VIMENTIN IS A PHOSPHORYLATED TARGET OF MCP-1-INDUCED
PKCβ ACTIVATION AND AN ENDOGENOUS LIGAND FOR THE INNATE
IMMUNE RECEPTOR DECTIN-1

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by

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# TABLE OF CONTENTS

LIST OF FIGURES .......................................................................................................................... viii

LIST OF TABLES ............................................................................................................................ xi

LIST OF ABBREVIATIONS .............................................................................................................. xii

DEDICATION ..................................................................................................................................... xiii

ACKNOWLEDGEMENTS ................................................................................................................ xiv

1 Introduction .................................................................................................................................. 1

1.1 Identification of MCP-1-induced Targets of PKCβ Phosphorylation in Human Monocytes ................................................................................................................................. 1

1.2 Identification of Endogenous Ligands for Dectin-1 in Atherosclerotic Plaques ................................................................................................................................. 3

2 Identification of MCP-1-induced Targets of PKCβ Phosphorylation in Human Monocytes ................................................................................................................................. 5

2.1 Background ................................................................................................................................ 5

2.1.1 Role of MCP-1 in atherogenesis .......................................................................................... 5
2.1.2 Role of protein kinase Cβ in MCP-1-induced primary human monocyte chemotaxis ................................................................. 8

2.2 Materials and Methods ......................................................................................................................... 11

2.2.1 Materials ........................................................................................................................................ 11

2.2.2 Isolation of primary human monocytes ......................................................................................... 11

2.2.3 Treatment of cells with oligodeoxyribonucleotides and PKCβ peptide inhibitor .......................... 12

2.2.4 Preparation of cell lysates ............................................................................................................ 13

2.2.5 Two-dimensional gel electrophoresis of PKCβ sense/anti-sense oligodeoxyribonucleotides treated primary human monocytes ........ 13

2.2.6 Liquid chromatography mass spectrometry ............................................................................. 15

2.2.7 Metabolic labeling and vimentin immunoprecipitation ............................................................ 16

2.2.8 Vimentin immunoprecipitation .................................................................................................. 17

2.3 Results .............................................................................................................................................. 18

2.3.1 Vimentin is a potential target of phosphorylation by PKCβ in MCP-1-activated human monocyte chemotaxis ........................................ 18

2.3.2 Phospho-(Ser) PKC substrate antibody identifies vimentin as a target of PKCβ phosphorylation in MCP-1-activated human monocytes ............................................................................. 23

2.3.3 PKCβ induces vimentin phosphorylation in MCP-1-activated human monocytes ................................. 26
2.3.4 PKCβ associates with vimentin upon treatment with MCP-1 in primary human monocytes ................................................................. 29

2.4 Discussion .................................................................................. 31

3 Identification of Endogenous Ligands for Dectin-1 in Atherosclerotic Plaques .......... 37

3.1 Background ................................................................................ 37

3.1.1 Role of NADPH oxidase in atherosclerosis .................................. 37

3.1.2 Role of immune-inflammatory mechanisms in atherogenesis ........... 38

3.1.3 DAMPS (Damage associated molecular patterns) and alarmins:

DAMPs (Damage associated molecular patterns) and alarmins:

Danger Signaling ............................................................................. 39

3.1.4 Pattern recognition receptors .................................................. 40

3.1.5 C-type lectin receptors and their functions ................................. 41

3.1.6 Dectin-1, a non-toll C-type lectin receptor: Its structure

and functions .................................................................................. 42

3.2 Materials and Methods ................................................................ 51

3.2.1 Materials ................................................................................ 51

3.2.2 Immunoblotting of human carotid atherosclerotic tissue samples ....... 51

3.2.3 Two-dimensional gel electrophoresis of human carotid atherosclerotic

  tissue samples .................................................................................. 52

3.2.4 Immunoaffinity purification of proteins that bind to anti-zymosan

  antibody .......................................................................................... 52
3.2.5 Binding studies using surface plasmon resonance and solution affinity kinetics.................................................................53

3.2.6 Superoxide anion assay ........................................................................................................................................55

3.3 Results........................................................................................................................................................................56

3.3.1 Detection of protein bands that react with anti-zymosan antibody in human carotid atherosclerotic tissue samples by immunoblotting........56

3.3.2 Mass spectrometry analysis of protein bands detected by SDS-PAGE of human carotid atherosclerotic tissue samples ..................58

3.3.3 Detection and identification of proteins that react with anti-zymosan antibody in human carotid atherosclerotic tissue samples by 2-dimensional gel electrophoresis.................................................................61

3.3.4 Detection of vimentin and galectin-3 binding protein as proteins that bind to anti-zymosan antibody by immunoaffinity purification .................................................................................................................62

3.3.5 Direct, high-affinity binding of vimentin and galectin-3 binding protein to dectin-1 were observed using surface plasmon resonance ..................................................................................................................65

3.3.6 Vimentin and galectin-3 binding protein induce superoxide anion production in human monocytes via the dectin-1 receptor ..........71

3.4 Discussion......................................................................................................................................................................78
Summary and Conclusions .................................................................89

4.1 Vimentin is a downstream target of phosphorylation of PKCβ in
MCP-1-activated monocytes ..............................................................90

4.2 Vimentin and galectin-3 binding protein are non-microbial endogenous
ligands present in human atherosclerotic lesions that bind to dectin-1 and
trigger NADPH oxidase activity to produce superoxide anion ...............93

4.3 Conclusions ..................................................................................96

BIBLIOGRAPHY ..................................................................................99
LIST OF FIGURES

2.1 MCP-1-induced subendothelial migration of circulating blood monocytes and differentiation into monocytes-derived macrophages ........................................7

2.2 Detection of potential PKCβ substrate proteins .............................................20

2.3 Detection of increased vimentin phosphorylation in MCP-1-treated monocytes by phospho-(Ser) PKC substrate antibody .................................................24

2.4 PKCβ anti-sense oligodeoxyribonucleotides inhibit vimentin phosphorylation in MCP-1-activated human monocytes .................................................................27

2.5 MCP-1 induces the association of vimentin with PKCβ in primary human monocytes ............................................................................................................30

3.1 Dectin-1 binds to zymosan and induces NADPH oxidase activity to produce superoxide anion .............................................................................................46

3.2 Detection of non-microbial endogenous ligands for dectin-1 in human atherosclerotic tissue samples using anti-zymosan antibody .........................................47

3.3 The model depicts our hypothesis that there are unidentified non-microbial endogenous ligands in human atherosclerotic tissue that bind to dectin-1 and trigger NADPH oxidase activity to produce superoxide anion .................50

3.4 Detection of protein bands in human carotid atherosclerotic tissue extracts on immunoblotting with anti-zymosan antibody .................................................57
3.5 Identification of proteins in human carotid atherosclerotic tissue samples that react with anti-zymosan antibody by two-dimensional gel electrophoresis .................................................................................................................59

3.6 Identification of vimentin in human carotid atherosclerotic tissue samples by two-dimensional gel electrophoresis .................................................................................................................................63

3.7 Detection of vimentin and galectin-3 binding protein in human atherosclerotic tissue samples as proteins that bind to anti-zymosan antibody by immunoaffinity purification .................................................................................................................................................66

3.8 Direct binding was observed between dectin-1 and vimentin by surface plasmon resonance using BIACORE ...........................................................................................................................................................68

3.9 No non-specific binding was observed between dectin-1 and anti-vimentin antibody using BIACORE ............................................................................................................................................................................69

3.10 No non-specific binding was observed between vimentin and CD36 ...............70

3.11 Direct binding was observed between dectin-1 and galectin-3 binding protein ........................................................................................................................................................................73

3.12 No direct binding was observed between dectin-1 and lumican .......................74

3.13 Vimentin induces superoxide anion production in primary human monocytes via the dectin-1 receptor ..................................................................................................................................................................76

3.14 Galectin-3 binding protein induces superoxide anion production in primary human monocytes via the dectin-1 receptor .......................................................................................................................................................77
3.15 Regulation of vimentin secretion by pro- and anti-inflammatory cytokines

4.1 Vimentin is a downstream phosphorylation target of PKCβ in MCP-1-activated primary human monocytes

4.2 Vimentin and galectin-3 binding protein are endogenous ligands found in human atherosclerotic lesions that trigger NADPH oxidase activity to produce superoxide anion via the dectin-1 receptor
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Identification of potential PKCβ substrate proteins</td>
<td>22</td>
</tr>
<tr>
<td>3.1</td>
<td>Identification of potential dectin-1 ligands from 1-dimensional gel electrophoresis</td>
<td>60</td>
</tr>
<tr>
<td>3.2</td>
<td>Identification of potential dectin-1 ligands from 2-dimensional gel electrophoresis</td>
<td>64</td>
</tr>
<tr>
<td>3.3</td>
<td>Identification of proteins that bind to anti-zymosan antibody</td>
<td>67</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-DIGE</td>
<td>1-dimensional gel electrophoresis</td>
</tr>
<tr>
<td>2-DIGE</td>
<td>2-dimensional gel electrophoresis</td>
</tr>
<tr>
<td>AS-ODN</td>
<td>Anti-sense-oligodeoxyribonucleotides</td>
</tr>
<tr>
<td>CnBr</td>
<td>Cyanogen bromide sepharose column</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Damage associated molecular patterns</td>
</tr>
<tr>
<td>Dectin-1</td>
<td>Dendritic cell-associated C-type lectin-1</td>
</tr>
<tr>
<td>Gal-3BP</td>
<td>Galectin-3 binding protein</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxyribonucleotide</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PKCβ</td>
<td>Protein kinase C β isoform</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
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<tr>
<td>S-ODN</td>
<td>Sense-oligodeoxyribonucleotides</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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</tbody>
</table>
DEDICATION

To Amma, Appa & Anna
(To Mom, Dad & Brother)
who mean the world to me
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xiv
CHAPTER 1

INTRODUCTION

Atherosclerosis is a multifactorial disease wherein the causes of atherosclerosis are not exactly known but over the years, certain risk factors have been associated with the disease which contribute to damage of the inner layers of the artery. Some of these factors are aging (>45 for men, >55 for women), gender (male), smoking, high fat and cholesterol diet, diabetes, hypertension, obesity, sedentary lifestyle, and family history of heart disease. Atherogenesis is an insidious process lasting for many years which eventually leads to thrombosis compromising oxygen flow to different organs such as heart and brain resulting in heart attack and stroke, respectively. The major clinical outcomes of atherosclerosis are coronary heart disease, cardiovascular disease and cerebrovascular disease. By 2020, it will be the major cause of death in the entire world (1). Though risk factors could be modified, the causative factors are not clearly understood. It is critical that the causes are known, so that atherosclerosis can be prevented.

1.1 Identification of MCP-1-induced targets of PKCβ phosphorylation in human monocytes

Chemokines are chemotactic cytokines that promote directed migration of inflammatory cells playing a key role in the pathogenesis of atherosclerosis. MCP-1 (monocyte chemoattractant protein-1) is a predominant chemokine in the development of
atherosclerosis. It functions by recruiting monocytes into the sub-endothelial cell layer of the vessel wall (2, 3). Atherosclerosis, the cause of ischemic heart disease, myocardial infarction, stroke, and peripheral arterial disease is characterized by chronic inflammation in the artery wall. Infiltration of monocyte-derived macrophages (MDM) is central to atherosclerotic plaque development. Strategies to disrupt mechanisms involved in the recruitment of monocytes represent potential therapeutic targets (4). Our lab has been working on the signaling pathways involved in primary human monocyte chemotaxis, predominantly on MCP-1-induced signaling pathways. Protein kinase Cβ (PKCβ), one of the two major classical isoforms of protein kinase C in primary human monocytes, has been shown to be required for MCP-1-mediated chemotaxis of primary human monocytes.

We designed a study to detect substrates of PKCβ phosphorylation in MCP-1-activated primary human monocytes. We hypothesized that MCP-1-activation of PKCβ results in the phosphorylation of substrate(s) that regulate monocyte chemotaxis to MCP-1. This hypothesis was driven by our previously published work demonstrating that PKCβ expression and activity are required for MCP-1-induced signaling pathways. Detection of the downstream targets of PKCβ phosphorylation would make restriction of monocyte extravasation (one of the key events during atherogenesis) into the arterial intima possible. We designed a study to identify the downstream targets of PKCβ phosphorylation in MCP-1-activated primary human monocytes to understand how PKCβ regulates monocyte chemotaxis. Two-dimensional electrophoreses (2-DIGE) were performed to find phosphoproteins that differ in intensity on phosphoprotein staining. For
this experiment, two monocyte groups were used, namely a group with and a group without inhibition of PKCβ expression. A complementary method was also performed using phospho-(Ser) protein kinase C substrate antibody on two groups with and without inhibition of PKCβ activity. Further specific experiments such as in vivo: orthophosphate metabolic labeling and immunoprecipitation were conducted to confirm that the identified protein is indeed a PKCβ phosphorylation substrate in MCP-1-activated human monocytes.

1.2 Identification of endogenous ligands for dectin-1 in atherosclerotic plaques

Oxidation of low density lipoprotein (LDL) by reactive oxygen species (ROS) plays a crucial role in the pathogenesis of atherosclerosis. In atherosclerotic lesions, MDM are the primary source of ROS. Superoxide anion (O$_{2}^-$) is one of the ROS that is involved in LDL oxidation. NADPH (nicotinamide adenine dinucleotide phosphate) oxidase is one of the primary sources of O$_{2}^-$ in monocytes and MDM.

Recent work in our laboratory has indicated that engagement of the pattern recognition receptor (PRR) dectin-1 induces a profound activation of NADPH oxidase and significant O$_{2}^-$ production. Although previously identified ligands for dectin-1 are microbial in derivation (e.g. zymosan, β-glucans), no non-microbial endogenous ligands had been identified for dectin-1. We hypothesized that non-microbial endogenous ligands for the PRR, dectin-1 may contribute to the pathogenesis of chronic inflammatory diseases such as atherosclerosis. Our hypothesis was driven by the finding that other PRRs like toll-like receptors (TLRs) can also bind endogenous ligands, yet no such
ligands had been identified for dectin-1. Our prediction was that these potential ligands might have repetitive structures similar to known ligands of PRRs and that inflammation may generate such potent endogenous ligands for dectin-1.

