Experimental study on the role of lysophosphatidic acid in mediating cytokine/chemokine production and on the signaling pathways involved in inflammatory arthritis

Thèse

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Résumé

La polyarthrite rhumatoïde (PR) est l'une des maladies auto-immunes qui atteint les articulations. Les symptômes principaux de la PR incluent une inflammation chronique de la synoviale dépendante de diverses cytokines et chimiokines inflammatoires, ainsi que de nombreux médiateurs de caractère lipidique, sécrétés par les cellules immunitaires et par les synoviocytes. L'acide lysophosphatidique (LPA) et son enzyme productrice, l'autotaxine (ATX) ont été détectés dans le liquide synovial. Les niveaux des ARNm de deux des six récepteurs du LPA, soit LPA_1 et LPA_3, sont aussi plus élevés dans les fibroblastes synoviaux des patients PR comparé à ceux d'arthrose ou suite avec une incubation avec le TNFα. Le LPA est aussi reconnu pour sa capacité à induire une production de cytokines et de chimiokines par des synoviocytes de type B (RAFLS) provenant de patients PR, ainsi que dans le modèle murin de poche d'air. D'ailleurs, la pré-incubation avec le facteur de nécrose tumorale alpha (TNFα) *in vitro* ou bien *in vivo* rehaussa cette sécrétion de cytokines et de chimiokines induite par la LPA. Dans le cadre de cette étude, nous avons démontré qu'une chimiokine appelée CXCL13 est impliquée dans le recrutement lymphocytaire induit par le LPA après une pré-incubation au TNFα, dans le modèle de poche d'air. Le mécanisme par lequel le LPA peut faire sécréter des cytokines et des chimiokines et par lequel la TNFα peut contribuer à une superproduction de cytokines et de chimiokines dépendante du LPA a aussi été investigué. Nos résultats indiquent que la p38MAPK, la cascade signalétique ERK-MSK-CREB, la Rho kinase, et la PI3K sont toutes des voies de signalisation stimulées par le LPA qui modulent une sécrétion de l'interleukine-8 (IL-8) chez les synoviocytes de type B. En revanche, nous avons remarqué que la sécrétion de l'IL-8 par les synoviocytes stimulés par le LPA devenait insensible aux inhibiteurs des dites kinases lorsque traités au préalable au TNFα. Il n'en demeure pas moins qu'une inhibition simultanée de la p38MAPK et d' ERK a mené à une diminution

efficace de la sécrétion de l'IL-6 et de l'IL-8. Cette étude a permis de mieux comprendre le mécanisme de la réponse inflammatoire déclenchée par le LPA et d'identifier les principales voies de signalisation intracellulaire contribuant à la sécrétion de cytokines et de chimiokines en présence ou en absence de TNFα. Le travail pourrait avoir des retombées majeures en ce qui concerne les stratégies pour développer des drogues ciblant l'axe ATX-LPA et les kinases mentionnées ci-dessus pour le traitement de la polyarthrite rhumatoïde.

Abstract

Rheumatoid Arthritis (RA) is one of the most severe inflammatory arthritides. The main features of RA include chronic inflammation in the synovium mediated by inflammatory cytokines and chemokines as well as lipid mediators, secreted by immune cells and synoviocytes. Lysophosphatidic acid (LPA), a monoacyl phospholipid mediator, and its producing enzyme autotaxin (ATX) were detected in synovial fluid. LPA_1 and LPA_3 receptor mRNA levels were also higher in synovium from RA patients than from normal individuals. LPA was previously reported to induce cytokine and chemokine production in RA fibroblast-like synoviocytes (RAFLS) and in a mouse air pouch model. In addition, TNFα pretreatment *in vivo* or *in vitro* enhanced this cytokine and chemokine secretion induced by LPA. In this study, we demonstrated that an LPA-induced chemokine named CXCL13 is also involved in LPA-induced leukocyte recruitment in the mouse air pouch model after TNFα pretreatment. The mechanism whereby LPA induces cytokine/chemokine secretion and whereby TNFα pretreatment induces cytokine/chemokine super-production mediated by LPA was also investigated in this study. Our data demonstrated that p38MAPK, the ERK-MSK-CREB axis, Rho kinase, and PI3K were all involved in the LPA signaling resulting in IL-8 secretion in RAFLS. However, we found that, after pretreatment with TNFα, LPA-induced IL-8 secretion became insensitive to inhibitors of those kinases mentioned above. Notwithstanding, blocking both the p38MAPK and ERK pathways could effectively decrease IL-8 and IL-6 secretion. This study allowed a deeper understanding of the mechanism of the LPA-induced inflammatory response, including the signal pathways regulating cytokine/chemokine secretion with or without the exacerbating effect of TNFα. The study may have important implications for the development of drugs targeting ATX-LPA axis and the aforementioned signaling pathways for the treatment of RA.

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Liste des abréviations

AC	adenylyl cyclase
ACKR	Atypical chemokine receptor
acyl-CoA	acyl coenzyme A, cofactor formed of pantothenate, cysteamine and adenosine 3'-phosphate 5'-pyrophosphate
AGC group	a kinase group named after the protein kinase A, G, and C families (PKA, PKC, PKG)
Akt	Protein kinase B (PKB), a serine/threonine-specific protein kinase
APC	allophycocyanin
ApoM	Apolipoprotein M a.k.a. Apom, component of low-and high-density lipoproteins
ATF-1	activating transcription factor-1
ATX	autotaxin, lysophospholipase D that produces LPA from LPC; a.k.a. ENPP2, NPP2
B103 ASSAY	bioassay on a rat central nervous system neuronal cell line B103
BSA	bovine serum albumin
C/EBP-1	CCAAT/enhancer binding protein
CBP	CREB-binding protein
CCP	cyclic citrullinated peptide
CCR	receptors for CC chemokines
CD	The cluster of differentiation
CIA	collagen-induced arthritis
COX-2	cyclo-oxygenase 2 or prostaglandin endoperoxide synthase
CREB	cAMP response element-binding protein
CXCL	chemokine (C-X-C motif) ligand
CXCL1/KC	C-X-C chemokine motif ligand 1/Keratinocyte chemoattractant
CXCL10/IP-10	C-X-C chemokine motif ligand 10/Interferon gamma-induced protein 10
CXCL8/IL-8	C-X-C chemokine motif ligand ligand 8/Interleukin 8
CXCR	receptors for CXC chemokines
DAG	diacylglycerol
DCs	dendritic cells
DMARD	disease modifying anti-rheumatic drug
DUSP	Dual Specificity Phosphatase
EDG	endothelial differentiation genes, family of receptors for LPA and for S1P

eNOS	endothelial nitric oxide synthase
ENPP	nucleotide or ectonucleotide pyrophosphatase/ phosphodiesterase; a.k.a. Enpp; see ATX.
ERK	extracellular signal-related kinase; a.k.a. p44/42
FAB-MS	fast-atom bombardment mass spectrometry, a technique for the analysis of protein sequence and structure
FABP3	fatty-acid binding protein 3
FBS	fetal bovine serum
FLS	fibroblast-like synoviocyte, also named synovial fibroblast (SF)
Foxp3	forkhead box P3, a member of the forkhead transcription factor family
FPP	farnesyl (trimethyldodecatrienyl) pyrophosphate
GC-MS	gas chromatography–mass spectrometry
GM-CSF	granulocyte and macrophage colony-stimulating factor
GPAT	glycerophosphate acyl transferase
GPCR	G-protein-coupled seven-transmembrane-helix receptor
HDL	high-density lipoprotein
HEV	high-endothelial venule
HLA-DRB1,4	human major histocompatibility class II complex, locus DR, beta chain, variants 1 and 4
HMGN1	high mobility group nucleosome binding domain 1
HPTLC	High performance thin layer chromatograph
HTLV	human T-cell lymphotropic virus type 1; a.k.a. human T-cell leukemia type 1
IFNgamma	interferon gamma (type II interferon)
IKK	The I kappa B kinase
IL-1	interleukin 1
IL-17RA	the interleukin-17 receptor subunit
IL-1Ra	The interleukin-1 receptor antagonist
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
K/BxN	Mice expressing both the T cell receptor (TCR) transgene KRN and the MHC class II molecule A(g7) (develop severe inflammatory arthritis)
KRN mice	Mice expressing the transgenic T cell receptor (TCR)
LCAT	Lecithin–cholesterol acyltransferase
LC-MS	liquid chromatography–mass spectrometry
LDL	low-density lipoprotein

LPA	lysophosphatidic acid, the subject of this thesis
LPAAT	LPA acyl-transferase
LPC	lysophosphatidylcholine, precursor of LPA
LPE	lysophosphatidylethanolamine
LPG	lysophosphatidylglycerol
LPI	lysophosphatidylinositol
LPL	lysophospholipid
LPP	lipid phosphate phosphatase
LPS	lipopolysaccharide
LPT	lysophosphatidylthreonine
LTB4	leukotriene B4
lysoPS	lysophosphatidylserine
MAG	monoacyl glycerol
MAPK	mitogen-activated protein kinase
MAPKAP-2	MAPK-activated protein kinase 2; a.k.a. MK2
MAPKK/MAP2K	MAP kinase kinase (mitogen-activated protein kinase kinase)
MAPKKK/MAP3K	MAP kinase kinase kinase
MCP-1/CCL2	monocyte chemoattractant protein 1/C-C motif chemokine ligand 2
MEK	MAP/ERK kinase; a.k.a. MKK, MAP2K, see MAP2K
MIP-2/CXCL2	macrophage inflammatory protein 2/C-X-C motif chemokine ligand 2
MK2/3	MAPK-activated protein kinase 2/3
MMP	matrix metalloproteinase
MSK	mitogen- and stress-activated protein kinase; a.k.a. ribosomal protein S6 kinase A5
NF-κB	nuclear factor kappa-B, a transcription factor
NLD domain	nuclease-like domain; a.k.a. NUC doamin
NPP	nucleotide pyrophosphatase/phosphodiesterase
OA	osteoarthritis
OPG	osteoprotegerin
PA	phosphatidic acid
PDE domain	phosphodiesterase domain
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	Protein kinase C
PLA	phospholipase A
PLC	phospholipase C
PLD	phospholipase D
PPAR	peroxisome proliferator-activated receptor

RA	rheumatoid arthritis
Rac protein	monomeric GTPase, belongs to Rho family GTPases
RANK/RANKL	receptor activator of nuclear factor κB ligand, a.k.a. osteoprotegerin ligand (OPGL)
RANTES/CCL5	regulated on activation, normal T cell expressed and secreted/Chemokine (C-C motif) ligand 5
Rho kinase/ROCK/ROK	kinase for small G protein Ras homolog A (RhoA); a.k.a. Rock1, Rock2
RSK/S6K	ribosomal s6 kinase
S1P	sphingosine 1-phosphate
SKG mice	a genetic model of RA due to altered signal transduction in T-cells
SMB domain	somatomedin B-like domain
SPC	sphingosylphosphatidylcholine
STAT	signal transducer and activator of transcription protein
TAK1	transforming growth factor-β-activated kinase 1, a MAP3K
TAZ	a transcriptional co-activator (with PDZ-binding motif)
Th17	type 17 T helper cell
TLC-GC	thin-layer chromatography- gas chromatography
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAP	tartrate-resistant acid phosphatase
Treg cells	regulatory T cells
VEGF	vascular endothelial growth factor
VLDL	very-low-density lipoprotein
5-ZO	(5z)-7-oxozeaenol

Dédicaces

Dedicated to my parents, my colleagues and all the friends who support me during my studies…

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Zhao C, Hui W, Fernandes MJ, Poubelle PE, Bourgoin SG. 2014. Lysophosphatidic acid-induced IL-8 secretion involves MSK1 and MSK2 mediated activation of CREB1 in human fibroblast-like synoviocytes. *Biochem Pharmacol.* Jul 1; 90(1):62-72. (10%; 50%; 40%; 10%)

Chapter 4

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Appendix

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Chapter 1

Introduction

1.1 Lysophosphatidic Acid

1.1.1 Lysophospholipids

The term lysophospholipid (LPL) refers to any derivative of a phospholipid in which one acyl derivative is removed by hydrolysis. Lysophospholipid mediators, such as sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA), can be synthesized from membrane phospholipids. Other LPLs include lysophosphatidylserine (lysoPS), lysophosphatidylinositol (LPI), lysophosphatidylglycerol (LPG), lysophosphatidylethanolamine (LPE), lysophosphatidylthreonine (LPT) and lysophosphatidylcholine (LPC). Some of these have been found to be important signaling molecules mediating diverse effects by binding to and activating their cognate receptors [1-3] (Figure 1). The actions of those LPLs as lipid mediators including LysoPS, LPT, LPE and LPG have been reviewed in [3]. The history of LPLs and discovery of their receptors have been reviewed elsewhere (Figure 2). They will not be further discussed here as this is not the scope of this thesis. In this thesis, we focus on the study of LPA.

