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P120 CATENIN REGULATES INFLAMMATION IN MACROPHAGE

by

XIAOQING GUAN

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2017

MAJOR: BIOCHEMISTRY

Approved By:

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Advisor Date

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DEDICATION

To the memory of my father. His words of encouragement will forever support me. Because of him, I can follow my dream.

To my mother, my sister and the rest of my extended family for their unconditional love and endless support.

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TABLE OF CONTENTS

Dedication ii

Acknowledgments iii

List of Tables vii

List of Figures viii

List of Abbreviations ix

Chapter 1 Introduction 1

1.1 p120 catenin 1

1.1.1 Isoforms of p120 catenin 1

1.1.2 Structure of p120 catenin 3

1.1.3 Functions of p120 catenin 4

1.1.4 Phosphorylation of p120 catenin 7

1.2 LPS/TLR4 mediated inflammation in macrophage 9

1.2.1 Toll-like receptor 9

1.2.2 TLR4-mediated signaling pathways 11

1.3 Autophagy 12

1.3.1 Process of autophagy 13

1.3.2 Signaling pathways for regulation of autophagy 15

1.3.3 Selective autophagy 16

1.3.4 General functions of autophagy 18

1.3.5 Autophagy in immunity 19

1.4 p120 Catenin in inflammation 25

1.5 Hypotheses of this study 28

1.6 Specific aims of this study 28

Chapter 2 Materials and Methods 29

المنارة للاستشارات

www.manaraa.com

2.1 Antibodies and Reagents 29
2.2 Cell culture 29
2.3 p120 Catenin knockdown inTHP-1 cells 30
2.4 Western blot 30
2.5 p120 Catenin isoform identification 30
2.6 Expression and distribution of p120ctn in macrophages upon LPS stimulation 31
2.7 Analysis of pro-inflammatory cytokine expression 32
2.8 Activation of signaling pathways 32
2.9 LPS-induced autophagy 32
Chapter 3 Results 33
3.1 Establishment of macrophage cell model 33
3.2 p120 Catenin isoforms in macrophages 33
3.3 p120 Catenin expression in macrophages in response to LPS stimulation 33
3.4 Activation of MAPK and NF-κB signaling pathways in THP-1 derived macrophages after LPS treatment 34
3.5 Src signaling responsible for the degradation of p120 catenin in THP-1 derived macrophages upon LPS stimulation 35
3.6 mRNA expression of proinflammatory cytokines in p120 catenin knockdown macrophages in response to LPS stimulation 35
3.7 NF-κB signaling in p120 catenin knockdown macrophages in response to LPS challenge 36
3.8 Loss of p120 catenin decreases LPS-induced autophagy in THP-1 derived macrophages 36
3.9 Autophagy-associated protein expression in p120 catenin knockdown macrophages in response to LPS challenge 37
Chapter 4 Discussion, Conclusions and Future Directions 56
4.1 Discussion 56
4.2 Conclusions 65
4.3 Future directions 66

المنارة للاستشارات
www.manaraa.com

References ____ 67

Abstract ____ 99

Autobiographical Statement ____ 101

المنارة للاستشارات

www.manaraa.com

LIST OF TABLES

Table 2.1. Primers used to identify p120ctn isoforms ________________________________ 38

Table 2.2. Primers used to examine cytokine expression ______________________________ 39

Table 3.1. The p120 catenin isoforms expressed in various cell lines ____________________ 40

المنارة للاستشارات

www.manaraa.com

LIST OF FIGURES

Figure 1.1 Candherin/catenin complex ____ 41

Figure 1.2 Schematic representation of human p120 catenin isoforms ____ 42

Figure 1.3 Toll-like receptor 4 (TLR4) signaling ____ 43

Figure 1.4 The process of macroautophagy in mammalian cells ____ 44

Figure 1.5 Two protein conjugation systems required for autophagosome formation ____ 45

Figure 3.1 THP-1 cells were induced to differentiate into macrophages ____ 46

Figure 3.2 p120 Catenin isoform expression in THP-1 cells and THP-1 derived macrophages_ 47

Figure 3.3 p120 Catenin expression in THP-1 derived macrophages in response to LPS stimulation ____ 48

Figure 3.4 Comparison of TLR4 signaling in THP-1 derived macrophages in response to the stimulation of LPS from *E. coli* and *P. aeruginosa.* ____ 49

Figure 3.5 Signaling of tyrosine kinase Src associated with downregulation of p120 catenin in THP-1 derived macrophages after LPS (*E. coli*) stimulation. ____ 50

Figure 3.6 Identification of p120 catenin knockdown in THP-1 derived macrophages ____ 51

Figure 3.7 mRNA expression of proinflammatory cytokines in p120 catenin knockdown macrophages in response to LPS stimulation ____ 52

Figure 3.8 NF-κB signaling in p120 catenin knockdown macrophages in response to LPS (*P. aeruginosa*) stimulation ____ 53

Figure 3.9 LPS-induced autophagy in p120 catenin knockdown THP-1 derived macrophages _54

Figure 3.10 Expression of autophagy-associated proteins Atgs in p120 catenin knockdown THP-1 derived macrophages after LPS treatment ____ 55

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LIST OF ABBREVIATIONS

AIM	Atg8-interacting motif
Ambra-1	activating molecule in Beclin-1-regulated autophagy protein 1
AP-1	activator protein 1
Arm	armadillo-repeat
ATG	autophagy-related
Bcl	B-cell lymphoma/leukemia
CBD	catenin-binding domain
CK	casein kinase
DAMP	danger-associated molecular patterns
EMT	epithelial mesenchymal transition
ER	endoplasmic reticulum
Erk	extracellular signal–regulated kinase
GAP	GTPase-activating protein
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine nucleotide exchange factors
GSK	glycogen synthase kinase
Hsc	heat shock cognate protein
IFN	interferon
IKK	IκB kinase
IL	interleukin
IRAK	IL-1 receptor-associated kinase
IRF3	interferon regulatory factor-3

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IκB	NF-κB inhibitor
JMD	juxtamembrane domain
JNK	c-Jun N-terminal kinase
LAMP-2A	lysosomal-associated membrane protein 2A
LIR	LC3-interacting region
LPS	lipopolysaccharide
MAP1LC3/LC3	microtubule-associated protein 1 light chain 3
MAPK	mitogen-activated protein kinase
mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation factor 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
p120ctn	p120 catenin
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PE	phosphatidylethanolamine
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PRR	pattern-recognition receptor
PTEN	phosphatase and tensin homolog
ROCK	Rho-associated protein kinase
ROS	reactive oxygen species
SNARE	soluble N-ethylmaleimide-sensitive factor attached protein receptor
SQSTM1	Sequestosome 1

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TBK	TANK-binding kinase
TDM	THP-1 derived macrophage
TIR	interleukin-1 receptor homology domain
TIRAP	TIR-domain-containing adapter protein
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α
TRAF6	tumor necrosis factor receptor-associated factor 6
TRAM	TRIF-related adapter molecule
TRIF	TIR-domain-containing adapter inducing interferon-β
TSC	tuberous sclerosis
TNF	tumor necrosis factor
UBA	ubiquitin-associated
VEGF	vascular endothelial growth factor
Vit D3	1α, 25-dihydroxyvitamin D3

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CHAPTER 1 INTRODUCTION

1.1 p120 Catenin

p120 Catenin (p120ctn) is the prototypic member of a subfamily of armadillo-repeat domain (Arm domain) proteins involved in intercellular adhesion. p120ctn was originally identified as a substrate of Src oncoprotein [1]. cDNA cloning revealed that it contains an Arm domain which shares 22% identity with that of β-catenin [2, 3]. Further experiments demonstrated its direct interaction with multiple types of the cadherins, including E-cadherin, V-cadherin, P-cadherin and VE-cadherin [4-7]. Cadherin function is modulated by a group of catenins, including α-catenin, β-catenin, γ-catenin (plakoglobin) and p120ctn, which interact with the cadherin intracellular domain in direct or indirect manner [8]. β-Catenin binds to the catenin-binding domain (CBD) of cadherins and functionally links cadherins with the actin cytoskeleton through α-catenin [9-11], while p120ctn is responsible for stabilizing cadherin-catenin complexes at the cell surface by interacting with the juxtamembrane domain (JMD) of cadherins (Figure 1.1) [12-14]. Through the interaction with cadherin JMD, p120ctn is implicated in various processes, including supporting lateral clustering of cadherin molecules and adhesive strength [15], as well as suppression of cell motility [16]. Besides its function in maintenance of the epithelial barrier, p120ctn also plays important roles in regulation of intracellular signal transduction and gene transcription [17].

1.1.1 Isoforms of p120 catenin

Most cell types are observed expressing multiple p120ctn isoforms which are derived from alternative splicing of a single gene [4, 18]. The human p120ctn gene (CTNND1), located on chromosome 11q12.1, is consist of 21 exons which potentially encode up to 32 protein isoforms due to alternative splicing. At N-terminus, the use of four different ATGs as the translational start

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site results in the expression of p120ctn isoform 1, 2, 3 or 4 [18]. Furthermore, alternative splicing of the C-terminal end leads to use of exon A (exon 18) and/or exon B (exon 20), or neither of them. On rare occasions, p120ctn contains a sequence encoded by exon C (exon 11), which is inserted within Arm domain. Therefore, the longest isoform of human p120ctn is 1ABC which comprises 968 amino acid residues (Figure 1.2) [19]. Various combinations of these N- and C-terminal exons could generate multiple p120ctn isoforms observed as diverse bands on SDS-PAGE. The tissue type-specific expression of p120ctn isoforms was obvious, and the presence of isoforms varies in abundance among different cell types [18]. These cell type-associated expression patterns of p120ctn isoforms implies that various isoforms of p120ctn might have distinct or preferential functions [20, 21]. The functional consequence of the multitude of p120ctn variants has not been very clear. The alternative splicing usually affects only the N- and C-terminal domains, so the Arm domain is intact and free to bind to cadherins. The mesenchymal and motile cells, such as macrophages and fibroblasts, preferentially express p120ctn isoform 1A, whereas the sessile cells, such as epithelial cells, predominantly express short isoforms, such as p120ctn 3A. Non-adherent cells, including B cells and T cells do not express detectable level of p120ctn. p120ctn isoform 1 and 3 are observed most frequently among different cells. p120ctn isoform 4 contains the Arm domain without the N-terminal regulatory (phosphorylation) domain. In contrast to other isoforms, p120ctn isoform 4 mRNA has been detected, but is rarely expressed as a protein [18, 22].

The expression level and pattern of p120ctn isoforms can be changed in the cells in response to variant factors. Heterogeneous expression patterns of p120ctn isoforms in human tumor cells revealed the association between functions and p120ctn isoforms [22]. Some poorly differentiated tumor cells failed to express particular p120ctn isoforms which were typically observed in well-differentiated cell lines. Hence, the distinct expression pattern of p120ctn

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isoforms in human tumors might contribute to malignancy, which may partially result from its functions in stabilization and regulation of membrane cadherins [22]. For example, different p120ctn isoforms showed a dramatic effect on the morphology of human pancreatic tumor cells (S2-013 cells) in culture. S2-013 cells originally expressed very low level of p120ctn and showed a moderate mesenchymal morphology. Re-expression of p120ctn 1A enhanced the mesenchymal phenotype with cells exhibiting elongated shapes and increased cell motility within the monolayer. Re-expression of p120ctn 3A and 4A induced epithelial changes in cell morphology [23]. Based on those observations, cell type-dependent expression of p120ctn isoforms is correlated to the cell morphology and functions. Different isoforms may regulate cadherin complex functions by recruiting distinct binding partners or signaling molecules to the cadherin complex in response to variant stimuli. The regulated expression of multiple p120ctn isoforms might provide a mechanism for fine-tuning the activities of cadherins in different cell types.

1.1.2 Structure of p120 catenin

p120ctn consists of four distinct functional regions, including a N-terminal coiled-coil domain, a regulatory or phosphorylation domain, an Arm domain containing ten armadillo repeats and a short C-terminal tail (Figure 1.2). The coiled-coil domain is only present in the long isoform (isoform 1). N-terminal regulatory domain is present in all except the shortest isoform (isoform 4). All p120ctn isoforms contain a central Arm domain, a repeating ~46 amino acid motif composed of three α-helices, which was first characterized in the *Drosophila* segment polarity protein Armadillo Tandem [2]. Armadillo repeat units fold together as a superhelix, which provides a versatile platform for interactions with other protein partners [24]. p120ctn interacts via its central Arm domain with its partners including cadherins, RhoA, transcription factor Kaiso and microtubules [25]. The N-terminal regulatory domain is important for signal integration. It

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contains multiple tyrosines, serines and threonines which can be modified by tyrosine kinases and serine/threonine kinases, as well as phosphatases. In addition to being a substrate for the kinases and phosphatases, p120ctn can also bind to and regulate the function of non-receptor tyrosine kinases such as Fer, Fyn and Yes, and the tyrosine phosphatases PTPμ and SHP-1 [25].

1.1.3 Functions of p120 catenin

Stabilization of adherens junction by association with cadherins

Cadherins comprise a superfamily of transmembrane cell–cell adhesion molecules involved in tissue development, morphogenesis, wound healing, and tumor malignancy [26, 27]. The extracellular domains of cadherins interact in a homophilic and Ca^{2+}-dependent fashion to form adherens junctions between adjacent cells. The cytoplasmic domains interact with the catenins. C-terminal catenin binding domain interacts with α-, β- and γ-catenin, which bridge and regulate the interaction of the cadherin complex with the actin cytoskeleton [10, 28]. Cadherins bind through the juxtamembrane domain to the Arm domain of p120ctn (Figure1.1) [15, 29]. In cadherin-deficient cell model systems, p120ctn-uncoupled E-cadherin mutants failed to induce the strong cell adhesion observed in cells transfected with wild-type E-cadherin [29, 30]. Moreover, the dissociation of p120ctn from the cadherin complex resulted in endocytic internalization of cadherins [12, 31, 32]. Endocytosis of cadherins has been observed in cells during development, epithelial to mesenchymal transitions, and in response to growth factors and tumorigenic signaling agonists [33-35]. E-cadherin trafficking plays a pivotal role in E-cadherin-mediated cell–cell adhesions. Endocytosed cadherins can be either degraded in the lysosomes, or recycled to the plasma membrane via a process that involves p120ctn and Rap1 GTPase [36, 37]. p120ctn binding to the cadherin regulates cadherin membrane levels by preventing cadherin endocytosis. The structure of p120ctn in association to E-cadherin revealed that both “dynamic” and “static”

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interactions contribute to the binding of p120ctn Arm domain with the cadherin JMD. The residues of E-cadherin involved in this interaction are also implicated in clathrin-mediated endocytosis and Hakai-dependent ubiquitination of E-cadherin [14]. Thus, p120ctn binding to cadherin physically impedes the interaction of cadherin with either clathrin or E3 ligases, which results in suppression on cadherin endocytosis and degradation [38, 39]. p120ctn may also serve as a scaffold for molecules which directly regulate the functions of cadherin/catenin complex proteins. For example, p120ctn can recruit a number of kinases and phosphatases that could affect the formation and stability of adherens junctions by changing the phosphorylation status of the entire cadherin/catenin complex, including p120ctn itself [34, 40]. Thus the membrane-associated p120ctn is a master regulator which is responsible for stabilizing cadherin expression at the plasma membrane and maintaining the structure of adherens junctions [41].

Regulation of Rho GTPase signaling

Rho GTPases (such as RhoA, Rac and Cdc42) are a subgroup of the Ras superfamily of small GTP-binding proteins that are involved in regulating a wide spectrum of cellular functions. They act as intracellular switches that control multiple signaling cascades by switching between active GTP-binding and inactive GDP-binding states. Exchanges in the GTP- and GDP-state cycle are generally regulated by guanine nucleotide dissociation inhibitors (GDIs), GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). GDIs associate with GDP-bound Rho GTPases to prevent their membrane-association and thus sequester them in an inactive state. GEFs activate Rho GTPases by catalyzing nucleotide exchange from GDP to GTP. GAPs inactivate Rho GTPase by promoting GTP hydrolysis. In their active state, Rho-GTPases interact with over 60 different target effectors [42]. Rho GTPases and their effectors are mediators of cytoskeletal dynamics [43] and cadherin-mediated adhesion [44-47] by transducing extracellular

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signals to the actin cytoskeleton. They play important roles in regulating cell migration, motility and morphology [48, 49]. They also regulate gene transcription by activating MAPK and NF-κB signaling pathways [50-55].