Immunoblot analysis on 1- and 2-DIGE, using anti-zymosan antibodies, was employed to identify similar epitopes in human carotid atherosclerotic tissue samples. Specific experiments like immunoaffinity purification using anti-zymosan antibody were also performed to identify the proteins in human carotid atherosclerotic lesions that directly bind to anti-zymosan antibody. Direct binding of potential ligands to dectin-1 was analyzed by surface plasmon resonance (SPR) using the BIACORE. The potential ligands were evaluated for their ability to induce monocyte NADPH oxidase activity to produce $\text{O}_2^-$.
CHAPTER 2

IDENTIFICATION OF MCP-1-INDUCED TARGETS OF PKCβ PHOSPHORYLATION IN HUMAN MONOCYTES

2.1 Background

2.1.1 Role of MCP-1 in atherogenesis

Directed migration of inflammatory cells under the influence of chemoattractant cytokines, defined as chemotaxis, is one of the key events in the pathogenesis of atherosclerosis. MCP-1, a chemotactic cytokine plays an important role in atherogenesis by recruiting monocytes into the subendothelial cell layer. Recruitment of monocytes from the flowing blood into the arterial vessel wall is one of the primordial events in atherogenesis. MCP-1 is critical for the initiation and progression of atherosclerotic lesions. The presence of excess local LDL influences monocyte recruitment leading to enhanced transmigration of monocytes, adhesion molecule upregulation, chemoattractant production and release. Upon monocyte extravasation into the subendothelial intimal space, they differentiate into macrophages and become functionally active as shown in the model in Figure 2.1 (2, 3). Accumulation of macrophages within plaques is a hallmark of this disease (5). MCP-1-deficient mice showed minimal lipid deposition and fewer macrophages within the artery walls (6). Signaling pathways regulating the processes by which chemokines dynamically attract monocytes, modulate adhesion and transmigration are still poorly understood.

Though multiple proteins and lipids have been shown to possess chemotactic activity, a group of small peptides known as chemokines are primarily responsible for
this chemotaxis (7). Chemokines or chemotactic cytokines are divided into subgroups (C, CC, CXC, CX3C) according to the number and spacing of cysteine residues in the NH2-terminal region. The families include α, β, lymphotactin, and fractalkine. α-chemokines have one amino acid separating the first two cysteines (CXC). β-chemokine cysteines are adjacent to each other (CC). In contrast, lymphotactin has only one cysteine (C) in this region, and fractalkine is a membrane-bound protein that has three amino acids separating the first two cysteines (CXXXC) (8). These structurally related small protein molecules are critically involved in directing activation and trafficking of leukocytes in both acute and chronic inflammation (4, 9).
Figure 2.1  MCP-1-induced subendothelial migration of circulating blood monocytes and differentiation into monocyte-derived macrophages.

This model shows the recruitment of circulating monocytes from the flowing blood into the arterial blood vessel wall under the influence of MCP-1, which is one of the crucial events in atherogenesis. MCP-1 has been shown to be critical for the initiation and progression of atherosclerotic lesions. Upon extravasation into the subendothelial intimal space, transmigrated monocytes differentiate into macrophages and become functionally active. Accumulation of MDM within the atherosclerotic plaques is the hallmark of this disease.
Our lab has been investigating the signaling pathways involved in primary human monocyte chemotaxis, predominantly on MCP-1-induced signaling pathways. MCP-1 is a secreted 76 amino acid peptide belonging to the CC or β-chemokine family with potent monocyte activating and chemotactic properties. The β-chemokine family also includes regulated upon activation, normal T cell expressed and secreted (RANTES), macrophage inflammatory protein-1, monocyte chemoattractant proteins-2 and -3. MCP-1 has been found to be expressed by endothelial cells, smooth muscle cells and monocytes/macrophages mainly within the tunica media of the arterial wall during various stages of atherosclerosis in various cultured cells like fibroblasts, keratinocytes, synovial cells, pulmonary alveolar type-II cells, various cancer cell lines and peripheral blood mononuclear cells (10-12). CCR1, CCR2 and CCR5 are the three different receptors found on monocytes. CCR2 serves as the primary receptor for MCP-1. CCR2 is a G-protein coupled receptor characterized by seven transmembrane domains and coupled to a GTP-binding protein (13, 14). CCR2 expression is upregulated by LDL and a variety of regulators including platelet-derived growth factor, interleukins IL-1 and IL-4, tissue necrosis factor, vascular endothelial growth factor, bacterial LPS and interferon γ (7).

2.1.2 Role of protein kinase Cβ in MCP-1-induced primary human monocyte chemotaxis

MCP-1-induced monocyte chemotaxis was first shown to be regulated by signaling pathways mainly involving serine/threonine protein kinases (3, 6, 15, 16). For
MCP-1-induced arachidonic acid release, extracellular calcium was found to be strictly required. Release of arachidonic acid seems to play the major role in chemotaxis induced by the β chemokine family as a cofactor for protein kinase C (PKC) activation (17). PKC is an enzyme that phosphorylates protein substrates on serine (Ser) or threonine (Thr) residues. The PKC family is a group of multifunctional isoenzymes that play key roles in signal transduction and intracellular crosstalk by phosphorylating serine/threonine residues in an array of substrates, including cell surface receptors, enzymes, contractile proteins, transcription factors, and other kinases (18). So far, 12 serine/threonine kinase isoforms have been identified in the PKC family of enzymes. PKC isoforms are classified based on their structure and substrate requirements into a) conventional PKCs (α, βI, βII and γ), which are Ca\(^{2+}\)-dependent and activated by both phosphatidylserine, the second messenger diacylglycerol and phorbol esters; Protein kinase C βI and βII are generated by alternative splicing from a single gene, their C-terminal 50 (βI) or 52 (βII) residues are different (19); b) novel PKCs (δ, ε, η, and θ), which are Ca\(^{2+}\)-independent and regulated by diacylglycerol, phosphatidylserine and phorbol esters; and c) atypical PKCs (ζ and λ), which are Ca\(^{2+}\)-independent and do not require diacylglycerol for activation (20, 21). Of the known mammalian PKC isoforms, 8 are activated by diacylglycerol. In monocytes, PKCα and β were the two predominant isoforms identified. PKCε and ζ were also present in minimal amounts. PKCβ was found to be distributed equally in the cytosol and membrane, whereas PKCα was found predominantly in the cytosol (22). Translocation of PKC from the cytosol to the membrane and phosphorylation of substrate proteins is induced by PKC activation.
When cells were treated with MCP-1, CCR2 was phosphorylated at serine/threonine residues (6, 23, 24). Using selective pharmacological inhibitors of PKC, our group has previously shown that there is significant inhibition of MCP-1-induced monocyte chemotaxis. For specific PKC isoforms, both sense (S) and anti-sense (AS)-oligodeoxyribonucleotides (ODN) were employed to observe the changes in specific PKC isoform expression. ODN were initially used to target conventional PKC isoforms. Significant inhibition of MCP-1-induced chemotaxis with conventional PKC AS-ODN and no change with control conventional PKC S-ODN were observed. For a more specific followup experiment, AS- and S-ODN targeting PKCα and β isoforms were used. Significant inhibition of MCP-1-induced monocyte chemotaxis was observed with PKCβ AS-ODN in its highest dose. Little or no effect was observed with PKCβ control-S-ODN, PKCα AS- and S-ODN. It was also shown that addition of PKCβ S-and AS-ODN did not alter the calcium response triggered by interaction of MCP-1 with its receptor CCR2, indicating that PKCβ is essential for post-receptor signal transduction downstream of the calcium signal (7). Our aim was to identify the downstream phosphorylation targets of PKCβ in MCP-1-activated human monocytes. In this study, we used the myristoylated PKCβ peptide inhibitor and PKCβ S- and AS-ODN that were checked for efficacy and used in our previous work (7).
2.2 Materials and Methods

2.2.1 Materials

PKCβ peptide inhibitor was purchased from Promega (Madison, WI). PKCβ S- and AS-ODNs were custom ordered from Invitrogen (Carlsbad, CA). MCP-1 was purchased from BD Biosciences (San Jose, CA). [³²P]-orthophosphate radionuclide with specific activity (314-337 TBq/mMole) was purchased from Perkin Elmer (Waltham, MA). Primary antibodies used were V9 monoclonal antibodies from Sigma (St. Louis, MO), anti-vimentin antibody from Cell Signaling (Danvers, Ma) and anti-PKCβ antibody from Novus Biologicals (Littleton, CO). Secondary antibodies were Alexafluor 488 goat anti-mouse and Alexafluor 594 goat anti-rabbit from Invitrogen (Carlsbad, CA).

2.2.2 Isolation of primary human monocytes

Monocytes were isolated according to the method of Kumagai et al (25). 240 mL of blood was withdrawn from healthy volunteers with 60 mL syringes containing ethylenediaminetetraacetic acid (EDTA) (3-4 mM final concentration). Collected blood was diluted 1:1 with phosphate buffered saline (PBS) and layered on Ficoll-Paque density solution. The mononuclear layer was isolated and then washed twice with PBS. In order to remove contaminating platelets, 100% bovine calf serum (BCS) was overlaid with the mononuclear cells and centrifuged at 150 x g for 10 min. This step was repeated if platelet contamination was evident. 75 cm² flasks were pre-coated with 10% BCS for 16 hr and then the BCS was replaced with 10% BCS in Dulbecco’s modified eagle medium (DMEM). Platelet-free mononuclear cells were added to the flasks and incubated at 37°C in 10% CO₂ for 2 h. Flasks were washed gently with warm DMEM to remove non-
adherent cells and these cells were plated in separate flasks containing 10% BCS/DMEM after centrifugation at 200 x g for 10 min. Flasks with both adherent and nonadherent cells were incubated at 37°C in 10% CO₂ for 16 h. In order to obtain adherent cells, the media with nonadherent cells was removed and 5 mM EDTA was added for 30 min after incubating the flasks at 37°C in 10% CO₂ for 10 min. The cells were collected after tapping the flasks a few times and washing the flasks several times with warm DMEM. The media was removed by centrifugation and the cells were suspended in 10% BCS/DMEM solution and counted. Monocytes in 10% BCS/DMEM were prepared in Falcon 5 mL polypropylene round bottomed tubes and incubated at 37°C in 10% CO₂ for at least 2 hr before use in our experiments.

2.2.3 Treatment of cells with oligodeoxyribonucleotides and PKCβ peptide inhibitor

The efficacies of the PKCβ isoenzyme-specific S- and AS-ODN used in our experiments were demonstrated in our previously published work (7). The PKCβ isoenzyme-specific antisense ODN sequence was 5’-AGC GCA CGG TGC TCT CCT CG-3’. The PKCβ isoenzyme-specific control-sense ODN sequence was 5’-CGA GGA GAG CAC CGT GCG CT-3’. Phosphorothioate-modified ODN were used for these experiments to prevent ODN degradation (7). Either S- or AS-ODNs (10µM) were added to the isolated human monocytes (2.5 x 10⁶ cells/mL) suspended in 10% BCS/DMEM and incubated at 37°C in 10% CO₂ for 24 h. The PKCβ peptide inhibitor (10µM) was added 30 min prior to the addition of MCP-1 (50 ng/mL). MCP-1 was added during the last 30 min of incubation.
2.2.4 Preparation of cell lysates

After PKCβ AS- or S-ODN treatment for 24 h, MCP-1 (50 ng/mL) in DMEM with 0.1% bovine serum albumin (BSA) was added to each tube and incubated for 30 min at 37°C in 10% CO₂. Cells were then treated with 1mM sodium orthovanadate (Ipswich, MA) for 15 min to inhibit phosphatases, harvested, centrifuged and washed three times with PBS. The cells were then resuspended in lysis buffer (1% Triton X-100 (Sigma, St. Louis, MO), 150 mM NaCl, 50 mM NaF, 50 mM Tris, pH 7.4, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 500 µM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO), and 1:100 diluted protease inhibitor mixture (Sigma, St. Louis, MO)). After 30 min on ice, the extracts were centrifuged at 9300 x g for 15 min at 4°C and the supernatants were collected as cell lysates.

2.2.5 Two-dimensional gel electrophoresis of PKCβ sense/anti-sense oligodeoxyribonucleotides treated primary human monocytes

In order to get good separation of proteins for identification, 2-DIGE of primary human monocyte lysates was performed using a Bio-Rad (Hercules, CA) 2-D IEF system using 11 cm Immobilized pH gradient strips. Immobilized pH gradient (IPG) strips were used for the first dimension and 10% sodium dodecyl sulfate polyacrylamide electrophoreses gels (SDS-PAGE) were used for the second dimension. Cells were treated with MCP-1 in the presence or absence of PKCβ AS-ODN. Cell lysates were boiled for 5 min, then cooled to room temperature and treated for 10 min with 2 µg DNAase and 2 µg RNAase with 50% protease inhibitor mixture prepared in 0.5 M Tris, pH 7.8, 50 mM MgCl₂ solution. Protein concentrations were determined by the BCA
Protein Assay Kit (Pierce, Rockford, IL) and the 2-D Clean-Up Kit (Amersham Biosciences, Piscataway, NJ) was used to reduce non-protein impurities and to improve the quality of 2-DIGE results after double acetone precipitation (80% v/v) of the proteins. Protein precipitates were resuspended in the solubilization buffer freshly prepared according to the Bio-Rad instructions. Rehydration was performed on the IPG strips for 12 -16 hr at room temperature applying 50 V. Isoelectric focusing was carried out with the following voltage program: 250 V (gradient over 15 min), 8000 V (gradient over 30 min), 8000 V (fixed for 4 hr 30 min), 500 V (gradient over 5 min), 500 V (fixed for 3 h). The strips were then equilibrated and immediately subjected to SDS-PAGE for the second dimension and the gels were fixed for 30 min at room temperature (26). The gels were stained with Pro-Q Diamond phosphoprotein gel stain Invitrogen (Carlsbad, CA) for 90 min with gentle agitation in the dark and then destained for 30 min three times and washed with distilled water for another 10 min. After imaging the gels, they were placed directly into SYPRO Ruby protein gel stain (Invitrogen, Carlsbad, CA) for total protein staining in the dark for overnight. The gels were then washed twice for 30 min. Gel imaging was performed after rinsing the gels with distilled water. Finally, the gels were stained with GelCode Blue (Pierce, Rockford, IL) to aid in locating the spots that are identified by comparison of the two gels stained with Pro-Q Diamond. Molecular masses were determined by simultaneously running standard protein markers. Selected spots were cut out, trypsinized, digested and analyzed by LC-mass spectrometry.

2-DIGE was performed as described above using monocytes treated with MCP-1 in the presence/absence of the PKCβ peptide inhibitor. Both the groups were probed with
phospho-(Ser)-PKC substrate antibody from Cell Signaling (Danvers, MA) to detect and compare the proteins phosphorylated at serine residues in PKC substrate proteins. The potential phosphoprotein was identified using liquid chromatography mass spectrometry (LC-MS).

