⁺-activated K⁺ channel is essential for innate immunity. *Nature* 427:853.
17. Geiszt, M., J. Witta, J. Baffi, K. Lekstrom, and T. L. Leto. 2003. Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *Faseb J* 17:1502.
18. Forteza, R., M. Salathe, F. Miot, and G. E. Conner. 2005. Regulated hydrogen peroxide production by Duox in human airway epithelial cells. *Am J Respir Cell Mol Biol* 32:462.

19. Heinecke, J. W., W. Li, G. A. Francis, and J. A. Goldstein. 1993. Tyrosyl radical generated by myeloperoxidase catalyzes the oxidative cross-linking of proteins. *J Clin Invest* 91:2866.
20. Grandvaux, N., A. Soucy-Faulkner, and K. Fink. 2007. Innate host defense: Nox and Duox on phox's tail. *Biochimie* 89:1113.
21. Park, H. S., H. Y. Jung, E. Y. Park, J. Kim, W. J. Lee, and Y. S. Bae. 2004. Cutting edge: direct interaction of TLR4 with NAD(P)H oxidase 4 isozyme is essential for lipopolysaccharide-induced production of reactive oxygen species and activation of NF-kappa B. *J Immunol* 173:3589.
22. Snelgrove, R. J., L. Edwards, A. J. Rae, and T. Hussell. 2006. An absence of reactive oxygen species improves the resolution of lung influenza infection. *Eur J Immunol* 36:1364.
23. Fink, K., A. Duval, A. Martel, A. Soucy-Faulkner, and N. Grandvaux. 2008. Dual role of NOX2 in respiratory syncytial virus- and sendai virus-induced activation of NF-kappaB in airway epithelial cells. *J Immunol* 180:6911.
24. Vignais, P. V. 2002. The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. *Cell Mol Life Sci* 59:1428.
25. Pao, M., E. A. Wiggs, M. M. Anastacio, J. Hyun, E. S. DeCarlo, J. T. Miller, V. L. Anderson, H. L. Malech, J. I. Gallin, and S. M. Holland. 2004. Cognitive function in patients with chronic granulomatous disease: a preliminary report. *Psychosomatics* 45:230.
26. Clark, R. A., H. L. Malech, J. I. Gallin, H. Nuno, B. D. Volpp, D. W. Pearson, W. M. Nauseef, and J. T. Curnutte. 1989. Genetic variants of chronic granulomatous disease: prevalence of deficiencies of two cytosolic components of the NADPH oxidase system. *N Engl J Med* 321:647.
27. Francke, U., H. D. Ochs, B. T. Darras, and A. Swaroop. 1990. Origin of mutations in two families with X-linked chronic granulomatous disease. *Blood* 76:602.
28. Segal, A. W., P. G. Heyworth, S. Cockcroft, and M. M. Barrowman. 1985. Stimulated neutrophils from patients with autosomal recessive chronic granulomatous disease fail to phosphorylate a Mr-44,000 protein. *Nature* 316:547.
29. Francke, U., C. L. Hsieh, B. E. Foellmer, K. J. Lomax, H. L. Malech, and T. L. Leto. 1990. Genes for two autosomal recessive forms of chronic granulomatous disease assigned to 1q25 (NCF2) and 7q11.23 (NCF1). *Am J Hum Genet* 47:483.
30. Leto, T. L., M. C. Garrett, H. Fujii, and H. Nuno. 1991. Characterization of neutrophil NADPH oxidase factors p47-phox and p67-phox from recombinant baculoviruses. *J Biol Chem* 266:19812.
31. Goldstein, J. L., Y. K. Ho, S. K. Basu, and M. S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci U S A* 76:333.
32. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1983. Enhanced macrophage degradation of biologically modified low density lipoprotein. *Arteriosclerosis* 3:149.

33. Hessler, J. R., A. L. Robertson, Jr., and G. M. Chisolm, 3rd. 1979. LDL-induced cytotoxicity and its inhibition by HDL in human vascular smooth muscle and endothelial cells in culture. *Atherosclerosis* 32:213.