RhoA, Rac and Cdc42 are the most well studied members of Rho GTPases. Initial experiments demonstrated that p120ctn can inhibit RhoA and activate Rac and Cdc42, resulting in disruption of stress fibers and focal adhesions, as well as promoting migration of cells [56-58]. The interaction of p120ctn with Rho GTPase extends the effects of p120ctn to additional events, including cell survival, growth, and invasiveness because of Rho GTPase-mediated signaling cascades. Strong evidence revealed that cytosol p120ctn acts mainly through its direct interaction with RhoA. The mechanism by which p120ctn inhibits RhoA was revealed by in vitro GTP exchange assays. It suggested that p120ctn could inhibit the intrinsic GDP/GTP exchange activity of RhoA in a manner comparable to that of GDI [57]. The direct interaction between p120ctn and RhoA sequesters RhoA in an inactive GDP-bound state. Two domains of p120ctn have been identified being essential for the inhibitory interaction with RhoA: a central region (including amino acids 622-628) [57, 59] and an N-terminal region (including amino acids 131-156) [60, 61]. The interaction of p120ctn with RhoA is regulated by phosphorylation of p120ctn. For example, the interaction is promoted via tyrosine phosphorylation of p120ctn at Tyr217 and Tyr228 by Src, or destabilized by phosphorylation at Tyr112 by Fyn [61].

p120ctn could activate Cdc42 and Rac1, which might result from its interaction with the guanine nucleotide exchange factor Vav2 [58]. The activation of Rac1 by p120ctn might be another mechanism involved in its inhibitory effect on RhoA, since activated Rac1could inhibit RhoA activities indirectly in some cells via reactive oxygen species (ROS), p190 RhoGAP and cadherin homophilic ligation [62-64].

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Regulation of gene transcription via interaction with Kaiso

p120ctn is also a regulator of gene transcription via its interaction with transcription suppressor Kaiso. Kaiso binds to both a specific consensus Kaiso binding site (TCCTGCNA) and methylated CpG dinucleotides within target gene promoters [65]. Kaiso mediates DNA methylation-dependent transcriptional repression through its interaction with the nuclear receptor corepressor (NCoR) [66, 67]. p120ctn is capable of nucleocytoplasmic trafficking since it has both nuclear localization signal and nuclear export signal [68, 69]. Its interaction with zinc finger domain of Kaiso interrupts Kaiso-DNA binding, followed by translocation and sequestration of Kaiso into cytosol. The promoters of some Wnt/β-catenin target genes contain the sequence-specific binding sites for Kaiso. Kaiso acts as a transcriptional repressor to antagonize β-catenin-mediated transcriptional activation [70, 71]. Hence the interaction of p120ctn with Kaiso could facilitate Wnt/β-catenin signaling. Interestingly, Wnt signaling also affects transcriptional activity of Kaiso by regulating the phosphorylation and degradation of p120ctn [72]. Like β-catenin, p120ctn could transport into nucleus to regulate gene expression. However, the signals that trigger p120ctn nucleocytoplasmic trafficking has not been very clear. The transmembrane adhesion protein Mucin-1 might be one of the triggers for p120ctn nuclear localization [73].

1.1.4 Phosphorylation of p120 catenin

p120ctn is an efficient substrate for tyrosine kinases and serine/threonine kinases [1, 74, 75]. N-terminal regulatory domain contains multiple sites identified for phosphorylation by tyrosine kinases and serine/threonine kinases. p120ctn is highly tyrosine phosphorylated in Src-transformed cells [1, 4], and in response to a group of growth factors including platelet-derived growth factor, colony-stimulating factor 1, epidermal growth factor [76, 77], hepatocyte growth factor [78, 79], and vascular endothelial growth factor (VEGF) [80]. Hence, p120ctn is a substrate

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of both non-receptor- and ligand-activated transmembrane receptor tyrosine kinases. p120ctn is not tyrosine phosphorylated in cells expressing non-myristylated alleles of Src [1, 77] or in cadherin-deficient cells when p120ctn is residing in the cytoplasm. These studies indicated that p120ctn phosphorylation requires the recruitment of the kinases to the membrane and the intact structure of cadherin-catenin complex [30].

The stability and dynamics of the adherins junction are regulated by protein tyrosine kinases and phosphatases which are able to modulate the phosphorylation state of adherins junction components such as β-catenin and p120ctn. In some cell contexts the phosphorylation is associated with loss of epithelial differentiation and increased invasiveness in human cancer cells [78]. p120ctn regulates the stability of adherens junction not only through its direct interaction with cadherins, but also via its interactions with multiple non-receptor tyrosine kinases and phosphatases. Some non-receptor protein tyrosine kinases such as Fer and Fyn are constitutively associated with p120ctn which in turn is their substrate [81, 82]. Interfering with the Fer-p120ctn interaction would result in the disruption of adherins junction complex in areas of cell-cell contacts [83]. Tyrosine phosphorylation of p120ctn increases the binding affinity of Fer or Fyn to adherens junction complex [81]. Therefore, p120ctn is not only the major substrate for kinases but also plays as a scaffolding and regulatory protein in adherens junctions by recruiting tyrosine kinases and phosphatases which can further modulate α-catenin/β-catenin and β-catenin/cadherin interactions [81, 84].

p120ctn is also found phosphorylated on Ser/Thr residues in different cell lines [85-87]. Multiple residues of serine and threonine of p120ctn have been identified as phosphorylation sites [88]. For example, Protein kinase C (PKC) can promote phosphorylation of Ser879, and

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dephosphorylation of several sits, such as Ser268, Ser288, Thr310 and Thr916, which is associated with stabilization of cell–cell adhesion [88-91].

Significant dephosphorylation of p120ctn has been observed in endothelial cells in response to inflammatory agents, such as histamine, thrombin and lysophosphatidic acid [92, 93], via PKC-dependent and PKC-independent pathways in modulating its serine/threonine phosphorylation. VEGF stimulates both tyrosine phosphorylation and serine/threonine dephosphorylation of p120ctn in endothelial cells. Histamine and activators of PKC have a similar effect to that of VEGF on phosphorylation of p120ctn. Since VEGF and histamine could increases vascular permeability, these studies suggested that the phosphorylation state of p120ctn may contribute to regulating vascular permeability via controlling cell–cell adhesion [94].

The functions of combinatorial phosphorylation of p120ctn have not been very clear. A systematic identification and subsequent mutational analysis of tyrosine and serine/threonine residues targeted by kinases and phosphatases might be possible to address the physiological role of phosphorylation of p120ctn.

1.2 LPS/TLR4 mediated inflammation in macrophage

1.2.1 Toll-like receptor

Toll-like receptors (TLRs) are human homologue of the Drosophila toll protein which has been shown to induce the innate immune response in adult Drosophila [95]. TLRs are prototype of pattern-recognition receptors (PRRs) that can detect both pathogen-associated molecular patterns (PAMPs) from microorganisms, and danger-associated molecular patterns (DAMPs) from damaged tissues [96, 97]. They belong to the type-1 transmembrane receptor family and are evolutionarily conserved proteins among vertebrates and invertebrates [95, 98]. TLRs are characterized by multiple copies of leucine-rich repeats in the extracellular domain and a

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cytoplasmic Toll/ interleukin-1 receptor homology (TIR) domain. Mammalian TLRs comprise a large family including 13 members. TLR1–9 are conserved between human and mouse. TLR10 is presumably functional in the human. TLR11-13 are functional in mouse [99]. TLRs primarily recognize invading pathogens and initiate an immediate innate immune response, followed by a long-lasting adaptive immune response. Activation of TLRs provokes a variety of immune responses, including the expression of proinflammatory cytokines such as interleukin (IL)-1 and IL-12, chemokines such as IL-8, and inflammatory effector substances such as nitric oxide (NO) [98].

TLR4 was first identified as the receptor recognizing lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria [100]. In addition to the recognition of LPS, TLR4 from various species could recognize several other components of pathogens such as mannuronic acid polymers from Gram-negative bacteria [101], teichuronic acid from Gram-positive bacteria [102], and viral components such as the F protein of respiratory syncytial virus [102, 103]. It also binds to endogenous molecules, such as heat shock proteins [104, 105], type III extra domain A of fibronectin [106, 107] and saturated fatty acids [108, 109]. Hence, both exogenous pathogens and endogenous molecules produced during abnormal situations, such as tissue damage, are able to trigger TLR4-dependent pathways [110].

TLR4 can be detected in multiple types of tissue, such as lung, colon and spleen [111]. Analyses of total RNA showed that the predominant TLR4 expressing cells are monocytes in human peripheral blood mononuclear cells (PBMCs) [112, 113]. Exposure of monocytes or macrophages to LPS or to proinflammatory cytokines increases the expression of TLR4 [111] . In addition, TLR4 expression may also be up-regulated by high concentration of glucose in human

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monocytes, which revealed one of the possible mechanism associated with high glucose-induced NF-κB activity and proinflammatory cytokine secretion [114].

1.2.2 TLR4-mediated signaling pathways

TLR4 forms a complex on the cell surface with other proteins including CD14 and myeloid differentiation factor 2 required for ligand recognition and signal transduction in high efficiency [115-117]. LPS is initially bound in the serum by LPS binding protein which transfers LPS to CD14 [118]. Upon LPS binding at the cell surface, TLR4 receptors dimerize through interactions between their intracellular TIR domains, resulting in conformational changes. The subsequent signaling process involves the recruitment of TIR-domain-containing adapter molecules to the cytoplasmic tail of TLR4 cluster via homophilic interactions between the TIR domains, which provide a platform enabling the recruitment and activation of the downstream kinases. The further propagation of signals ultimately leads to the activation of specific transcription factors, such as activator protein 1 (AP-1) and NF-κB, as well as inflammatory gene expression (Figure 1.3) [98].

Four TIR-domain-containing adapter molecules are recruited to mediate TLR4 signaling pathways: myeloid differentiation factor 88 (MyD88); TIR-domain-containing adapter protein (TIRAP); TIR-domain-containing adapter inducing interferon-β (TRIF), and TRIF-related adapter molecule (TRAM). Through these four adaptors, TLR4 initiates intracellular signaling by at least two major pathways (Figure 1.3): (1) MyD88-dependent/TIRAP–MyD88 pathway. Upon stimulation, MyD88 recruits a cytoplasmic adapter protein IL-1 receptor-associated kinase (IRAK). Activated IRAK then associates with tumor necrosis factor receptor-associated factor 6 (TRAF6), resulting in the activation of two signaling pathways. One pathway leads to activation of AP-1 transcription factors through activation of MAPK cascade. Another pathway activates IκB kinase (IKK) complex. Activated IKK complex induces phosphorylation and subsequent

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degradation of IκB, which leads to nuclear translocation of transcription factor NF-κB. MyD88-dependent pathway regulates the activation of MAPK and NF-κB signaling, and the production of related inflammatory cytokines such as IL-1 and IL-12. (2) MyD88-independent TRIF–TRAM pathway, which activates the transcription factor interferon regulatory factor-3 (IRF3) that leads to the subsequent expression of the genes encoding type I interferons (IFNs) and co-stimulatory molecules. This TRIF–TRAM pathway also increases tumor necrosis factor (TNF)-α production and secretion. The subsequent binding of secreted TNF-α to its receptors leads to NF-κB activation. Thus, the TRIF–TRAM pathway is also responsible for the late phase NF-κB activation through TNF-α signaling [99, 116, 119, 120].

1.3 Autophagy

Autophagy refers to the conserved catabolic process in which cytoplasmic components are delivered to lysosome for degradation [121, 122]. Three types of autophagy have been identified, including macroautophagy, microautophagy and chaperone-mediated autophagy. In macroautophagy, the cytoplasmic components are sequestered in the double-membrane vesicles (autophagosome), followed by fusion with the lysosome to form autolysosome where the cargo is degraded [123]. Microautophagy refers to cytosolic components being directly taken up by the lysosome through invagination of the lysosomal membrane [124]. Both macroautophagy and microautophagy are capable of engulfing large molecules and organelles through selective and non-selective mechanism. In chaperone-mediated autophagy, targeted proteins are translocated across the lysosomal membrane with chaperone proteins (such as heat shock cognate protein Hsc-70) through the lysosomal membrane receptor lysosomal-associated membrane protein 2A (LAMP-2A), resulting in unfolding and degradation of the target protein [125].

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Autophagy occurs at low basal levels in cells to maintain homeostasis [126]. It is upregulated when cells suffer nutrient starvation and growth factor withdrawal, or undergo structural remodeling during developmental transitions, or remove the damaging cytoplasmic components during oxidative stress, infection and protein aggregate accumulation [121, 127]. Macroautophagy (henceforth referred to as 'autophagy') has been profoundly studied in various research areas since it plays important roles in both health and multiple diseases [128-130].

1.3.1 Process of autophagy

Autophagy is initiated by formation of a curved membrane structure, phagophore, in the cytoplasm. The phagophore elongates to form a double-membrane structure named autophagosome by recruiting and accumulating additional proteins. A portion of the cytoplasm along with the cellular components is enclosed in the autophagosome which is further fused with lysosome to form an autolysosome. The enclosed cargo is then degraded via proteolysis. In order to study the molecular mechanism, the process of autophagy is divided into five stages described as initiation, elongation, maturation, fusion and degradation (Figure 1.4) [131]. The process of autophagy is mediated and regulated by several molecular complexes which are composed of proteins encoded by AuTophaGy-related (ATG) genes [132]. More than 30 products of these genes have been originally characterized in yeast, and many orthologs have subsequently been identified as autophagy-related proteins in higher eukaryotes [133, 134].

Initiation and elongation

During the initiation stage, the autophagic membrane forms a phagophore, and then elongates. Several different cellular organelles have been considered as the autophagic membrane source, including the endoplasmic reticulum (ER), Golgi, mitochondria and plasma membranes [135-138]. It's suggested that SNARE (soluble N-ethylmaleimide-sensitive factor attached protein

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receptor) proteins mediate homotypic fusion of phagophore precursors, which contributes to recruitment of proteins that enable subsequent maturation of autophagosome [139]. The initiation and elongation stages are mediated by two major steps of protein processing and conjugation (Figure 1.5). The mechanisms of both conjugation systems resemble protein ubiquitination with corresponding enzymes to the E1 and E2 in ubiquitin ligation. Atg12 is conjugated to Atg5 by Atg7 (an E1-like protein) and Atg10 (an E2-like protein). Atg5 interacts further with Atg16L1 to form Atg12–Atg5-Atg16L1 complex (also called the Atg16L1 complex), a multimeric complex through the homo-oligomerization of Atg16L1. The Atg12–Atg5–Atg16L1 complex is recruited to the membrane to function as an E3-like ligase to mediate the lipidation of microtubule-associated protein 1 light chain 3 (MAP1LC3/LC3, an ortholog of yeast Atg8) in the process assisted by Atg7 (an E1-like protein) and Atg3 (an E2-like protein). A pro-LC3 is first cleaved at C-terminus by Atg4 to generate LC3-I, followed by conjugation to the lipid phosphatidylethanolamine (PE) to form LC3-II (the lipid-conjugated form). LC3-II is anchored into the membrane of autophagosome to mediate the expansion and fusion of the isolated autophagic membrane [140, 141]. Furthermore, LC3 molecules form a platform on the autophagic membrane to influence autophagosome formation by mediating assembly and disassembly of protein complexes at the membrane. [142, 143].

Maturation, fusion and degradation

The autophagosome may fuse with a late endosome to form an amphisome [144], or fuse directly with the lysosome to generate an autolysosome in mammals [145]. In yeast, the autophagosome fuses with the vacuole and then releases the inner vesicle termed an autophagic body [146]. The inner vesicle of the autophagosome, together with the sequestered cargoes, is degraded, followed by the release of the breakdown products into the cytosol for reuse [147, 148].

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Before the closure of the membrane of an autophagosome, the Atg proteins bound to the membrane dissociate from autophagosome. LC3-II is present on both sides of the phagophore and the early autophagosome. It is released from the outer membrane by Atg4-dependent deconjugation during autophagosome maturation [142]. Since LC3-II is found on the inner and outer surfaces of the autophagosome and remains bound to the membrane even after lysosomal fusion, LC3-II is widely used as a marker for identifying autophagosomes in cells [133].

1.3.2 Signaling pathways for regulation of autophagy

Several protein complexes containing Atg proteins are involved in the signaling responsible for initiation and elongation stages of autophagy. Different reasons for autophagy are mediated through various signaling pathways. One of the regulators is mammalian target of rapamycin (mTOR). mTOR is the major negative regulator that shuts off autophagy when the growth factors and nutrients are abundant [130]. The activity of mTOR depends on a variety of upstream signals that include the energy and nutrient status of the cells. In nutrient-rich conditions, mTOR interacts with the ULK1 kinase complex (ULK1-Atg13-FIP200 complex) and phosphorylates ULK1 and Atg13 to suppress autophagosome formation. Upon starvation, the ULK1 kinase complex is activated due to the inhibition of mTOR activity. Activated ULK1 complex leads to the activation of Beclin-1/Vps34 complex which consists of Vps34 (the class III PI3 kinase) and Beclin-1 (a mammalian ortholog of yeast Atg6). The Beclin-1/Vps34 complex can also be activated by other proteins that interact with Beclin-1, including UV radiation resistance protein and Ambra-1 (activating molecule in Beclin-1-regulated autophagy protein 1) [149]. Once being activated, Beclin-1/Vps34 complex can further activate the Atg proteins, and phosphorylate phosphatidylinositol to generate phosphatidylinositol 3-phosphate which is an essential lipid component of the autophagosome [150-153]. The activity of Beclin-1 is inhibited by its interaction

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with B-cell lymphoma/leukemia (Bcl)-2 or Bcl-XL. This interaction can be disrupted by phosphorylation of Bcl-2 and/or Beclin-1, or ubiquitination of Beclin-1. For example, under the condition of nutrient starvation, the stress-activated JNK1 phosphorylates Bcl-2, which induces the dissociation of Bcl-2 from Beclin-1. Upon stimulation of TLR4, TRAF6 and the deubiquitinating enzyme A20 can also control the initiation of autophagy through regulating lysine (K) 63-linked ubiquitination of Beclin-1 [154]. Thus dissociated Beclin-1 can interact with other members of the autophagic machinery and induce autophagy [155]. Autophagy can be induced by some drugs such as rapamycin which inhibits mTOR activity [156].