1.1.2 History of LPA

The discovery of LPA and its function date back to the 1950s and 1960s, when many lipid mediators with pharmacological activities were identified [4]. In 1954, Arneil and Dekanski detected an unknown vasopressor principle (named Arneil factor) in heparinized plasma (incubated at room temperature for clinical purposes) in patients or healthy subjects [5]. It was suggested to be LPC at first in 1960 [6], but was demonstrated later to be LPA produced from LPC by the action of a lysophospholipase D (lyso-PLD)-like activity in 1986 by the group of Tokumura [7]. LPA was first isolated and identified in 1978 as a vasopressor phospholipid in crude soybean lecithin

[8] and later was also identified in feline plasma incubated at body temperature [9]. Early reports showed that LPA was able to cause contraction of smooth muscle cells [10] and induce an increase in intracellular Ca^{2+} levels in 3T3 mouse embryonic fibroblast cells [11] and myeloma cells [12], indicating the existence of LPA receptors. In addition, LPA had been implicated as a bioactive lipid mediator with hormone- and growth factor-like activities [13]. Though many earlier reports demonstrated the involvement of putative LPA receptors [11, 14], the first high-affinity, cognate cell surface receptor for LPA was identified (LPA_1) in 1996 [15] (Figure 2) by the group of Jerold Chun while they were identifying novel GPCR genes related with neurodevelopment. Until now, six LPA receptors have been identified [16-21], and an intracellular receptor of LPA was also identified (peroxisome proliferator-activated receptor gamma, PPARγ) [22]. Recent reviews have summarized the diverse roles of LPA in the past twenty years and updated nomenclature of LPA receptors [23-28]. As evidenced by the booming number of publications since the twentieth century, LPA has been implicated in various physiological and pathological states.

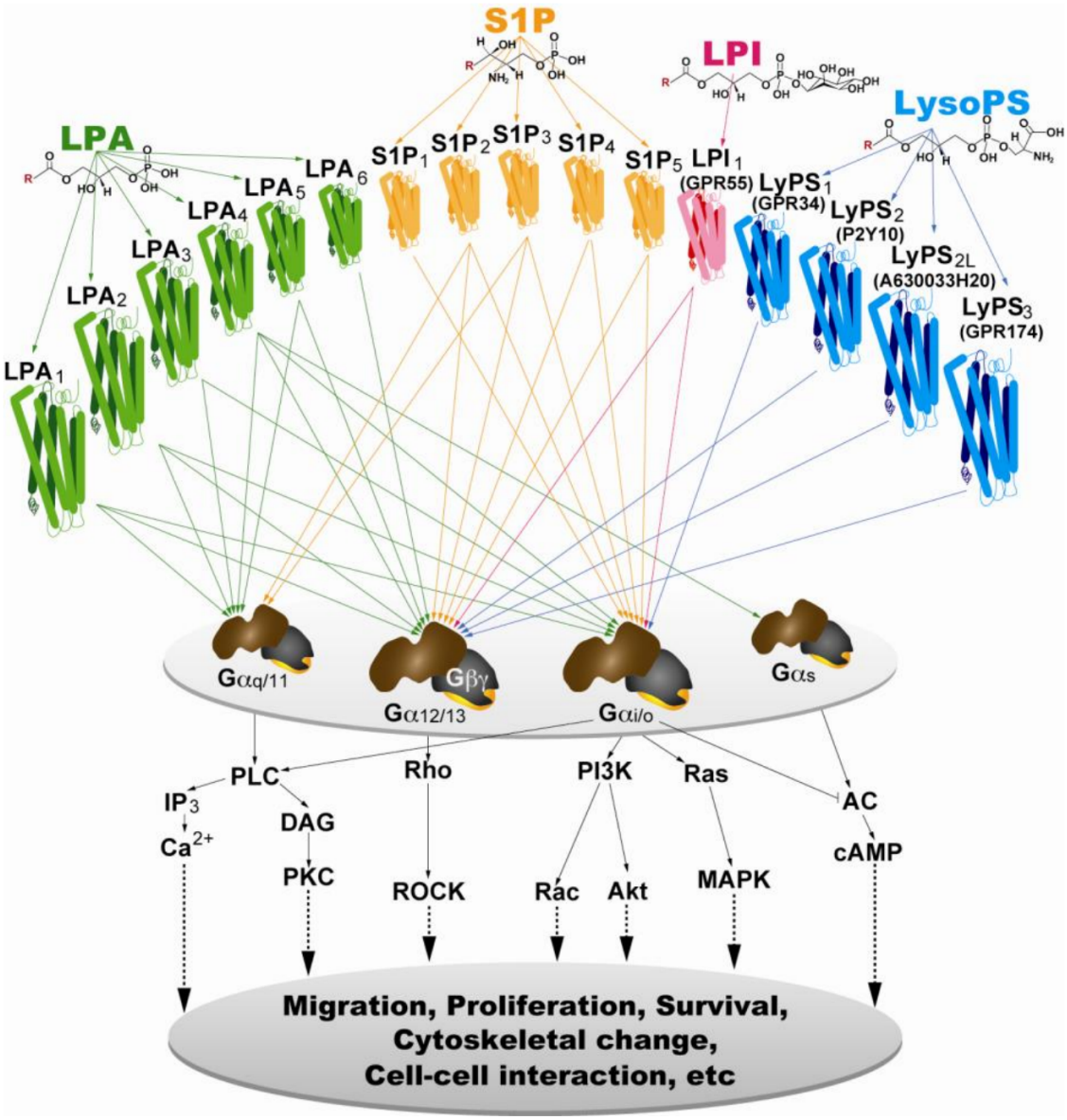


Figure 1. Lysophospholipid receptors and their intracellular signaling pathways (from [24])

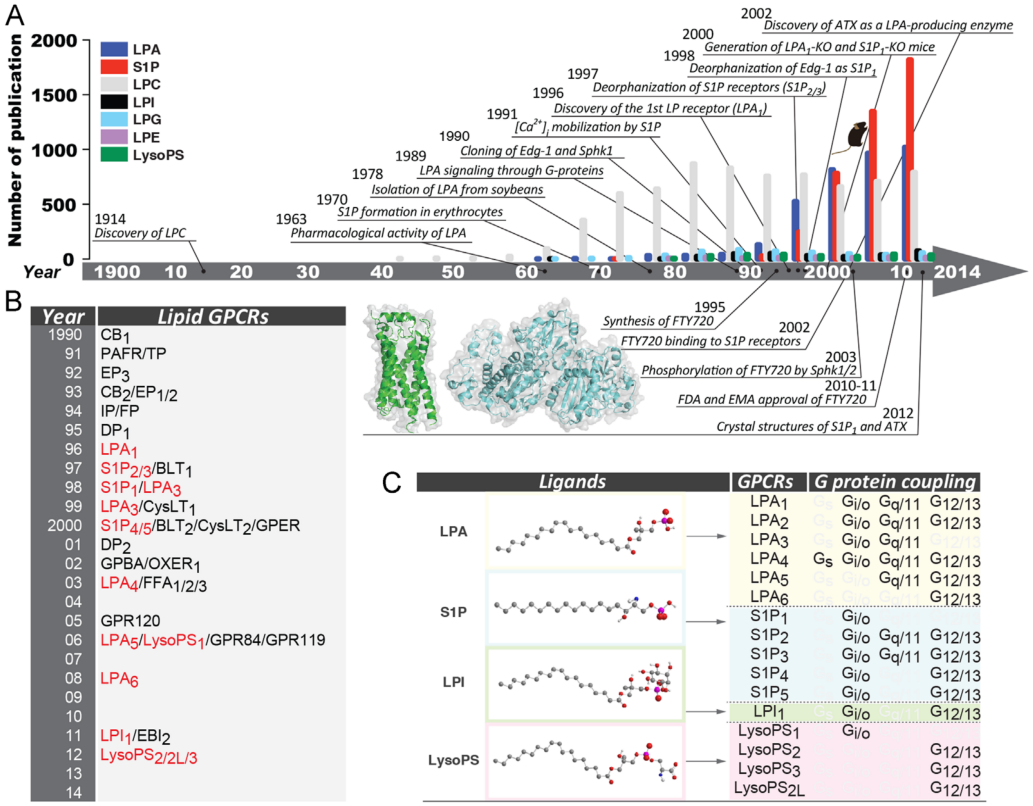
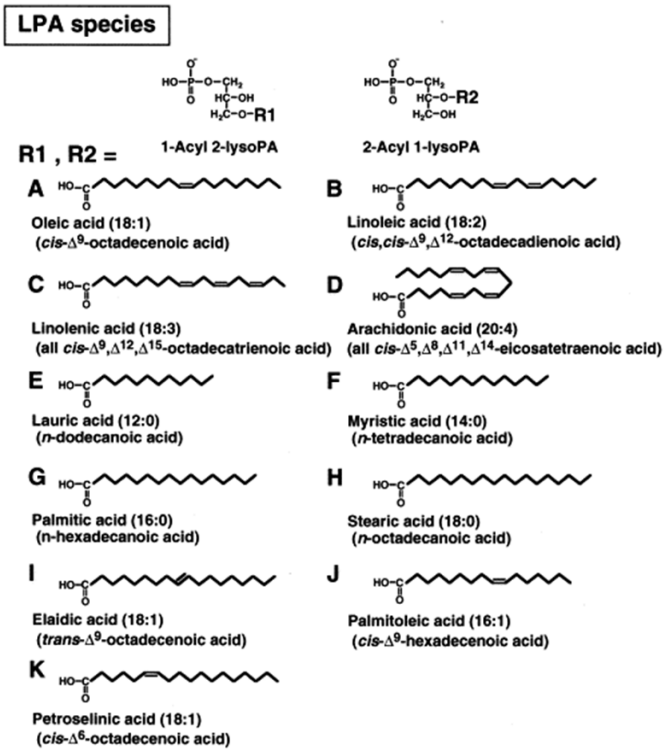


Figure 2. Chronology of the LPL field, LPLs and other lipid receptors, and overview of proximal LPL signaling features (From [29])

1.1.3 The Structure of LPA Species

LPA can be generated naturally during platelet activation [30-32], thus platelets are a very important source of LPA species. Many other mammalian cell types are also known to produce LPA, such as adipocytes, neutrophils, fibroblasts, ovarian and prostate cancer cells, and neuronal cells [33, 34]. Many species of LPA are present in various biological fluids in different forms, due to the ester and ether linkages, the variety of fatty acid structure (fatty acid chain length and degree of saturation), and fatty acid linkage to sn-1 or sn-2 position of the glycerol backbone. The widely used species of LPA for signaling studies in the laboratory is 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate [23] (Figure 3). Different forms of LPA include saturated to

polyunsaturated fatty esters, alkyl, alkenyl ether, as well as analogs 2, 3-cyclic phosphates [35] (Figure 3). The rank order of the acyl species of LPA in normal human plasma differs from the rank order in serum or in resting and thrombin-stimulated platelets, indicating that serum LPA and plasma LPA species have distinct precursors [31, 36]. The majority of serum or plasma LPA is in the form of acyl-LPA with palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and arachidonic acid (20:4) [36]. The 18:2 and 20:4 species make up 84% of the LPA found in serum [32].