2.2.6 Liquid chromatography mass spectrometry

To identify the protein spots on gel pieces, LC-MS was performed. For the protein digestion, the bands were cut from the gel as closely as possible from the gel with a punch and washed/destained in 50% ethanol, 5% acetic acid. The gel pieces were then dehydrated in acetonitrile, dried in a Speed-vac, and digested with trypsin by adding 5 µL (of 10 ng/µL trypsin) in 50 mM ammonium bicarbonate and incubating overnight at room temperature. The peptides that were formed were extracted from the polyacrylamide in two aliquots of 30 µL 50% acetonitrile with 5% formic acid. These extracts were combined and evaporated to ~10 µL in Speedvac and then resuspended in 1% acetic acid to make up a final volume of 30 µL for LC-MS analysis. The LC-MS system was a Finnigan LTQ linear ion trap mass spectrometer system. The HPLC column was a self-packed 9 cm x 75 µm (inner diameter) Phenomenex Jupiter C18 reversed-phase capillary chromatography column. 10 µL volumes of the extract were injected and the peptides eluted from the column by an acetonitrile/0.1% formic acid gradient at a flow rate of 0.25 µL/min were introduced into the source of the mass spectrometer on-line. The microelectrospray ion source is operated at 2.5 kV. The digest was analyzed using the data dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid
sequence in successive instrument scans. This mode of analysis produces approximately 2500 collisionally-induced dissociation (CID) spectra of ions ranging in abundance over several orders of magnitude. Please note that not all CID spectra are derived from peptides. The data were analyzed by using all CID spectra collected in the experiment to search the NCBI non-redundant database with the search program Mascot using a human taxonomy filter. All matching spectra were verified by manual interpretation. The interpretation process was aided by additional searches using the programs Sequest and Blast as needed.

2.2.7 Metabolic labeling and vimentin immunoprecipitation

Isolated primary human monocytes (5 x 10^6 cells/2mL/well) were incubated in 10% BCS/DMEM in the presence or absence of PKCβ S- or AS-ODN for 20 hr at 37°C in 10% CO₂. Cells were then preincubated in phosphate-free MEM for 1 hr at 37°C in 10% CO₂. Cells were labeled with [32P]-orthophosphate 100 µCi/mL for 3 hr. MCP-1 (50 ng/mL) was added to respective groups for 30 min, sodium orthovanadate (1 mM) was added at the last 15 min incubation. For preparation of cell lysates, the cells were collected and washed with Tris-buffered saline after treatment. The cells were then resuspended in lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM NaF, 50 mM Tris, pH 7.4, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 500 µM phenylmethylsulfonyl fluoride, phosphatase inhibitor cocktail and 1:100 diluted protease inhibitor mixture). After 30 min on ice, the extracts were centrifuged at 9300 x g for 15 min at 4°C. The supernatants were used as cell lysates. Cell lysates were
immunoprecipitated with vimentin V9 antibody for 2 hr and protein G agarose beads (Roche diagnostics, Indianapolis, IN) were added overnight at 4°C, both with constant rotation. Beads were washed and sample buffer was added prior to boiling for 5 min, followed by electrophoresis on 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (26). Incorporation of $^{32}$P was determined by analysis with a phosphorimager before vimentin loading was verified by immunoblotting using anti-vimentin antibody and detected by enhanced chemiluminescence.

2.2.8 Vimentin Immunoprecipitation

Human monocytes ($5 \times 10^6$ cells/2 mL/well) were incubated in the presence or absence of the PKCβ peptide inhibitor for 30 min followed by treatment with MCP-1 for 30 min and sodium orthovanadate (1 mM) was added for the final 15 min of incubation. The cell lysates were immunoprecipitated with anti-vimentin antibody followed by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (26). Anti-PKCβ antibody was used to detect the presence of PKCβ. The membrane was stripped using a 20 mL stripping solution (Stock solution: 12.5 mL of 62.5 M Tris pH 6.8, 20 mL of 20% SDS, 165 mL distilled water and 141.7 µL of β-mercaptoethanol was added right before use to prepare 20 mL stripping solution) and reprobed with anti-vimentin antibody.
2.3 Results

The focus of these studies was centered on identifying downstream target(s) of PKCβ phosphorylation as a result of MCP-1-activation of primary human monocytes.

2.3.1 Vimentin is a potential target of phosphorylation by PKCβ in MCP-1-activated human monocyte chemotaxis

Prior studies in our lab have shown that PKCβ is required for human monocyte chemotaxis to MCP-1 (7). In this study, 2-DIGE was performed for two groups of monocytes. The groups were treated with MCP-1 in the presence or absence of PKCβ AS-ODN. ProQ Diamond and SYPRO Ruby stains were used to stain phosphorylated proteins and all the proteins, respectively. The gels with and without PKCβ AS-ODN were compared after the ProQ Diamond stain and proteins that stained with less intensity in the presence of PKCβ AS-ODN were identified, which were normalized for the total protein stain. These proteins were localized after Coomassie Blue staining of the gels as in Figure 2.2. Figure 2.2 shows 2-DIGE of human monocytes after staining with ProQ Diamond and SYPRO Ruby stains (experiment performed by Dr. Ayse C. Akbasli). Proteins are separated by isoelectric point (pI) 5 to 8 horizontally (left to right) and by molecular weight vertically (top to bottom). Figure 2.2 (A, B) represents the 2D-gel image of MCP-1 treated human monocytes. The gel is stained with ProQ Diamond phosphoprotein gel stain 2.2 (A) and total proteins are stained with Sypro Ruby 2.2 (B). Figure 2.2 (C, D) represents the 2D-gel image of MCP-1 treated monocytes in the presence of PKCβ AS-ODN. Phosphoproteins are stained with ProQ Diamond phosphoprotein gel stain 2.2 (C) and total proteins are stained with Sypro Ruby 2.2 (D). Figure 2.2 (E)
shows the Coomassie stained two-dimensional gels after electrophoresis with protein extracts prepared from monocytes treated with MCP-1. The arrows point to the potential PKCβ substrate protein spots that stained with less intensity on phosphoprotein staining in the PKCβ AS-ODN treated group. The spots were sequenced using mass spectrometry and proteins identified are listed out in Table 2.1 (experiment performed by Dr. Ayse C. Akbasli). Twelve potential PKCβ substrate proteins were localized and identified. These proteins were cut out of the gel, washed, digested with trypsin and finally analyzed by LC-MS. MASCOT was used to analyze the data. Among the twelve proteins, four of them included vimentin. Vimentin, an intermediate filament protein was observed consistently on sequencing. Two of the proteins (spot number 5 and 6) were identified as the capping protein gelsolin and two of the others were identified as enolase 1 (spot number 8 and 10). The rest of the proteins were identified as biliverdin reductase, transaldolase, lasp-1 protein, annexin 1, lamin B1, L-plastin. Based on these data, we decided to further analyze vimentin as it was identified consistently, showed very marked inhibition and also has been reported to be regulated by PKC in different cell types (27).
Figure 2.2 Detection of potential PKCβ substrate proteins.

2-DIGE of human monocyte lysates was performed and stained with ProQ Diamond phosphoprotein stain and SYPRO Ruby total protein stain. Proteins are separated by isoelectric point (pI) horizontally (left to right) and by molecular weight vertically (top to bottom). (A), (B) represent the 2D-gel images of MCP-1 treated human monocytes. The gel is stained with ProQ Diamond phosphoprotein gel stain (A) and total protein Sypro Ruby stain (B). (C), (D) represent the 2D-gel images of MCP-1 treated monocytes in the presence of PKCβ antisense ODN. Phosphoproteins are stained with ProQ Diamond phosphoprotein gel stain (C) and total proteins are stained with Sypro Ruby total protein stain (D). Figure (E) shows the Coomassie Blue stained two-dimensional gel after electrophoresis with protein extracts prepared from monocytes treated with MCP-1. The arrows point to the potential PKCβ substrate protein spots that showed decreased intensity on phosphoprotein staining in PKCβ AS-ODN treated group compared to the MCP-1 treated group. The spots were sequenced using LC-MS and the identified proteins are listed in Table 2.1. Vimentin, an intermediate filament protein was observed consistently on sequencing. (experiment performed by Dr. Ayse C. Akbasli)
Table 2.1 Identification of potential PKCβ substrate proteins

(experiment performed by Dr. Ayse C. Akbasli)

<table>
<thead>
<tr>
<th>Spot number</th>
<th>Protein name</th>
<th>NCBI accession number</th>
<th>Mol. mass (kDa)</th>
<th>Isoelectric point (pl)</th>
<th>No. of peptides (% seq. coverage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vimentin Tubulin, alpha</td>
<td>37582 13436317</td>
<td>54</td>
<td>5.0</td>
<td>33 (73%) 3 (11%)</td>
</tr>
<tr>
<td></td>
<td>ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide</td>
<td>37582 16741373</td>
<td>54</td>
<td>5.0</td>
<td>28 (54%) 18 (49%)</td>
</tr>
<tr>
<td>3</td>
<td>Vimentin NF-M protein</td>
<td>37582 35046</td>
<td>54</td>
<td>5.0</td>
<td>29 (55%) 4 (4%)</td>
</tr>
<tr>
<td>4</td>
<td>Vimentin Tubulin, alpha</td>
<td>7576229 13436317</td>
<td>54</td>
<td>5.0</td>
<td>46 (73%) 11 (31%)</td>
</tr>
<tr>
<td>5</td>
<td>Lamin B1</td>
<td>576840</td>
<td>66.6</td>
<td>5.1</td>
<td>18 (29%)</td>
</tr>
<tr>
<td>6</td>
<td>Lymphocyte cytosolic protein 1 (L-plastin)</td>
<td>8217500</td>
<td>71</td>
<td>5.3</td>
<td>13 (26%)</td>
</tr>
<tr>
<td>7</td>
<td>Capping protein gelsolin-like</td>
<td>60655417</td>
<td>39</td>
<td>5.8</td>
<td>14 (34%)</td>
</tr>
<tr>
<td>8</td>
<td>Capping protein gelsolin-like</td>
<td>60655417</td>
<td>39</td>
<td>5.8</td>
<td>14 (34%)</td>
</tr>
<tr>
<td>9</td>
<td>Annexin I Aflatoxin aldehyde reductase AFAR</td>
<td>55959292 2736256</td>
<td>39</td>
<td>6.5</td>
<td>17 (47%) 2 (8%)</td>
</tr>
<tr>
<td>10</td>
<td>ENO1 (Enolase 1 variant)</td>
<td>62896593</td>
<td>47</td>
<td>7.0</td>
<td>25 (53%)</td>
</tr>
<tr>
<td>11</td>
<td>ENO1 (Enolase 1 variant) Lasp-1 protein</td>
<td>29792061 2135552</td>
<td>47</td>
<td>7.0</td>
<td>6 (18%) 5 (18%)</td>
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<tr>
<td>12</td>
<td>Bilverdin reductase A Transaldolase 1</td>
<td>13543489 14603290</td>
<td>34</td>
<td>6.0</td>
<td>6 (21%) 2 (5%)</td>
</tr>
</tbody>
</table>
2.3.2 Phospho-(Ser) PKC substrate antibody identifies vimentin as a target of PKCβ phosphorylation in MCP-1-activated human monocytes

Upon identifying vimentin in the mass spectrometry sequencing results of phosphoprotein staining, we wanted to employ another complementary method to determine if vimentin indeed is a target of PKCβ in MCP-1-activated human monocytes. 2-DIGE were performed with monocytes treated with MCP-1 as shown in Figure 2.3 (A) and monocytes treated with MCP-1 and a PKCβ peptide inhibitor as shown in Figure 2.3 (B). The phospho-(Ser) PKC substrate antibody detects endogenous levels of many cellular proteins only when phosphorylated at serine residues on PKC substrate proteins. A few proteins were observed to show more intense reactivity with the phospho-(Ser) PKC substrate antibody in monocytes treated with MCP-1 as compared to monocytes treated with MCP-1 and the PKCβ peptide inhibitor as in Figure 2.3. The arrow points to a prominent protein spot in the MCP-1 treated group that showed higher intensity with the phospho-(Ser) PKC substrate antibody in the MCP-1 treated monocyte group as compared to the group that was treated with MCP-1 and the PKCβ peptide inhibitor. This spot was sequenced using mass spectrometry and the protein that showed increased phosphorylation was identified as vimentin. Thus, vimentin was identified in one of the prominent spots signifying the important role of vimentin phosphorylation in MCP-1-activated monocytes.
A  Monocytes + MCP-1

B  Monocytes + MCP-1 + PKCβ inhibitor

Immunoblot: Phospho (Ser) PKC substrate antibody
Figure 2.3 Detection of increased vimentin phosphorylation in MCP-1-treated monocytes by phospho-(Ser) PKC substrate antibody.

Phospho-(Ser) PKC substrate antibody detects endogenous levels of many cellular proteins only when phosphorylated at serine residues specifically in conventional PKC substrate motifs. Proteins were separated on the basis of their isoelectric point in the first dimension and on SDS-PAGE in the second dimension. The arrow points to a protein spot on the blot of the MCP-1 treated group that showed higher intensity with the phospho-(Ser) PKC substrate antibody as compared to the monocytes that were treated with MCP-1 and PKCβ inhibitor. This spot was sequenced using mass spectrometry and the protein that showed increased phosphorylation in the MCP-1 treated group was identified as vimentin.
2.3.3 PKCβ induces vimentin phosphorylation in MCP-1-activated human monocytes

To further examine whether vimentin phosphorylation is regulated by PKCβ in MCP-1-activated human monocytes, the effect of PKCβ on vimentin phosphorylation was examined in primary human monocytes labeled with $^{32}$P-orthophosphate as in Figure 2.4. Monocytes were incubated with PKCβ S- or AS-ODN followed by $^{32}$P labeling. In Figure 2.4 (A), phosphorylation of vimentin was detected using phosphorimage analysis. In Figure 2.4 (B), the vimentin content was analyzed by immunoblotting using ECL detection. The figure shows 4 lanes: Lane 1, monocytes; lane 2, monocytes treated with MCP-1; lane 3, monocytes treated with MCP-1 and PKCβ S-ODN; lane 4, monocytes treated with MCP-1 and PKCβ AS-ODN. The vimentin phosphorylation levels in lane 2 and lane 3, wherein the monocytes were treated with MCP-1 in the absence and presence of PKCβ S-ODN, respectively, showed a marked increase as compared to lanes 1 and 4. Immunoprecipitation of vimentin from monocyte extracts revealed a PKCβ-induced increase in its phosphorylation relative to the control. Vimentin phosphorylation in MCP-1 treated human monocytes in the presence or absence of PKCβ S-ODN is comparable. In groups of untreated monocytes and monocytes treated with PKCβ AS-ODN, vimentin phosphorylation levels were significantly less as compared to groups treated with MCP-1 in the presence or absence of PKCβ S-ODN. Figure 2.4 (C) shows quantitative results of phosphorylation of vimentin in primary human monocytes upon activation with MCP-1 are compared with unactivated cells.
A  IP: anti-vimentin
   Phosphorimage
   
   Phospho-vimentin

B  Immunoblot: Anti-vimentin
   Vimentin (~58 kDa)

MCP-1  PKC-β sense  PKC-β antisense
-     +     +     +     -
-     -     +     -     +

C  

Relative fold phosphorylation

-  *  **  **

*  P < 0.05
**  P < 0.05
Figure 2.4 PKCβ anti-sense oligodeoxyribonucleotides inhibit vimentin phosphorylation in MCP-1-activated human monocytes.