LC3 is another regulating point of autophagy. Phosphorylation of LC3 by protein kinase A inhibits autophagy by suppressing recruitment of LC3 molecules to autophagosome [157]. It was also observed that both rapamycin and pathological inducers of autophagy can cause dephosphorylation of endogenous LC3, which further proved that phosphorylation of LC3 might be an important regulatory mechanism during autophagy [157].

1.3.3 Selective autophagy

The balance between the biosynthesis and the degradation of cellular components is of importance for normal cellular homeostasis and health [158]. Autophagy has been thought as an essential non-selective pathway for bulk degradation induced by nutrient deprivation [159, 160] . However, accumulating evidence has shown selective autophagy contributes to intracellular homeostasis in non-starved cells by selective degradation of components including aberrant protein aggregates, lipid droplets, dysfunctional and superfluous organelles and intracellular pathogens [161-164]. In selective autophagy, the cargo has to be specifically recognized and effectively tethered to a nascent autophagosome. Selectivity in autophagy is achieved by cargo receptor proteins, which are able to tether the selected cargo to a nascent autophagosome by

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simultaneous interaction with both the cargo and LC3-family proteins on the isolation membrane of autophagosome [161]. The post-translational modifications such as protein ubiquitination, have an important role in ensuring substrate recognition and selectivity [165]. The recognition of ubiquitinated substrates is achieved by molecular adaptors including p62, NBR1, NDP52, VCP and optineurin, which target ubiquitinated cargo to autophagy-associated proteins, such as the LC3 family members [166, 167].

A variety of cargo receptors are responsible for recognition of different cargoes. For example, in mammalian cells, nuclear receptor coactivator 4 (NCOA4) is the cargo receptor for ferritin during ferritinophagy [168]. Ferritin forms complex to sequester excessive intracellular iron [169]. Under conditions of low intracellular iron, NCOA4 receptor binds to ferritin heavy chains and deliver the ferritin complexes into the lysosomal compartment via selective autophagy [168, 170]. Degradation of ferritin in the lysosomes ultimately leads to the release of iron back into the cytosol [171].

Another well-studied cargo receptor p62, also known as sequestosome 1 (SQSTM1) mediates the degradation of ubiquitinated molecules such as aggregated proteins or cytosolic bacteria [172, 173]. The cargo receptor p62 binds to ubiquitin through its ubiquitin-associated (UBA) domain with a relatively low affinity. The affinity of p62 for polyubiquitin chain is increased by phosphorylation of serine 403 within its UBA domain [174]. The N-terminal Phox/Bem 1 (PB1) domain of p62 mediates its homo-oligomerization. Oligomerization of p62 stabilizes binding to the cargo material on which ubiquitin is concentrated [175]. Different ubiquitin chains trigger different cellular degradation processes. The preferential interaction of p62 with certain types of ubiquitin chain might contribute to selectivity regulation during selective autophagy. K48-linked ubiquitin chains are recognized by the proteasome and therefore mediate

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proteasomal degradation of the substrates. K63-linked ubiquitin chains are associated with selective autophagy [176, 177]. It has been shown that oligomeric p62 preferentially binds to K63-linked ubiquitin chains, as well as mono-ubiquitin [178].

The selected cargoes target to a nascent autophagosome via the interaction between LC3-family proteins and cargo receptors through their conserved LC3-interacting region (LIR) motif, also known as Atg8-interacting motif (AIM). The consensus for the core LIR motif is (W/F/Y)XX(L/I/V), where X is any other residue. This sequence is surrounded by at least one acidic residue. Structural studies have revealed that the hydrophobic residues of the core LIR motif dock into two hydrophobic pockets in the ubiquitin-like domain of LC3, whereas adjacent acidic residues form electrostatic interactions with the N-terminal arm of LC3 molecules. The core LIR motif adopts an extended β-conformation and forms an intermolecular parallel β-sheet with the β2 strand of LC3 [179].

1.3.4 General functions of autophagy

Autophagy has been found associated with a broad spectrum of biological processes including aging, development, degenerative diseases, and cancer [130, 180]. Autophagy is important in mammals for development and differentiation such as preimplantation [181], survival during neonatal starvation [159], as well as cell differentiation during erythropoiesis [182], lymphocyte differentiation [183, 184], and adipogenesis [185].

Autophagy is also associated with the pathogenesis of various diseases. Tissue-specific knockout studies in mice indicate autophagy plays a critical role in intracellular protein and organelle quality control, which is associated with the pathogenesis of several genetic diseases including liver disease, neurodegenerative diseases, and myodegenerative diseases. They may be caused due to either the failure of autophagosomes to fuse with lysosomes or the aggregation of

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misfolded proteins that exceed the autophagic clearance capacity [186-188]. Autophagy is also found associated with tumorigenesis. The expression of Beclin-1 has been found decreased in human breast, ovarian, and brain tumors [189, 190]. Gene transfer of Beclin-1 can inhibit the growth of tumor cell lines and the tumorigenicity of human breast carcinoma cells in mouse xenograft models [191, 192]. Thus, Beclin-1 might be a candidate of tumor suppressor, which is the first specific link between the autophagy and human cancer. Moreover, several tumor suppressors involved in the inhibition of mTOR signaling, such as phosphatase and tensin homolog (PTEN), tuberous sclerosis (TSC) 1, and TSC2, could stimulate autophagy [193]. Conversely, oncogene products such as class I PI3K and Akt could inhibit autophagy through activating mTOR. Tumor suppressor p53 regulates autophagy under genotoxic and other stresses [194].

1.3.5 Autophagy in immunity

The functions of autophagy in immunity have been profoundly studied recently [195]. Autophagy not only maintains cellular homeostasis under conditions of endogenous distress, but also plays a primordial role in controlling intracellular pathogens in different species from unicellular organisms to human [196]. Autophagy represents one of the most primitive examples of innate immune responses. In mammals, the functions of autophagy have evolved to incorporate into multiple pathways of innate and adaptive immunity, including direct elimination of microorganisms [197, 198], as well as regulating inflammation [199], MHC II-restricted endogenous antigen presentation [200, 201], lymphocyte homeostasis [202], the secretion of immune mediators [203], and central tolerance [184]. The functions of autophagy in regulating immunity has been also proved by the evidence that autophagy contributes to chronic inflammatory diseases [204-206].

Direct elimination of microorganisms

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The antimicrobial functions of autophagy provide barriers against invading microorganisms. It includes xenophagy which is the selective uptake and degradation of intracellular microorganisms via autophagy [207], and LC3-associated phagocytosis which involves the engagement of the autophagic machinery as the bacterium is confined in the phagosome [208, 209]. Some invading microorganisms or the damaged host membranes associated with the pathogen express some molecular tags, such as ubiquitin, galectin and membrane phospholipid modifications. Those tags can be recognized by autophagy receptors which recruit the autophagic machinery and initiate autophagy [166, 210-212]. Autophagy-associated proteins have been shown to directly target microbial proteins, such as Atg5 which can target surface protein VirG of bacteria Shigella and promote selective autophagy of bacteria [213].

Regulation of innate immunity

Many signals which can initiate immune response also induce autophagy, such as PAMPs [214-216], DAMPs (including ATP, ROS and misfolded proteins) [217], JNK [218], and IKK [219]. Pathogen can initiate autophagy at multiple stages [220]. Autophagy can be initiated during adhesion and uptake of bacteria by the host cells, or during phagocytosis of bacteria by macrophages [209, 214, 221]. After bacterial uptake, autophagy is induced by pathogen-inflicted cell damage or the intracellular bacteria [211-213, 222, 223]. The initiation of autophagy after virus infection occurs at different stages of the virus life cycle [224-228]. The proinflammatory cytokines, including IL-1, IL-2, IL-6, TNF-α, transforming growth factor-β and IFN-γ, can induce autophagy; whereas TH2 cell-associated cytokines, such as IL-4, IL-10, and IL-13, can inhibit autophagy [229, 230]. IL-1β could induce autophagy leading to autophagic elimination of Mycobacterium tuberculosis in macrophages, which was dependent on TANK-binding kinase 1 (TBK-1) [231]. TBK1 is necessary for autophagic maturation and autophagic machinery

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assembly. It can phosphorylate p62 on Ser403, which is essential for p62 in autophagic clearance. IL-1β activates NF-κB signal pathway through TRAF6 [232] which is also involved in the activation of autophagy via the subsequent TRAF6-dependent K63-linked ubiquitination of Beclin-1 [154].

In response to infection, both innate and adaptive immune system are activated to control infection. At the same time some specific responses that limit detrimental immune activation and inflammation are also initiated. Research on autophagy reveals autophagy proteins function in both the activation and inactivation of innate immune signaling, such as type I IFN signaling and inflammasome activation [220].

The autophagy pathway can activate the production of type I IFN in plasmacytoid dendritic cells by delivering viral nucleic acids to endosomal TLRs [227]. Single-stranded RNA viruses are recognized by the RIG-I-like receptors (RLRs) in the cytosol. RLRs signaling results in the production of antiviral cytokine type I IFNs. Atg5-deficient cells exhibited enhanced RLR signaling and increased IFN secretion. In the absence of autophagy, accumulated dysfunctional mitochondria, as well as dysfunctional mitochondria-associated ROS were mainly responsible for the enhanced RLR signaling [233]. Double-stranded DNA (dsDNA) can also induce potent innate immune responses via the induction of type I IFN and IFN-inducible genes. Upon sensing dsDNA, stimulator of IFN genes (STING) moves from ER to the Golgi apparatus and finally to the cytoplasmic to assemble with TBK1. The translocation and assembly of the signal transducers, STING and TBK1, are required for dsDNA-triggered innate immune responses. However, STING also co-localizes with the autophagy protein LC3 and Atg9a after dsDNA stimulation. The loss of Atg9a greatly enhances the assembly of STING to TBK1 by dsDNA, resulting in aberrant

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activation of the innate immune response. Thus autophagy could limit dsDNA-induced innate immune responses by regulating the assembly of STING and TBK1 [234].

The inflammasomes are multiprotein complexes activated by infection or stress. Inflammasomes in immune cells are fundamental in the innate immunity against microbes. They are composed of nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) proteins and functions as signaling platforms for the activation of caspase-1 and subsequent maturation of the proinflammatory cytokines IL-1β and IL-18 [235]. The aberrant activation of inflammation is involved in the pathogenesis of autoimmune and inflammatory diseases , as well as metabolic disorders [236]. Several studies have revealed a link between autophagy and inflammasome activation, and the potential roles in disease progression [237-239]. For example, Atg16L1-deficient mice suffer severe colitis induced by dextran sulfate sodium, which can be alleviated by neutralization of IL-18 and IL-1β. This research indicated that the severe colitis probably resulted from the enhanced inflammasome activity resulted from defective autophagy [199]. Activation of inflammasomes in macrophage could lead to autophagosome formation via the activation of small G protein RalB, a direct trigger of autophagosome formation. Activated autophagy is able to recruit p62 and then target ubiquitinated inflammasomes for degradation via autophagy [240]. Hence, autophagy and inflammasome activation are mutual regulated.

In addition to directly regulating inflammatory signaling, the autophagy pathway might prevent tissue inflammation through its role in apoptotic corpse clearance. The efficient clearance of apoptotic cells prevents secondary necrosis and excess inflammation triggered by released danger signals from dead cells. Mice lacking Atg5 display a defect in apoptotic corpse engulfment during embryonic development. Autophagy contributes to the clearance of dead cells by a mechanism that involves the generation of ATP-dependent engulfment signals [241]. Involution

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is the process of post-lactational mammary gland regression which involves the death of secretory epithelial cells and stroma remodeling. It was revealed that Beclin-1- or Atg7-deficient mammary epithelial cells were defective in engulfment of apoptotic bodies, which resulted in higher levels of involution-associated inflammation in Atg-deficient tissues. Beclin-1 or Atg7 knockdown could compromise apoptotic body clearance. One possible mechanism is associated with decreased activation of GTPase Rac1 which mediates actin reorganization and phagocytic cup formation for effective dead cell engulfment [242].

Autophagy in adaptive immunity

Autophagy is also a cell biological pathway affecting adaptive immune responses. Studies revealed that dendritic cells utilize autophagy machinery for optimal phagosome-to-lysosome fusion and subsequent processing of antigen for MHC class II loading [243]. Another study indicates that autophagy-dependent antigen presentation is defective in dendritic cells with Crohn's disease-associated Atg16L1 risk variants [221].

In addition to its role in antigen presentation, autophagy affects both the homeostasis and the functions of T cells. Autophagy and clearance of mitochondria are required for normal haematopoietic stem cell maintenance and function [244]. After exiting the thymus, naive T cells depend on autophagy and mitochondrial content reduction for their maturation [183]. Autophagy also has a role in the selection of naive T cell repertoires in the thymus. Thymic epithelial cells had a high level of constitutive autophagy. Atg5 deficiency in thymic epithelial cells led to altered selection of MHC-II-restricted T-cell specificities and resulted in severe colitis and multi-organ inflammation. These effects have been attributed to the defective presentation of cytoplasmic antigens by autophagy-deficient thymic epithelial cells [184]. Autophagy is also involved in the NF-κB activation in antigen-activated T cells. The adaptor protein Bcl-10 is a mediator of T cell

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receptor (TCR)/NF-κB signaling. TCR activation promotes K63 polyubiquitination of Bcl-10, followed by Bcl-10 association with the autophagy adaptor p62. p62 binding was required for both Bcl-10 signaling to NF-κB and gradual degradation of Bcl-10 by autophagy. The blockade of Bcl-10 degradation via autophagy can enhance TCR activation of NF-κB. Hence, selective autophagy is involved in the degradation of Bcl-10, which can reduce NF-κB activation by TCR in antigen-activated T cells [245].

Autophagy is required for plasma cell homeostasis and long-lived humoral immunity. Mice with conditional deficiency of Atg5 in B cells showed plasma cells had a larger ER and more ER stress signaling which led to more antibody secretion and fewer antigen-specific long-lived bone marrow plasma cells. The enhanced antibody synthesis was associated with lower level of intracellular ATP and more death of mutant plasma cells. Autophagy reduces immunoglobulin secretion from plasma cells, which seems to be a mechanism preventing excessive antibody production [246].

Autophagy and inflammatory disease

Perturbations in autophagy-dependent functions in immunity contribute not only to increased susceptibility to infection, but also to chronic inflammatory diseases and autoimmune diseases. The well-characterized link between autophagy and inflammatory disease is the variations in autophagy proteins in association with Crohn's disease, a chronic inflammatory disorder of the intestine [247]. Other emerging associations include the autoimmune diseases such as systemic lupus erythematosus (SLE) [248] , and inflammation-associated metabolic disorders such as obesity and diabetes [249, 250].

Genome-wide association studies identified that Crohn's disease susceptibility genes, such as Atg16L1, are involved in autophagy [247]. Antigen presentation has been found defective in

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dendritic cells from patients with the Crohn's disease-associated Atg16L1 risk variant [221]. Studies using mouse model with Atg16L1 mutation further proved that defective autophagy is relevant to pathogenesis of Crohn's disease. Loss of Atg16l1 function in mice results in enhanced proinflammatory cytokine production by macrophages induced by TLR agonists [199], enhanced chemical-induced colitis [199, 251] and altered inflammatory gene transcriptional profiles in Paneth cells [205, 251].

Genome-wide association studies have also linked several single nucleotide polymorphisms in Atg5 to SLE susceptibility [248, 252, 253]. SLE is characterized by autoimmune responses against self-antigens. Autophagy protein Atg5 is critical for T-cell selection which is responsible for the generation of a self-tolerant T-cell repertoire in thymus. Atg5-depleted thymic epithelial cells are defective in negative thymic selection, resulting in generation of autoimmunity and multi-organ inflammation in mice [184]. Other Atg5-dependent effects, including regulation of IFN and proinflammatory cytokine secretion [233, 254], clearance of dying cells [254] and dendritic cell antigen presentation [243], might also be relevant to the autoimmunity and inflammation associated with SLE.

Defects in autophagy may contribute to some metabolic diseases, such as diabetes and obesity, which are linked to inflammation and insulin resistance [255]. Hepatic suppression of Atg7 in mice results in increased ER stress and insulin resistance [249]. p62 deficient mice develop mature-onset obesity and insulin resistance [250]. Thus, the defective autophagy-dependent regulation of ER stress, as well as immune cell homeostasis and activation may contribute to inflammatory responses that underlie the pathogenesis of metabolic diseases [196].