M

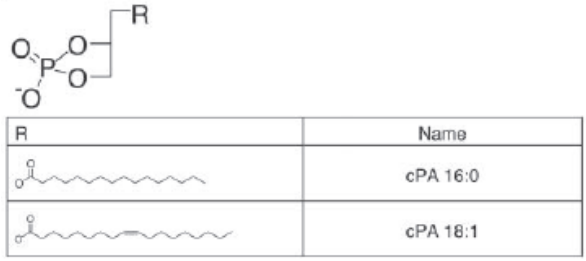


Figure 3. Chemical structure of LPA species, analogs and cyclic derivatives

A-L, Chemical structures of LPA species and analogs; **M,** 2,3-cyclic derivatives of LPA (From [35, 37])

1.1.4 Metabolism of LPA

1.1.4.1 Synthesis of LPA

LPA is an important intermediate product in the biosynthesis of phospholipids and triacylglycerol, and as a signaling molecule. Thus far, several hypothetical pathways of LPA production have been postulated. LPA is present in blood with plasma concentration ranges from 0.1 to 1μM, and serum concentrations up to 10μM [38]. Serum LPA can be generated by platelet-dependent and platelet-independent pathways [39]. However, the mechanism by which LPA is produced in different biological fluids (saliva, seminal fluid, ascites, synovial fluid, follicular fluid) is still not very clear. The concentration of LPA in different biological fluids has been reviewed elsewhere (Table 1) [25, 40]. LPA can be produced: (1) intracellularly from glycerol-3-phosphate and acyl-CoA by a glycerophosphate acyl transferase (GPAT) [39, 41]; (2) from monoacylglycerol (MAG) by a monoacylglycerol kinase in mitochondria and microsomes [4, 42]; (3) from phosphatidic acid (PA) by PLA_1- or PLA_2-(phospholipase A) mediated hydrolysis [43, 44], which is the main mechanism in activated platelets (PA can be generated from hydrolysis of phospholipids by a phospholipase D (PLD) or from phosphorylation of diacylglycerol by a diacylglycerol kinase); (4) from lysophospholipids by lyso-PLD-mediated hydrolysis (autotaxin, ATX) [45] which is the primary source of extracellular LPA; (5) from oxidative modification of low density lipoprotein (LDL) [42]. Serum LPA can be produced mainly through pathway (3) and (4) [39] (Figure 4). The enzymes involved in these two pathways have been thoroughly discussed in the review of Aoki [39]. As pathway (1) is conserved in lower organisms without evidence of extracellular LPA production, it is not considered to be involved in extracellular LPA-dependent signaling events [39].

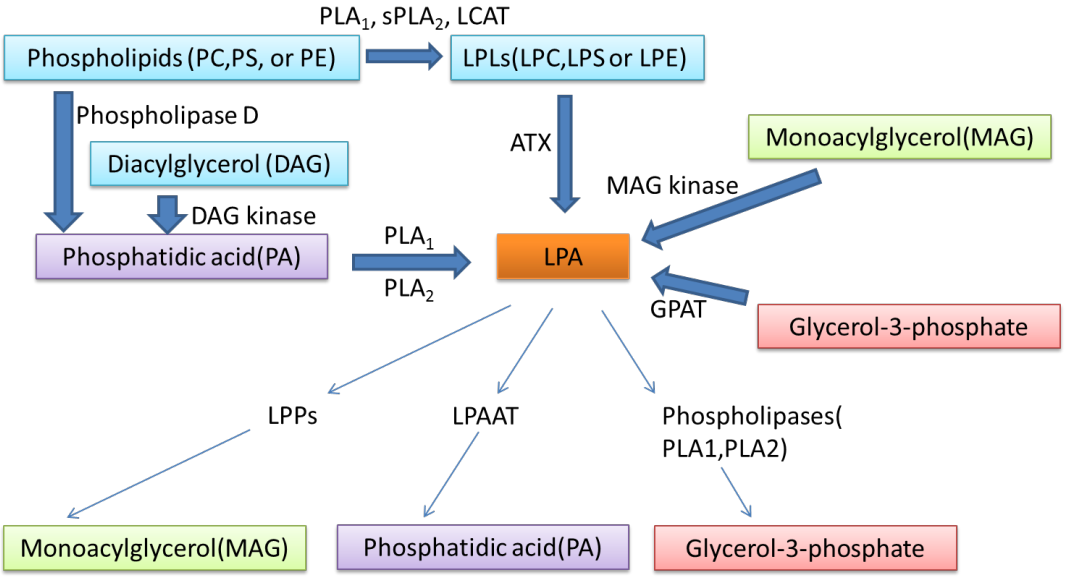


Figure 4. LPA metabolism schematic

LCAT: Lecithincholesterol acyltransferases

LPPs: lipid phosphate phosphatases

LPAAT: LPA acyltransferases

	Tissues/Fluids	[LPA]	[LPC]	Method of measurement
Physiological Conditions	Embryonic brain	0.32-0.35 pmol/mg	Not available	LC-MS
	Adult brain	3.7-35 pmol/mg	Not available	GC-MS, LC-MS-MS
	Nerve (spinal cord)	0.79 pmol/mg	Not available	B103 bioassay
	Plasma	0.7 μM / 0.17-0.63μM	100-140 μM / ~440μM	LC-MS, RH7777 bioassay
	Serum	4-15.5 μM	234 μM	TLC-G, enzymatic assay, LC-MS-MS
	Cerebrospinal fluid	0.025-0.2 pM	Not detected	RH7777 bioassay
	Seminal fluid	Negligible	8-19 μM	RH7777 bioassay, enzymatic cycling
	Saliva	0.785 nM	Not available	FAB-MS
	Lacrimal gland fluid	1.3 μM	Not available	MS
	Aqueous humor	0.2 μM	Not available	MS
	Follicular fluid	25.3 μM	157 μM	TLC-GC
	Acites	0.62 μM	Not available	RH7777 bioassay
	Fertilized hen white	8.0-9.6 μM	Not available	LC-MS
Pathophysiological Conditions	Nerve (injury)	74.8 pmol/mg	Not available	B103 bioassay
	Serum (systemic sclerosis)	5.5 μM	130 μM	LC-MS-MS
	Serum (sepsis)	Not available	43.5 μM	Enzymatic assay
	Acites (pancreatic cancer patients)	2.7 μM	Not available	RH7777 bioassay
	Plasma (chronic liver injury)	0.3-0.4 μM	Not available	Enzymatic assay
	Plasma (obesity)	Not available	83.5-84.4 μM / ~420-460 μM	LC-MS
	Synovial fluid(RA)	3.7 ± 2.2 μM	30-250 μM	RH7777 bioassay HPTLC

Table 1. Determination of LPA in different biological fluids. (Adapted from [25, 40])

1.1.4.2 The Enzyme Autotaxin

Autotaxin was first found to be secreted by melanoma cells and to be a chemoattractant for cancer cells [46]. Later autotaxin was proven to be the main enzyme responsible for LPA production which contributes to cancer cell migration (reviewed in [46, 47]).

(1) General

Autotaxin (ATX) is a member of the NPP (nucleotide pyrophosphatase/phosphodiesterase) family, and is also referred to as ENPP2 (ectonucleotide pyrophosphatase/phosphodiesterase). Similar to NPP1/ENPP1 and NPP3/ENPP3, ATX is a multi-domain protein consisting of two N-terminal Cys-rich somatomedin B-like (SMB) domains, a central catalytic phosphodiesterase (PDE) domain and a C terminal nuclease-like domain (NLD) that is catalytically inactive (Figure 5A) [46]. ENPP1 and ENPP3 are membrane-bound enzymes which can be cleaved into soluble protein functionally hydrolyzing nucleotides, while ENPP2 is a secreted form [47]. Though ATX also hydrolyzes nucleotides, similar to ENPP1 and ENPP3, it shows 10-fold enhanced affinity for LPC than for nucleotides. LPC enhances the ATX-dependent cell migration [48], demonstrating that ATX could hydrolyze LPC to produce LPA as a cell response stimulus. Besides the catalytic function to produce LPA as a lysophospholipase D (lysoPLD), ATX is also reported to hydrolyze other LPLs, such as LPE, LPS, LPI [49] and SPC [50].

(2) Isoform

The alternative splicing of ATX mRNA results in distinct isoforms, of which α, β, γ, and δ have been identified with similar lysoPLD activities and substrate preferences [47](Figure 5B). ATXβ is the most widely expressed and is the predominant isoform that has been thoroughly investigated. ATXγ is brain specific. But whether distinct ATX isoforms are associated with specific (patho) physiological conditions is not clear. One report showed that ATXα but not ATXβ, by specific binding to heparin and

heparan sulfate, has increased lysoPLD activity toward LPC [51]. The demonstration of the crystal structure of ATX (Figure 5C) will allow us to better understand the interaction between ATX and its substrates, and protein-protein interactions, as well as the modulation of enzyme activity.

(3) Structure/Function Relationships

As shown in Figure 5C, ATX crystal structure studies have elucidated its domain organization and interaction, including interaction between the central catalytic PDE domain and SMB domains, as well as interaction between the PDE domain and the NLD which binds Ca^{2+} by an EF hand-like motif. ENPP1 and ATX/ENPP2 both harbor a shallow pocket for accommodating and binding the nucleotide for breaking the phosphodiester bond, which also accommodates LPLs (Figure 5C). Compared with ENPP1 or other phospholipases, ATX displays a distinct deep hydrophobic lipid-binding pocket (Figure 5C and Figure 6) unique for hydrolyzing LPLs and a tunnel through which LPA exits. The function of the deep pocket is confirmed by mutational and biochemical studies. This deep pocket is distinct from the autoinhibitory loop of secreted phospholipase A2 (sPLA2) which functions as an interfacial enzyme. The fourth feature of ATX is a SMB domain with integrin binding properties. In ATX, this domain interacts with the catalytic domain. The SMB domain of ATX has been found to bind β1 and β3 integrins in activated platelets and other cells for localizing LPA synthesis to the cell surface (Figure 6) [52]. As one example of this, ATX binds to integrins, then releases LPA which itself stimulates lymphocytes through their LPA receptors, enhancing their motility [53]. Some researchers suggest that ATX may interact with integrins to exert non-catalytic signaling functions like stimulating cell proliferation, which is independent of LPA signaling [54].

(4) Genetics

ATX showed physiological importance in embryonic development from the evidence that ATX knockout mice (*Enpp2*-/-) die before embryonic day 10 with clear vascular and neural tube defects [55]. *Enpp2*-/+ heterozygotes, survive until adulthood, but have only half the plasma LPA level of wild-type mice [56], and showed attenuated neuropathic pain [57]. Since the phenotypes of ATX knockout mice are more severe than those of any LPA receptor knockout mice (listed in Table 2), it is possible that other as yet unidentified LPA receptors may be involved in ATX function, or certain LPA receptors may have redundant functions, or ATX could function through other mechanisms unrelated to LPA signaling.