Cells were labeled with [³²P]-orthophosphate as described in the Materials and Methods section. The cell lysates were immunoprecipitated with vimentin antibody followed by SDS-PAGE and transfer onto a polyvinylidene fluoride membrane. In (A), phosphorylation of vimentin was detected using phosphorimage analysis. In (B), vimentin loading was analyzed by immunoblotting using ECL detection. The figure shows 4 lanes: Lane 1, monocytes; lane 2, monocytes treated with MCP-1; lane 3, monocytes treated with MCP-1 and PKCβ S-ODN; lane 4, monocytes treated with MCP-1 and PKCβ AS-ODN. The vimentin phosphorylation levels in lane 2 and lane 3 showed marked increase compared to lanes 1 and 4. Figure (C) shows quantitative results of relative vimentin phosphorylation of the 4 groups of monocytes. The data are the averages of three similar experiments and the error bars indicate standard deviation values. The data were derived from band densitometry of the phosphorylated protein signal and were corrected for the amount of protein detected by immunoblotting.
2.3.4 PKCβ associates with vimentin upon treatment with MCP-1 in primary human monocytes

Upon confirming that vimentin is a downstream phosphorylation target of PKCβ in MCP-1 treated primary human monocytes, we wanted to check whether vimentin associates with PKCβ upon MCP-1 treatment in monocytes. To check this, immunoprecipitation of primary human monocytes was performed with anti-vimentin antibody and the immunoblotting was performed with anti-PKCβ antibody as in Figure 2.5. Three different groups of monocytes were prepared, wherein the first group was untreated monocytes, the second was monocytes treated with MCP-1 and the third was monocytes treated with MCP-1 and the PKCβ peptide inhibitor. The lysates were immunoprecipitated using anti-vimentin antibody. In Figure 2.5 (A), association between vimentin and PKCβ was observed in monocytes treated with MCP-1, whereas the untreated monocytes did not show association between vimentin and PKCβ as shown in Figure 2.5(A). The membrane was stripped and reprobed with anti-vimentin antibody as shown in Figure 2.5 (B). It is clear that PKCβ becomes increasingly associated with vimentin upon treatment of monocytes with MCP-1. No association between PKCβ and vimentin was observed in untreated monocytes thereby indicating the essential role of MCP-1 in inducing PKCβ binding with vimentin.
Figure 2.5 MCP-1 induces the association of vimentin with PKCβ in primary human monocytes.

Human monocytes were incubated in the presence or absence of the myristoylated PKCβ peptide inhibitor for 30 min followed by treatment with MCP-1 for 30 min as described in the Materials and Methods section. The cell lysates were immunoprecipitated with anti-vimentin antibody followed by immunoblotting with anti-PKCβ antibody. Figure (A) shows that upon MCP-1 treatment, increased association between PKCβ and vimentin occurs. No association of PKCβ with vimentin was observed in untreated monocytes. Upon PKCβ inhibitor treatment, the association between PKCβ and vimentin was not altered. The membrane was stripped and reprobed with anti-vimentin antibody to check loading as shown in (B).
2.4 Discussion

MCP-1-induced monocyte chemotaxis is believed to be regulated by signaling pathways mainly involving serine/threonine protein kinases. Using isoform specific PKCα and β AS-ODN, we have shown that PKCβ, not PKCα, mediates MCP-1-activated human monocyte chemotaxis (7). PKCβ activation has been shown to augment mechanisms involved in the progression of atherosclerosis. Activation of PKCβ is triggered by transient increases in a variety of factors including oxidant stress, ischemia and phorbol esters (28-30). Endothelial dysfunction in atherosclerosis has been observed to be strongly correlated with PKCβ activation in features like increased adhesion of monocytes to the vessel wall (31). It has been well established through cell culture studies that PKC plays a crucial role in mediating leukocyte-endothelial interactions (32-34). And also, adhesion and transmigration of monocytes into the sub-endothelial space and differentiation of monocytes to macrophages have been shown to be dependent on PKC activation (35). Studies showed that mice lacking both apoE and PKCβ displayed significantly decreased atherosclerosis compared with apoE-null mice. And also apoE-null mice, fed chow containing the PKCβ inhibitor ruboxistaurin, displayed significantly decreased atherosclerosis compared with the mice fed chow containing vehicle as a control (36, 37).

We designed a study to detect the downstream targets of phosphorylation by PKCβ in MCP-1-activated primary human monocytes. 2-DIGE was performed for two groups, monocytes treated with MCP-1 in the presence or absence of PKCβ AS-ODN, followed by phoso- and total protein staining. Proteins that stained less intensely with
the phospho-stain in the PKCβ AS-ODN treated group were sequenced by mass spectrometry. Detection of proteins and their post-translational modifications that utilize different fluorescent dyes have greatly enhanced the utility of 2-DIGE. A newly developed fluorescent phosphosensor technology is the ProQ Diamond post-staining method that detects phosphoserine-, phosphotyrosine-, and phosphothreonine-containing proteins in gels. It has been shown that the phosphoprotein staining procedure with ProQ Diamond is compatible with the total protein staining procedure using SYPRO-Ruby (38). Of the proteins identified that stained with lesser intensity with the phosphostain, vimentin was consistently present. Vimentin was also identified on 2-D gels in similar groups using a phospho-(Ser) PKC substrate antibody on lysates from monocytes activated with MCP-1.

Vimentin is the most abundant intermediate filament protein. Monocytes/macrophages contain a rich filamentous vimentin network. Intermediate filaments are a major component of the cytoskeleton of eukaryotic cells. During developmental stages, vimentin shows dynamically altered expression patterns and high sequence homology throughout all vertebrates from fish and Xenopus to humans (39). This strongly suggests that vimentin is physiologically important. Recent work has shown its involvement in cell adhesion, migration, signaling and wound healing (40-42). Recent studies also show its involvement in the pathogenesis of viral infections like virion assembly and its transport (43). A recent study has shown that novel vimentin-containing structures aid in motility on cell spreading. These structures, termed ‘vimentin dots’, contain non-filamentous vimentin and appear to be precursors of
vimentin intermediate filaments (44). One of the key observations was the targeting of vimentin intermediate filament precursors to areas of the spreading cytoplasm. Earlier, studies have shown close interactions between intermediate filaments and microtubules predominantly on spreading and mitotic cells. In the process, retraction of vimentin intermediate filaments occurs after dissociation of microtubule-associated proteins and de-polymerization of microtubules (45-48). But studies have also shown that polymerization and depolymerization of vimentin filaments occur in the presence and absence of microtubules. Cells that are motile send out vimentin dots into the new cytoplasmic extensions towards the moving direction. Based on our fluorescence imaging experiments (data not shown), there are signs of evidence that the vimentin network may get redistributed or polarized depending on the stimulus, like in our case, the chemokine, MCP-1. It has been observed that intermediate filament precursors constantly move towards the extensions where the non-filamentous forms gets converted to the filamentous form (44).

Factors that keep vimentin in the non-filamentous precursor state or induce its polymerization are still not clearly known. There is evidence that phosphorylation of vimentin induces its reorganization during mitosis. Organization of the intermediate filament networks is observed to be primarily regulated and modulated by phosphorylation. The state of phosphorylation regulates the interconversion between the non-filamentous and the filamentous forms. Vimentin has been shown to be phosphorylated \textit{in vitro} by cAMP-dependent protein kinase (49) and is also phosphorylated \textit{in situ} by an unidentified kinase upon treatment with chemotactic factors.
such as formyl-peptides (50). Also, vimentin in neutrophils is phosphorylated upon stimulation with phorbol myristate acetate, strongly suggesting that it may be a substrate for PKC (51). Several protein kinases like PKC and cyclic AMP have played significant roles in phosphorylating vimentin at different sites (52). The phosphorylation pattern of vimentin is highly complex involving different sites and kinases specific for different cellular processes like differentiation, stress and mitosis (53). Organization of vimentin filaments and the role of phosphorylation on assembly-disassembly remain obscure (40, 54).

In our studies using \(^{32}\text{P}\) labeling and vimentin immunoprecipitation, MCP-1 treatment increased vimentin phosphorylation in control monocytes and monocytes treated with PKC\(\beta\) S-ODN. In contrast, increased phosphorylation was not observed in the absence of MCP-1 and in monocytes treated with MCP-1 and PKC\(\beta\) AS-ODN. Immunoprecipitation of monocyte lysates with an antibody to vimentin and immunoblotting with a PKC\(\beta\) antibody revealed that increased PKC\(\beta\) becomes associated with vimentin upon MCP-1 activation and this binding is independent of PKC\(\beta\) functional activity.

In the past decade, an emerging interest on the regulatory nature of intermediate filaments in signal transduction has developed. Reversible phosphorylation brings about this reversible exchange between small depolymerized and fully polymerized fractions of intermediate filaments. This active exchange appears to be for other purposes than just restructuring. Knockout models of animals that do not express vimentin showed no abnormalities in phenotype, (31) but abnormalities were observed in special
physiological and pathological conditions like injury (40). Studies focus on the emerging concept of targeting of regulatory enzymes such as protein kinases and phosphatases to subcellular structures and organelles containing substrates for these enzymes. Conventional wisdom is that kinases phosphorylate substrates by recognizing specific primary sequence motifs within the substrate. As for monocytes, the effects of vimentin phosphorylation on its regulation and control of monocyte functions such as chemotaxis remain unknown, as further studies are most certainly in order (55). Based on our findings in our studies, we conclude that vimentin is a target of PKCβ phosphorylation in MCP-1-activated human monocytes.

The functional significance of the assembly mechanism for vimentin filaments could be to offer dynamic support to maintain the cell shape in different physiological and adverse conditions. Study of factors regulating their assembly into polymers and their delivery to different regions of the cell will further help to elucidate its functional significance in living cells. Cells infected with reoviruses, picornaviruses, vaccinia viruses and human immunodeficiency virus show a collapse of vimentin around the perinuclear area. This is believed to be similar to the protective process of aggresome formation by which the cells enclose the potentially toxic aggregates of proteins that are not folded properly. In addition to the protective covering, vimentin is also believed to play a dynamic role in this protective mechanism that is yet to be discovered (56-58). Apart from offering mechanical support, vimentin also interacts with nucleic acids. Both processes involve the non-α helical, arginine-rich N-terminal head domains of vimentin.
Different isoforms of PKC have also been observed to regulate vimentin phosphorylation. The expression of vimentin is upregulated during myeloid cell differentiation and PKCδ involved in differentiation by its association with vimentin. PKCδ colocalized with vimentin upon indirect immunostaining in the cytosol and perinuclear region of these cells. Upon treatment of HL60 cells with the PKC-specific inhibitor chelerythrine showed decreased phosphorylation of vimentin. These data strongly suggest that vimentin is a substrate for different isoforms of PKC like PKCβ and PKCδ in different cell types under different conditions (59-61).

Studies have shown that upon phosphorylation, vimentin is secreted by activated macrophages. We have data showing that vimentin can induce monocyte O₂⁻ production. PKC regulation of neutrophil NADPH oxidase was suggested two decades ago when it was shown to be stimulated by phorbol esters (62). Continuous infusion of the nonselective PKC inhibitor chelerythrine decreased superoxide production and also prevented approximately half the increase in NADPH oxidase subunit expression (63). The PKCβ inhibitor ruboxistaurine is currently being tested in ongoing clinical trials with microvascular endpoints. Clinical trials may determine whether PKCβ inhibition can prevent atherosclerosis in the future (35).
CHAPTER 3

IDENTIFICATION OF ENDOGENOUS LIGANDS FOR DECTIN-1 IN Atherosclerotic Plaques

3.1 Background

Atherosclerosis, the major cause of morbidity and mortality in developed countries, claims more lives than all cancers combined (64). It is a chronic inflammatory disorder characterized by cholesterol deposition in macrophages in medium and large-sized arteries within the wall leading to gradual impingement on the lumen resulting in heart attack, stroke, peripheral vascular disease and other major life threatening complications. Oxidation of LDL by ROS plays a central role in atherogenesis. MDM are the primary source of ROS in atherosclerotic lesions. One ROS involved in LDL oxidation is $O_2^-$. $O_2^-$ comes primarily from NADPH oxidase in monocytes and MDM.

3.1.1 Role of NADPH oxidase in atherosclerosis

It is well known that phagocytes, including neutrophils, monocytes and macrophages contain a plasma membrane bound NADPH oxidase that catalyzes the reduction of molecular oxygen to $O_2^-$. NADPH oxidase is a multi-component enzyme complex that is unassembled in the inactivated state and its components are located both in the cytoplasm and membrane. Cytosolic components include p47phox, p67phox, Rac1 and the membrane components are cytochrome b558, p91phox, and p22phox. Upon activation, the cytosolic components are translocated to the plasma membrane followed by activation of $O_2^-$ production. Monocytes, in immediate response to activating stimuli,
produce \( \text{O}_2^- \). Monocytes are the primary source of NADPH oxidase-derived \( \text{O}_2^- \) in atherosclerotic lesions, which has been shown to oxidize LDL, marking its significant role in the pathogenesis of atherosclerosis since oxidized LDL contributes to foam cell formation and lipid accumulation within the vessel wall. Superoxide is primarily produced to curb infections by killing invading microorganisms. For many years, our laboratory has focused on the regulation of NADPH oxidase activity in primary human monocytes (65). Our lab was the first to show that monocytes could oxidize LDL in a superoxide-anion-dependent fashion (66).

### 3.1.2 Role of immune-inflammatory mechanisms in atherogenesis

Chronic inflammation characterizes the pathogenesis of the cardiovascular disease, atherosclerosis (67). Immune-inflammatory mechanisms have been shown to play a significant role in the development and regulation of atherosclerosis and its complications. Studies suggest that there might be interactions between the innate immune defense mechanisms and the pro-inflammatory pathways contributing to development of atherosclerotic lesions (68). Production of cytokines and other inflammatory mediators by the inflammatory process at atherogenic sites leads to cell migration, proliferation, extracellular matrix production, and eventually, development of the plaque. Only a part of the epidemiological features of atherosclerosis are explained by the risk factors such as hypercholesterolemia, smoking, hypertension, and diabetes. Emerging data indicates that autoimmune mechanisms comprised of autoantigens and autoantibodies also play a crucial role in atherogenesis (69). Risk of myocardial infarction associated with these antibodies was independent of other well-established risk
factors (70). For example, serum antibodies against oxidized LDL are present in atherosclerosis and also in a variety of inflammatory and autoimmune conditions (71). A study of patients with carotid artery atherosclerosis showed a positive correlation between the amount of antibodies in serum and the rate of progression of atherosclerotic plaques (70).