1.4 p120 Catenin in inflammation

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The evidence that p120ctn might be involved in the regulation of inflammation was first observed in p120ctn knockout animal models. Conditional knockout of p120ctn in mice results in a chronic inflammatory response in intestines [256, 257] and epidermis [258, 259], which is characterized by tissue infiltration of immune cells including lymphocytes, mast cells, granulocytes, and macrophages, as well as expression of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, macrophage inflammatory protein 3 α (MIP3α), and macrophage chemotactic protein 1 (MCP1), even in the absence of exogenous inflammatory stimuli. In human endothelial cells, depletion of p120ctn increased transcription factor activities (including NF-κB, AP-1, and Kaiso), adhesion molecules expression, neutrophil adhesion, and Erk1/2 signaling [260]. Overexpression of p120ctn in human umbilical vein and dermal microvascular endothelial cells inhibited neutrophil transendothelial migration [261]. p120ctn-depleted epidermal cells have elevated activities of NF-κB, which triggers a cascade of proinflammatory responses both in vivo and in vitro. The activation of RhoA/Rho-associated protein kinase (ROCK) pathway resulting from the loss of p120ctn might be partially responsible for NF-κB over-activation and proinflammatory cytokine production [259]. There is also a significant correlation between p120ctn loss and increased incidence of inflammatory bowel disease in both human patients and mouse models [257, 262]. Multiple researches demonstrated that p120ctn depletion could cause a chronic inflammatory response which might be mediated by the mechanism that does not require some specific exogenous stimuli under some circumstances.

Immune functions of p120 catenin in LPS/TLR4-dependent inflammatory response

p120ctn knockdown alone in mouse lung endothelial cells did not significantly affect NF-κB activation but enhanced LPS-induced NF-κB activation, which suggests an important role of endothelial p120ctn in the regulation of LPS/TLR4-dependent NF-κB signaling. In LPS-induced

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lung injury mouse model, p120ctn was rapidly degraded in response to LPS challenge. Moreover, the level of p120ctn was correlated inversely with the severity of lung inflammation. Hence, endothelial p120ctn degradation during endotoxemia might be one of the reasons for the amplification of pulmonary inflammation. Depletion of p120ctn in pulmonary endothelial cells could increase the sensitivity to LPS and induced inflammatory response characterized by increased production of proinflammatory cytokines (such as TNF-α and IL-6), elevated neutrophil infiltration into the lung, hyperpermeability of pulmonary vasculature. In contrast, overexpression of p120ctn could suppress those LPS-induced effects [263]. Hyperpermeability of pulmonary vasculature induced by LPS might be associated with LPS-induced tyrosine phosphorylation of VE-cadherin, γ-catenin, and p120ctn through Src family tyrosine kinases [264]. Therefore, endothelial p120ctn may be a negative regulator of sepsis-induced lung inflammatory injury.

p120ctn can also regulate LPS/TLR4 signaling by facilitating TLR4 endocytic trafficking in mouse bone marrow-derived macrophages. p120ctn silencing could diminish LPS-induced TLR4 internalization. Genetic and pharmacological inhibition of RhoA could rescue the decrease in endocytosis of TLR4 and TLR4-MyD88 signaling in p120ctn null cells. This might be another reason for LPS-induced over-inflammatory response in p120ctn-depleted cells [265].

p120 Catenin regulating inflammation via RhoA/ROCK-dependent signaling

RhoA/ROCK signaling associates with the activation of IKK/NF-κB [266, 267]. Since cytosol p120ctn can interact with RhoA and inhibit its activation, RhoA/ROCK pathway would be one of the mechanism to explain how p120ctn regulates NF-κB-mediated inflammatory response. In mouse p120ctn-depleted epidermis, chronic inflammation and increased NF-κB activity were observed even without specific stimuli [259]. Re-expression of wild-type p120ctn, overexpression of dominant-negative RhoA or inhibition of ROCK reduced NF-κB activity. In contrast,

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expression of a mutant form of p120ctn (Δ622–628) which is unable to bind to RhoA failed to suppress NF-κB activity. Expression of a cadherin-uncoupled p120ctn mutant in keratinocytes still could reduce NF-κB activity, suggesting that cadherin binding is not required for the ability of p120ctn to inhibit NF-κB activity in human epidermis [259].

1.5 Hypotheses of this study

p120 Catenin suppresses LPS-induced inflammatory response via its inhibitory effects on NF-κB pathway activation in THP-1 derived macrophages. Decreased autophagy is associated with abnormal proinflammatory reactions in p120 catenin-depleted macrophages in response to LPS stimulation.

1.6 Specific aims of this study:

1. To examine the expression of p120 catenin in THP-1 derived macrophages in response to LPS challenge.
2. To study the functions of p120 catenin in NF-κB signaling in THP-1 derived macrophages in response to LPS challenge.
3. To investigate the effects of p120 catenin depletion on LPS-induced autophagy in THP-1 derived macrophages.

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CHAPTER 2 MATERIALS AND METHODS

2.1 Antibodies and reagents

Anti-human p120 catenin (G7) (targeting N-terminus) and Na^+/K^+ ATPase antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Antibodies including anti-phosphorylated and total Erk, p38, JNK, Akt, Src, IKK, and NF-κB p65 were from Cell Signaling Tecnology (Danvers, MA, USA). Anti-IκBα, LC3, Atg3, Atg7, Atg12, Atg16L1 and lamin B1 antibodies were purchased from Cell Signaling Tecnology (Danvers, MA, USA). Anti-p62 antibody was acquired from Abcam (Cambridge, MA, USA). Anti-GAPDH antibody, M-PER protein extraction reagent and subcellular protein fractionation kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA). RPMI 1640 media, HEPES and fetal bovine serum (FBS) were obtained from Thermo Scientific HyClone (Logan, UT, USA). LPS (*Escherichia coli*, O111:B4, and *Pseudomonas aeruginosa* 10), phorbol 12-myristate 13-acetate (PMA) and MG132, goat serum were acquired from Sigma-Aldrich (St. Louis, MO. USA). Bafilomycin A1 was acquired from Cell Signaling Tecnology (Danvers, MA, USA). PP2 was purchased from Cayman (Ann Arbor, MI, USA). Lentivirus p120ctn shRNA and control shRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PCR primers for cytokines and p120ctn isoforms were synthesized by Sigma-Aldrich (St. Louis, MO. USA). ReliaPrep™ RNA Cell Miniprep System was from Promega (Madison, WI, USA). ProtoScript II reverse transcriptase, murine RNase inhibitor, dNTP mix and TopTaq DNA polymerase were from New England Biolabs (Ipswich, MA, USA). Laemmli sample buffer and Clarity Western ECL reagents were purchased from Bio-Rad Life Science (Hercules, CA, USA).

2.2 Cell culture

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Human monocytic THP-1 cells were obtained from the ATCC (TIB-202™). THP-1 cells were cultured in RPMI 1640 medium containing 10% of FBS, 10 mM HEPES, 50 μM ß-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. THP-1 monocytes were differentiated into THP-1 derived macrophages (TDMs) by culturing with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) in RPMI 1640 medium containing 10% of FBS and 10 mM HEPES for 3 days. TDMs were then treated with other reagents after cultured in RPMI 1640 medium without PMA overnight. Lung cancer cell lines A549 and Calu-3 cells were from ATCC. A549 cells were cultured in RPMI 1640 medium containing 10% of FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Calu-3 cells were cultured in DMEM/F12 medium containing 10% of FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3 p120 Catenin knockdown inTHP-1 cells

Lentivirus p120ctn shRNA and control shRNA were used to knock down p120ctn expression in THP-1 cells. Stable p120ctn knockdown THP-1 cell line was selected with 1 μg/ml puromycin for 3 days. p120ctn knockdown THP-1 cells were further differentiated to macrophages with PMA. The expression of p120ctn was tested via PCR and Western blot.

2.4 Western blot

Culture cells were lysed with M-PER protein extraction reagent after washed with cold PBS twice. The total proteins were resolved by SDS-PAGE, followed by transfer to polyvinylidene fluoride membranes. The membranes were then blocked with 1% goat serum, followed by overnight incubation with antibodies anti-target proteins at 4°C and then with goat anti-mouse/rabbit IgG at room temperature for 1 hour. The protein bands were visualized by using Clarity Western ECL reagents.

2.5 p120 Catenin isoform identification

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p120ctn isoforms were identified via reverse transcription PCR (RT-PCR) and Western blot. Total RNA was isolated from the cells using ReliaPrep™ RNA Cell Miniprep System, in accordance with the manufacturer's recommendations. The total RNA was then synthesized to cDNA by reverse-transcription. PCR was used to identify the isoforms of p120ctn expressed in THP-1 and THP-1 derived macrophages by using specific primers targeting different mRNA variants. The primers and the PCR conditions were described previously (Table 2.1) [268]. GAPDH was used as internal control. The products were analyzed on a 1-2% agarose gel and compared to a 100 bp DNA ladder. The protein expression was analyzed using Western blot. Epithelial cells A549 and Calu3 were used as control.

2.6 Expression and distribution of p120ctn in macrophages upon LPS stimulation.

TDMs were stimulated with LPS (both *E. coli* and *P. aeruginosa*) in RPMI 1640 medium containing 5% of FBS and 10 mM HEPES for indicated time points. Total protein was extracted with M-PER protein extraction reagent. Subcellular proteins, including membrane, cytosolic and nuclear protein, were prepared by using Subcellular protein fractionation kit. Western Blot was then used to test the total expression and subcellular localization of p120ctn in macrophages upon LPS stimulation for indicated time intervals. Relative protein levels were compared in reference to internal controls including Na^{+}/K^{+} ATPase, GAPDH, and lamin B1. The macrophages were pretreated with Src inhibitor PP2 (10 µM) for 30 minutes, followed by stimulation with 1 µg/ml of LPS (*E. coli* O111:B4) for 2 hours. The total p120ctn expression was tested via immunoblot. To test the pathways responsible for p120ctn degradation, the macrophages were stimulated with 1 µg/ml of LPS (*E. coli* O111:B4) for 30 min, followed by treatment with or without bafilomycin A1 (1 µM) or MG132 (10 µM) for 2 hours (LPS was not removed). The total p120ctn expression was tested via immunoblot.

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2.7 Analysis of proinflammatory cytokine expression

p120ctn knockdown and control macrophages were stimulated by 100 ng/ml of LPS in RPMI1640 medium containing 5% FBS for indicated time. Proinflammatory cytokines (IL-1β, IL-8, IL-6 and TNF-α) were examined by RT-PCR. PCR primers and conditions were described in Table 2.2.

2.8 Activation of signaling pathways

Macrophages were cultured in RPMI 1640 medium containing 1% FBS overnight, followed by stimulation with LPS for indicated time. Activation of signaling pathways, including NF-κB, MAPK, Src and Akt pathways, was investigated by immunoblot.

2.9 LPS-induced autophagy

THP-1 derived macrophages were stimulated with 100 ng/ml of LPS (*E. coli*, O111:B4) in RPMI1640 medium containing 5% FBS for indicated time. LC3, P62 and Atgs were tested by Western blot. In order to test autophagy flux, the macrophages were stimulated with 100 ng/ml of LPS (*E. coli* O111:B4) for 8 hours, followed by treatment with or without bafilomycin A1 (1 μM) for 1 hour. LC3 and p62 were tested by Western blot.

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CHAPTER 3 RESULTS

3.1 Establishment of macrophage cell model.

THP-1 cells, a human monocytic cell line, were induced to differentiate into THP-1 derived macrophages after exposure to PMA for 3 days. During the differentiation, suspension THP-1 monocytes adhered to culture plates accompanied by differentiating into a macrophage phenotype: flat, spreading and amoeboid in shape (Figure 3.1A). Prior to examing p120 catenin functions, THP-1 derived macrophages were stimulated with 1 µg/ml of LPS (*P. aeruginos*a 10) for 12 and 24 hours. mRNA expression of proinflammatory cytokines including IL-1β, IL-8 and TNF-α was tested via RT-PCR. mRNA level of IL-1β, IL-8 and TNF-α was significantly increased in macrophages upon LPS stimulation (Figure 3.1B).

3.2 p120 Catenin isoforms in macrophages

p120ctn has multiple isoforms due to alternative splicing. In order to identify the isoforms expressed in THP-1 monocytes and mcrophages, RT-PCR was used to analyze the mRNA expression of p120ctn isoforms, using specific primers targeting different mRNA variants of isoforms. Lung cancer cell lines A549 and Calu-3 were used as control. In combination with the protein bands analyzed via Western blot, THP-1, THP-1 derived macrophages, and A549 expressd isoform 1A and 3A, whereas Calu-3 expressed isoform 3A and 3AB. The protein levels of two isoforms were similar in macrophages (Figure 3.2 and Table 3.1).

3.3 p120 Catenin expression in macrophages in response to LPS stimulation.

The expression of p120 catenin in THP-1 derived macrophages was determined by Western blot analysis following stimulation of LPS. p120ctn protein level was reduced in a time-dependent manner following *E. coli* LPS challenge, whereas p120ctn expression in macrophages was not changed significantly after *P. aeruginosa* LPS stimulation (Figure 3.3A). p120ctn decreased to the

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lowest level within 4-hour treatment of LPS from *E. coli* and then gradually recovered within 24 hours after LPS exposure (1 μg/ml). The effects of LPS (*E. coli*) on p120ctn mRNA expression was examined by RT-PCR and no significant difference was observed (Fig. 3.3B). These results suggest that the rapid loss of p120ctn after LPS (E. coli) challenge might be due to the enhanced degradation of p120ctn protein. Next, the proteasome inhibitor MG132 and autophagy inhibitor bafilomycin A1 were used to identify the pathways for degradation of p120ctn protein in macrophages following LPS challenge. The loss of p120ctn protein following LPS treatment was not suppressed in macrophages after inhibition of proteasomal pathway, whereas p120ctn degradation after LPS stimulation was inhibited by autophagy inhibitor bafilomycin A1. Thus these results indicate that the autophagic pathway might be the major mechanism responsible for p120ctn protein degradation in macrophages following *E. coli* LPS challenge (Fig. 3.3C). The total protein of p120ctn was not significantly changed in macrophages after stimulation with *P. aeruginosa* LPS. Therefore, the subcellular distribution of p120ctn protein was analyzed and no significant change was observed in macrophages after *P. aeruginosa* LPS treatment (Figure 3.3D).

3.4 Activation of MAPK and NF-κB signaling pathways in THP-1 derived macrophages after LPS treatment.

Since LPS from *E. coli* and *P. aeruginosa* could induce different effects on p120ctn protein expression in macrophage, the signaling pathways were compared in macrophages upon both LPS (1 μg/ml) stimulation. Both LPS could activate MAPK pathways including Erk, p38 and JNK, and no significant difference was observed in the activation pattern of these signaling pathways in macrophages after both LPS stimulation (Figure 3.4, A and B). The basal level of activated Akt was very high and the activation of Akt was not very strong in THP-1 derived macrophages following stimulation of LPS from both *E. coli* and *P. aeruginosa* (Figure3.4, A and B). The

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activation of NF-κB signaling pathway was also examined in macrophages upon LPS stimulation. As the Figure 3.4, C and D showed, the time-dependent pattern of IKKγ activation and IκBα degradation was similar in macrophages after treatment with LPS from both *E. coli* and *P. aeruginosa.*

3.5 Src signaling responsible for the degradation of p120 catenin in THP-1 derived macrophages upon LPS stimulation

There are two major phosphorylation sites on Src: Tyr416 and Tyr527. Tyr416 phosphorylation activates Src by allowing the substrate to gain access to the catalytic domain. Phosphorylation of Tyr527 inactivates Src through changing Src conformation into a closed inaccessible bundle [269]. Upon *E. coli* LPS stimulation, Src was phosphorylated at Tyr416 (Figure 3.5B), whereas the phosphorylation of Src Tyr416 was not observed in macrophage after *P. aeruginosa* LPS challenge (Figure 3.5A). The phosphorylated state of Src at Tyr527 was not changed in macrophages after the stimulation by LPS from both *E.coli* and *P. aeruginosa*. These data indicated that Src signaling might be responsible for protein degradation of p120ctn in macrophages upon LPS stimulation. In order to test this hypothesis, PP2, an inhibitor for Src-family kinases, was used to pretreat the macrophages, followed by stimulation of *E. coli* LPS. As Figure 3.5C displayed, PP2 could partially inhibit the downregulation of p120ctn in response to LPS challenge.

3.6 mRNA expression of proinflammatory cytokines in p120 catenin knockdown macrophages in response to LPS stimulation.