Receptors	**Lethality**	**Phenotypes of KO mice**
LPA_1	Semi-lethality	Impaired suckling behaviour Decreased postnatal growth rate, reduced size Craniofacial dysmorphism Frontal hematoma Increased apoptosis in sciatic nerve Schwann cells Changes in neurotransmitters (schizophrenic-like pathology) Inhibition of the neuropathic pain (inhibition of demyelination) Proliferation of preadipocyte and inhibition of adipocyte differentiation Cerebral cortex growth and folding *ex vivo* (LPA_1/LPA_2-deficient mice) Inhibition of renal tubulointerstitial fibrosis Inhibition of pulmonary fibrosis
LPA_2	Viable	Inhibition of cholera toxin-induced secretory diarrhea Inhibition of tumour formation in an colitis-associated cancer
LPA_3	Viable	Disruption of spacio-temporal blastocyst implantation in uterus
LPA_4	Viable	Enhanced migratory response stimulated with LPA in fibroblasts
LPA_5	N.D.	N.D.
LPA_6/P2Y5	N.D.	Hypotrichosis (human)

Table 2. Phenotypes of LPA receptor knockout mice (from [46])

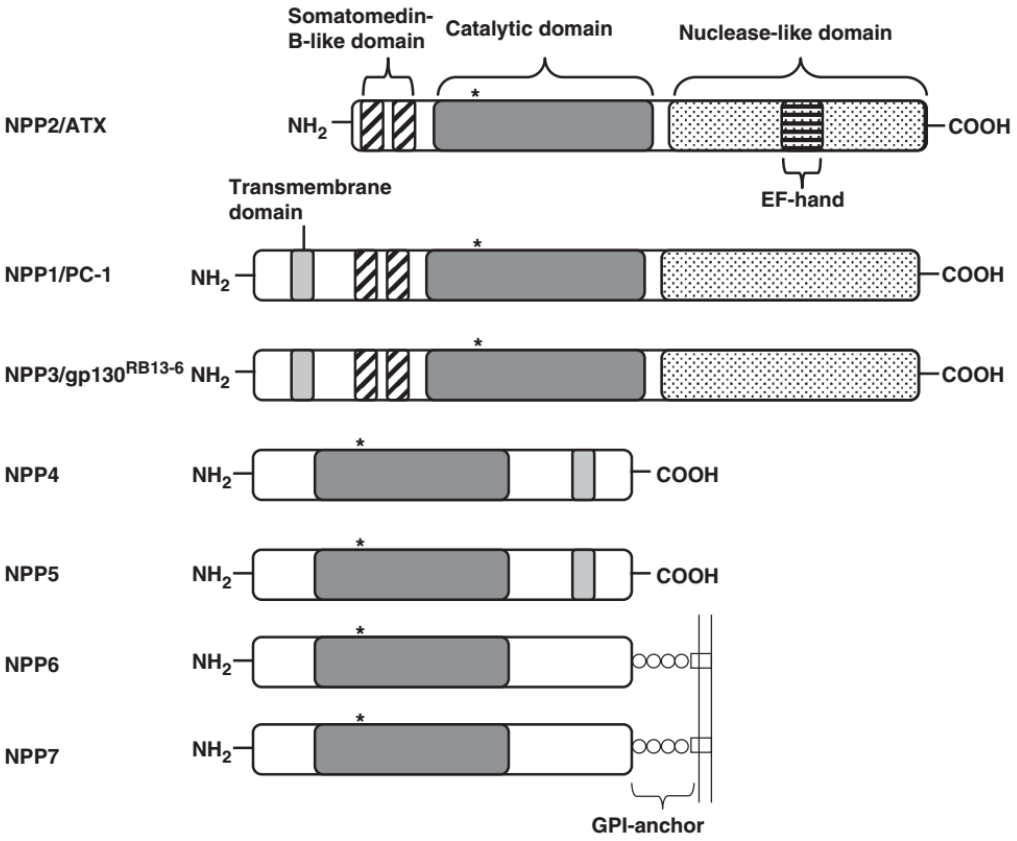


Figure 5A. Domain organization of NPP/ENPP family members (from [46])

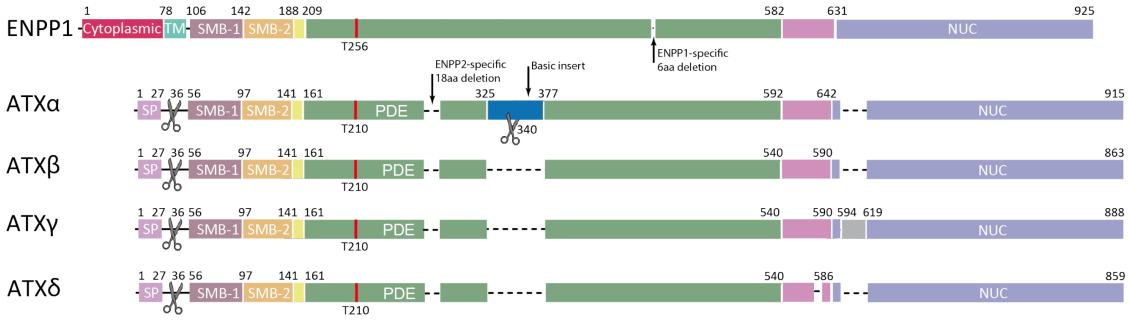


Figure 5B. Different domain organizations of ATX isoforms compared with that of ENPP1.

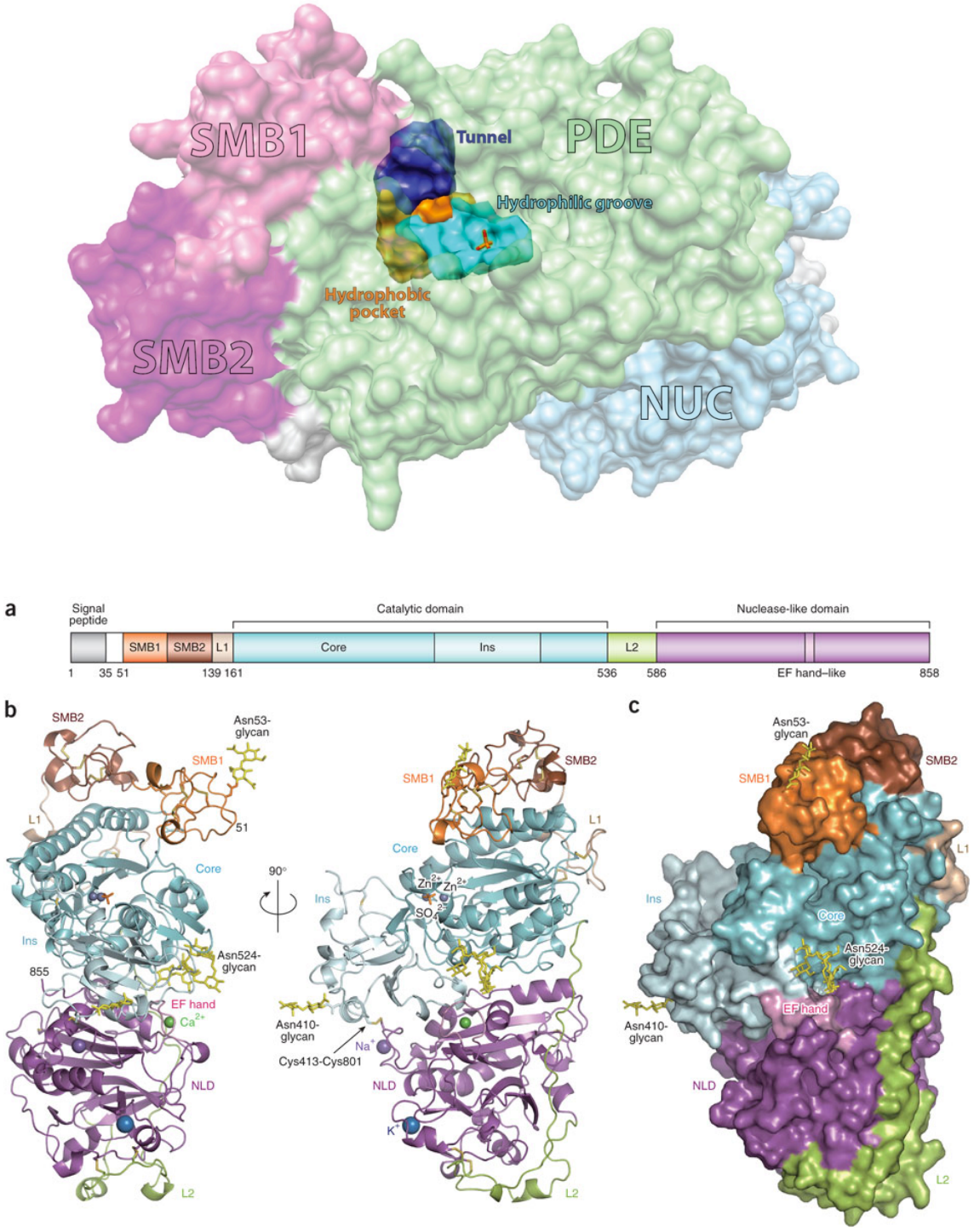


Figure 5C. ATX crystal structure

Figure 5. Domain organization and crystal structure of ATX (From [47, 58])

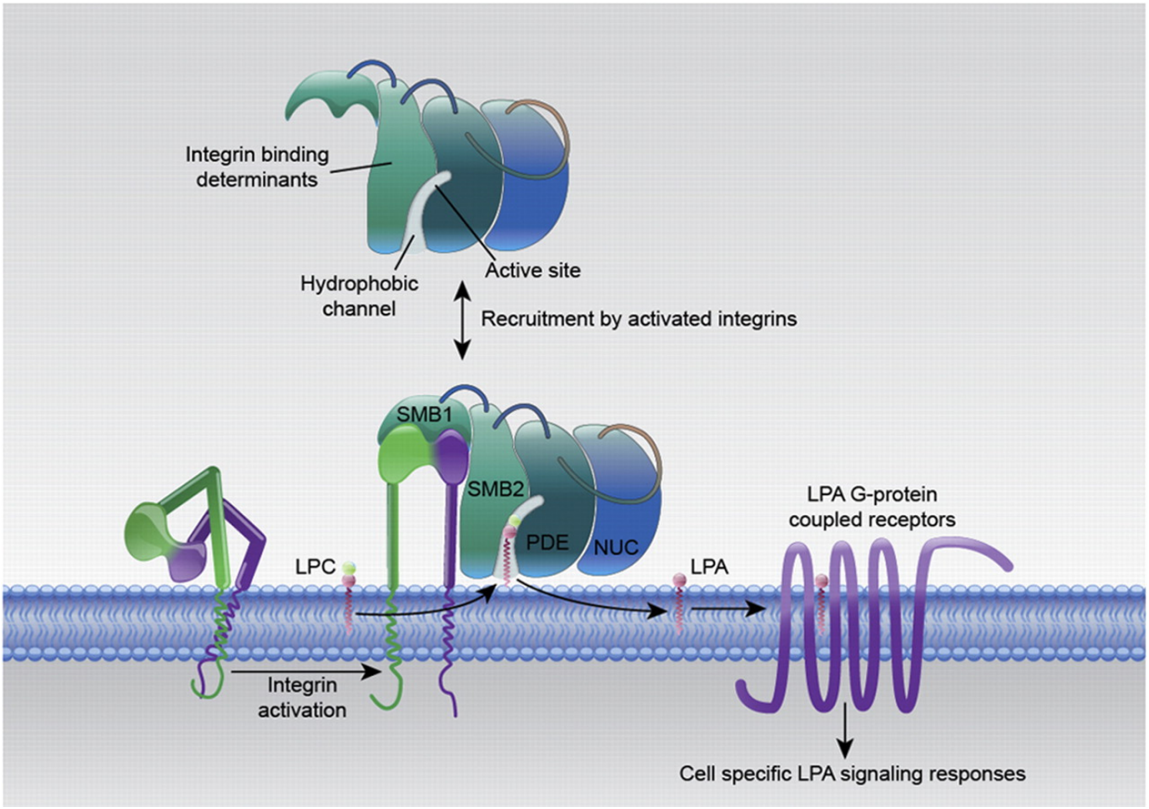


Figure 6. Illustration of the interaction between ATX, activated integrin and LPC (From [52])

1.1.4.3 Degradation of LPA

As shown in Figure 4, LPA can be degraded through three distinct pathways: dephosphorylation into MAG by a family of lipid phosphate phosphatases (LPPs, including isoforms LPP1, LPP1a, LPP2, LPP3), which also dephosphorylate sphingosine-1-phosphate, phosphatidic acid and ceramide 1-phosphate in a non-specific manner; conversion back to PA by LPA acyltransferases (LPAAT, including five members named LPAATα, β, γ, δ, ε); hydrolysis into glycerol phosphate through LPA-specific phospholipases [42].