34. Cathcart, M. K., D. W. Morel, and G. M. Chisolm, 3rd. 1985. Monocytes and neutrophils oxidize low density lipoprotein making it cytotoxic. *J Leukoc Biol* 38:341.
35. Cathcart, M. K., A. K. McNally, D. W. Morel, and G. M. Chisolm, 3rd. 1989. Superoxide anion participation in human monocyte-mediated oxidation of low-density lipoprotein and conversion of low-density lipoprotein to a cytotoxin. *J Immunol* 142:1963.
36. Aqel, N. M., R. Y. Ball, H. Waldmann, and M. J. Mitchinson. 1984. Monocytic origin of foam cells in human atherosclerotic plaques. *Atherosclerosis* 53:265.
37. Llodra, J., V. Angeli, J. Liu, E. Trogan, E. A. Fisher, and G. J. Randolph. 2004. Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. *Proc Natl Acad Sci U S A* 101:11779.
38. Boring, L., J. Gosling, M. Cleary, and I. F. Charo. 1998. Decreased lesion formation in CCR2^{-/-} mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* 394:894.
39. Gosling, J., S. Slaymaker, L. Gu, S. Tseng, C. H. Zlot, S. G. Young, B. J. Rollins, and I. F. Charo. 1999. MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. *J Clin Invest* 103:773.
40. Gu, L., Y. Okada, S. K. Clinton, C. Gerard, G. K. Sukhova, P. Libby, and B. J. Rollins. 1998. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell* 2:275.
41. Cathcart, M. K., G. M. Chisolm, 3rd, A. K. McNally, and D. W. Morel. 1988. Oxidative modification of low density lipoprotein (LDL) by activated human monocytes and the cell lines U937 and HL60. *In Vitro Cell Dev Biol* 24:1001.
42. Li, Q., A. Tallant, and M. K. Cathcart. 1993. Dual Ca²⁺ requirement for optimal lipid peroxidation of low density lipoprotein by activated human monocytes. *J Clin Invest* 91:1499.
43. Li, Q., V. Subbulakshmi, C. M. Oldfield, R. Aamir, C. M. Weyman, A. Wolfman, and M. K. Cathcart. 2007. PKC α regulates phosphorylation and enzymatic activity of cPLA2 in vitro and in activated human monocytes. *Cell Signal* 19:359.
44. Li, Q., V. Subbulakshmi, A. P. Fields, N. R. Murray, and M. K. Cathcart. 1999. Protein kinase calpha regulates human monocyte O-2 production and low density lipoprotein lipid oxidation. *J Biol Chem* 274:3764.
45. Zhao, X., E. A. Bey, F. B. Wientjes, and M. K. Cathcart. 2002. Cytosolic phospholipase A2 (cPLA2) regulation of human monocyte NADPH oxidase activity. cPLA2 affects translocation but not phosphorylation of p67(phox) and p47(phox). *J Biol Chem* 277:25385.
46. Zhao, X., B. Xu, A. Bhattacharjee, C. M. Oldfield, F. B. Wientjes, G. M. Feldman, and M. K. Cathcart. 2005. Protein kinase C δ regulates p67phox phosphorylation in human monocytes. *J Leukoc Biol* 77:414.
47. Bey, E. A., B. Xu, A. Bhattacharjee, C. M. Oldfield, X. Zhao, Q. Li, V. Subbulakshmi, G. M. Feldman, F. B. Wientjes, and M. K. Cathcart. 2004. Protein

- kinase C delta is required for p47phox phosphorylation and translocation in activated human monocytes. *J Immunol* 173:5730.
48. Bey, E. A., and M. K. Cathcart. 2000. In vitro knockout of human p47phox blocks superoxide anion production and LDL oxidation by activated human monocytes. *J Lipid Res* 41:489.
 49. Brown, G. D. 2006. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat Rev Immunol* 6:33.
 50. Pyz, E., A. S. Marshall, S. Gordon, and G. D. Brown. 2006. C-type lectin-like receptors on myeloid cells. *Ann Med* 38:242.
 51. Brown, G. D., and S. Gordon. 2001. Immune recognition. A new receptor for beta-glucans. *Nature* 413:36.
 52. Taylor, P. R., G. D. Brown, D. M. Reid, J. A. Willment, L. Martinez-Pomares, S. Gordon, and S. Y. Wong. 2002. The beta-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages. *J Immunol* 169:3876.