To study the effects of p120ctn ablation on inflammation in macrophages, p120ctn knockdown THP-1 cell line was generated by transduction of cells with p120ctn-specific short hairpin RNA (shRNA). Random shRNA was used as control. p120ctn knockdown in THP-1 cell

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line was verified via RT-PCR and Western blot. PCR and Western blot analyses revealed a dramatic reduction of both mRNA and protein levels of p120ctn compared with shRNA control cells (Figure 3.6). As expected, p120ctn loss led to an increase in mRNA levels of proinflammatory cytokines in macrophages upon LPS treatment, especially within 8 hours after LPS stimulation. The effects of p120ctn ablation on mRNA expressions of IL-1β, IL-8 and TNF-α were similar in macrophages stimulated by LPS from both *E. coli* and *P. aeruginosa*. p120ctn loss resulted in an upregulation of IL-6 expression in macrophages after *E. coli* LPS stimulation. However, *P. aeruginosa* LPS did not induce significant expression of IL-6 mRNA in THP-1 derived macrophages despite the loss of p120ctn (Figure 3.7).

3.7 NF-κB signaling in p120 catenin knockdown macrophages in response to LPS challenge

NF-κB is the master transcription factor for expression of proinflammatory cytokines in cells in response to LPS stimulation. Upon stimulation of LPS, IKKγ was phosphorylated and activated more rapidly in p120ctn knockdown macrophages (within 10 minutes), as compared with shRNA control cells. However, the loss of p120ctn did not increase the period of IKKγ activation. (Figure 3.8A). No significant differences were observed in IκBα degradation within 30 minutes after LPS stimulation in p120ctn knockdown macrophages as compared with control cells. (Figure 3.8A). The nuclear translocation of NF-κB p65 was not significantly changed by the loss of p120ctn in macrophages upon LPS stimulation. The phosphorylation of nuclear NF-κB p65 at Ser536 in p120ctn knockdown macrophages seemed augmented within one hour after LPS treatment (Figure 3.8, B and C).

3.8 Loss of p120 catenin decreases LPS-induced autophagy in THP-1 derived macrophages

Western blot analysis of p62 and LC3 was used to monitor the levels of autophagy. When autophagy is induced, LC3-I is conjugated to PE to form LC3-II, which is recruited to the

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phagophore. p62 delivers ubiquitinated proteins to the autophagosome and itself is degraded in the lysosome. LPS (*E. coli*) could induce autophagy in both p120ctn knockdown and shRNA control macrophages (Figure 3.9A). The decreased basal level of p62 was observed in p120ctn knockdown macrophages as compared with shRNA control macrophages without LPS treatment. Upon stimulation of LPS, p62 was decreased in control macrophages for a short period (within 60 minutes), followed by upregulated expression. In p120ctn knockdown macrophages, LPS induced time-dependent upregulation in p62 protein expression, and degradation of p62 was not significant (Figure 3.9B). In order to compare the difference of the autophagy flux in p120ctn knockdown and shRNA control macrophages, an inhibitor bafilomycin A1 was used to block the autophagy. Bafilomycin A1 blocks maturation of autolysosome by inhibiting fusion of autophagosomes with lysosomes [270]. Macrophages was stimulated by LPS for 8 hours followed by treatment of bafilomycin A1 for another 1 hour. The decreased level of LC3-I and LC3-II was observed in p120ctn knockdown cells compared with shRNA control macrophages (Figure 3.9C). The change of p62 protein level was not observed in both p120ctn knockdown and control cells in response to inhibition of autophagy (Figure 3.9D).

3.9 Autophagy-associated protein expression in p120 catenin knockdown macrophages in response to LPS challenge

The process of LC3-I to LC3-II is regulated by several autophagy-associated proteins. The macrophages were stimulated by LPS for indicated time and Atg protein level was tested by Western blot. The protein level of Atg3, Atg7 and Atg16L1was not changed in both p120ctn knockdown and control macrophages upon LPS challenge (Figure 3.10, A, B and C). Conjugated Atg12-Atg5 was gradually downregulated 8 hours after LPS stimulation (Figure 3.10 D).

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Table 2.1. Primers used to identify p120ctn isoforms [268]

Primers	Sequence 5'→3'	Tm (°C)	Fragment size (bp)
Total p120ctn:	CAGCATGAGCGAGGAAGTTT ATGGCGTGGCTTACAGTCTT	55	540
Isoforms 1, 2, 3:	TGCCCTGCTGGATTTGTCTT CGAGTGGTCCCATCATCTG	56	705 (1), 477 (2), 225/297/319/369 (3)
Isoforms A, B:	AAACCTGATCGGGAAGAAAT ACTGGCAAAAAGAACTAATC	52	470 (A), 557 (B)
Isoform C:	TTGCCTTCTTCGGAACTTAT CACTGTATCGTTTGCTGGAT	52	193
GAPDH	ATCTTCCAGGAGCGAGATC ACCACTGACACGTTGGCAGT	58	503

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Table 2.2. Primers used to examine cytokine expression

Primers	Sequence 5'→3'	Tm (℃)	Fragment size (bp)
IL-1β	ACGCTCCGGGACTCACAGCAA TTGAGGCCCAAGGCCACAGGTATT	58	164
IL-8	CGTGGCTCTCTTGGCAGCCTTC TTTCTGTGTTGGCGCAGTGTGGTC	58	179
IL-6	CAATGAGGAGACTTGCCTGGTG GGTTGGGTCAGGGGTGGTTA	52	193
TNF-α	GCTGCACTTTGGAGTGATCG GCTTGAGGGTTTGCTACAACA	52	144
GAPDH	ATCTTCCAGGAGCGAGATC ACCACTGACACGTTGGCAGT	58	503

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Table 3.1. The p120 catenin isoforms expressed in various cell lines

Cell line	Isoforms	Length (aa)	MW (kD)
THP-1	1A, 3A	933, 832	104, 93
THP-1 derived macrophage	1A, 3A	933, 832	104, 93
A549	1A, 3A	933, 832	104, 93
Calu3	3A, 3AB	832, 861	93, 96

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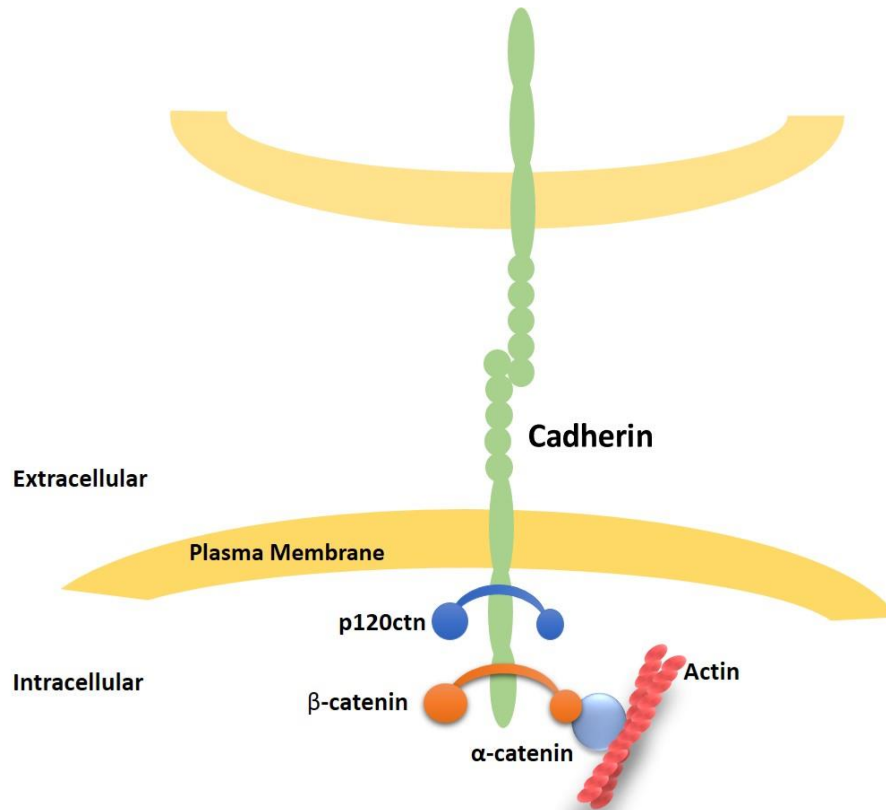

Figure 1.1. Candherin/catenin complex. The extracellular domain of cadherin interacts in a homophilic fashion to form adherens junction between adjacent cells. Cadherin function is modulated by a group of catenins. β-Catenin binds to the catenin-binding domain of cadherin and functionally links cadherin with the actin cytoskeleton through α-catenin, while p120ctn is responsible for stabilizing cadherin-catenin complexes at the cell surface by interacting with the juxtamembrane domain of cadherin.

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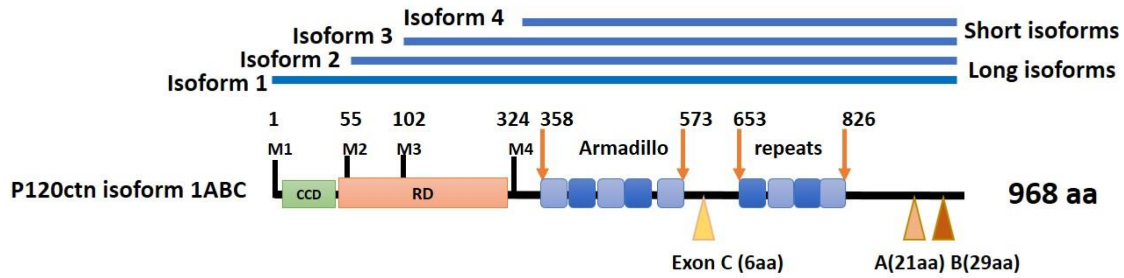

Figure 1.2. Schematic representation of human p120 catenin isoforms. Multiple isoforms of p120ctn result from the use of alternative start codons (M) and alternative splicing exons A (880-900, 21aa), B (937-965, 29aa) and C (626-631, 6aa). Sizes are indicated in amino acid residues (aa). Arm 6: 583-624aa. M: Methionine start codons. CCD: coiled coil domain (10-46, 37aa). RD: regulatory domain. This figure was sketched based on the information from Uniprot (UniProtKB - O60716 CTND1_HUMAN).

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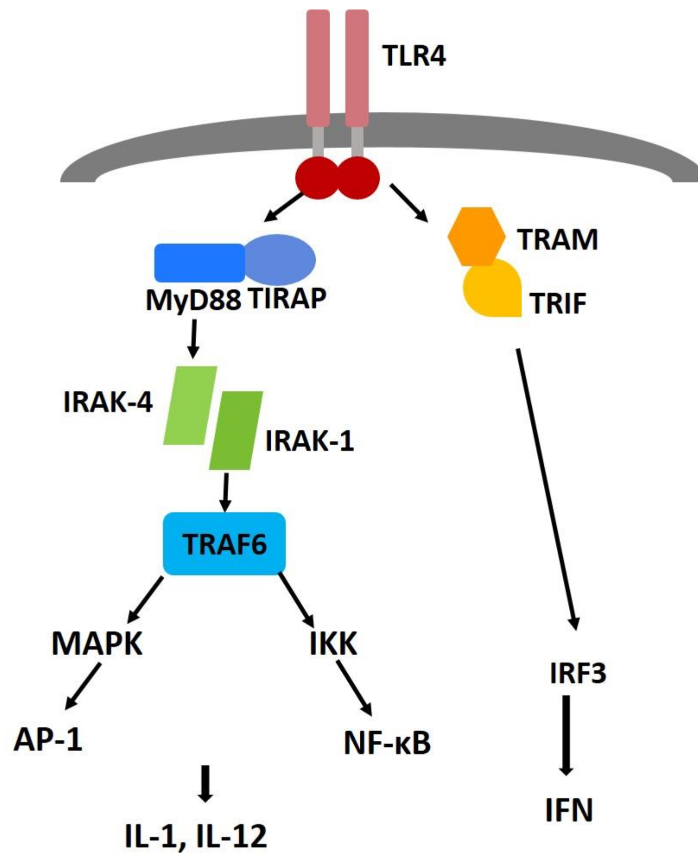

Figure 1.3. Toll-like receptor 4 (TLR4) signaling. TLR4 initiates intracellular signaling by at least two major pathways: (1) MyD88-dependent/TIRAP–MyD88 pathway.TLR4 signals through the key adaptors MyD88/TIRAP to recruit downstream kinase IRAK. Activated IRAK then associates with TRAF6, resulting in the activation of two signaling pathways. One pathway leads to activation of transcription factor AP-1 through MAPK activation cascade. Another pathway activates IKK complex, leading to nuclear translocation of transcription factor NF-κB. MyD88-depedent pathway regulates the production of inflammatory cytokines, such as IL-1 and IL-12. (2) MyD88-independent TRIF–TRAM pathway, which activates IRF3 transcription factor, leading to the subsequent expression of the type I IFN.

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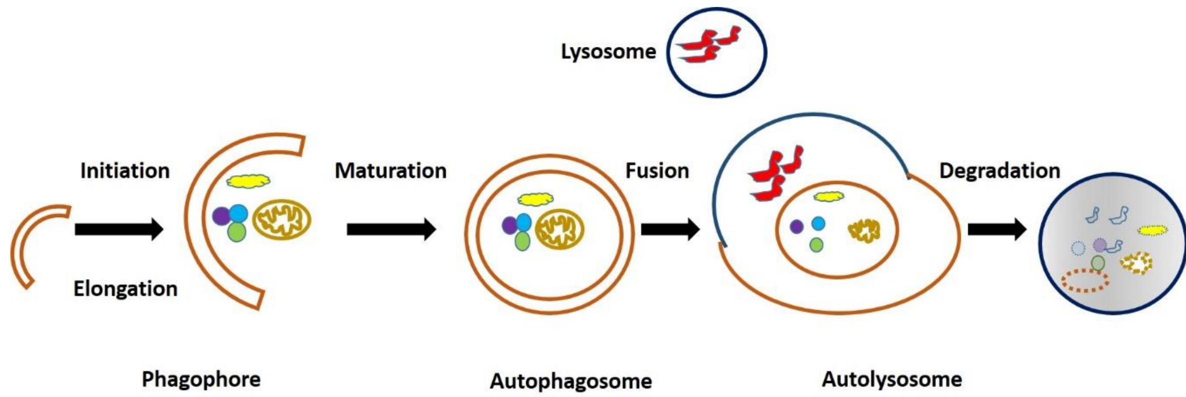

Figure 1.4. The process of macroautophagy in mammalian cells. A portion of cytoplasm is enclosed by an isolation membrane (phagophore) to form an autophagosome. The outer membrane of the autophagosome subsequently fuses with the lysosome, and the internal material is degraded. The nomenclature for various autophagic structures and stages of autophagy are indicated.

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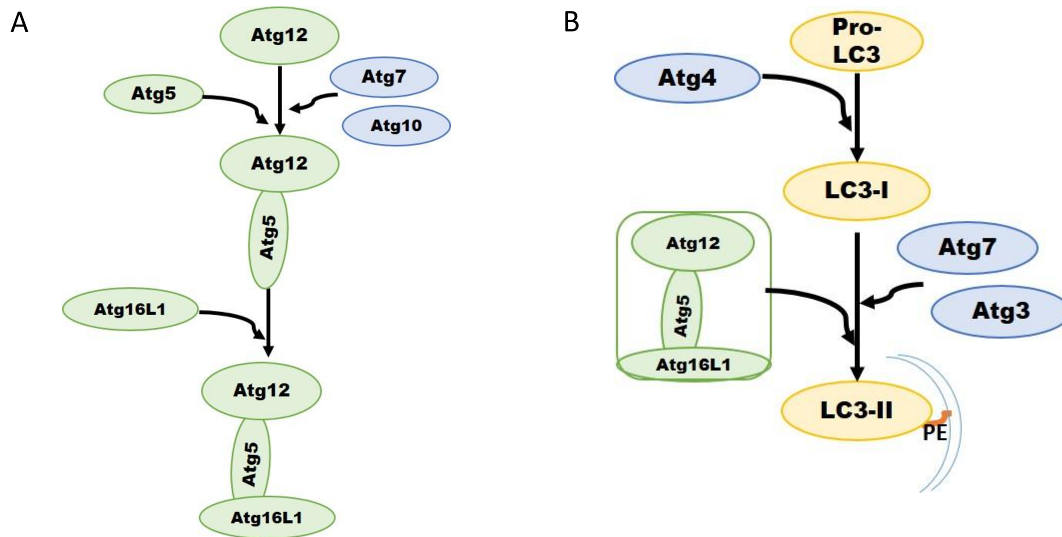

Figure 1.5. Two protein conjugation systems required for autophagosome formation. Atg12 is covalently conjugated to Atg5 in a process assisted by the enzymes Atg7 and Atg10. Atg12–Atg5 conjugates form a complex with Atg16L1 (Atg12-Atg5-Atg16L1 complex). Pro-LC3 is first cleaved by Atg4 at the carboxyl terminus to expose a glycine residue. Then LC3-I is ligated to the lipid phosphatidylethanolamine (PE) to form LC3-II in the process assisted by Atg7, Atg3 and Atg12-Atg5-Atg16L1 complex.