1.1.5 Carrier Proteins of LPA

Early studies related to LPA identified bovine or human serum albumin (BSA or HSA) [4, 42, 59], and liver fatty acid-binding protein [60] as endogenous LPA-binding proteins. Plasma gelsolin, the secreted form of gelsolin which is an intracellular actin binding protein, also binds LPA with nanomolar affinity [42]. In addition, FABP3 (fatty acid binding protein 3) has been identified as an intracellular LPA carrier protein in human coronary artery endothelial cells, regulating LPA-induced PPARγ activation in the nucleus [61]. Lipoprotein is known to be a lipid transportation complex. LDL is one of the major lipoproteins that plays a role in atherosclerosis [62]. In the same study, LPA is reported to be produced during oxidation of LDL, which actively initiates platelet activation. Phosphatidylcholine (PC), the major phospholipid component of VLDL [63], has been mentioned previously to generate LPA by two pathways through hydrolysis by PLA and PLD. High density lipoprotein (HDL) was identified as a carrier protein for several lysophospholipids including S1P, sphingosylphosphorylcholine (SPC) and lysosulfatide (LSF) [64]. S1P has been shown to bind to apolipoprotein M (ApoM) [65]. ApoM-bound S1P can activate $S1P_1$ receptor on endothelial cells [66]. About 55% S1P in plasma is bound to HDL via ApoM and 35% to albumin [67]. Only ~10% of plasma S1P resides in other lipoproteins such as LDL and VLDL. It is possible that ApoM could function as a carrier protein of LPA species in a similar way as S1P, since there is LPA production during LDL oxidization and from VLDL. A novel approach was reported for measuring S1P and LPA binding to carrier proteins using monoclonal antibodies and the Kinetic Exclusion Assay [68], which will help to understand more about potential LPA carrier proteins and their role in controlling LPA levels. One research group found that a low concentration of albumin (15μM) could inhibit activation of LPA receptor, implying an important role for LPA carrier protein in LPA-mediated cell responses [69]. LPA in biological fluids may also form micelles

with proteins on the lipid droplet surface that can increase the stability of the micelles [70], which is important for LPA transportation and half-life in biological fluids.

1.1.6 LPA Receptors and Their Signaling

As an extracellular signaling molecule, LPA stimulates cellular responses through binding to the 7-transmembrane domain of G protein-coupled receptors (GPCRs) and activating downstream G proteins [24]. Early reports named the first three LPA receptors LPA_1, LPA_2, and LPA_3 as EDG-2, EDG-4, and EDG-5 respectively (the endothelial differentiation, G-protein-coupled receptor gene family, EDG) (Table 3). The six LPA receptors and five sphingosine-1-phosphate (S1P) receptors constitute lysophospholipid receptors (LPL-R) [71]. GPCRs that bind specifically LPA have had their nomenclature updated recently: protein names LPA_1 – LPA_6, and italicized gene names *LPAR1-LPAR6* (human) and *Lpar1-Lpar6* (non-human) [24]. These GPCRs are believed to couple to one or more of the four classes of heterotrimeric G proteins ($G_{\alpha 12/13}$, $G_{\alpha q/11}$, $G_{\alpha i/o}$, and $G_{\alpha s}$) [72], though signaling without this coupling may also be possible [73]. Activation of these receptors and G proteins in turn exerts a broad range of biological and pathological effects by activating various downstream signaling pathways (some of them are shown in Table 3) [25, 33, 74, 75].

Name (gene)	Synonyms	Chromosomal location	Coupled G proteins	Cellular function
LP_{A1} (*lp_{A1}*)	VZG-1 MREC1.3 GPCR26 EDG-2	Mouse 4, close to *vc* Human 9q31.3-32	$G_{i/o}$ $G_{q/11/14}$ $G_{12/13}$	AC inhibition SRE activation DNA synthesis PLC activation Actomyosin stimulation
LP_{A2} (*lp_{A2}*)	Nonmutated EDG-4	Mouse 8, close to *kat* Human 19p12	$G_{i/o}$ $G_{q/11/14}$ $G_{12/13}$	AC inhibition MAP kinase activation SRE activation PLC activation Actomyosin stimulation
LP_{A3} (*lp_{A3}*)	EDG-7 (EDG-5)	Mouse 3, close to *va* Human 1	$G_{i/o}$ $G_{q/11/14}$	AC stimulation or inhibition MAP kinase activation PLC activation

AC, adenylyl cyclase; SRE, serum response element; PLC, phospholipase C.

Table 3. Characteristics of LPL receptors (from [33])

1.1.6.1 LPA_1

LPA_1 (previous name: VZG-1/EDG-2/mrec1.3) was the first identified and the best characterized high affinity receptor for LPA [15]. The mouse cDNA of LPA_1 encodes a 41-kDa protein consisting of 364 amino acids with 7 transmembrane domains, which is highly conserved in other mammalian (human and rat) *Lpar1* genes [33]. It shares ~50-60% amino acid sequence identity with LPA_2 and LPA_3, and 30%, 32% and 37% amino acid identity with the cannabinoid receptors, melanocortin receptor and $S1P_1$ receptor, respectively [76, 77]. The unique feature of LPA_1 gene structure is that it contains five exons with one conserved intron (shared within *Lpar1-3*) located within the middle of transmembrane domain VI [76]. Of note, a variant of *Lpar1* (mrec1.3) with a 18-amino acid deletion of the N terminus exists, but its biological significance is still unknown [78].

LPA_1 is widely expressed in mouse and human tissues, particularly in the developing nervous system (reviewed in [23]). The gene expression chart file of LPA_1 can be found at the BioGPS website (http://biogps.org/). After binding to LPA_1, LPA signals through the G proteins $G_{i/o}$, G_q, and $G_{12/13}$. In this way, cellular responses such as cell proliferation and cell migration can be initiated by LPA. These activated G proteins activate phospholipase C (PLC), mobilize Ca^{2+}, activate the kinases MAPK, Akt, and Rho kinase, and induce binding to the serum-response element (SRE) (Table 3, Figure 1) (reviewed in [23, 33, 79]). One interesting function of LPA_1 is that LPA mediates cell rounding in neuronal cells through $G_{12/13}$ by Rho kinase activation [80], but mediates cell adhesion in other cell types at least in part through the same receptor [81]. Other cellular responses mediated by LPA_1 have also been reviewed in [25].

Half of LPA_1 gene knockout mice die perinatally [23, 82]. A defective sense of smell, smaller body size, a blunted snout, and brain defects including cortical thinning are those main phenotypes reported in LPA_1 gene knockout mice [82, 83].

1.1.6.2 LPA_2

LPA_2 was identified through GenBank searches of orphan GPCR genes after LPA_1 identification. The amino acid sequence and molecular weight of LPA_2 are very similar to that of LPA_1 (Table 3). *Lpar2* mRNA expression is relatively restricted, with high expression levels in kidney, uterus, and testis; but lower expression in brain, heart, thymus, spleen and stomach compared with that of *Lpar1* in mice [84]. Human *LPAR2* mRNA is highly expressed in testis and leukocytes [25]. LPA_2 has been reported to be involved in cancer development with aberrant expression in several cancer cell lines [85-87]. Moreover, *LPAR2* gene expression was also reported in dendritic cells and unstimulated T cells (reviewed in [24]), which is different from *LPAR1* that is predominantly expressed in stimulated T cells.

LPA_2 was reported to couple to the same heterotrimeric G proteins as LPA1 (Figure 1 and Table 3), yielding similar cellular responses through the same downstream signaling pathway. LPA_2 signaling is reported to be mainly involved in cell migration and survival, especially tumor metastasis [24, 88].

Lpar2−/− mice are viable and normal, which is different from *Lpar1* knockout mice. However, double-null mutants (*Lpar1*−/−/*Lpar2*−/−) present exacerbated frontal hematomas, and decreased response to LPA stimulation in primary fibroblasts and embryonic cortical neurons, suggesting the existence of redundant functions for LPA_1 and LPA_2 [89]. *Lpar2*−/− mice also have a defect in CD4+ T cell early migration through HEVs and in lymph nodes [90], indicating a role for LPA_2 in T cell migration from the blood stream into lymphoid organs.

1.1.6.3 LPA_3

LPA_3 (EDG7) was also discovered and identified as LPA receptor using the same strategy as for LPA_2. Compared with LPA_1 and LPA_2 which can be stimulated by LPA with both saturated and unsaturated fatty acids, LPA_3 has a higher affinity for 2-acyl-LPA with unsaturated fatty acid chains (18:1, 18:2 and 18:3) when assessed in

Ca^{2+} mobilization assays [35, 91, 92]. LPA_3 gene expression is also more restricted compared with that of LPA_1. Though human *LPAR3* mRNA is highly expressed in heart, testis, prostate, and pancreas; and mouse *Lpar3* mRNA is highly expressed in lung, kidney, uterus and testis [23, 93], the function of LPA_3 was reported more related to reproduction through studies using gene knockout mice. One report showed that progesterone and estrogen cooperatively regulate LPA_3 expression in mouse uteri [94]. Another report showed that female *Lpar3*−/− mice are viable but have a defect in embryo implantation and exhibit smaller litter size [95, 96], demonstrating a role in mammalian reproduction. LPA_3 can couple to heterotrimeric G proteins $G_{\alpha i/o}$ and $G_{\alpha q}$, but is unable to couple to $G_{\alpha 12/13}$ (Table 3), which is shown in LPAR overexpression systems: LPA_3 does not mediate cell rounding in neuronal cells which requires signaling through $G_{\alpha 12/13}$ and Rho kinase [97]. Although the efficacy and potency of LPA varies from LPA_1 to LPA_3, these three receptors could mediate LPA-dependent inositol phosphate production, PLC activation, modulation of adenylyl cyclase, Ca^{2+} mobilization, MAPK activation, and arachidonic acid release through multiple G proteins [97] (Table 3, Figure 1). In addition, LPA_3 is involved in promoting erythropoiesis and megakaryopoiesis [98, 99], which contrasts with LPA_2 that was found to suppress erythropoiesis and megakaryopoiesis using pharmacological blockers and knockdown experiments *in vitro*.

1.1.6.4 LPA_4

Analysis of the expressed sequence tag (EST) database successfully identified the orphan receptor p2y9/GPR23 [100, 101], which was later reclassified and renamed LPA_4 after ligand screening data demonstrated specific LPA binding [18]. Distinct from LPA_1, LPA_2 and LPA_3, which belong to the Edg family, LPA_4 shares more homology with the P2Y purinergic receptor named p2y9. However, LPA_4 shows no nucleotide or nucleoside ligand binding affinity [18, 100]. LPA_4 has more specific binding affinity for

1-oleoyl-LPA (18:1-LPA) compared to other LPA species [18]. Human *LPAR4* mRNA is highly expressed in the ovaries compared to other tissues [18, 23]. In mouse, *Lpar4* mRNA is mainly expressed in bone marrow stromal cells and in mesenchymal stem cells (data available in BioGPS gene expression database: http://www.biogps.org) [102]. LPA_4 mediates morphological changes including neurite retraction, cell rounding and stress fiber formation through the $G_{\alpha 12/13}$ and Rho/Rho-kinase pathways (the same observation was reported in LPA_1-, LPA_2-, and LPA_5-expressing cells), and promotes intracellular cAMP accumulation through $G_{\alpha s}$ (Fig. 4) [23, 103, 104]. Notably, $G_{\alpha s}$-coupling is only reported for LPA_4 signaling but not for $LPA_{1\text{-}3}$ signaling (Fig. 4) [103, 104]. In contrast to LPA_1-LPA_3 that stimulate cell motility, LPA_4 suppresses LPA-dependent cell migration and invasion in fibroblasts and colon cancer cells [105].

LPA_4-deficient mice display no apparent abnormalities [105], except increased trabecular bone volume and thickness [106]. LPA_4 deficiency in a human mesenchymal stem cell line demonstrated the role of LPA_4 in the inhibition of osteoblastic differentiation [106]. LPA_1 and LPA_4 have opposite effects on bone metabolism, where LPA_1 hinders bone resorption and LPA_4 promotes bone resorption [107], indicating that both receptors are involved in the regulation of osteoblast and osteoclast functions. *Lpar4*-/- mice showed partial embryonic lethality due to the abnormal development of blood and lymphatic vessels [108]. One report showed that LPA_4-deficient mice also exhibit a delay in the recovery of hematopoietic stem cell numbers in the bone marrow and spleen, and were highly sensitive to myelosuppression [102], suggesting a role in hematopoiesis. Another report showed that in LPA_4 knockout mice, lymphocytes accumulated in the high endothelial venule (HEV) endothelial cell layer and there was a mild reduction of lymphocyte transmigration across HEVs, indicating a role for LPA_4 in regulating lymphocyte extravasation across HEV and entry into the peripheral lymph node [109].