### 3.1.3 DAMPS (Damage associated molecular patterns) and alarmins: Danger signaling

It has long been observed that injuries of both infectious and inflammatory origins induce a similar inflammatory response. Recent studies have shown that the mechanisms involved in innate immune system activation has singled out PRRs as a common pathway for recognition of the immune mechanisms of both microbial and tissue injuries. As the immune system recognizes any danger signal, either exogenous or endogenous, these receptors are key in recognizing both pathogens and endogenous warning signals which are released during cellular stress or insult. Upon recognition of a danger signal, these alert the host defense system by activating the innate immune system. Classical definitions of immunity as discrimination of non-self from self is suitable to explain the inflammatory mechanisms and immune activation during microbial invasion and rejection of transplants, but it fails to explain the inflammatory response to a variety of conditions where no non-self agents are involved like trauma and autoimmunity. This led to the evolution of the concept of danger signals (72). This danger signal model states that the inflammatory response is initiated in response to molecular patterns that are associated with pathogens and normal cellular components that are released during either
infectious or non-infectious conditions (73). The inflammatory response to a sterile injury closely resembles that of an infectious injury, with involvement of similar cytokine and chemokine production (74). Danger signals are cell constituents that are present in normal conditions but are released either actively by cells under stress in response to cellular injury or passively by necrotic cells. These endogenous ligands that act as danger signals are termed ‘alarmins’ (73). They perform diverse functions such as activation of the immune system, are released during necrosis, are sequestered in apoptotic conditions and secreted by immune cells. Alarmins are found in a variety of conditions, for example, sepsis, burns, infection, arthritis and cancer (75-78). They have been found to play key roles in both infectious and non-infectious or sterile inflammation.

3.1.4 Pattern recognition receptors

PRRs are germline-encoded receptors that recognize evolutionarily conserved structures expressed by various invading pathogens that are not present in mammalian cells termed pathogen-associated molecular patterns (PAMPs). PAMPs are exogenous and their endogenous analogues, alarmins, are collectively termed danger associated molecular patterns (DAMPs) which can be either self or non-self (79, 80). Higher animals have a limited set of PRRs (81). PRRs are located in three different cellular compartments: extracellular, membrane, and cytoplasmic. The major extracellular PRRs are the complement components. Major membrane-associated PRRs are toll-like and C-type lectin like receptors, and cytoplasmic receptors are cytoplasmic caspase recruiting domain helicases, nucleotide-binding oligomerization domain-like receptors (82). The best known and characterized classes of PRRs are the TLRs and C-type lectin receptors.
C-type lectin receptors possess one or more C-type lectin-like domains. Initial characterization was for their calcium dependent carbohydrate-binding property but they recently have been found to have diverse functions (84).

### 3.1.5 C-type lectin receptors and their functions

C-type lectins are either transmembrane proteins or secreted soluble proteins. C-type lectins like receptors consist of a carbohydrate-recognition-like domain that might bind proteins or lipids, apart from carbohydrates, in a calcium-dependent manner. Ligands for C-type lectin and lectin-like receptors have not been thoroughly characterized yet. There are type I and type II C-type lectins, which have their N termini pointing outwards or into the cytoplasm of the cell, respectively. The type I surface lectins are produced by dendritic cells (DC) and contain several carbohydrate-recognition domains or carbohydrate-recognition-like domains, whereas the type II C-type lectins identified so far have a single domain of this type. The macrophage mannose receptor (CD206), DEC-205 (CD205) are members of type I C-type lectin-like family of homologous proteins and has extensively been studied in dendritic cells (85, 86). The second group of C-type lectins contains only a single carbohydrate-recognition domain at the carboxyl (C)-terminus. Langerin (CD207), dendritic cell-immunoreceptor are members of type II C-type lectin-like family of proteins (87, 88). Several other new DC-type II C-type lectins have been revealed by molecular analysis, including the DC-associated C-type lectins 1 and 2 (dectin-1, dectin-2) (89). The major function of the TLR family is to alert DCs and induce their maturation, but the C-type lectin receptors function as powerful antigen capture receptors and also in the uptake mechanism of DCs.
C-type lectins, especially the membrane-bound are designed to trap pathogens for intracellular destruction, degradation and antigen loading of major histocompatibility complex (MHC) molecules (83).

3.1.6 Dectin-1, a non-toll C-type lectin receptor: Its structure and functions

Although the TLRs play a crucial role in recognizing PAMPs and development of pathogen-specific immune response, a recent resurgence of interest in non-toll receptors has been observed. Of the non-toll PRRs, dectin-1 is the archetypical member. Dectin-1 is the first non-toll receptor to induce its own signaling (90). Dectin-1 was recently identified primarily as the receptor for β-glucans in addition to scavenger receptors, complement receptor 3 (CD11b/CD18) and lactosyl-ceramide. It is a nontypical C-type lectin that works in a calcium-independent manner since it lacks residues for calcium binding. We have prior data showing that dectin-1 is a potent receptor for activating NADPH oxidase in primary human monocytes as shown in Figure 3.1 (91). Dectin-1 is a glycosylated type II transmembrane receptor that contains a single extracellular C-type lectin-like recognition domain which recognizes β1,3- and/or β1,6-linked glucans (92), followed by a short stalk region and a short 40 aminoacid cytoplasmic N-terminal domain containing a single immunoreceptor tyrosine-based (ITAM-like) activation motif. The ITAM-like motif mediates signaling by becoming phosphorylated providing an important breakthrough in understanding of activation signaling through single tyrosine-based motifs (81). Human dectin-1 (hdectin-1), 33 kDa, is similar to murine dectin-1 in structure. The murine and human dectin-1 isoforms differ in the number and position of N-glycosylation sites. hDectin-1 mRNA is alternatively spliced, resulting in two
predominant forms, hdectin-1 A which has only one N-glycosylation site in its stalk region between the carbohydrate recognition and the transmembrane domains, whereas hdectin-1 B does not have the stalk region as well as the N-glycosylation sites. The only isoforms that are functional for β-glucan binding are the two major isoforms. But the function of the other six isoforms of dectin-1 is not clearly understood yet (93). Of the six isoforms, the one that is different and unique is hdectin-1E which contains a complete C-type lectin-like domain and also as an ITAM-like sequence. hdectin-1E is different from hdectin-1A in its lacking a part of the putative cytoplasmic domain, transmembrane region and the stalk. hdectin-1E was not found to be secreted but remained primarily in the cytoplasm. Both in vitro and in vivo studies show that a binding protein called Ran-binding protein, RanBPM, was found to interact with hdectin-1E strongly suggesting that hdectin-1E may function as an adaptor molecule (94).

β-glucans, upon binding with dectin-1 trigger various cellular responses, namely, endocytic and phagocytic uptake of ligand, activation of the respiratory burst, and production of arachidonic acid metabolites, cytokines and chemokines. These responses triggered by dectin-1 are cell-type dependent and may or may not require TLR signaling. These differences in cellular responses have been partly attributed to the actions of cytokines like TNF, IFN-γ, and GM-CSF (81). Dectin-1 also binds T-lymphocytes at a site distinct from the β-glucan binding site, indicating that this receptor may recognize both endogenous and exogenous ligands (95-98). Dectin-1 is implicated in the innate immune recognition of yeasts such as Saccharomyces cerevisiae and fungal pathogens like Candida albicans and Pneumocystis carinii in a glucan-dependent fashion. β-glucan
binding has been shown to be mediated by the lectin-like domain independent of calcium (102). C-type lectin-like domain mediates ligand binding and though the domain structure and functionality has been examined by mutational analysis, the actual mechanism of carbohydrate recognition remains unknown. Dectin-1 has also been shown to bind to ligands on mycobacteria and endogenous ligands, though the speculated ligands have not been identified yet (81). It is related to members of the lectin-like NK-cell receptors (it is closely related to NK2D and LOX-1) and is encoded on human chromosome 12 in the NK-gene complex. The residues that require calcium binding are the ones that mediate carbohydrate binding in the classical lectins (99-101).

For the major part of our studies, we have used anti-zymosan antibody and human carotid atherosclerotic tissue samples. Anti-zymosan antibodies were used to identify similar, cross-reactive epitopes on extracts from human atherosclerotic tissue. The reason being that zymosan is a well-known ligand for dectin-1 and when anti-zymosan antibody is added, it binds to zymosan, prevents zymosan from binding to Dectin-1 and blocks the production of $O_2^-$ as shown in the model in Figure 3.2. We hypothesized that inflammation and stress may generate potent molecules that act as endogenous danger signals to promote and exacerbate the inflammatory response. As described above, some of these endogenous ligands might bind to dectin-1 with the ability to activate $O_2^-$ production and contribute to atherogenesis as shown in Figure 3.3.

In our studies, we have employed zymosan, a cell wall preparation of *Saccharomyces cerevisiae* composed primarily of β-glucans, mannans, mannoproteins, and chitin. Each of these compounds has been implicated in recognition of yeast by the
innate immune system. Zymosan stimulates phagocytosis in myeloid cells and production of inflammatory cytokines, chemokines, reactive oxygen and nitrogen species, and is a potent adjuvant in stimulating adaptive immune responses (96). Zymosan has been used for over 50 years primarily as a model phagocytic and inflammatory stimulus both in vivo and in vitro. Zymosan is a well-known ligand for dectin-1 and is known to induce the oxidative burst (NADPH oxidase) through dectin-1 and TLRs in several cell systems. Prior data from our lab show that in primary human monocytes, zymosan induces NADPH oxidase activity to produce O$_2^-$ exclusively in a dectin-1 dependent manner, without collaboration with the TLRs as shown in Figure 3.1 (91). Binding of dectin-1 to zymosan is inhibited by soluble glucan polysaccharides such as laminarin and glucan phosphate.
Figure 3.1 Dectin-1 binds to zymosan and induces NADPH oxidase activity to produce superoxide anion.
C. Endogenous ligands

D. Endogenous ligands

- gp91
- p22
- p67
- p47
- Rac1

$O_2^-$
Figure 3.2 Detection of non-microbial endogenous ligands for dectin-1 in human atherosclerotic tissue samples using anti-zymosan antibody.

Zymosan, a well-known ligand for dectin-1 potently activates NADPH oxidase activity to produce $O_2^-$ as shown in Figure (A). Upon adding anti-zymosan antibody, zymosan binds to this antibody which prevents its binding to dectin-1, thereby blocking the induction of NADPH oxidase to produce $O_2^-$ as in Figure (B). We hypothesized that there are non-microbial endogenous ligands for dectin-1 in human atherosclerotic tissue samples that bind to dectin-1 and trigger NADPH oxidase activity as in Figure (C). Figure (D) shows a potential endogenous ligand present in human atherosclerotic tissue that binds to dectin-1 and triggers NADPH oxidase activity to produce $O_2^-$. 
Figure 3.3 The model depicts our hypothesis that there are unidentified non-microbial endogenous ligands in human atherosclerotic tissue that bind to dectin-1 and trigger NADPH oxidase activity to produce superoxide anion.
3.2 Materials and Methods

3.2.1 Materials

Human carotid atherosclerotic tissue samples were obtained from the Co-operative Human Tissue Network (NIH), the Department of Vascular Surgery at the Cleveland Clinic and Biochain (Hayward, CA). Antibodies used were anti-zymosan antibody from Invitrogen (Carlsbad, CA), anti-dectin-1 antibody from R&D Systems (Minneapolis, MN) and V9 monoclonal antibody from Sigma (St. Louis, MO). The cyanogen bromide sepharose column (CnBr) was ordered from GE Healthcare (Piscataway, NJ). Proteins used in various experiments were vimentin from American Research Products (Belmont, MA), recombinant human dectin-1 from R&D systems (Minneapolis, MN), galectin-3-binding protein from Genway Biotech (San Diego, CA) and recombinant human lumican from R&D systems (Minneapolis, Minnesota). Recombinant CD36 protein, with and without a His-tag, were generous gifts from Dr. Roy Silverstein’s lab. Laminarin, superoxide dismutase and cytochrome C were ordered from Sigma (St. Louis, MO).

3.2.2 Immunoblotting of human carotid atherosclerotic tissue samples

Human carotid atherosclerotic tissue samples were homogenized in a 1% SDS solution and diluted to 0.1% SDS. Protein concentration of the tissue lysates were measured using the Bio-Rad DC (detergent compatible) assay (Hercules, CA). Homogenized carotid atherosclerotic tissue lysates were subjected to immunoblot analysis. 50 µg of tissue extracts were run on 10% SDS-PAGE, transferred onto polyvinylidene fluoride membranes and probed with anti-zymosan antibody (26).
SDS-PAGE was performed simultaneously in two gels wherein 10 µg of the same carotid atherosclerotic tissue extract was loaded in lane 1, 30 µg in lane 2 and 50 µg in lane 3 of both the gels (26). One gel was Coomassie Blue stained and the other gel was transferred onto a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA) and probed with anti-zymosan antibody. Observed bands were superimposed on the Coomassie Blue stained gel to select the protein bands on the gel for LC-MS sequencing.

3.2.3 Two-dimensional gel electrophoresis of human carotid atherosclerotic tissue samples

2-DIGE were performed. Human carotid atherosclerotic tissue extracts were processed as described above. Two 10% SDS gels were run simultaneously loaded with 50 µg of the same tissue extract in each gel (26). One gel was stained with Coomassie Blue and the other gel was transferred onto a polyvinylidene fluoride membrane and probed with anti-zymosan antibody. The bands observed on immunoblotting with anti-zymosan antibody were superimposed on the gel stained with Coomassie Blue and the spots marked were sequenced using LC-MS.

3.2.4 Immunoaffinity purification of proteins that bind to anti-zymosan antibody

To detect proteins in human carotid atherosclerotic tissue samples that bind to anti-zymosan antibody, the antibody was immobilized on a CnBr 4B from GE (Piscataway, NJ). CnBr-activated sepharose is a pre-activated 4% agarose medium for immobilization of proteins. It is stable to all commonly used aqueous solutions. The immunoaffinity column was prepared as per instructions from the manufacturer in 0.8 cm
x 4 cm columns. 5 mg of anti-zymosan antibody was coupled to the column overnight at 4°C. After coupling, the column was washed with the coupling buffer (0.5 M sodium phosphate pH 7.5) and blocked using 0.1 M Tris-HCl pH 8.0 for 4 h at 4°C. After blocking, the column was washed again. Tissue extraction was performed in phosphate buffered saline containing 1% Triton-X overnight at 4°C and it was diluted 10 times before passing through the column. Once the column is washed after blocking, the diluted tissue extracts was passed through the column continuously overnight at 4°C using a peristaltic pump. Proteins coupled to the antibody were eluted and the eluate was run on an SDS-PAGE and stained with Coomassie Blue (26). The observed protein bands were sequenced using mass spectrometry.