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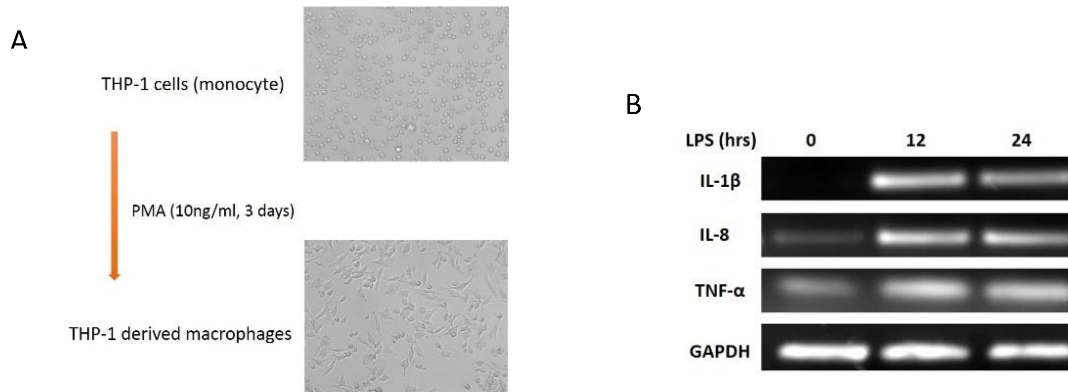

Figure 3.1. THP-1 cells were induced to differentiate into macrophages (THP-1 derived macrophages). (A). The human monocytic cell line THP-1 cells were differentiated into THP-1 derived macrophages by culturing with PMA (10 ng/ml) for 3 days. The morphology of monocytic cell THP-1 and macrophages was analysed by microscope. (B). THP-1 derived macrophages were stimulated with LPS from *Pseudomonus aeruginosa* 10 (1 μg/ml) for 12 hours and 24 hours. The mRNA expression of pro-inflammatory cytokines including IL-1α, IL-8 and TNF-α was tested via reverse transcription PCR.

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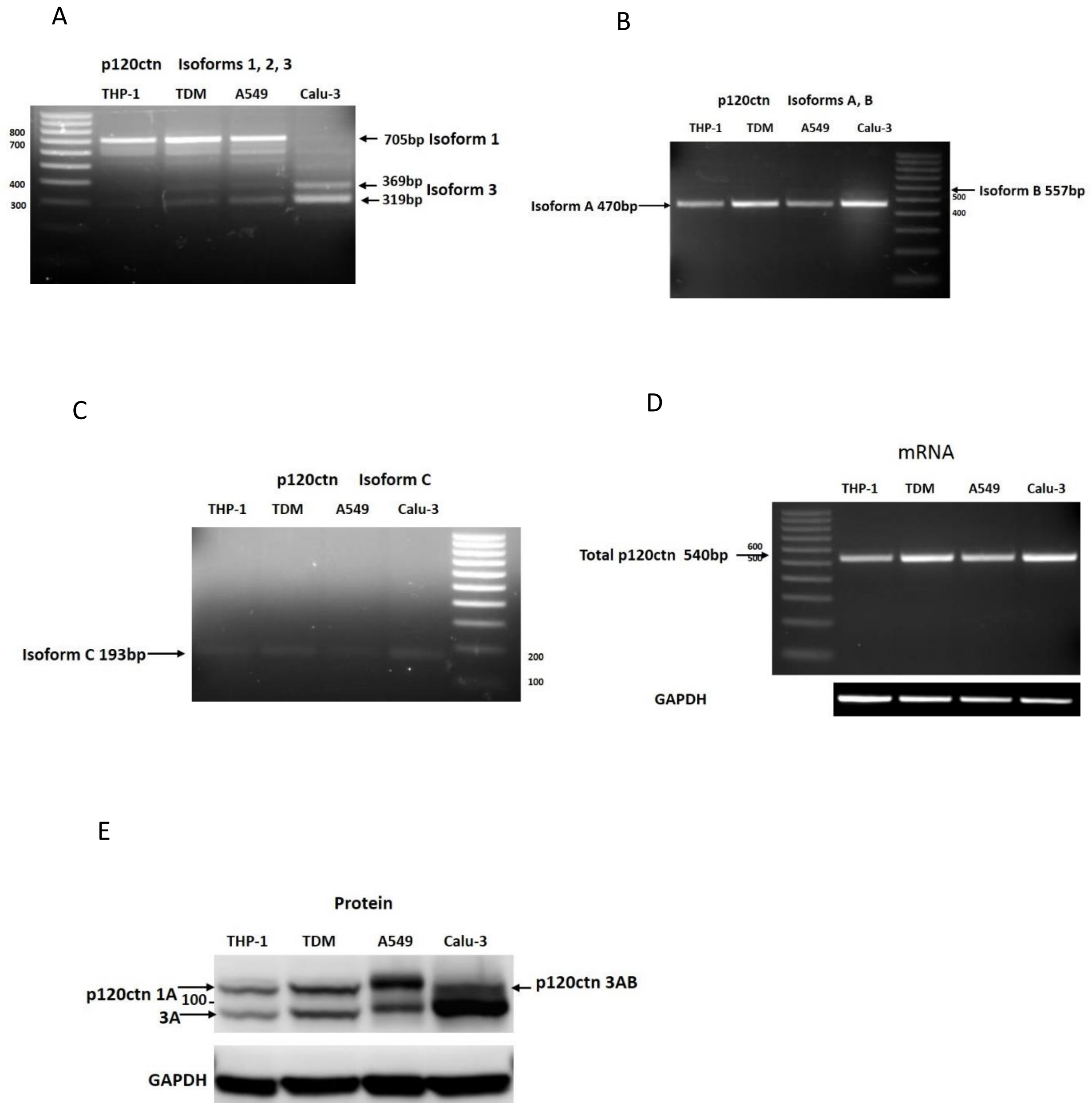

Figure 3.2. p120 Catenin isoform expression in THP-1 cells and THP-1 derived macrophages. mRNA expression of p120ctn isoforms 1, 2 and 3 (A), isoforms A and B (B), as well as isoforms C (C) was analyzed by RT-PCR in THP-1 cells, THP-1 derived macrophages (TDM), and lung cancer cell lines including A549 and Calu-3. The total mRNA expression (D) and protein expression (E) of p120ctn were tested in those four cell lines. The isoform codes and their fragment sizes are depicted.

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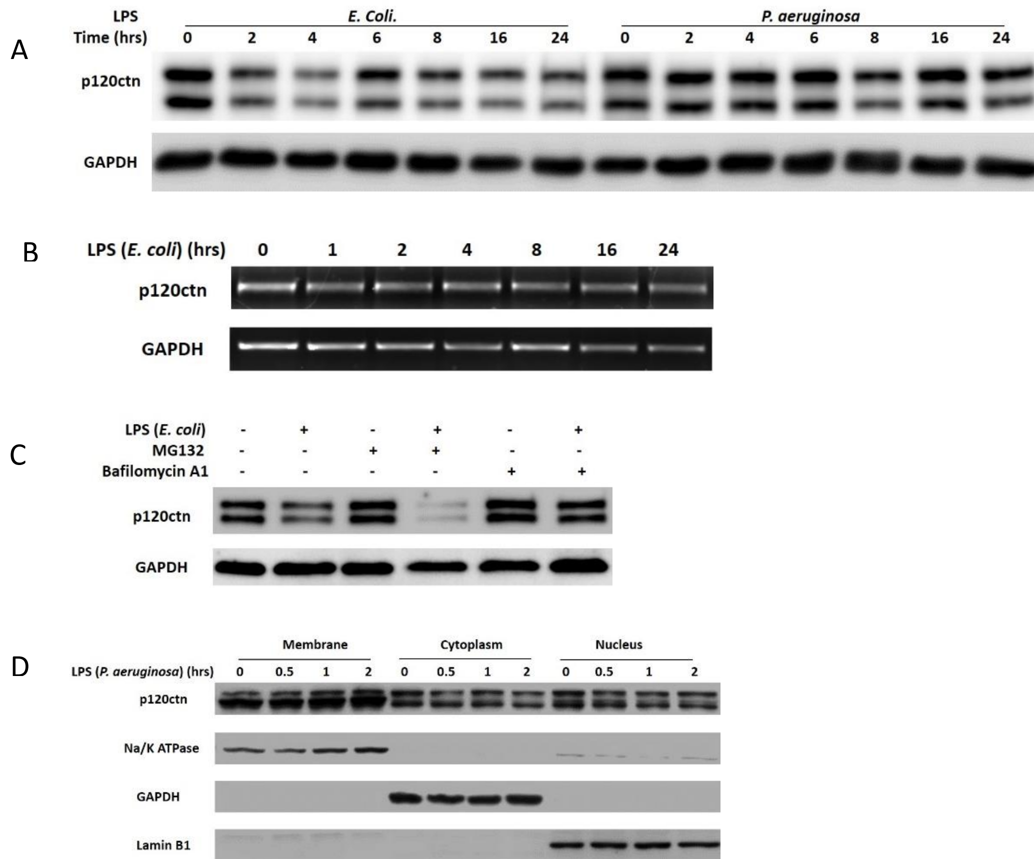

Figure 3.3. p120 Catenin expression in THP-1 derived macrophages in response to LPS stimulation. (A). THP-1 derived macrophages were stimulated with 1 µg/ml of LPS from *E. coli* and *P. aeruginosa* for indicated time intervals. Total expression of p120ctn was analyzed via Western blot. (B). mRNA expression of p120ctn was tested via RT-PCR in macrophages after treatment with 1 µg/ml of LPS (*E. coli*). (C). p120ctn degradation was inhibited by autophagy inhibitor bafilomycin A1 in macrophages in response to the stimulation of 1 µg/ml of LPS from *E. coli*. (D). Subcellular distribution of p120ctn was analyzed in THP-1 derived macrophages after stimulation with 1 µg/ml of LPS from *P. aeruginosa*.

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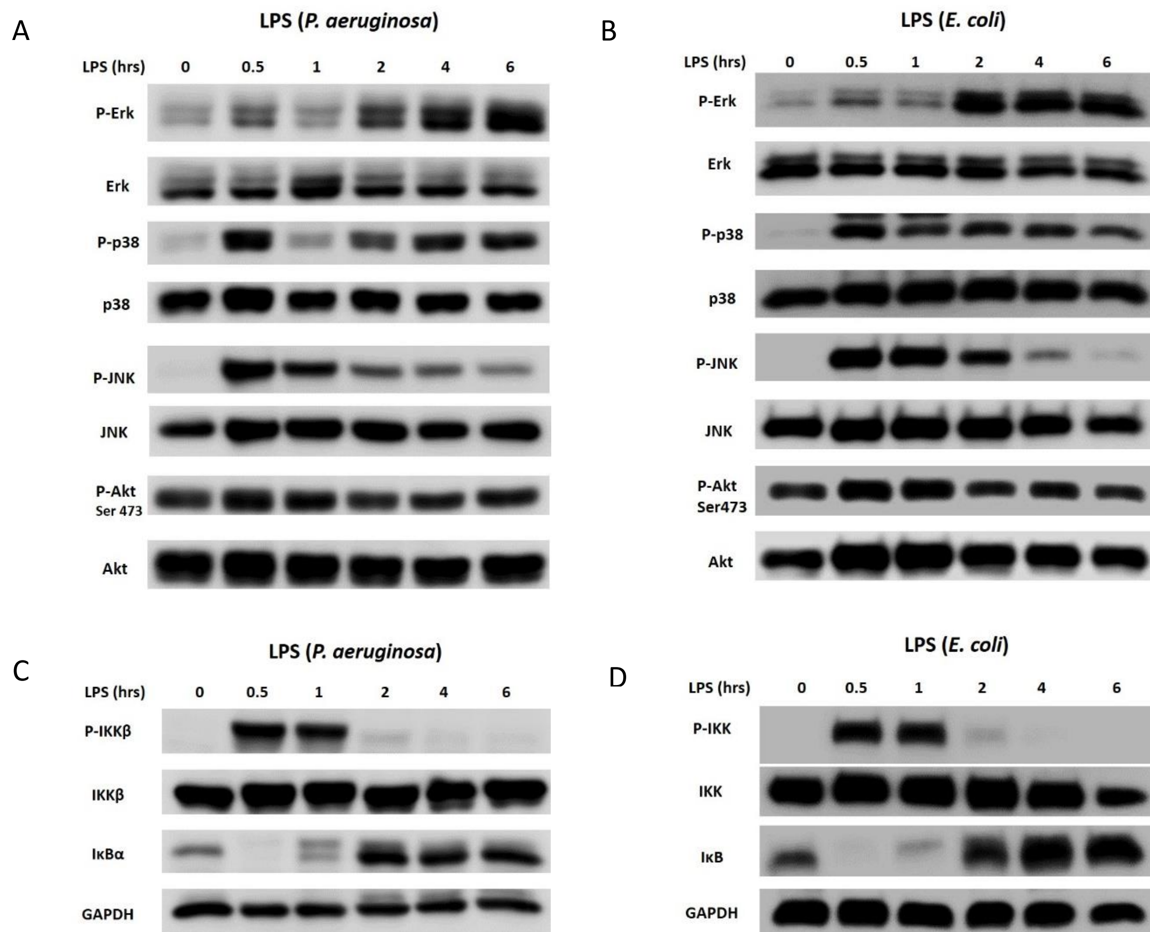

Fig 3.4. Comparison of TLR4 signaling in THP-1 derived macrophages in response to the stimulation of LPS from *E. coli* and *P. aeruginosa*. Western blot analysis of MAPK and Akt signaling in macrophages upon stimulation of LPS (1 µg/ml) from *P. aeruginosa* (A) and *E. coli* (B). NF-κB signaling was compared in macrophages after stimulation with *P. aeruginosa* LPS (C) and *E. coli* LPS (D)

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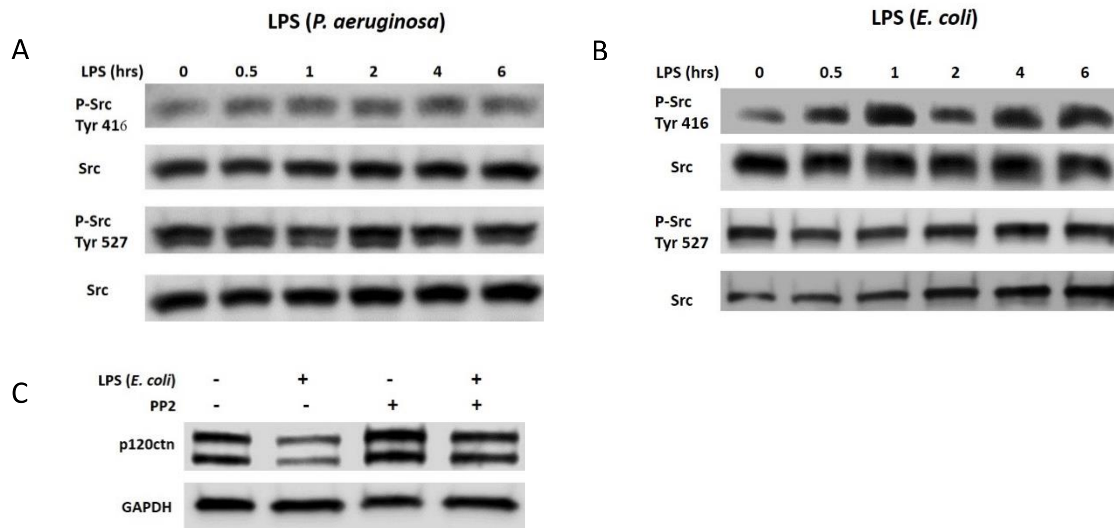

Fig 3.5. Signaling of tyrosine kinase Src associated with downregulation of p120 catenin in THP-1 derived macrophages after LPS (*E. coli*) stimulation. Src activation was analyzed in macrophages after treatment with *P. aeruginosa* LPS (A) and *E. coli* LPS (B). (C). Macrophages were pretreated with Src inhibitor PP2 (10 μM) for 30 minutes, followed by stimulation of 1 μg/ml of LPS (*E. coli*) for 2 hours. p120ctn expression level was examined by Western blot.

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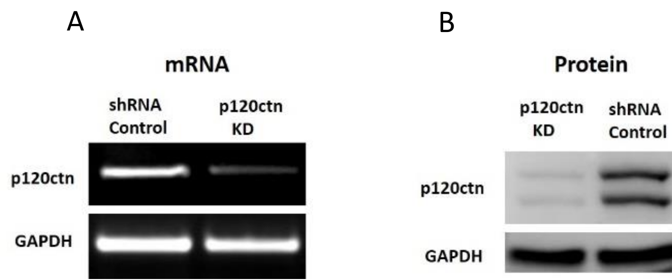

Figure 3.6. Verification of p120 catenin knockdown in THP-1 derived macrophages. shRNA lentivirus targeting p120ctn was used to knock down the expression of p120ctn in THP-1 derived macrophages. The mRNA level (A) and protein expression (B) of p120ctn were tested after shRNA transfection.