1.1.6.5 LPA_5

LPA_5 (GPR92) was identified in 2006 [19, 21], and is structurally closer to LPA_4 (35% homology), and dissimilar to LPA_{1-3} (~22% homology) [21]. LPA_5 exhibits a higher affinity (~6.4 nM) than that of LPA_4 (45 nM) for 1-oleoyl 18:1-LPA [18, 19]. Oh *et al.* in 2008 [110] suggested that two other naturally occurring ligands, farnesyl pyrophosphate (FPP) and N-arachidonoylglycine (NAGly), are more potent LPA_5 agonists than LPA 18:1. However, the data of Williams *et al.* in 2009 [111] showed that LPA18:1 is a more potent ligand of LPA_5 than farnesyl phosphate analogs (EC50 = 8.9 ± 0.7nM for LPA 18:1; 40 ± 15nM for FPP; 49 ± 13nM for farnesyl monophosphate), thus confirming LPA_5 as a member of the non-Edg receptor family. LPA_5 mRNA is widely expressed in mouse tissues, including placenta, brain, gut, spleen, and dorsal root ganglion neurons, and is highly expressed in heart and gastrointestinal tract [19, 112]. Recently, *Lpar5* expression was also reported in the early embryonic mouse brain, thereby suggesting a role in brain development [25]. In humans, *LPAR5* mRNA is highly expressed in the spleen, moderately expressed in the small intestine, heart, and placenta, and to a lesser degree in the colon, and liver [23]. LPA_5 and LPA_4, are the most abundant LPA receptor mRNA transcripts in human platelets [69, 113].

Cell lines expressing LPA_5 show that LPA induces neurite retraction, stress fiber formation, and receptor internalization through $G\alpha_{12/13}$; increased intracellular calcium levels and cAMP accumulation through Gq [21]. LPA_5 expressed by CD8 T cells was reported to suppress antigen receptor signaling, cell activation and proliferation *in vitro* and *in vivo* [114]. Interestingly, LPA_5 also negatively regulates BCR signaling, B cell activation and immune response via a $G\alpha_{12/13}$-Arhgef1 pathway [115]. LPA_5 is found to be highly expressed in human mast cells and microglia cells [116]. Inhibition of LPA_5 by its small molecular inhibitor (specific non-lipid LPA_5 antagonist) prevents the activation of these cells [116, 117], indicating its role in chronic neuroinflammation. LPA_5 knock-out mice were less sensitive to pain, were more hyperactive at night, and

were less anxious and less socially connected, implying a role for LPA_5 in pain reception and normal brain function [112, 118]. Recent reports showed that LPA_5 is also expressed in an increased level in mouse melanoma cells [119]. *In vitro* and *in vivo* data from gene silencing and gene knockout demonstrate that LPA_5 inhibits melanoma cell invasion, whereas LPA_2 stimulates cell invasion [114, 119-122].

1.1.6.6 LPA_6

LPA_6 (P2Y5) is a member of the P2Y receptor family. LPA6 plays a role in hair growth and hair follicle development as a mutation in LPA_6 was found in a family with hypotrichosis simplex, a disease with familial hair loss [20, 123]. A method detecting GPCR activation called TGF-α shedding assay, identified LPA_6 as an LPA receptor [124]. LPA_6 has some preference for 2-acyl-LPA vs. 1-acyl-LPA, and requires high concentrations of LPA to mediate cell responses [125]. LPA_6 stimulates cAMP accumulation and morphological change through G_{13}-Rho signaling (Figure 1).

LPA_6 knockdown inhibited pancreatic cancer cell and endothelial cell motility, as well as cancer cell invasiveness, which is an observation opposite to LPA_5 knockdown [122]. LPA_6 KO mice also showed lymphocyte accumulation in HEV endothelial cell layers, but did not have a defect in lymphocyte extravasation, indicating a different role for LPA_6 in regulating lymphocyte extravasation across HEV and entry into the peripheral lymph nodes [109].

1.1.7 Other Proposed LPA Receptors

LPA was also reported to be an agonist of other GPCRs including GPR87 [69, 126], GPR35 [127] and GPR45/PSP24 [128], as well as nuclear hormone PPARγ receptors [22] in cell lines overexpressing these receptors. However, these receptors bind many other ligands such as uridine diphosphate-glucose and cysteinyl-leukotrienes for GPR87 [129], and kynurenic acid for GPR35 [130]. It is still unclear whether these

receptors are physiologically related LPA receptors. Further investigation of their physiological significance is proposed in the future by some researchers [131].

1.1.8 Intracellular signaling pathways- MAPK cascades

The mitogen-activated protein kinases (MAPKs) are one of the best-known signal transduction families involved in LPA signaling. MAPKs are critical for regulating cellular processes such as growth, differentiation and apoptosis [132]. Stimuli that are capable of activating MAPKs include growth factors, cytokines, neurotransmitters and hormones. These signaling molecules transmit their signals either through receptor tyrosine kinases, G-protein coupled receptors or hormone receptors. They function as effector kinases of MAPK cascades composed of three kinases: The above-mentioned environmental stimuli transmit the signals through their receptors to activate a MAP kinase kinase kinase (MAPKKK/MAP3K), leading to activation of a MAP kinase kinase (MAPKK/MAP2K), and finally MAPK that modulates a cellular response by phosphorylation of protein substrates (Figure 7). The dominant multifunctional effector of this MAPK cascade is the MAPK element. Mammalian MAPK subgroups include: ERK1/2; ERK3/4; JNK1/2/3; p38 proteins (α/β/δ/γ); ERK5, ERK8 (ERK7 is the rat homolog) and NLK (Nemo-Like Kinase, Serine/threonine protein kinase).

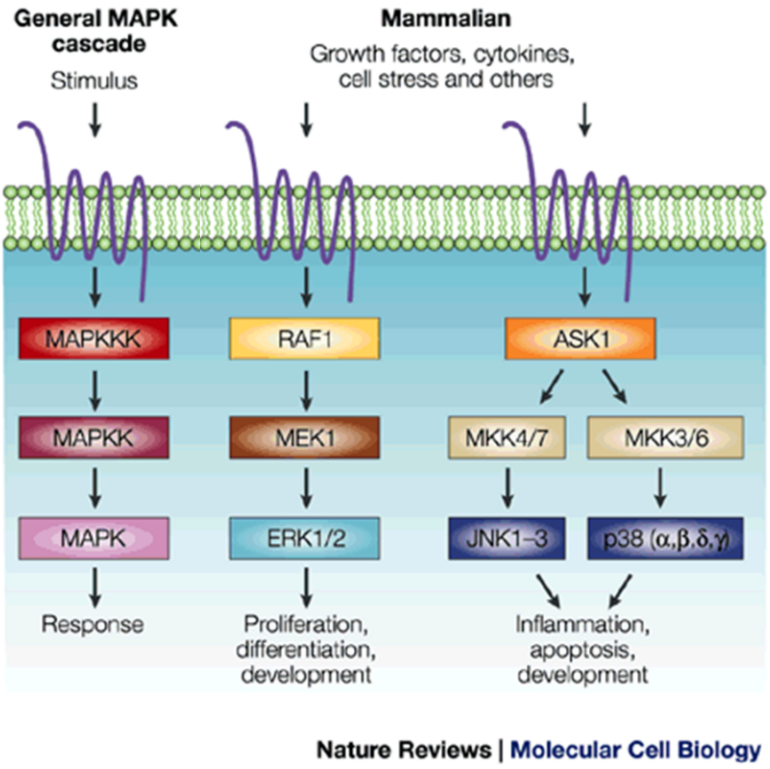


Figure 7. Schematic overview of MAPK cascades (From [133])

As an evolutionally conserved kinase, extracellular-signal-regulated kinase (ERK) phosphorylates various substrates (enzymes, transcription factors and cytoskeletal proteins). ERK signaling is well known to play a crucial role in cell growth, proliferation, differentiation, migration and survival [133]. Differences in the duration (sustained or transient activation), magnitude (strong or weak activation) and subcellular compartmentalization (cytoplasmic or nuclear activation) of ERK activity determine signaling specificity [134]. In addition, several proteins were found to act as spatial, temporal or strength-controlling regulators of ERK activity (such as Sef, PEA-15 and KSR) [134]. ERK1 and ERK2 (ERK1/2) are known to be activated by the upstream MAP/ERK kinase 1 (MEK1) and MEK2 (MEK1/2, MAPKK family members) [135]. First, MAPKKK-mediated phosphorylation leads to MEK1/2 activation; activated MEK1/2 then phosphorylates threonine and tyrosine residues of ERK1/2

(ERK activation). Activated ERK1/2 then phosphorylates many substrates (transcription factors, such as Elk1 and c-Myc; and protein kinases, such as RSK and MSK) [136]. Subsequently, expression of immediate early genes, such as c-Fos, is induced [137]. ERK1 gene knockout mice are viable and fertile [138] but ERK2 knockout is embryonic lethal [139]. B-cell-specific double ERK1 and ERK2 null mice have defect in B cell development [140], while T cell development defect is observed in either ERK1 or ERK2 T cell-specific deletion [141], indicating a pivotal role of ERK in lymphocyte development.

The p38 MAPK cascade is activated by a broad spectrum of cellular stress stimuli, such as UV-C or ionizing radiation, heat or oxidative stress, hypoxia, PAMPs, heavy metals and some antibiotics [136, 142]. It is also activated by a variety of physiological signaling molecules, including pro-inflammatory cytokines and retinoic acid [143]. These stimuli employ several distinct MAPKKKs to activate MKK3 and MKK6 (MAPKKs), which finally activates the p38 family members p38α, p38β, p38γ and p38δ. These four kinases share approximately 60% amino acid sequence similarity. They also differ highly in their expression patterns, substrate specificities and sensitivities to inhibitors [144]. MKK3, 4 and 6 are kinases known to activate p38 MAPKs [145]. The p38MAPK pathway regulates cytokine synthesis and several inhibitors of p38MAPK have been evaluated in clinical trials for the treatment of autoimmune and inflammatory diseases [146-148].

Methods to identify physiological substrates for p38α and p38β include using specific inhibitors such as SB203580 and SB202190, as well as using mice deficient for each p38 MAPK. Pharmacological inhibitors, siRNA and shRNA are widely used and effective tools for elucidating these signaling pathways. Knockout of p38α in mice results in embryonic lethality, whereas p38β, p38γ and p38δ and double p38γ/p38δ knockout mice are viable and fertile [144]. p38α and p38β are able to phosphorylate transcription factors directly or activate transcription factors through phosphorylating

other protein kinases, other structural proteins or metabolism-related enzymes (reviewed in [144]).

The mitogen and stress-activated kinases-1 and -2 (MSK1 and MSK2) were discovered as protein kinases that mediate signal transduction by MAPK cascades [149]. MSK1 and MSK2 are activated by ERK1/2 and the p38 family (Figure 8) [150]. MSKs contain several different domains, motifs and phosphorylation sites (See Appendix Figure 1). MSK phosphorylation ultimately activate the N-terminal kinase domain (reviewed in [142]). In stage 1, MSK is locked in its inactive state due to autoinhibitory elements and lack of activating phosphorylation. In stage 2, active ERK1/2 or p38 bound to the MAPK docking site in MSK, phosphorylates Ser360, Thr581, and Thr700. Phosphorylation of these site activates the C-terminal domain. In stage 3, the activated C-terminal domain phosphorylates Ser212, Ser376, and Ser381. This phosphorylation leads to N-terminal domain activation, then phosphorylation of other auto-phosphorylation sites in MSK and phosphorylation of the downstream substrates of MSK. Well validated MSK substrates include CREB, ATF-1, Histone H3, HMGN1, NF-κBp65 and RARα1 (Retinoic acid receptor). MSK1 and MSK2 knockout mice are viable and fertile in pathogen-free conditions [151]. MSK1 and MSK2 double knockout mice were found to be hypersensitive to lipopolysaccharide (LPS)-induced endotoxic shock [152]. MSK1 and MSK2 are believed to down-regulate Toll-like receptor-driven inflammation, especially by limiting the production of proinflammatory cytokines and promoting DUSP1 expression (MAPK phosphatase DUSP1 deactivates p38) in response to LPS in macrophages, implying a role for MSKs in regulating innate immunity. MSKs are also implicated in neurodegeneration and synaptic plasticity [153]. The impact of MSKs in RA has been summarized and highlighted in the appendix.