 53. Adachi, Y., T. Ishii, Y. Ikeda, A. Hoshino, H. Tamura, J. Aketagawa, S. Tanaka, and N. Ohno. 2004. Characterization of beta-glucan recognition site on C-type lectin, dectin 1. *Infect Immun* 72:4159.
 54. Ariizumi, K., G. L. Shen, S. Shikano, S. Xu, R. Ritter, 3rd, T. Kumamoto, D. Edelbaum, A. Morita, P. R. Bergstresser, and A. Takashima. 2000. Identification of a novel, dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning. *J Biol Chem* 275:20157.
 55. Underhill, D. M., E. Rossnagle, C. A. Lowell, and R. M. Simmons. 2005. Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. *Blood* 106:2543.
 56. Underhill, D. M. 2007. Collaboration between the innate immune receptors dectin-1, TLRs, and Nods. *Immunol Rev* 219:75.
 57. Oldberg, A., A. Franzen, and D. Heinegard. 1986. Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. *Proc Natl Acad Sci U S A* 83:8819.
 58. Nemir, M., M. W. DeVouge, and B. B. Mukherjee. 1989. Normal rat kidney cells secrete both phosphorylated and nonphosphorylated forms of osteopontin showing different physiological properties. *J Biol Chem* 264:18202.
 59. Chang, P. L., and C. W. Prince. 1991. 1 alpha,25-dihydroxyvitamin D3 stimulates synthesis and secretion of nonphosphorylated osteopontin (secreted phosphoprotein 1) in mouse JB6 epidermal cells. *Cancer Res* 51:2144.
 60. Flores, M. E., M. Norgard, D. Heinegard, F. P. Reinholt, and G. Andersson. 1992. RGD-directed attachment of isolated rat osteoclasts to osteopontin, bone sialoprotein, and fibronectin. *Exp Cell Res* 201:526.
 61. Reinholt, F. P., K. Hultenby, A. Oldberg, and D. Heinegard. 1990. Osteopontin--a possible anchor of osteoclasts to bone. *Proc Natl Acad Sci U S A* 87:4473.
 62. Horton, M. A., M. L. Taylor, T. R. Arnett, and M. H. Helfrich. 1991. Arg-Gly-Asp (RGD) peptides and the anti-vitronectin receptor antibody 23C6 inhibit dentine resorption and cell spreading by osteoclasts. *Exp Cell Res* 195:368.
 63. Weber, G. F., S. Ashkar, M. J. Glimcher, and H. Cantor. 1996. Receptor-ligand interaction between CD44 and osteopontin (Eta-1). *Science* 271:509.

64. Chellaiah, M. A., N. Kizer, R. Biswas, U. Alvarez, J. Strauss-Schoenberger, L. Rifas, S. R. Rittling, D. T. Denhardt, and K. A. Hruska. 2003. Osteopontin deficiency produces osteoclast dysfunction due to reduced CD44 surface expression. *Mol Biol Cell* 14:173.
65. Koh, A., A. P. da Silva, A. K. Bansal, M. Bansal, C. Sun, H. Lee, M. Glogauer, J. Sodek, and R. Zohar. 2007. Role of osteopontin in neutrophil function. *Immunology* 122:466.
66. Yokosaki, Y., K. Tanaka, F. Higashikawa, K. Yamashita, and A. Eboshida. 2005. Distinct structural requirements for binding of the integrins α v β 6, α v β 3, α v β 5, α 5 β 1 and α 9 β 1 to osteopontin. *Matrix Biol* 24:418.
67. Liaw, L., D. E. Birk, C. B. Ballas, J. S. Whitsitt, J. M. Davidson, and B. L. Hogan. 1998. Altered wound healing in mice lacking a functional osteopontin gene (spp1). *J Clin Invest* 101:1468.
68. Wang, K. X., and D. T. Denhardt. 2008. Osteopontin: role in immune regulation and stress responses. *Cytokine Growth Factor Rev* 19:333.