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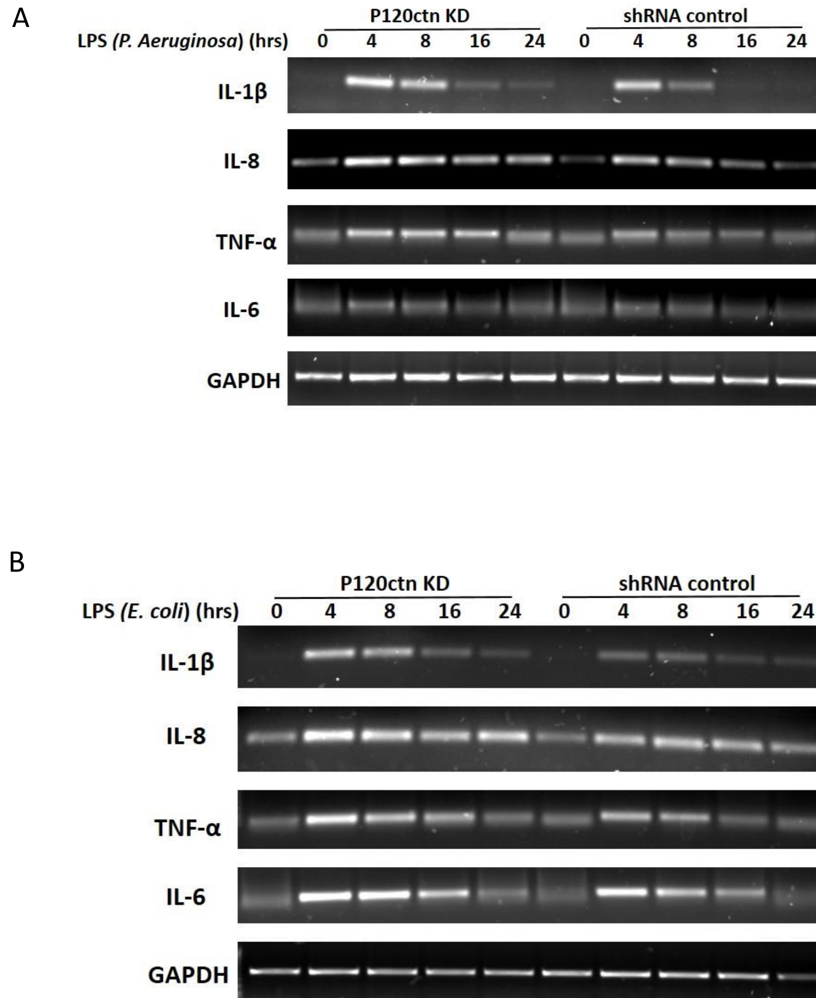

Figure 3.7. mRNA expression of proinflammatory cytokines in p120 catenin knockdown macrophages in response to LPS stimulation. The mRNA expression of proinflammatory cytokines, including IL-1β, IL-8, TNF-α and IL-6, was analyzed via RT-PCR in p120ctn-knockdown and shRNA control macrophages upon stimulation of LPS (100 ng/ml) from *P. aeruginosa* (A) and *E. coli* (B).

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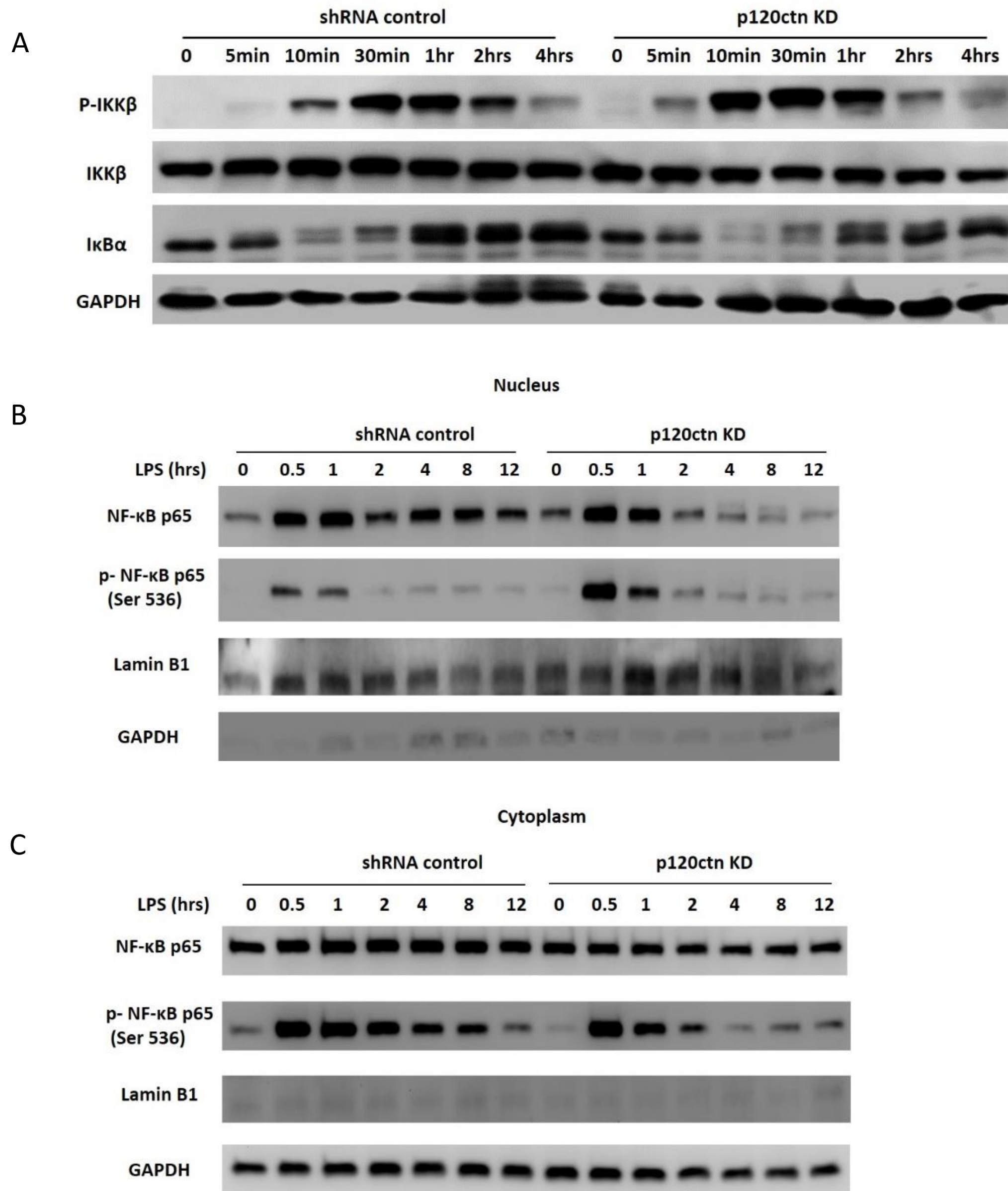

Figure 3.8. NF-κB signaling in p120 catenin knockdown macrophages in response to LPS (*P. aeruginosa*) stimulation. (A). The phosphorylation of IKKβ and degradation of IκBα were detected via Western blot in p120ctn-knockdown and shRNA control macrophages upon stimulation of LPS. After stimulation of LPS in p120ctn-knockdown and shRNA control macrophages, the translocation and phosphorylation NF-κB p65 at Ser536 (B) and (C) was analyzed by western blot. GAPDH and Lamin B1 were used as cytoplasmic and nulcear internal controls respectively.

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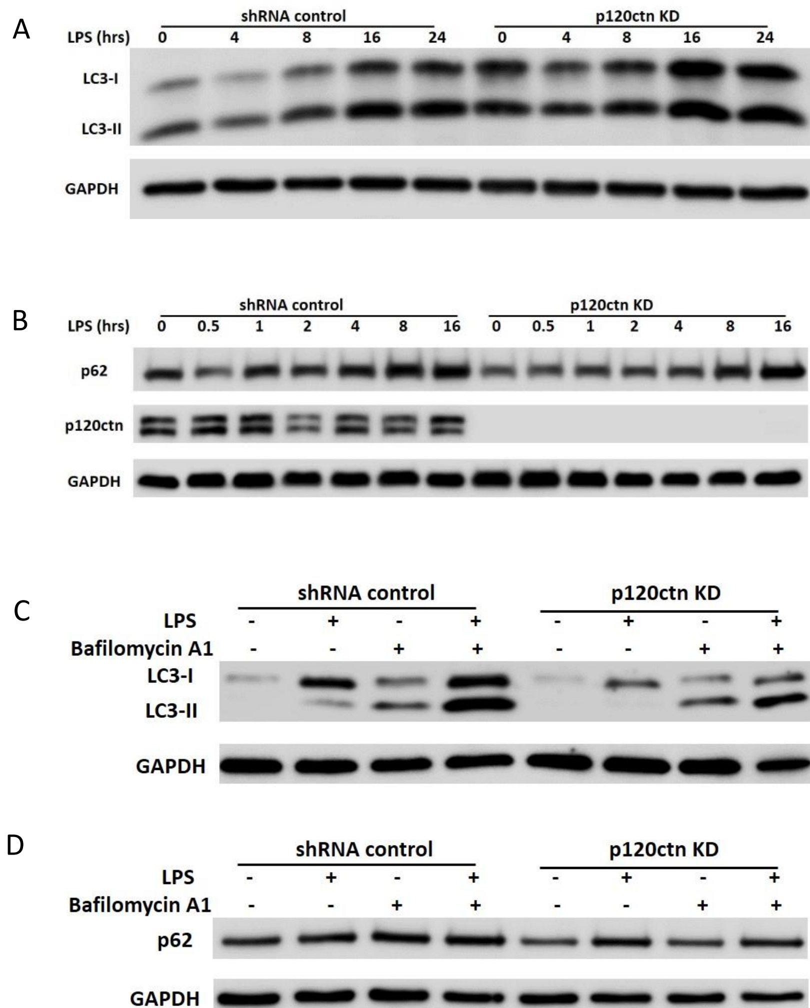

Figure 3.9. LPS-induced autophagy in p120 catenin knockdown THP-1 derived macrophages. p120ctn-knockdown and shRNA control macrophages were treated with 100 ng/ml of LPS (*E. coli*) for indicated time. Autophagy was tested by Western blot analysis of LC3 (A) and p62 (B). The macrophages were stimulated with 100 ng/ml of LPS (*E. coli*) for 8 hours, followed by treatment with autophagy inhibitor bafilomycin A1 (1μM) (DMSO as vehicle control) for 1 hour. LC3 (C) and p62 (D) was then analyzed by Western blot.

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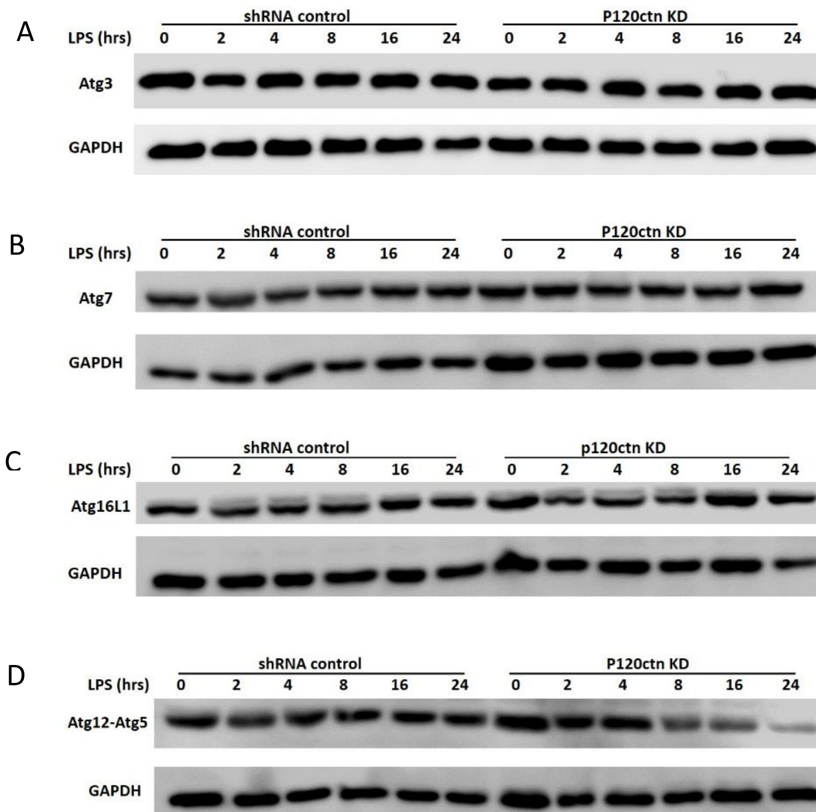

Figure 3.10 Expression of autophagy-associated proteins Atgs in p120 catenin knockdown THP-1 derived macrophages after LPS treatment. p120ctn-knockdown and shRNA control macrophages were treated with 100 ng/ml of LPS (*E. coli*) for indicated time. Autophagy-associated proteins including Atg3 (A), Atg7 (B), Atg16L1 (C) and conjugated Atg12-Atg5 (D) were compared between p120ctn-knockdown macrophages and shRNA control macrophages by Western blot.

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CHAPTER 4 DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Discussion

The incidence of severe sepsis is estimated to be 300 cases per 100,000 population in the United States [271]. European Prevalence of Infection in Intensive Care (EPIC II) study reported that the predominant organisms were Staphylococcus aureus (20.5%), Pseudomonas species (19.9%), Enterobacteriaceae (mainly *E. coli*, 16.0%), and fungi (19%), with Gram-negative organisms being still prevalent [272]. Sepsis associated with Gram-negative bacteria is a persistent problem all over the world. In spite of the development of new antibiotics, mortality from Gram-negative bacteria sepsis remains very high [273]. The outer membrane component of Gram-negative bacterial, LPS, is recognized as the most potent microbial mediator implicated in the pathogenesis of sepsis and septic shock [274]. The levels of circulating LPS can predict development of multi-organ failure, including acute respiratory distress syndrome [275]. LPS as a component of Gram-negative bacterial membrane has been used as a model representing Gram-negative bacteria-induced inflammation [276, 277]. In order to study the effects of p120ctn on inflammatory reactions in macrophages, LPS was used to stimulate macrophages in my studies.

Cell model for studying human p120 catenin functions in macrophages

There are several cell models for studying human macrophage, such as THP-1, U937, and HL-60. THP-1 cell line was established from the peripheral blood of a 1-year old male patient with acute monocytic leukemia [278]. This cell line has been widely used to study immune responses in the monocyte state and in the macrophage-like state [279, 280]. THP-1 cells can be differentiated into a macrophage-like phenotype by using either PMA, or 1α, 25-dihydroxyvitamin D3 (Vit D3) [279]. Vit D3-induced differentiation of THP-1 cells resulted in macrophages less similar to human monocyte derived macrophages in terms of phagocytic activity and production

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of IL-1β and TNF-α, as compared with THP-1 derived macrophages generated by using PMA [279]. Compared with heterogeneous PBMC monocytes from individual donors, the homogeneous genetic background of THP-1 could minimize the degree of phenotype variability, which facilitates reproducibility of studies. Moreover, PBMC monocytes require cytokines, such as IL-1β or TNF-α, granulocyte-monocyte-colony stimulating factor or IFN-γ to function as survival factors to prevent apoptosis, whereas, THP-1 cells can be easily cultured, as well as be stocked in liquid nitrogen [281, 282]. Therefore, THP-1 cell line is the optimal monocyte model. PMA is the most effective and universally used differentiation agent to acquire THP-1 derived macrophages with similarities to human monocyte derived macrophages [281]. In my study, 10 ng/ml of PMA was used to induce THP-1 cells to differentiate into macrophage-like cells. After 3-day culturing with PMA, THP-1 monocytes acquired typical morphological features of macrophage: adhesive to culturing plate and flatten into irregular and elongated cells with short protrusions. The production of proinflammatory chemokines and cytokines was also used to identify one of the typical functions of macrophages in response to LPS stimulation. As shown in Figure 3.1B, PMA-induced THP-1 derived macrophages could respond to LPS challenge in terms of the proinflammatory cytokine expression.

Isoforms of p120 catenin in THP-1 derived macrophages

Divergent expression patterns of p120ctn isoforms have been observed in a tissue- and cell-type-specific manner [22, 283, 284]. The cell type-specific expression of p120ctn isoforms was evident. The expression of p120ctn isoforms varies in abundance among different cell types [18]. It has been reported that the mesenchymal and motile cells, such as macrophages and fibroblasts, express p120ctn long isoforms, whereas the epithelial or endothelial cells predominantly express short isoforms [22]. In my study, the RT-PCR and Western blot analysis showed that both THP-1

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monocytes and THP-1 derived macrophages express p120ctn isoform 1A and 3A. Compared with THP-1 monocytes, PMA-induced macrophages express higher level of p120ctn isoform 1A and 3A . The increased expression of p120ctn might be a marker for monocyte differentiation. But this would require using multiple monocyte/macrophage cell models to figure out the PMA effect on the expression of p120ctn. My study demonstrated that monocytes or macrophages could express more than one isoforms, possibly both long isoform (isoform 1A) and short isoform (isoform 3A). Moreover, the preferential expression of either isoform was not observed in THP-1 cells and THP-1 derived macrophages at least by the antibody used in this study. However, the subcellular distribution of p120ctn showed that the membrane would predominantly express the short isoform 3A, and no significant difference of isoform expression was observed in cytoplasmic and nuclear part in macrophages (Figure 3.3D). It has been reported that in tumor cell lines, p120ctn isoform 1A promoted the epithelial mesenchymal transition (EMT) and increased tumor cell invasiveness, while p120ctn isoform 3A showed the opposite effects on EMT and invasiveness [285]. But the functions of different p120ctn isoforms have not been very clear in macrophages. In addition, the profile of p120ctn isoform expression was not switched by LPS challenge (Figure 3.3A). p120ctn isoform 4 is rarely expressed as a protein [17], so this study didn't examine the expression of isoform 4 in macrophages.