CREB (cAMP response element-binding protein) is a transcription factor capable of binding DNA via the bZIP domain (Basic Leucine Zipper Domain) and regulating gene expression. CREB is comprised of several conserved domains, including the bZIP, Q1, KID and Q2/CAD domains (Figure 9) [142]. The KID domain of CREB is the

critical domain for the regulation of CREB activity. The KID domain contains several sites (Ser 133, Ser129, Ser142) for posttranslational modification. Ser 133 phosphorylation has been the most extensively studied and was considered to be critical for the regulation of CREB activity. CREB can be activated downstream of a number signaling pathways, including cAMP, Ca2+, and MAPK-dependent pathways [154]. These pathways all promote the phosphorylation of CREB on Ser133. Three groups of MAPK activated kinases have been linked to CREB phosphorylation: RSKs, MSKs, and MK2/3 [155]. MSKs seem to be the major MAPK activated CREB kinases in cells. Studies using a specific MSK inhibitor SB747651A [156], MSK1/2 double knockout mice [151] and knockin mice with mutation of Ser133 in CREB have confirmed a role for MSK in phosphorylating CREB [157]. In macrophages, TLR agonist LPS induces CREB phosphorylation also via MSK1 and MSK2 [152].

Rho kinase (also named ROCK/ROK) is a serine/threonine kinase that was identified as one effector of Rho, a small GTPase [158]. It plays a dominant role in regulating cytoskeletal reorganization downstream of GPCR. Rho kinase also plays various roles in regulating cell migration, morphology, proliferation, and gene expression. The Rho kinase family has two members: Rho kinase α and β (alternative names ROCK2 and ROCK1, respectively). Both are composed of a catalytic domain (N-domain), a coiled coil domain with Rho binding domain (middle-domain), and a pleckstrin homology (PH) domain (C-domain) [159]. ROCK1 and ROCK2 knock-out mice die in early postnatal life [159]. Double heterozygous offspring created by crossing ROCK1+/- with ROCK2+/- mice are fertile, but their numbers are less than expected by Mendelian inheritance, showing some synthetic lethality [160]. Y-27632 is a broadly used inhibitor of Rho kinase. It selectively inhibits Rho kinase in an ATP-competitive manner. Studies on the structure of the Rho-kinase-Y-27632 complex showed the mechanism of action of this inhibitor: induced-fit conformational change of this complex increased the binding of Y-27632 with the phosphate loop of Rho kinase [161]. The substrates of Rho kinase include myosin light chain, ERM

(Ezrin/Radixin/Moesin) domain proteins, MAP-2/Tau, Calponin, endophilin, LIM-kinase, eNOS, etc.[162]. Some recent studies indicate a role of Rho kinase on cytokine [163] and chemokine [164, 165] regulation in immune responses. A previous study in our lab found that Y-27632 could inhibit p38MAPK to decrease cytokine/chemokine secretion. It would be interesting to investigate the involvement of Rho kinase in regulating actin organization and cytokine/chemokine expression simultaneously.

PI3Ks (phosphoinositide 3-kinases) are capable of converting its substrate phosphatidylinositol (3, 4)-bisphosphate (PIP2) to phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3) by adding a phosphate at the 3-OH group of the inositol ring [166]. Receptor tyrosine kinases and GPCRs can activate PI3Ks, stimuli of which are growth factors and cytokines. Activated PI3Ks then generate phospholipids to activate AKT and other downstream effector pathways, mediating cell survival, proliferation and differentiation. Based on their structure, PI3Ks are divided into three classes: Class I (A and B), Class II and Class III [167]. The only difference between Class IA and Class IB PI3Ks in the structures situates in adaptor/regulatory subunit. Class IA catalytic subunits include three isoforms p110α, β and δ (110–120 kDa), these constitutively bind the adaptor subunit p85 (in one of the two isoforms, α and β), which contains the lipid substrate binding activity [168]. Class IB consists of a catalytic subunit p110γ and a regulatory subunit p101 or p84 [168, 169]. Class II PI3Ks (PI3K-C2α, β or γ) are large (170–210 kDa) proteins that share similar catalytic domain with Class I PI3Ks (45–50% similarity) and also have distinct domains: a coiled-coil domain, a proline-rich domain, and a Phox domain [170, 171]. Class II PI3Ks preferentially phosphorylate PI and PIP, while Class I PI3Ks are able to phosphorylate PI, PIP, or PIP2 [170]. Class III PI3K can only phosphorylate PI and is capable of forming a heterodimer with Vps15 (vacuolar protein sorting-associated protein) bound to the intracellular membrane [167]. Potential downstream effectors of PI3K include p70S6 kinase, Akt kinase, RAC, GSK3 and protein kinase C [167, 168]. Wortmannin,

discovered as a fungal metabolite with anti-inflammatory activities, is a relatively more selective PI3K inhibitor [172]. The IC50 values for Class I and Class III PI3K are in the range of 1-10 nM, for Class II PI3Ks, in the range of 50-450nM [170]. Some PI3K inhibitors have been processed through clinical phases for treatment of cancer and inflammation.

As mentioned above, LPA receptors may mediate cell responses through a MAPK cascade or even Rho kinase or PI3K signaling. Depending on the cell type, regulation of LPA-induced IL-8 expression depends on the G_i protein, p38MAPK, ERK1/2, JNK, PKCδ, phosphoinositide-3-kinase and/or Rho, as well as on activation of several transcription factors, such as AP-1 (c-Jun) and NF-κB [173-175]. In this thesis, we will focus on the role of LPA in cytokine/chemokine secretion and the involved signaling pathways, in the context of rheumatoid arthritis.

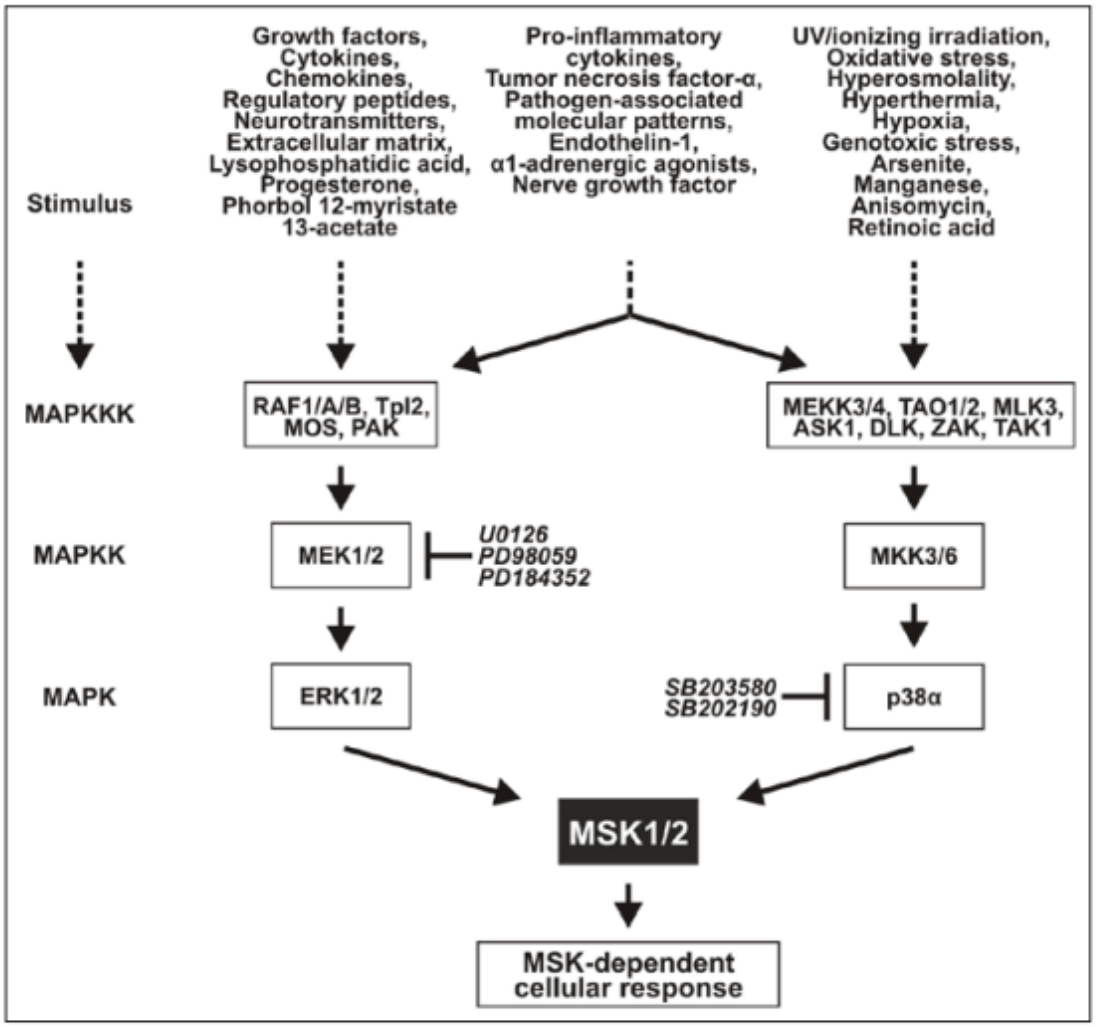


Figure 8. Stimuli and MAPK pathways that activate MSK

Figure 9. The domain structure of CREB [142]

1.2 Rheumatoid Arthritis

1.2.1 Symptoms and Features

Rheumatoid arthritis (RA) is a chronic autoimmune disease. The predominant symptoms of RA are progressive, symmetrically distributed joint deformation, ankylosis and pain. It mainly affects the wrist and small joints of the hand (Figure 10), as well as other peripheral joints in the late phase. RA is more frequent in women than men [176]. There is ~1% of the population affected by RA in the world. As an autoimmune disease, it features the existence of autoantibodies (named rheumatoid factor for RA diagnosis) against the Fc portion of IgM or IgG, and autoantibodies against cyclic citrullinated peptides [177]. One of the main features of RA is the constantly and gradually worsening inflammation of peripheral joints and adjacent tissues resulting in the destruction of cartilage and bone. Another typical feature of the RA synovium is hyperplasia of synovial lining cells along with infiltration of leukocytes, forming a "pannus". The "pannus" covers and invades the articular surface, releasing pro-inflammatory mediators and matrix degrading enzymes, that contribute to cartilage damage and bone loss (Figure 11 and 12). Though the mechanism of synovial hyperplasia is not fully explained, the roles of the various immune cells and inflammatory mediators involved have been thoroughly investigated in past decades. Angiogenesis in the synovial membrane is another feature of RA in the early phase [178]. The pathologic process of RA in different phases has been reported and summarized in Table 4.