69. Denhardt, D. T., M. Noda, A. W. O'Regan, D. Pavlin, and J. S. Berman. 2001. Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. *J Clin Invest* 107:1055.
70. Scatena, M., L. Liaw, and C. M. Giachelli. 2007. Osteopontin: a multifunctional molecule regulating chronic inflammation and vascular disease. *Arterioscler Thromb Vasc Biol* 27:2302.
71. Singh, M., S. Ananthula, D. M. Milhorn, G. Krishnaswamy, and K. Singh. 2007. Osteopontin: a novel inflammatory mediator of cardiovascular disease. *Front Biosci* 12:214.
72. Li, Q., and M. K. Cathcart. 1997. Selective inhibition of cytosolic phospholipase A2 in activated human monocytes. Regulation of superoxide anion production and low density lipoprotein oxidation. *J Biol Chem* 272:2404.
73. Di Carlo, F. J., and J. V. Fiore. 1958. On the composition of zymosan. *Science* 127:756.
74. Le Cabec, V., C. Cols, and I. Maridonneau-Parini. 2000. Nonopsonic phagocytosis of zymosan and *Mycobacterium kansasii* by CR3 (CD11b/CD18) involves distinct molecular determinants and is or is not coupled with NADPH oxidase activation. *Infect Immun* 68:4736.
75. Le Cabec, V., S. Carreno, A. Moisand, C. Bordier, and I. Maridonneau-Parini. 2002. Complement receptor 3 (CD11b/CD18) mediates type I and type II phagocytosis during nonopsonic and opsonic phagocytosis, respectively. *J Immunol* 169:2003.
76. Sato, M., H. Sano, D. Iwaki, K. Kudo, M. Konishi, H. Takahashi, T. Takahashi, H. Imaizumi, Y. Asai, and Y. Kuroki. 2003. Direct binding of Toll-like receptor 2 to zymosan, and zymosan-induced NF-kappa B activation and TNF-alpha secretion are down-regulated by lung collectin surfactant protein A. *J Immunol* 171:417.
77. Ozinsky, A., D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder, and A. Aderem. 2000. The repertoire for pattern

- recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci U S A* 97:13766.
78. Underhill, D. M. 2003. Macrophage recognition of zymosan particles. *J Endotoxin Res* 9:176.
 79. Nilsen, N., U. Nonstad, N. Khan, C. F. Knetter, S. Akira, A. Sundan, T. Espevik, and E. Lien. 2004. Lipopolysaccharide and double-stranded RNA up-regulate toll-like receptor 2 independently of myeloid differentiation factor 88. *J Biol Chem* 279:39727.
 80. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu Rev Immunol* 21:335.
 81. Gantner, B. N., R. M. Simmons, S. J. Canavera, S. Akira, and D. M. Underhill. 2003. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J Exp Med* 197:1107.
 82. Roy, B., and M. K. Cathcart. 1998. Induction of 15-lipoxygenase expression by IL-13 requires tyrosine phosphorylation of Jak2 and Tyk2 in human monocytes. *J Biol Chem* 273:32023.
 83. Wahl, S. M., I. M. Katona, B. M. Stadler, R. L. Wilder, W. E. Helsel, and L. M. Wahl. 1984. Isolation of human mononuclear cell subsets by counterflow centrifugal elutriation (CCE). II. Functional properties of B-lymphocyte-, T-lymphocyte-, and monocyte-enriched fractions. *Cell Immunol* 85:384.
 84. Wahl, S. M., J. B. Allen, S. Dougherty, V. Evequoz, D. H. Pluznik, R. L. Wilder, A. R. Hand, and L. M. Wahl. 1986. T lymphocyte-dependent evolution of bacterial cell wall-induced hepatic granulomas. *J Immunol* 137:2199.
 85. Pick, E., and D. Mizel. 1981. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J Immunol Methods* 46:211.
 86. Arko-Mensah, J., E. Julian, M. Singh, and C. Fernandez. 2007. TLR2 but not TLR4 signalling is critically involved in the inhibition of IFN-gamma-induced killing of mycobacteria by murine macrophages. *Scand J Immunol* 65:148.