3.2.5 Binding studies using surface plasmon resonance and solution affinity kinetics

The direct binding between dectin-1 and various identified ligands was measured using SPR using the BIACORE 3000 instrument (BIACORE, Uppsala, Sweden). Both direct and indirect immobilization methods were employed. CM5 sensor chips (GE Healthcare, Piscataway, NJ) were used for all our binding studies. Covalent immobilization of dectin-1 was achieved by a negatively charged carboxylated dextran surface coupled to the gold film which is the standard surface medium in the BIACORE system. These chips are designed to reduce non-specific absorption of ligands at physiological ionic strength and at the same time, facilitate accessibility of the ligands in the subsequent study. It comes preactivated for coupling via four different covalent coupling strategies: amine, ligand thiol, surface thiol, or aldehyde. The dextran matrix
was first activated with a 1:1 mixture of 0.4 M 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) and 0.1 M N-hydorxysuccinimide (NHS) which creates reactive succinimide esters. The injected ligand acquires a positive charge and is effectively preconcentrated into the negatively charged carboxymethylated dextran matrix. Finally, unreacted esters are blocked with ethanolamine. The volume or concentration of ligand injected may be varied to adjust the immobilization level.

Different concentrations of the analytes were passed through the flow cell in modified Hanks (HBSS) buffer on the sensor chip with or without dectin-1 (reference cell). The buffer effect response observed on the blank reference flow cell was subtracted and corrected for the corresponding sensorgrams.

In another set of experiments, vimentin was indirectly immobilized. This approach is an alternative to covalent immobilization. The reason vimentin was indirectly immobilized was that vimentin, being a highly sticky protein didn’t freely flow through the flow cells when passed over a blank chip. To prevent this non-specific binding of vimentin to the chip and other structures along the way in the BIACORE system, vimentin was indirectly immobilized using a capture molecule. The capturing molecule, in our case monoclonal anti-vimentin antibody, was covalently immobilized using amine coupling. Vimentin was captured onto the anti-vimentin antibody with a high-affinity that was activated with EDC/NHS and then blocked with ethanolamine. Experiments were performed by injecting the analytes at 20 µl/min for 2 min. Data were analyzed using the BIAevaluation 3.1 program (BIACORE, Uppsala, Sweden).
3.2.6 Superoxide anion assay

Superoxide anion production by human monocytes was measured using the superoxide anion assay. This method is a modification of an assay previously published by Pick and Mizel (102). Human monocytes were plated in 24-well tissue culture plates pretreated with Hanks BSS containing 10% BCS for 1 h at 37°C (500 µL respectively, 1x10^6/mL in Hanks BSS) and allowed to adhere for at least two hr. This was followed by treatment with different inhibitors and controls at respective concentrations at indicated incubation times. Cytochrome C (160 U/mL, Sigma), with or without superoxide dismutase (SOD) (300 U/mL, Sigma), was added to the wells. This was followed by the addition of stimulants like zymosan, vimentin and galectin-3 binding protein in their respective groups. The cells were incubated for 1 hr at 37°C. The media was collected in tubes and the absorbance of the supernatant was read in cuvettes at 550nm. The SOD-inhibitable O_2^- 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 and expressed as nmol/hr/10^6 cells.
3.3 Results

The focus of the experiments in this chapter will be centered on identification of endogenous ligands for dectin-1 that induce NADPH oxidase activity.

3.3.1 Detection of protein bands that react with anti-zymosan antibody in human carotid atherosclerotic tissue samples by immunoblotting

Based on previous data from our lab, zymosan induced O$_2^-$ production through dectin-1 in primary human monocytes (91). In this experiment, our goal was to detect proteins in human carotid atherosclerotic tissue samples that have similar epitopes to zymosan and therefore, could be potential endogenous ligands for dectin-1. Immunoblotting was performed with the processed tissue samples with anti-zymosan antibody. Prominent bands around 50 kDa were seen consistently as in Figure 3.4 (A). As the bands were observed around 50 kDa, we wanted to make sure that the observed bands were not due to non-specific interactions e.g. heavy chain of human IgG. Upon stripping and reprobing with the secondary antibody, no bands were observed indicating that the observed bands on anti-zymosan antibody treatment are specific and not due to cross-reactivity between the human IgG heavy chain and the antibody as shown in Figure 3.4 (B). Similar protein bands were observed across different carotid atherosclerotic tissue samples from different donors as in Figure 3.4 (C). All the tissue samples showed similar consistent protein bands that reacted with anti-zymosan antibody. Identification of these proteins and understanding the significance of their presence in the lesions is important.
Figure 3.4 Detection of protein bands in human carotid atherosclerotic tissue extracts on immunoblotting with anti-zymosan antibody.

Homogenized carotid endarterectomy tissue samples were subjected to immunoblotting with anti-zymosan antibody. Figure (A) shows protein bands around 50 kDa. Figure (B) shows no bands upon stripping and reprobing with human preadsorbed anti-rabbit secondary antibody. Figure (C) shows two specimens obtained from different tissue sources. Both atherosclerotic tissue specimens were processed under similar conditions and immunoblotted with anti-zymosan antibody. Similar bands around 50kDa were observed in both of the tissue specimens confirming consistency across different tissue samples.
3.3.2 Mass spectrometry analysis of protein bands detected by SDS-PAGE of human carotid atherosclerotic tissue samples

On confirming the consistency of the detection of cross-reactive protein bands in human atherosclerotic tissue samples with anti-zymosan antibody, identification of the proteins by running an SDS-PAGE of the processed sample and LC-MS was done (26). For this experiment, a human carotid atherosclerotic tissue sample was processed and loaded in 3 different amounts of 10, 30 and 50 µg on 2 gels similarly. Immunblotting was performed on one gel with an anti-zymosan antibody as shown in Figure 3.5 (B) whereas the other gel was stained with Coomassie Blue on SDS-PAGE as shown in Figure 3.5 (A) (26). Bands (arrows marked) were picked from two lanes on the Coomassie stained gel which correlated with the immunoblot data. All of these bands were positively identified with multiple proteins identified in each band. A total of 30 unique proteins were identified. Vimentin was identified in both the lanes (marked with arrows) with 9 peptides covering 16% of the protein in the first lane, 14 peptides covering 30% and 17 peptides covering 36% in the last lane lane with a predicted molecular weight of 54 kDa as shown in Table 3.1. Other identified proteins included several collagen isoforms, several myosin isoforms, albumin, biglycan preprotein, serine (or cysteine) proteinase inhibitor, clade A, several immunoglobulins as well as others.
Figure 3.5 Identification of proteins in human carotid atherosclerotic tissue samples that react with anti-zymosan antibody by Two-dimensional gel electrophoresis.

SDS-PAGE was performed simultaneously in two gels where 10 µg of the same carotid atherosclerotic tissue extracts were run in lane 1, 30 µg in lane 2 and 50 µg in lane 3 of both the gels. One gel was stained with Coomassie Blue and the other gel was transferred onto a polyvinylidene fluoride membrane and probed with anti-zymosan antibody. Figure (A) shows the Coomassie Blue stained one-dimensional gel with three lanes. Figure (B) shows the bands in the immunoblot of 3 similar lanes of the same tissue extract probed with anti-zymosan antibody. These bands were superimposed on the Coomassie Blue stained gel to select the protein bands on the gel for LC-MS sequencing. The commonly identified proteins in both (marked by arrows) are shown in Table 3.1.
Table 3.1 Identification of potential dectin-1 ligands from 1-dimensional gel electrophoresis

<table>
<thead>
<tr>
<th>Lane</th>
<th>Identification</th>
<th>Lane 1 Peptides (% Coverage)</th>
<th>Lane 1 Mascot Score</th>
<th>Lane 3 Peptides (% Coverage)</th>
<th>Lane 3 Mascot Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 &amp; 3</td>
<td>Albumin Precursor (4502027, 71 kDa)</td>
<td>26 (48%)</td>
<td>1398</td>
<td>17 (30%)</td>
<td>708</td>
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<td></td>
<td>Lumican Precursor (4505047, 39 kDa)</td>
<td>7 (19%)</td>
<td>172</td>
<td>13 (36%)</td>
<td>523</td>
</tr>
<tr>
<td></td>
<td>Vimentin (62414289, 54 kDa)</td>
<td>9 (16%)</td>
<td>150</td>
<td>14 (30%)</td>
<td>308</td>
</tr>
</tbody>
</table>
3.3.3 Detection and identification of proteins that react with anti-zymosan antibody in human carotid atherosclerotic tissue samples by 2-dimensional gel electrophoresis

After obtaining the mass spectrometry analysis for the one-dimensional electrophoresis experiment, 2-DIGE was employed for protein analysis. Since 2-DIGE offers a better separation based on the size and isoelectric point (pI), we wanted to perform a more specific analysis of proteins in the atherosclerotic tissue sample that react with anti-zymosan antibody. The pI strip with the range 3-10 was used to cover a wider range of proteins with better distinction. In this experiment, 50 µg of the same processed atherosclerotic tissue sample was loaded onto two 2-D gels. One was stained with Coomassie Blue after SDS-PAGE (Figure 3.6 (A)) and the other gel was immunoblotted with anti-zymosan antibody (26). On 2-DIGE with anti-zymosan antibody, few well separated spots were observed as in Figure 3.6 (B). The anti-zymosan immunoblot analysis gave several distinct areas of staining in the gel. The spots were superimposed on the stained gel and the marked spots were sequenced. Vimentin was the major component identified in three (spots 2, 3 and 6) bands by the presence of 28-35 peptides covering 53-60% of the protein sequences as shown in Table 3.2. Chondroitin sulfate proteoglycan 2 was identified as a minor component of band 2 by the presence of 3 peptides covering 1% of the protein sequences. All of these peptides were mapped to the N-terminal portion of the protein sequence. This taken together with the discrepancy between the observed molecular weight and the molecular weight of chondroitin sulfate proteoglycan 2 (374 kDa) suggests that this protein may be C terminally truncated.
3.3.4 Detection of vimentin and galectin-3 binding protein as proteins that bind to anti-zymosan antibody by immunoaffinity purification

We also performed a complementary and a specific approach to identify potential dectin-1 ligands in atherosclerotic tissue samples. Anti-zymosan antibody was immobilized onto an activated CnBr sepharose column. Atherosclerotic tissue extracts were passed through the column overnight at 4°C for effective binding. After passing the tissue extracts through the column, the proteins in the atherosclerotic tissue extracts that bound to the anti-zymosan antibody was eluted out. A mock column was also used as a control where half the tissue extracts was passed through the activated CnBr sepharose column without the immobilized anti-zymosan antibody. The non-specifically bound proteins were eluted from the control mock column. The eluates were run on SDS-PAGE and stained with Coomassie Blue stain to detect a major band just above 50 kDa as shown in Figure 3.7 (26). The band was sequenced. Vimentin and galectin-3 binding protein were the potential proteins identified by LC-MS among other proteins present in human carotid atherosclerotic tissue samples that bound to anti-zymosan antibody.
Figure 3.6 Identification of vimentin in human carotid atherosclerotic tissue samples by two-dimensional gel electrophoresis

2-DIGE were performed on 2 gels simultaneously using the same human carotid endarterectomy tissue extract (100 μg each). One gel was transferred onto a polyvinylidene fluoride membrane and probed with anti-zymosan antibody as shown in Figure (B). The other gel shown in Figure (A) was stained with Coomassie Blue. The Coomassie Blue stained, circled spots (A) on the gel were picked out for sequencing by superimposing them on the spots (B) observed on immunoblotting with anti-zymosan antibody. Circled spots 2, 3 and 6 were identified as vimentin as shown in the Table 3.2.
Table 3.2 Identification of potential dectin-1 ligands from 2-dimensional gel electrophoresis

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Identification</th>
<th>Peptides (% coverage)</th>
<th>Mascot Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Vimentin (62414289, 53 kDa)</td>
<td>35 (57%)</td>
<td>2057</td>
</tr>
<tr>
<td>3</td>
<td>Vimentin (62414289, 53 kDa)</td>
<td>31 (53%)</td>
<td>1735</td>
</tr>
<tr>
<td>6</td>
<td>Vimentin (62414289, 53 kDa)</td>
<td>25 (63%)</td>
<td>1630</td>
</tr>
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</table>
3.3.5 Direct, high-affinity binding of vimentin and galectin-3 binding protein to
dectin-1 were observed using surface plasmon resonance

To explore whether vimentin could directly bind to dectin-1 we performed studies using SPR. Proteins were immobilized using amine coupling to a CM5 sensor chip. Indirect immobilization of vimentin was performed by capturing the monoclonal anti-vimentin antibody on the chip followed by the immobilization of vimentin over this antibody. Serial concentrations of dectin-1 (0.5 µM, 1 µM, 2 µM, 4 µM, 8 µM) were passed through the flow cell over the immobilized vimentin. The black line indicates the actual curve calculated after buffer effect correction and the red line is the line of fit (Figure 3.8). Relatively strong binding was observed between dectin-1 and vimentin using the single injection kinetic titration method with a dissociation constant $K_d = 4.03 \times 10^{-7}$ M. Single injection kinetic titration was the solution affinity kinetics method used to calculate the data (103). This method involves injection of an analyte in serial concentrations in a sequential manner without any regeneration in-between.

A control experiment was performed using dectin-1 and anti-vimentin antibody to check if dectin-1 non-specifically binds to anti-vimentin antibody. Anti-vimentin antibody (16 µM) was passed through a flow channel over dectin-1 immobilized on a CM5 sensor chip. No non-specific binding between the two was observed as shown in Figure 3.9. Also as positive and negative controls, 10 µM of dectin-1 and 10 µM of CD36 (both with and without the His-tag) were passed through indirectly immobilized vimentin, and no non-specific binding was observed between CD36 and vimentin as shown in Figure 3.10.
Figure 3.7 Detection of vimentin and galectin-3 binding proteins in human atherosclerotic tissue samples as proteins that bind to anti-zymosan antibody by immunoaffinity purification

Immunoaffinity purification was performed by immobilizing anti-zymosan antibody on an activated CnBr column. An atherosclerotic tissue sample was passed through and the proteins that bound to anti-zymosan antibody were eluted. The eluate was run on SDS-PAGE and stained with Coomassie Blue stain. A major band was detected just above 50 kDa (see arrow). The band was sequenced. Vimentin and galectin-3 binding protein were identified among proteins that bound to anti-zymosan antibody as shown in Table 3.3.
Table 3.3 Identification of proteins that bind to anti-zymosan antibody

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Identification</th>
<th>Peptides (% Coverage)</th>
<th>Mascot Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vimentin (62414289, 54 kDa)</td>
<td>8 (20%)</td>
<td>418</td>
</tr>
<tr>
<td>2</td>
<td>Galectin-3 binding protein (5031863, 66 kDa)</td>
<td>4 (9%)</td>
<td>288</td>
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</table>
Figure 3.8 Direct binding was observed between dectin-1 and vimentin by surface plasmon resonance using BIACORE.

Vimentin was captured on a CM5 sensor chip using a monoclonal anti-vimentin antibody for indirect capture. Serial concentrations of dectin-1 (0.5 μM, 1 μM, 2 μM, 4 μM, 8 μM) were passed through the flow cell over the immobilized vimentin with no regeneration after each injection. The black line indicates the actual curve calculated after correction for the buffer effect and the red line is the line of fit. Relatively strong binding was observed between dectin-1 and vimentin using the single injection kinetic titration method with a dissociation constant $K_d$ of $4.03 \times 10^{-7}$ M.
Figure 3.9  No non-specific binding was observed between dectin-1 and anti-vimentin antibody using BIACORE.