LPS-induced degradation of p120 catenin in THP-1 derived macrophages

Previous study revealed that LPS challenge could reduce p120ctn protein expression in mouse lungs and pulmonary endothelial cells [263]. The p120ctn protein level was correlated inversely with severity of lung inflammation. Knockdown of p120ctn also increased the neutrophil recruitment, production of cytokines TNF-α and IL-6, and pulmonary transvascular permeability in response to LPS [263]. p120ctn expression was also observed to be significantly reduced after

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LPS stimulation in human bronchial epithelial cell line [286]. My research revealed that *P. aeruginosa* LPS could not induce p120ctn downregulation or redistribution in macrophages, whereas *E. coli* LPS reduced the p120ctn expression in macrophages within 4 hours after LPS treatment. Moreover, the mRNA expression level of p120ctn was not decreased by *E. coli* LPS stimulation, which indicated that the decreased protein level was not attributed to reduced mRNA level of p120ctn. Next, the pathways responsible for p120ctn degradation were analyzed by using inhibitors targeting proteasome and autophagy respectively. As shown in Figure 3.3C, autophagy inhibitor bafilomycin A1, but not proteasome inhibitor MG132, suppressed the LPS-induced degradation of p120ctn, which suggests that autophagy pathway might contribute to the degradation of p120ctn in macrophage upon *E. coli* LPS challenge. One of the selective degradation by autophagy is mediated through autophagy adaptor protein p62 which could recognize K63-ubiquitinated proteins. Behrends *et al* reported a proteomic analysis of the autophagy interaction network in human cells under basal autophagy, which revealed a network of 751 interactions among 409 candidate interacting proteins [287]. In this research, p120ctn-p62 interaction in HEK293 cells was identified via affinity-capture mass spectrometry by using p62 as a bait [287]. Selective autophagy can also be mediated by the interaction between LIR motif of the target and the autophagy protein LC3, which targets selected cargo to a nascent autophagosome. The core LIR motif is (W/F/Y)XX(L/I/V), where X is any other residue. This sequence is surrounded by at least one acidic residue [179, 288]. Examination of p120ctn amino-acid sequence (reference sequence: NP_001078927.1) revealed a potential LIR motif "WAPL" at positions 336-339aa upstream of the N-terminal Arm domain. Also, an aspartic acid residue is located upstream of this motif (-4) together with two tyrosine residues and one serine residue nearby which might be associated with the mechanism of regulating the protein interactions. Another Arm domain

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family membrane β-catenin was reported to contain a potential LIR motif (WPLI) at positions 504–507 within the Arm domains (Arm 9) [289] . During nutrient deprivation β-catenin was selectively degraded via the formation of a β-catenin–LC3 complex mediated by the LIR motif. The interaction between LC3 and β-catenin was required for non-proteasomal degradation of β-catenin [289]. However, the further co-immunoprecipitation and mutation assays are required to confirm the pathway responsible for p120ctn degradation via selective autophagy in macrophage upon LPS stimulation. Some other studies reported that p120ctn was phosphorylated by casein kinase (CK)-1α and glycogen synthase kinase (GSK) 3β, and then degraded through the ubiquitin-proteasome pathway. The inhibition of proteasome pathway with MG132 could raise p120ctn levels in melanoma cells and 293T cells [72]. This indicated the pathways responsible for p120ctn degradation might be cell-context and signaling-pathway dependent. The activation of GSK3β or CK-1α signaling led to the proteasomal degradation of p120ctn [72], whereas, LPS-induced Src activation might be responsible for autophagy degradation of p120ctn.

Comparing the signaling pathways revealed the similar activation patterns of the MAPK, Akt, and IKK-NF-κB signaling pathways activated by *E. coli* and *P. aeruginosa* LPS. p120ctn was originally identified as a Src substrate from v-Src-transformed chicken embryo fibroblasts [1]. Multiple sites of p120ctn tyrosine phosphorylation by Src were identified [74]. But the functional consequences of p120ctn tyrosine phosphorylation are not completely understood. Under some specific circumstances, in vitro and in vivo assays indicated that tyrosine-phosphorylation of p120ctn was correlated with an altered affinity of p120ctn for cadherins and the structural integrity of the adherens junctions [75, 81, 290, 291]. The macrophages do not form a static cell-cell adhesion, therefore, the p120ctn phosphorylation by Src and its degradation induced by LPS might be associated with the mobility of macrophages in inflammatory responses.

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LPS is the major structural component present in the outer membrane of Gram-negative bacteria that can trigger innate immune responses through TLR4. LPS is composed of three distinct units: the hydrophobic phospholipid known as lipid A, a core oligosaccharide, and an outer polysaccharide called O antigen [292]. Studies employing synthesized lipid A have demonstrated that lipid A is the major inflammation-inducing moiety of LPS [293, 294]. The diversity of structural motifs in the polysaccharide and the lipid A moieties accounts for the highly variable *in vivo* and *in vitro* responses to LPS [295]. However, the relationships between diverse structure of LPS and biological responses are complex, and studies have indicated that LPS isolated from particular bacterial strains can preferentially induce MyD88- or TRIF-dependent expression of cytokines [293]. My study revealed that LPS from two different bacterial strains (*E. coli* and *P. aeruginosa*) could induce diverse response in macrophages with respect to p120ctn expression. At present, the functional significance related to LPS-induced degradation of p120ctn in macrophages remains elusive. Further studies are required to define whether LPS-induced degradation of p120ctn is associated with distinct profile of cytokine expression and macrophage mobility.

p120 Catenin suppresses LPS-induced NF-κB activation and proinflammatory cytokine expression

Previous observations showed that p120-null epidermal cells exhibited increased NF-κB activation, resulting in elevated expression of proinflammatory cytokines and chemokines in vitro and in vivo [258, 259]. Wang *et al* revealed p120ctn expressed in endothelial cells could inhibit TLR4-mediated NF-κB signaling and lung inflammation [263]. These findings raise the possibility that p120ctn might have important roles in regulating immunity. In my research, p120ctn knockdown in macrophages also led to elevated activation of NF-κB signaling and mRNA expression of proinflammatory cytokines including IL-1β, IL-8, IL-6 and TNF-α. The loss of

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p120ctn could not extend the time period of IKKβ activation, IκBα degradation or NF-κB nuclear translocation. Instead, p120ctn depletion resulted in more rapid activation of NF-κB signaling pathway.

A number of kinases have been identified that phosphorylate NF-κB p65 at Ser536 in the transactivation domain, including IKKα, IKKβ, IKKε and NF-κB activating kinase/TBK1 [296, 297]. The consequences of Ser536 phosphorylation of p65 would depend on the physiological context. For example, in 293T cells, TNF-α-induced phosphorylation at Ser536 of p65 displays enhanced transcriptional activity which is associated with increased acetylation of p65 [298]. Moreover, p65 Ser536 phosphorylation leads to the expression of a distinct set of target genes, which indicates phosphorylation of p65 might be associated with the selectivity of gene expression [299]. In my research, the phosphorylated p65 at Ser536 was increased transiently in nucleus in p120ctn knockdown macrophages after LPS stimulation. This might be associated with the upregulation of proinflammatory expression in p120ctn-depleted macrophages. Further studies are required to explain the functional association of p65 Ser536 phosphorylation with the cytokine expression.

p120 Catenin is associate with LPS/TLR4-induced autophagy in THP-1 derived macrophages

Pattern recognition receptor signaling that is induced following the recognition of PAMPs and DAMPs can activate autophagy [215, 216]. For example, activation of TLR4 leads to ubiquitination of the Beclin-1 by the E3 ligase TRAF6. Ubiquitinated Beclin-1 is released from its inhibitor Bcl-2 and becomes active [154]. TRAF6 also activates ULK1 through ubiquitination [300]. TLR activation also promotes the interaction of MyD88 and TRIF with Beclin-1, which reduces the binding of Beclin-1 to Bcl-2 [301].

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Autophagy also facilitates TLR signaling by transferring cytosolic pathogen components to their cognate endosomal TLRs [227]. Autophagosome plays an important role in delivery of antigen to antigen-loading compartments for MHC II presentation [302].

Autophagy is a dynamic process through which eukaryotic cells degrade long-lived or misfolded proteins, and impaired or superfluous cytoplasmic organelles, as well as recycle autophagy-derived nutrients [303]. Autophagy comprises sequential steps including the formation of autophagosomes, the fusion of autophagosomes with lysosomes and cargo degradation [121]. Autophagy is associated with a wide range of physiological and pathological processes, such as embryonic development, immunity, cancer, cardiovascular disease, muscular disease and neurodegenerative disease [130]. To date, the major methods used to monitor autophagic activation are the Western blot analysis of LC3 processing and detection of autophagosome formation by fluorescence or electron microscopy [304]. However, autophagy is a highly dynamic process with multiple steps. The increased LC3-II levels observed via Western blot or the accumulation of autophagosomes detected by microscope could indicate either the activation of autophagy or a blockage of downstream steps in autophagy, such as inefficient fusion or decreased lysosomal degradation. Therefore, the mere detection of the presence of LC3 processing is insufficient for an overall evaluation of the entire autophagic system. Evaluation of the rate of autophagic degradation, defined as autophagic flux, seems more accurate to assess the whole dynamic process of autophagy [305, 306]. In this research, LPS-induced autophagy was compared between p120ctn knockdown THP-1 derived macrophages and shRNA control macrophages. The conversion of LC3-I to LC3-II, an indicator of autophagy activation, was assessed by immunoblot analysis with LC3 antibody. As shown in Figure 3.9A, LC3-II abundance was increased by LPS challenge in a time-dependent manner in both p120ctn knockdown macrophages and shRNA

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control macrophages. Generally, without inhibition of autophagy process, LC3-II level was observed to be gradually increased 8 hours after LPS treatment, and reached the highest level around 16 hours of LPS treatment. Since the autophagic process is highly dynamic and autophagosomes turn over rapidly, the absolute level of LC3-II at specific time point after LPS stimulation might not represent the real rate of autophagy flux unless lysosomal degradation is blocked. By using bafilomycin A1 to block the fusion of autophagosome with lysosome, an increase of LC3-II level was observed in both p120ctn knockdown and control macrophages after LPS challenge (Figure 3.9C). But, the elevated level of LC3-II was significantly lower in p120ctn knockdown macrophages as compared with control cells. Without LPS stimulation, the inhibition of autophagy with bafilomycin A1 could also increase the abundance of LC3-II similarly in both macrophages, suggesting that basal level of autophagy might not be affected by the loss of p120ctn in macrophages.

p62 has an N-terminal PB1 domain followed by a zinc finger domain, and a C-terminal UBA domain [307]. p62 could bind to polyubiquitin via its UBA domain and thus recruit ubiquitinated protein aggregates [308, 309]. The N-terminal PB1 domain is used for the polymerization of p62 and for binding to other proteins containing PB1 domains [310]. p62, together with the bound cargoes, is degraded by autophagy, so p62 may also be used as an autophagic marker [173]. My data demonstrated that LPS could induce a transient downregulation of p62 followed by upregulation and maintaining at a high level in shRNA control macrophages (Figure 3.9B). The LPS-induced decrease of p62 was not observed in p120ctn knockdown macrophages, whereas the gradual increase was evident during the course of LPS challenge.

TRAF6 is the essential mediator for activation of NF-κB pathway induced by LPS, proinflammatory cytokines and growth factors [311]. p62 also functions to facilitate K63-

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polyubiquitination of TRAF6 and thereby mediates the activation of the NF-κB pathway [311]. As shown in Figure 3.9 B and D, p62 level was increased by LPS and could not be further elevated by bafilomycin A1. Hence, the increase of p62 might result from LPS-induced activation of NF-κB signaling in THP-1 derived macrophages. This is consistent with another study which demonstrated that LPS treatment led to induction of p62 mRNA and protein in mouse bone marrow-derived macrophages [312]. As an adaptor protein, p62 is involved in multiple biologic processes, such as NF-κB activation and protein degradation via autophagy. The predominant functions of p62 might be cell-context dependent. p62 may display distinctive functions in response to different stimuli. In this THP-1 derived macrophage model, instead of autophagic degradation, p62 might be mainly involved in the activation of NF-κB signaling pathway via interaction with TRAF6. As the Figure 3.9B showed, the basal level of p62 was lower in p120ctn knockdown macrophages as compared with control cells. The mechanisms responsible for p62 downregulation in p120ctn-depleted cells remains unclear, and further study is warranted.

Covalently conjugated Atg12-Atg5 forms a multimeric complex with Atg16L1, which functions as ubiquitin-protein ligase E3-like enzyme essential for LC3-PE conjugation reaction and therefore for autophagosome formation [313, 314]. Without any stimulation, Atg12 has been constitutively conjugated to Atg5 [315]. The level of conjugated Atg12-Atg5 was downregulated 8 hours after LPS treatment in p120ctn knockdown macrophages. This might be one of the reasons for the decreased activity of LPS-induced autophagy in p120ctn knockdown macrophages. The mechanism by which the loss of p120ctn affects the expression of conjugated Atg12-Atg5 needs further studies.

4.2 **Conclusions**

In conclusion, p120ctn has conserved anti-inflammatory functions across different cell types including epithelial cells, endothelial cells and macrophages. NF-κB signaling pathway

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might be the common regulatory target. The downregulation of p120 catenin in the macrophages in response to *E. coli* LPS stimulation might be regulated by Src signaling and autophagy. The depletion of p120ctn results in the decreased activity of LPS-induced autophagy in THP-1derived macrophages. The loss of p120ctn leads to downregulation of conjugated Atg12-Atg5 in macrophages upon LPS stimulation. The abnormal autophagy might contribute to the over-activation of LPS-induced inflammation in p120ctn knockdown macrophages.

4.3 Future directions

The LPS-induced downregulation of p120ctn was revealed in this study. Future research is required in order to explore how p120ctn is degraded via autophagy and whether the downregulation of p120ctn is associated with the distinct patterns of proinflammatory cytokine expression. The defective LPS-induced autophagy was displayed in p120ctn-depleted macrophages. Further studies will be performed to determine how p120ctn influences Atg12-Atg5 conjugation.

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ABSTRACT

P120 CATENIN REGULATES INFLAMMATION IN MACROPHAGE

by

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Advisor: Dr. Chunying Li

Major: Biochemistry

Degree: Doctor of Philosophy

Objective: p120 catenin (p120ctn) has been reported to play a critical role in maintenance of the stability of adherens junctions. It also has potential anti-inflammatory effects in epithelial and endothelial cells. This research was designed to evaluate the effects of p120ctn on inflammatory responses in human macrophages upon LPS stimulation, as well as the possible mechanism by which p120ctn regulates LPS-induced proinflammatory response in macrophages. **Methods**: THP-1 cells were induced to differentiate into macrophages by PMA. The isoforms of p120ctn were identified via RT-PCR and Western blot. The expression of p120ctn was examined by Western blot in THP-1 derived macrophages after LPS challenge. p120ctn was knocked down in THP-1 cells by using shRNA lentivirus particles. mRNA level of proinflammatory cytokines were evaluated by RT-PCR. The autophagy pathway was analyzed by Western blot in macrophages after LPS stimulation with or without Bafilomycin A1 treatment. **Results**: THP-1 derived macrophages expressed p120ctn isoform 1A and 3A. *E. coli* LPS could downregulate p120ctn expression in macrophage via autophagy pathway, which was suppressed by Src signaling inhibitor PP2. mRNA levels of proinflammatory cytokines including IL-1β, IL-8, IL-6 and TNF-α were elevated in p120ctn-delpeted macrophages in response to LPS stimulation, as compared with control cells. The activation of NF-κB signaling pathway was more rapid in p120ctn

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knockdown macrophages in response to LPS treatment. Decreased autophagy and downregulation of conjugated Atg12-Atg5 were observed in p120ctn knockdown macrophages after LPS challenge. **Conclusions**: The downregulation of p120ctn in the macrophages in response to *E. coli* LPS stimulation is regulated by Src signaling and autophagy. p120ctn has anti-inflammatory functions in macrophages. NF-κB signaling pathway might be the regulatory target. The loss of p120ctn results in the decreased activity of LPS-induced autophagy and downregulation of conjugated Atg12-Atg5 in macrophages upon LPS stimulation, which might contribute to the over-activation of LPS-induced inflammation in p120ctn knockdown macrophages.

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