 87. Esen, N., F. Y. Tanga, J. A. DeLeo, and T. Kielian. 2004. Toll-like receptor 2 (TLR2) mediates astrocyte activation in response to the Gram-positive bacterium *Staphylococcus aureus*. *J Neurochem* 88:746.
 88. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 11:443.
 89. Brown, G. D., J. Herre, D. L. Williams, J. A. Willment, A. S. Marshall, and S. Gordon. 2003. Dectin-1 mediates the biological effects of beta-glucans. *J Exp Med* 197:1119.
 90. Rogers, N. C., E. C. Slack, A. D. Edwards, M. A. Nolte, O. Schulz, E. Schweighoffer, D. L. Williams, S. Gordon, V. L. Tybulewicz, G. D. Brown, and C. Reis e Sousa. 2005. Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity* 22:507.
 91. Bijli, K. M., F. Fazal, M. Minhajuddin, and A. Rahman. 2008. Activation of Syk by PKC delta regulates thrombin-induced ICAM-1 expression in endothelial cells via tyrosine phosphorylation of RelA/p65. *J Biol Chem*.

92. Gantner, B. N., R. M. Simmons, and D. M. Underhill. 2005. Dectin-1 mediates macrophage recognition of *Candida albicans* yeast but not filaments. *Embo J* 24:1277.
93. Herre, J., A. S. Marshall, E. Caron, A. D. Edwards, D. L. Williams, E. Schweighoffer, V. Tybulewicz, C. Reis e Sousa, S. Gordon, and G. D. Brown. 2004. Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. *Blood* 104:4038.
94. Suram, S., G. D. Brown, M. Ghosh, S. Gordon, R. Loper, P. R. Taylor, S. Akira, S. Uematsu, D. L. Williams, and C. C. Leslie. 2006. Regulation of cytosolic phospholipase A2 activation and cyclooxygenase 2 expression in macrophages by the beta-glucan receptor. *J Biol Chem* 281:5506.
95. Valera, I., N. Fernandez, A. G. Trinidad, S. Alonso, G. D. Brown, A. Alonso, and M. S. Crespo. 2008. Costimulation of Dectin-1 and DC-SIGN Triggers the Arachidonic Acid Cascade in Human Monocyte-Derived Dendritic Cells. *J Immunol* 180:5727.
96. Slack, E. C., M. J. Robinson, P. Hernanz-Falcon, G. D. Brown, D. L. Williams, E. Schweighoffer, V. L. Tybulewicz, and C. Reis e Sousa. 2007. Syk-dependent ERK activation regulates IL-2 and IL-10 production by DC stimulated with zymosan. *Eur J Immunol* 37:1600.
97. Fuller, G. L., J. A. Williams, M. G. Tomlinson, J. A. Eble, S. L. Hanna, S. Pohlmann, K. Suzuki-Inoue, Y. Ozaki, S. P. Watson, and A. C. Pearce. 2007. The C-type lectin receptors CLEC-2 and Dectin-1, but not DC-SIGN, signal via a novel YXXL-dependent signaling cascade. *J Biol Chem* 282:12397.
98. Steele, C., L. Marrero, S. Swain, A. G. Harmsen, M. Zheng, G. D. Brown, S. Gordon, J. E. Shellito, and J. K. Kolls. 2003. Alveolar macrophage-mediated killing of *Pneumocystis carinii* f. sp. muris involves molecular recognition by the Dectin-1 beta-glucan receptor. *J Exp Med* 198:1677.
99. Cho, H. J., and H. S. Kim. 2009. Osteopontin: a multifunctional protein at the crossroads of inflammation, atherosclerosis, and vascular calcification. *Curr Atheroscler Rep* 11:206.
100. Weber, G. F., and H. Cantor. 2001. Differential roles of osteopontin/Eta-1 in early and late lpr disease. *Clin Exp Immunol* 126:578.
101. Lai, C. F., V. Seshadri, K. Huang, J. S. Shao, J. Cai, R. Vattikuti, A. Schumacher, A. P. Loewy, D. T. Denhardt, S. R. Rittling, and D. A. Towler. 2006. An osteopontin-NADPH oxidase signaling cascade promotes pro-matrix metalloproteinase 9 activation in aortic mesenchymal cells. *Circ Res* 98:1479.