16 μM of anti-vimentin antibody was passed through the flow cell over the immobilized dectin-1 to determine if the antibody directly binds to dectin-1. No non-specific binding between dectin-1 and anti-vimentin antibody was observed.
Figure 3.10 No non-specific binding was observed between vimentin and CD36.

10 μM of dectin-1 was passed through indirectly immobilized vimentin and strong binding was observed. As controls, 10 μM of CD36, both with and without a His tag were passed through a flow channel over the vimentin that was indirectly immobilized. No non-specific binding was observed between CD36 and vimentin.
The other potential ligands identified in our screen were galectin-3 binding protein and lumican. These proteins were also used to check their direct binding to dectin-1. On passing serial concentrations of galectin-3 binding protein (0.5 µM, 1 µM, 2 µM, 4 µM, 8 µM) through the flow channel where dectin-1 was immobilized, relatively strong binding was observed with the classic titration method with a dissociation constant of \(8.71 \times 10^{-6}\) M as shown in Figure 3.11. In contrast, lumican showed no direct binding to dectin-1 as shown in Figure 3.12.

### 3.3.6 Vimentin and galectin-3 binding protein induce superoxide anion production in human monocytes via the dectin-1 receptor

Once we confirmed the binding between vimentin and dectin-1, and galectin-3 binding protein and dectin-1, we wanted to check whether these ligands induce \(\text{O}_2^-\) production via the dectin-1 receptor. This assay measures \(\text{O}_2^-\) production by quantifying superoxide dismutase (SOD)-inhibitable cytochrome C reduction. Human monocytes (1x10^6/mL) were plated in 24-well tissue culture plates pretreated with Hanks BSS containing 10% BCS for 1 hr. This was followed by treatment with laminarin (500 µg/mL), a soluble β-glucan that is well known to bind dectin-1 with very high affinity and block the induction of \(\text{O}_2^-\) production. Blocking antibodies like anti-zymosan antibodies and anti-dectin antibodies (each 5 µg/mL) were added 20 min before the addition of stimulants or ligands. Control antibodies, rabbit non-immune IgG and mouse non-immune IgG (each 5 µg/mL), were added at the same time as the blocking antibodies. Cytochrome C (160 U/mL, Sigma), with or without superoxide dismutase
(300 U/mL, Sigma), was added to the wells. This was immediately followed by the addition of stimulants like zymosan (100 µg/mL), vimentin (5 µg/mL) and galectin-3 binding protein (5 µg/mL) in their respective groups. As a well-known ligand for dectin-1, zymosan significantly induces $O_2^-$ production and upon addition of dectin-1 blockers like laminarin, or anti-dectin antibody, there is significant inhibition of $O_2^-$ as shown in Figure 3.13. This clearly confirms our prior data indicating that zymosan induces $O_2^-$ production via the dectin-1 receptor in human monocytes. Similarly, vimentin also causes significant induction (of $O_2^-$ that is significantly inhibited by the dectin-1 blockers as in Figure 3.13. As shown in Figure 3.14, galectin-3 binding protein also causes significant induction of $O_2^-$ production and there is significant inhibition on blocking dectin-1. Both vimentin and galectin-3 binding protein, like zymosan, induce $O_2^-$ production in a dectin-1-dependent manner.
Serial concentrations of galectin-3 binding protein (0.5 μM, 1 μM, 2 μM, 4 μM, 8 μM) were passed through the flow cell over immobilized dectin-1 with regeneration after each injection. The curve was corrected for the buffer effect. Relatively strong binding was observed between dectin-1 and galectin-3 binding protein using the classic titration method with a dissociation constant of K_d of 8.71 X 10^{-6} M.
Figure 3.12 No direct binding was observed between dectin-1 and lumican.

10 μM of lumican was passed through the flow channel over the directly immobilized dectin-1 and no direct binding was observed between the two proteins.
The graph shows the nmol O₂/hr/million monocytes for Laminarin, Anti-Zymosan Ab, Anti-dectin-1 Ab, Rabbit non-immune IgG, and Mouse non-immune IgG under different conditions indicated by ‘-’ and ‘+’. The graph includes error bars indicating the standard deviation. The presence of an asterisk (*) indicates a significance level of P<0.01.
Figure 3.13 Vimentin induces superoxide anion production in primary human monocytes via the dectin-1 receptor.

Human monocytes (1x10^6 cells/ml) were plated in 24-well tissue culture plates pretreated with Hank’s BSS containing 10% BCS for 1 h. This was followed by treatment with the known dectin-1 inhibitor laminarin (500 μg/mL), dectin-1 blocking antibodies like anti-zymosan antibody and anti-dectin-1 antibody (each 5 μg/mL) in the corresponding groups. Negative controls used for anti-zymosan and anti-dectin-1 antibodies were rabbit non-immune IgG and mouse non-immune IgG (each 5 μg/mL) respectively. After the respective treatments, Zymosan (100 μg/mL) and vimentin (5 μg/mL) were incubated for an hour in the corresponding groups and O₂⁻ production was determined in each of these groups. Data are from a representative experiment of five that were performed. Data represent the mean of triplicate determinations ± standard deviation.
Figure 3.14 Galectin-3 binding protein induces superoxide anion production in primary human monocytes via the dectin-1 receptor.

Human monocytes (1x10^6 cells/ ml) were plated in 24-well tissue culture plates pretreated with Hank’s BSS containing 10% BCS for 1 hr. This was followed by treatment with or without the dectin-1 inhibitor laminarin (500 µg/ mL). After the respective treatments, Zymosan (100 µg/ mL) and galectin-3 binding protein (5 µg/ mL) were incubated for 1 hr in the corresponding groups and O₂⁻ production was calculated in each of these groups. Data are from a representative experiment of three that were performed. Data represent the mean of triplicate determinations ± standard deviation.
3.4 Discussion

The initiation and regulation of the innate immune responses were delineated by characterizing antigen-recognizing receptors of the innate immune system. PRRs including TLRs, lectins and scavenger receptors contribute to survival of the host by recognizing PAMPs on different invading pathogens. One such PAMP is expressed on β-glucans which are major components of yeast cell walls that become exposed only when the yeast buds or dies, as they are normally in the inner layer and the outer layer is composed primarily of mannoproteins (104, 105). Dectin-1 mediates the biological responses of β-glucan recognition both in vivo and in vitro by triggering a variety of immune responses. There is increasing evidence that dectin-1 is the most important receptor for β-glucan.

Dectin-1 was initially isolated from a murine epidermal dendritic cell line by subtractive cDNA cloning, hence named dendritic-cell associated C-type lectin-1 (89). Dectin-1 being the primary non-toll-like PRR is expressed primarily on myeloid cells with high expression on monocyte-macrophage and neutrophil lineages and at low levels on dendritic cells, natural killer cells, B cells and mast cells. Resident peritoneal macrophages express relatively low levels of dectin-1 but peritoneal inflammatory macrophages express high levels. It is also expressed in tissues, including liver, lungs, thymus, stomach, spleen, small intestine, kidneys, and heart (95). The diverse expression patterns of dectin-1 suggest that it may play different roles in the immune system. By far, the most critical function of dectin-1 is to eliminate infecting fungi and to offer protection for immunocompromised patients from opportunistic detrimental pathogenic infections.
Dectin-1 also binds to a number of fungal organisms other than *C. albicans*, including *Aspergillus fumigatus, Pneumocystis carinii, Coccidioides posadasii, Microsporum audouinii*, and *Trycophyton rubrum* (90) and helps to control these infections by triggering a variety of immune responses. Dectin-1 is also involved in inducing adaptive immune responses, autoimmune diseases and immune tolerance (106).

NADPH oxidase-derived O$_2^-$, a ROS produced by MDM mediates LDL oxidation in atherosclerotic lesions. Oxidation of LDL by O$_2^-$ is one of the key oxidative processes in atherogenesis as oxidized LDL is phagocytosed by macrophages leading to cholesterol accumulation and foam cell formation. Our data show that dectin-1 can mediate potent activation of NADPH oxidase in primary human monocytes. The yeast cell wall zymosan is a potent activator of monocyte NADPH oxidase and this activation is entirely dependent on its binding to dectin-1 (91).

Recent studies show that PRRs can bind endogenous ligands, in addition to their exogenous ligands. A new set of endogenous ligands, the alarmins have been identified that are analogous to the exogenous PAMPs. Damage-associated molecular patterns (DAMPs) composed of PAMPs and alarmins are collectively termed danger signals. The Danger model proposed by Matzinger suggests that the immune system functions to protect the host from damage rather than just discriminating self from non-self. The system is put into action by alarm signals from tissue injury (72). These alarm signals are diverse both in structure and location, these could be present inside the cell or secreted into the extracellular space, or could be constitutive or be synthesized after induction. During stressful conditions, cells release their intracellular contents that are not supposed
to be exposed making them danger signals. The key feature of these danger signals is that they are not released by normal cells or cells that undergo physiological phenomena. Receptors for endogenous and exogenous signals have evolved simultaneously in both vertebrates and bacteria. There is evidence that these receptors are often the same molecules. TLRs have been shown to bind certain specific endogenous ligands. TLR4 binds to an exogenous foreign bacterial product, lipopolysaccharide (LPS), and also to endogenous molecules like heat shock protein 70 (Hsp70) and the extracellular matrix breakdown product of hyaluron. In a similar fashion, TLR2 also binds to exogenous bacterial lipoproteins and endogenous Hsp60, TLR9 binds to DNA CpG sequences that are ubiquitously present in all living creatures. Not just the TLRs, but also other groups of PRRs like the nucleotide-binding oligomerization domain-like receptors seem to respond to injury/pathogen-related signals and also to the physiological signals of apoptosis. The most fascinating feature of the PRRs is the diversity of the ligands that these receptors bind. The ligands could be either endogenous or exogenous, and totally unrelated in structure, function and location. But so far, endogenous ligands, though speculated, have not been identified for dectin-1 (74).

The widely studied and the well-known DAMPs are High mobility group box protein-1, S1000A8/ S100A9 (also known as myeloid-related protein-8), heat shock proteins, uric acid and DNA. DAMPs are associated with many diseases like cancer, sepsis, rheumatoid arthritis, inflammatory bowel disease, stroke and atherosclerosis. But under stressful conditions, like injury or infection, they have been shown to be released by activated immune and non-immune cells and promote inflammation. These DAMPs
are present in abundance in monocytes and neutrophils and upon activation, are released at sites of inflammation. The list of DAMPs is increasing with granulysin, eosinophil-derived neurotoxin and serum amyloid A being the latest additions (107, 108). Even though it is clear that DAMPs interact with TLRs, it is still not clear whether they bind to them. However, the role of dectin-1, the primary non-toll PRR in atherosclerosis has not yet been investigated (109, 110). The aim of our study was to identify the potential endogenous ligands of dectin-1 in human atherosclerotic lesions that might have similar epitopes as zymosan and bind to anti-zymosan antibody, and thereby bind to dectin-1 and induce O$_2^-$ production.

We hypothesized that inflammation may generate potent endogenous ligands for dectin-1. Anti-zymosan antibodies were used to identify similar, cross-reactive epitopes on extracts from human atherosclerotic tissue. Immunoblot analysis revealed consistent antibody reactive protein bands on 1-and 2-DIGE. Proteins that bound to anti-zymosan antibody coupled to CnBr column were also identified. The potential ligands identified were vimentin, galectin-3 binding protein and lumican.

Gal-3BP was first characterized as a 90-kDa Mac-binding protein from the supernatant of the human breast cancer cell line. It is also called the 90 kDa tumor associated antigen. It is a secreted glycoprotein of the scavenger receptor cysteine rich superfamily (111, 112). It is highly expressed in extracellular fluids and pericellular areas of tissues (111-113). It has seven potential N-linked glycosylation sites (114). Gal-3BP can be proteolytically cleaved by an endogenous protease into a 70kDa N-terminal peptide and a 26-kDa C-terminal domain but according to published data, the N-terminal
(70 kDa) peptide is the only active peptide. Gal-3BP is also a regulator of the immune system. It has a stimulatory activity on natural killer cells and stimulates the secretion of many cytokines and interleukins in peripheral blood mononuclear cells, including tumor necrosis factor-α, IL-1, and IL-6 which play a proinflammatory role (111). Gal-3BP has been shown to have multiple functions. It is expressed in numerous cancer cells including non-small cell lung cancer, astrocytoma, and colon cancer and is a predictor of poor survival. Gal-3BP binds to multiple proteins of the extracellular matrix like collagens, fibronectin and nidogen, and to molecules mediating cell-matrix and cell-cell adhesions that are critical during tumor cell invasion and migration (115). Based on our BIACORE data, we confirmed strong binding between dectin-1 and galectin-3 binding protein. We further showed that galectin-3 binding protein induces O$_2^-$ production in human monocytes in a dectin-1-dependent fashion.

The other potential ligand was lumican. Lumican is the major keratan sulfate proteoglycan of the cornea. It is primarily distributed in interstitial collagenous matrices throughout the body. It is known to regulate collagen fibril organization and circumferential growth, corneal transparency, and epithelial cell migration and tissue repair (116, 117). Binding studies using the BIACORE did not show any binding between dectin-1 and lumican.

Vimentin was identified by mass spectrometry in the immunoreactive bands of 2-DIGE gels and was observed across different atherosclerotic tissue samples. Vimentin was also identified as one of the proteins that bound to anti-zymosan antibody by immunoaffinity purification. Direct binding of vimentin to dectin-1 was observed using
BIACORE. Further data revealed that vimentin induces $O_2^-$ production in human monocytes in a dectin-1-dependent manner. Identification of vimentin was surprising as it has been characterized as an intracellular intermediate filament protein expressed throughout the body.

Vimentin is the only cytoplasmic intermediate filament found in macrophages, lymphocytes, neutrophils, endothelial cells and fibroblasts (118). Until recently, vimentin was believed to be just an intracellular cytoskeletal protein. Though vimentin is a cytoskeletal protein, it has been detected on the cell surface and in the extracellular space (119). This strongly suggests possible surface or extracellular-associated functions. Vimentin secretion is observed in activated macrophages, activated platelets, apoptosing neutrophils, apoptosing T cells and also endothelial cells (120). Secretion of vimentin by macrophages upon activation was observed in as early as 3 hr (119). Recently, it was observed on the cell surface of monocytes and this cell surface expression was upregulated in tuberculosis infection. Vimentin-positive cells increased by 2-3 fold on tuberculosis-infected monocytes compared to uninfected monocytes after 48 hr of infection. Surface expression of vimentin observed in *Mycobacterium tuberculosis* infected monocytes binds on natural killer cells and triggers lysis of infected cells. It was also observed on the cell surface of Epstein-Barr transformed B lymphocytes and endothelial cells in the form of diffuse patches rather than filaments (120).

Vimentin knockout mice did not show phenotypic defects that have been associated with the knockout of other intermediate filament proteins. But they showed defects in fibroblasts migration impairing wound healing, decreased flow-induced dilation
of resistance arteries reflecting a role in mechanotransduction of shear stress, reduced transcellular migration of leukocytes, and lack of integrity of the vascular endothelium (121). Vimentin was also found to be essential for strengthening endothelial-matrix adhesions as siRNA-mediated knockdown of vimentin in cells fails to retain their adhesions under flow conditions. Vimentin-deficient cells also display reduced motility, mechanical stability and severely impaired ability to reorganize collag enous matrices (40). Vimentin knockout mice also showed defects in cell-cell adhesions, cell interactions and exhibited leakiness (41). Vimentin has been observed to play a key role in migration as vimentin filaments have been shown to reside in filopodia and podosomes of adherent macrophages. Constant active subunit exchange between vimentin polymer and soluble subunits has been shown to occur. Vimentin also has been shown to act as a scaffold for signaling molecules and protein kinases, thereby playing a key role in signaling pathways (52). Vimentin also has been shown to potently regulate transcription by interacting with specialized DNA structures (122).

Vimentin has been reported to be secreted by activated macrophages by a process requiring PKC-mediated phosphorylation (119). Phosphorylation of vimentin seemed to enhance its secretion. Upon activation of neutrophils using phorbol 12-myristate 13-acetate, a PKC activator, vimentin was tyrosylated and the carboxyterminal part of the protein was phosphorylated at Thr-425. It has been shown that phosphorylation of vimentin is necessary for its translocation to the plasma membrane (51). The crucial role of phosphorylation in the regulation of vimentin secretion and its functions in vascular cells is not clear. The functions of many phosphorylation motifs in
vimentin have been identified, but there is a major part of N-and C-terminal phosphorylation sites for which the functions have not yet been identified. Studies are underway to better understand the regulatory functions of vimentin by functional analysis of phosphomimetic mutations of these phosphorylation sites (121). It requires further analysis for a complete understanding by which vimentin is secreted or released. The physiological significance of released or secreted vimentin remains unknown. Secretion of vimentin was found to be up and down-regulated by pro-and anti-inflammatory conditions respectively shown in Figure 3.15. Secreted vimentin has also been shown to induce the oxidative burst (119). Anti-vimentin antibody added to mature MDM reduced the oxidative burst in these cells. Surface expression of vimentin has also been speculated to be an ‘eat me’ signal. Extracellular vimentin is also speculated to be involved in the response to pathogens. It also interacts with complement cascade favoring bacterial killing (119).
Figure 3.15 Regulation of vimentin secretion by pro- and anti-inflammatory cytokines.
Susceptibility of vimentin to interact with the phospholipid bilayer indicates a possible direct interaction with one or several regions of the protein molecule. It is of importance to note that this interaction is with the highly positively charged N-terminal polypeptide by which it associates with the endoplasmic reticulum (123). Studies have shown that cholesterol, cholesterol fatty esters and diglycerides improve the stability of the interaction between vimentin filaments and phospholipid vesicles (124). Vimentin has been shown to interact with the plasma membrane of human erythrocytes in a membrane skeletal protein, ankyrin-dependent fashion (125). It is crucial to know whether the interaction is directly with the lipid bilayer or mediated by integral and peripheral membrane proteins. The mechanism by which vimentin gets secreted is a mystery, since it does not possess a secretory signal. Some groups suggest that it might bind covalently to another protein that aids in its secretion. The carboxyl terminus of vimentin contains a di-acidic motif (Asp-X-Glu) that is used by proteins for export from the endoplasmic reticulum to the golgi complex (126).

Intermediate filaments provide cellular integrity and resistance against mechanical stress. In the recent past, it has been discovered that these filaments assemble and disassemble and are highly dynamic (127-129). The cytoskeleton is composed of a dynamic structure of numerous proteins that collectively form networks. Key functions include chemotaxis, phagocytosis and apoptosis which require rearrangement of the cytoskeleton (130). The cytoskeleton has been observed to play a key role in many autoimmune disorders. Anti-cytoskeletal autoantibodies have been found in patients suffering from various rheumatic diseases. Cytoskeletal proteins are implicated as auto-
antigens in proinflammatory conditions and the presence of autoantibodies in many autoimmune diseases support this observation (131, 132). Recent studies have shown that vimentin is citrullinated during macrophage cell death suggesting that its arginine residues are determined to citrulline residues (133). Citrullinated vimentin is found predominantly in rheumatoid arthritis (132, 134).

It is very interesting to note that vimentin was also observed on the surface of activated platelets where it was found to bind to vitronectin and plasminogen activator inhibitor complexes. Platelets are known to internalize many circulating proteins that are secreted once the platelets are activated (135). Vimentin found on the surface of apoptotic T lymphocytes binds to secreted human group IIA phospholipase A₂ (PLA₂). This interaction enhanced the activity of PLA₂ suggesting that vimentin regulates PLA₂-mediated cellular arachidonic acid release (136). A population of endothelial cells was found to express vimentin on the cell surface and to secrete it into bloodstream. The expression of surface vimentin is restricted to endothelial cell subsets that monitor entry of circulating cells from the blood into the tissues like the high endothelial venules of lymph nodes and capillaries. Secreted vimentin is shown to be important in mediating the movement of cells in the blood circulation across the endothelium (137).

Apoptotic cells are found to be important sources of autoantigens. A couple of studies have shown that injection of apoptotic cells into normal mice induced production of various antibodies especially anti-vimentin antibodies (138). Human apoptotic neutrophils also express vimentin on the cell surface (139). Anti-vimentin staining observed on the cell surface is not due to secondary necrosis as all cytoskeletal proteins
other than intermediate filaments were not detected on the cell surface of neutrophils (140). The mechanisms involved in cell surface expression of vimentin are not known yet though caspases or proteases have been known to be important. Advanced atherosclerotic lesions are characterized by a necrotic core and ligands for PRRs, such as vimentin, would be expected to be present in this area. Vimentin is an abundant autoantigen that is exposed as a result of tissue damage and apoptosis. Secreted vimentin explains the reason for the development of auto-antibodies against this intermediate filament in chronic inflammatory diseases like systemic lupus erythematosus, rheumatoid arthritis (120). Its presence in chronic inflammatory diseases indicates that it may contribute to the chronic nature of the inflammatory disorders. Also during apoptotic body formation, vimentin-positive blebs appeared at the cell surface (118). Clinical studies show that after heart/renal transplantation, patients make antibodies to tissue-specific antigens like vimentin. Auto-immune response to vimentin, anti-vimentin antibodies actively contribute to graft rejection. Studies are underway by research groups to understand the functional significance of intermediate filaments. Development of methodologies to limit exposure of vimentin may lead to better therapeutic strategies to limit tissue damage in diseases of autoimmunity and transplant rejection (120).
CHAPTER 4

SUMMARY AND CONCLUSIONS

4.1 Vimentin is a downstream target of phosphorylation of PKCβ in MCP-1-activated monocytes

Our laboratory has shown that the protein kinase, PKCβ, mediates MCP-1-induced human monocyte chemotaxis and we designed a study to detect its downstream targets of phosphorylation. 2-DIGE was performed for two groups, monocytes treated with MCP-1 in the presence or absence of PKCβ AS-ODN, followed by phospho- and total protein staining. Proteins that stained less intensely with the phospho-stain, after normalization with the total protein stain, in the PKCβ AS-ODN treated group were sequenced by mass spectrometry. Of the proteins identified, vimentin was consistently present. Vimentin was also identified on 2-D gels in similar studies using a phospho-(Ser) PKC substrate antibody.

Upon $^{32}$P metabolic labeling and vimentin immunoprecipitation (IP), increased phosphorylation of vimentin was observed in monocytes upon treatment with MCP-1 as compared to untreated monocytes. PKCβ AS-ODN reduced MCP-1-induced vimentin $^{32}$P phosphorylation compared to MCP-1 treated primary human monocytes in the presence and absence of PKCβ S-ODN. IP of monocytes with an antibody to vimentin and immunoblotting with a PKCβ antibody revealed that increased PKCβ becomes associated with vimentin upon MCP-1 activation. Reversible enzymatic phosphorylation
of vimentin is involved in reorganization of vimentin. Vimentin, a major intermediate filament protein, has been reported previously to regulate the speed of cell migration (121). In summary, vimentin is a downstream target of phosphorylation by PKCβ in MCP-1-activated primary human monocytes. This finding is featured in the model presented in Figure 4.1.

The past decade has witnessed a growing interest in our understanding of the role of chemokines in the recruitment of leukocytes to sites of inflammation. Chemokine antagonists may stabilize established atherosclerotic plaques or cause them to regress in experimental animals. This will be followed by human clinical trials if the antagonists appear effective. Given the importance of chemokines in vascular diseases and the success in developing potent chemokine therapeutics, it seems likely that this area of research will remain a focus for basic and clinical scientists for some time to come (141).
Figure 4.1 Vimentin is a downstream phosphorylation target of PKCβ in MCP-1-activated primary human monocytes.
4.2 Vimentin and galectin-3 binding protein are non-microbial endogenous ligands present in human atherosclerotic lesions that bind to dectin-1 and trigger NADPH oxidase activity to produce superoxide anion

Prior data from our lab show that the non-toll pattern recognition receptor, dectin-1, potently activates NADPH oxidase in primary human monocytes (91). Upon treatment with zymosan, a known dectin-1 ligand, NADPH oxidase is activated to produce $O_2^-$. In atherosclerotic lesions, MDM are likely the primary source of $O_2^-$ that can mediate LDL oxidation. ROS-induced oxidation of LDL plays a central role in atherogenesis as it can be taken up by macrophages in an unregulated manner. PRRs, especially TLRs have been observed to bind endogenous ligands that are released during extracellular matrix remodeling and cellular injury, in addition to exogenous ligands. These endogenous ligands are termed DAMPs as they act as ‘danger signals’ activating the innate immune system receptors. So far endogenous ligands have been discovered only for the widely studied group of PRRs, the TLRs. No endogenous ligands for dectin-1 had yet been identified, though there was speculation suggesting their presence.

We hypothesized that inflammation may generate potent endogenous ligands for dectin-1. Employing anti-zymosan antibodies to identify similar epitopes in extracts of human atherosclerotic tissue samples, we observed consistent antibody reactive bands on 1- and 2-DIGE. Vimentin was consistently identified by mass spectrometry and across different human atherosclerotic tissue samples. Immunoaffinity purification, wherein anti-zymosan antibody was coupled to an activated CnBr sepharose column, also pulled out vimentin and Gal-3BP as potential proteins that bound to anti-zymosan antibody.
Using SPR, relatively strong direct binding of both vimentin and Gal-3BP to dectin-1 was observed. Following the binding studies, O$_2^-$ assays performed using primary human monocytes showed induction of O$_2^-$ production by vimentin and Gal-3BP via the dectin-1 receptor. In summary, vimentin and Gal-3BP are non-microbial endogenous ligands present in human atherosclerotic lesions that bind to dectin-1 and trigger NADPH oxidase activity as shown in the model in Figure 4.2.

We conclude that vimentin binds to the non-toll PRR, dectin-1 and triggers NADPH oxidase activity to produce O$_2^-$ which plays a major role in inducing oxidative stress during atherogenesis. The emergence, functions and the physiological significance of secreted vimentin remain mysterious (41, 121). These findings indicate a possible need for secreted vimentin to promote innate immunity.
Figure 4.2 Vimentin and galectin-3 binding protein are endogenous ligands found in human atherosclerotic lesions that trigger NADPH oxidase activity to produce superoxide anion via the dectin-1 receptor.

Presence of these proteins in human atherosclerotic lesions may suggest their contribution to the oxidative stress and chronic nature of atherosclerosis.
4.3 Conclusions

For over half a century, immunity was believed to offer protection against foreign antigens but now it is widely believed and accepted that immunity offers protection from any kind of danger, both foreign and self (142). This explains the rationale behind autoimmune mechanisms involved in various conditions like chronic inflammatory diseases and severe allergic conditions. Higher animals respond to a diverse group of exogenous PAMPs and endogenous alarmins by employing similar immune mechanisms. Any kind of damage or stress to the living cells in the body, irrespective of the source, induces a thorough system of warning signals that aid the organism to detect, control and repair the damage caused (79). This primarily requires a robust group of innate immune recognition receptors which bind to a whole spectrum of ligands, be it exogenous microbial or endogenous stress-related molecules. Of the crucial PRRs, dectin-1, the primary non-toll receptor has gained increasing importance in the recent past.

For the first time, we conclude that vimentin is a non-microbial endogenous ligand for dectin-1. Vimentin has been observed to possess a wide array of functions like regulation of cell attachment, speed of cell migration, subcellular organization, signal transduction from the plasma membrane to the nucleus, but the key function that has been discovered recently, is its function as an endogenous stress indicator. It is an intermediate filament protein that is found inside the cells under normal physiological conditions, but upon any trauma or stress to the cell, it gets released by one or more mechanisms to the extracellular space. Phosphorylation has been observed to induce secretion of vimentin (119). We have shown that in primary human monocytes, vimentin
is a phosphorylation target of PKCβ in MCP-1-activation. Studies are underway to explore whether phosphorylation of vimentin by PKCβ in MCP-1-activated primary human monocytes induces release of vimentin. The typical feature of vimentin being an intracellular intermediate filament protein and its release, under special circumstances into the extracellular space is the principal characteristic of all alarmins that have been discovered so far. This clearly suggests that vimentin may also function as an alarmin.

Chronic inflammatory diseases like atherosclerosis, rheumatoid arthritis, systemic lupus erythematosus and many others all involve auto-immune mechanisms clearly suggesting that the damage caused is of an endogenous etiology rather than foreign. Vimentin is a major autoantigen capable of inducing anti-vimentin antibodies thereby triggering auto-immune mechanisms during tissue damage. There is a strong correlation between the severity of chronic inflammatory disorders like rheumatoid arthritis, systemic lupus erythematosus, pulmonary fibrosis and the titer of anti-vimentin antibodies (143-145). Vimentin is also a major contributor to graft rejection after transplantation as it causes significant induction of anti-vimentin antibodies against the graft tissue. Therapeutic strategies are being planned to control the release of vimentin during tissue damage to the extracellular space to control auto-immunity and graft rejection in transplantation (120).

Understanding and modulating the activities of endogenously generated danger signals (alarmins) and their receptors in a chronic inflammatory disease such as atherosclerosis may enable us to bring the inflammatory process under control. Therapeutic strategies are being developed to modulate the expression of these
endogenous danger signals to prevent and treat atherosclerosis and other diseases like sepsis, arthritis, lupus, Crohn's disease and cancer. Our studies suggest that dectin-1 blockade may help to attenuate disease pathogenesis in chronic inflammatory diseases.


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