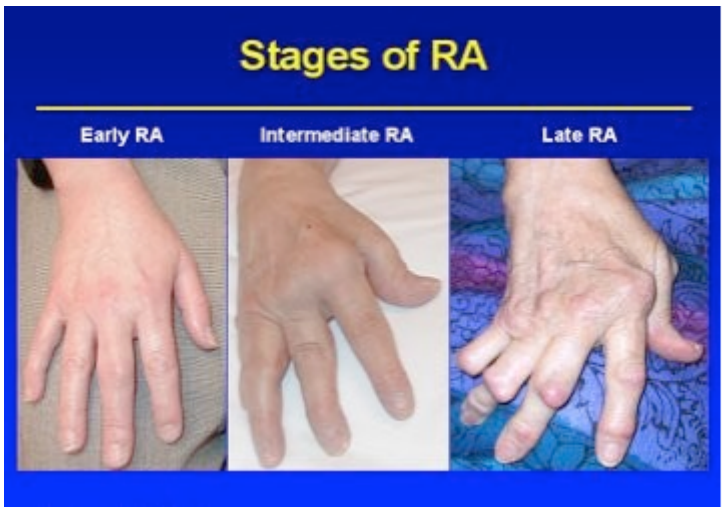


Figure 10. Joint deformation of RA patients

(From: http://www.physio-pedia.com/RA)

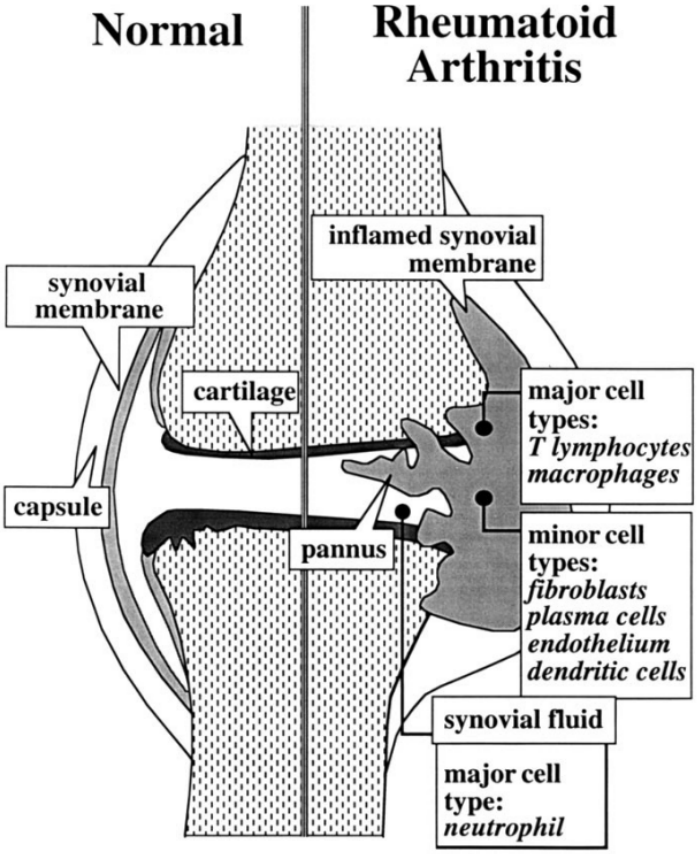


Figure 11. Diagrammatic representation of synovial joint

(normal joint in the left, rheumatoid arthritis joint in the right; from [179])

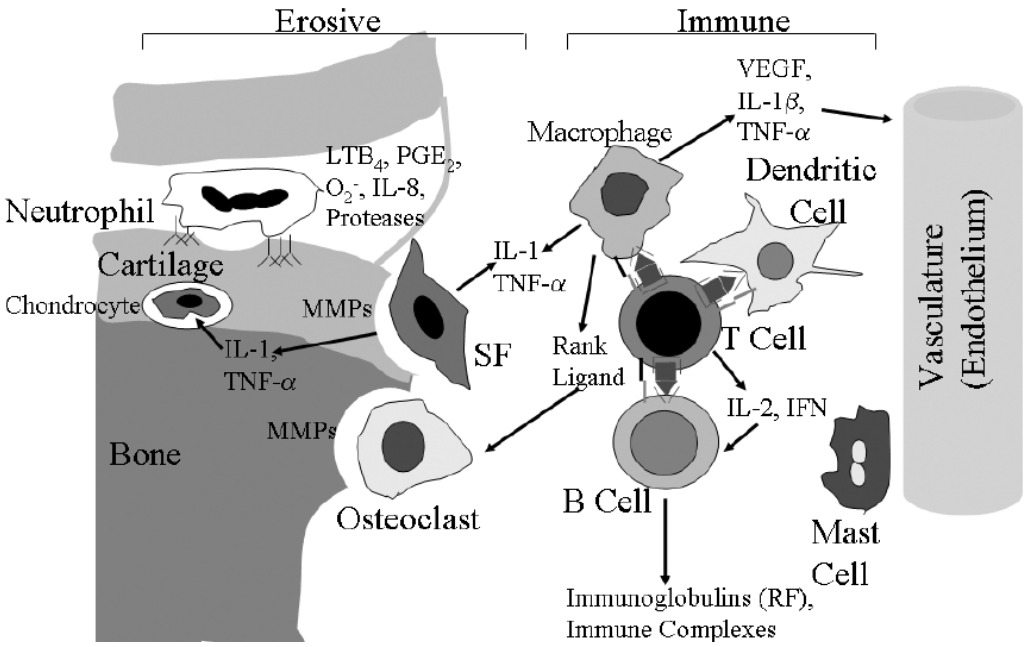


Figure 12. Pathophysiology of rheumatoid arthritis (From [180])

Stage	Pathologic Process	Symptoms	Physical Signs	Radiographic Changes*
1	Presentation of antigen to T cells	Probably none	—	—
2	T-cell proliferation B-cell proliferation Angiogenesis in synovial membrane	Malaise, mild joint stiffness and swelling	Swelling of small joints of hands or wrists, or pain in hands, wrists, knees, and feet	None
3	Accumulation of neutrophils in synovial fluid Synovial-cell proliferation without polarization or invasion of cartilage	Joint pain and swelling, morning stiffness, malaise and weakness	Warm, swollen joints, excess synovial fluid, soft-tissue proliferation within joints, pain and limitation of motion, rheumatoid nodules	Soft-tissue swelling
4	Polarization of synovitis into a centripetally invasive pannus Activation of chondrocytes Initiation of enzyme (proteinase) degradation of cartilage	Same as stage 3	Same as stage 3, but more pronounced swelling	MRI reveals proliferative pannus; radiographic evidence of periarticular osteopenia
5	Erosion of subchondral bone Invasion of cartilage by pannus Chondrocyte proliferation Stretched ligaments around joints	Same as stage 3, plus loss of function and early deformity (e.g., ulnar deviation at metacarpophalangeal joints)	Same as stage 3, plus instability of joints, flexion contractures, decreased range of motion, extraarticular complications	Early erosions and narrowing of joint spaces

*MRI denotes magnetic resonance imaging.

Table 4. The stages of Rheumatoid Arthritis (from [178])

1.2.1.1 T Cells in RA

Systemic reviews on T cell phenotypes in antigen recognition and their contribution to persistence of the chronic inflammatory response, as well as other roles of T cells in RA have been published [181, 182]. Here we will summarize the main features and roles of T cells in the pathogenesis of RA according to the two reviews:

1) Lymphocyte infiltration in the affected RA synovial joints is directed by expression of cytokines and chemokines, such as lymphotoxin-α1β2 and CXCL13.

2) The distinguishing phenotype of RA T cells involves: a, expression of cell surface antigens reflecting prior antigen experience (principally, markers of effector memory cells including specific chemokine receptors and integrins); b, a premature aging phenotype determined by telomere erosion in T cells, which may be associated with HLA-DRB4 status, a risk factor for RA; c, resistance to apoptosis; d, enhanced expression of inflammatory cytokines such as IFN-γ and TNFα and expression of the chemokine receptors CCR4, CCR5, CXCR3, and CXCR5.

3) RA T cells recognize the shared epitope, a stretch of DR β chain α-helix sequence encoded by many RA disease-associated HLA-DRB1 alleles. T cells may also recognize citrullinated peptide epitopes or a subset of autoantibodies to citrullinated fibrin, vimentin, α-enolase, histones and collagen II.

4) In the early phase of RA, the profile of cytokines is dominated by IL-2, IL-4, IL-13, IL-17, IL-15, basic fibroblast growth factor, and epidermal growth factor. However, there is a transition of the profile of cytokines expressed by synovial T-helper cells in the later phase of RA, which is dominated by expression of IFN-γ, TNF and IL-10 (with or without low expression of IL-2, IL-4, IL-5, IL-13), contributing to the chronicity of the inflammatory process in RA.

5) Th17 cells (characterized by the expression of IL-17A and IL-17F) are an important effector T-cell subset in RA. In rodent models of arthritis, IL-17 production was detected in synovial fluid and expression of IL-17, IL-17RA and IL-17RC was

reported in RA synovial tissue by immunohistochemistry. In addition, the cytokine composition of the inflammatory environment favors Th17 cell differentiation.

6) Reciprocal interactions between activated T cells and B cells, macrophages, resident stromal cells and adipocytes within the inflamed synovium result in induction of inflammatory cytokines, chemokines and matrix metalloproteinases but inhibition of collagen synthesis, leading to joint and bone matrix destruction.

7) Tregs can be selectively recruited into inflamed joints, and possibly play a protective role in arthritis: 1) CD4+CD25+Foxp3+ T cells in the inflamed joints in either arthritic KRN mice (mice transgenic for an RNase-specific T cell receptor) or K/BxN serum transfer mice, constituted more than 40% of the CD4+ T cells in the synovial fluid of mice; 2) Foxp3 mutant KRN mice had faster and more severe arthritis onset. In addition, more robust markers of Tregs may still be required to further validate their role in RA.

8) Aberrant pathways of T cell activation play a key role in RA. This is supported by the following observations: 1) RA is associated with mutations in several genes involved in regulation of lymphocyte activation including *HLA-DRB1*, *PTPN22, CTLA-4, IL-2RA, IL-2RB, STAT4, PTPN2 and PADI4*; 2) spontaneous arthritis that develops in various rodent models of arthritis like K/BxN, IL-1Ra -/-, gp130 mutant and SKG mice is T cell- dependent.

1.2.1.2 Synoviocytes in RA

The soft tissue lining the joint cavities is called the synovium, and is covered by a layer of synovial intimal cells comprising type A and type B synoviocytes [183]. Type A synoviocytes are macrophages that can actively phagocytose cell debris and waste in the joint cavity, and possess an antigen-presenting ability [183]. Type B synoviocytes are fibroblast-like synoviocytes (FLS, also named synovial fibroblasts, SFs) involved in the synthesis of extracellular matrix and proteoglycan [183]. In a healthy joint, FLS are responsible for the production of hyaluronic acid (a major component of synovial fluid

necessary for lubrication of the joint surface), matrix components and matrix-degrading enzymes to maintain the structure and function of the joint capsule [180]. In the RA joint, FLS switch to an inflammatory phenotype, and are considered to be not only passive responders to inflammatory mediators, but also invasive aggressors that independently participate in inflammation of the synovium [184]. Though the mechanism of the FLS inflammatory transformation is not completely understood, the interaction between FLS and the immune system [180], and activation by platelet-derived microparticles [185], growth factors, and matrix degradation [186] are reported to be involved. The proliferation of FLS is favored by the joint environment where there is recruitment of leukocytes. In terms of cell survival and growth RA FLS appear more resistant than OA FLS to apoptosis [187]. Progressive inflammation in the joint cavity of RA has been suggested to be orchestrated by a complex interplay of various inflammatory mediators and matrix-degrading enzymes released by synoviocytes and leukocytes. TNFα, IL-1 and IL-6 play a critical role as pro-inflammatory cytokines in the pathogenesis of RA. Other chemokines recruiting inflammatory leukocytes, including CXCL1/KC, CXCL5, CXCL8/IL-8, MCP-1/CCL2, RANTES/CCL5, CXCL13/BCA-1 and CXCL10/IP-10 produced by FLS or leukocytes also play roles in RA [188, 189]. The roles of cytokines and chemokines will be discussed in paragraphs 1.2.2 and 1.2.3.

1.2.1.3 Chondrocytes in RA

Chondrocytes are also involved in pannus formation (hypertrophied synovium) [190]. Chondrocytes undergo a switch from an anabolic to a catabolic state by synthesizing and releasing matrix-degrading enzymes instead of producing matrix, which contributes to rapid loss of cartilage matrix components [191]. In addition, chondrocytes also have the potential to produce pro-inflammatory cytokines/chemokines in an autocrine or paracrine mode [192]. Another main cause of cartilage degradation is chondrocyte apoptosis, which results in chondrocyte lacunae

formation [191]. The proliferating synovial cells invade the chondrocyte lacunae and enlarge the lacunar space, thereby contributing to accelerated joint erosion. Recent reports highlight the necessity of investigating therapeutic strategies limiting chondrocyte apoptosis [193], as inhibition of cartilage and bone damage could be a promising direction for the treatment of RA with respect to joint deformation.

1.2.1.4 Osteoblasts and Osteoclasts in RA

Osteoblasts and osteoclasts play crucial roles in bone remodeling. In a severe inflammatory arthritic state, there is an imbalance between bone formation (osteogenesis) by osteoblasts and bone absorption by osteoclasts, resulting in bone loss. Bone erosion develops rapidly at the early phase of RA if the patient is not given treatment [194]. The RANK/RANKL signaling pathway in the osteoclast plays a key role in regulating bone remodeling, since RANKL knockout mice present decreased bone erosion and no cartilage erosion in a mouse model of arthritis [195]. In contrast, osteoblasts produce osteoprotegerin (OPG, a soluble decoy receptor with homology to TNF-R) that interferes with RANK/RANKL signaling in osteoclasts. By binding to and sequestering RANKL, OPG reduces osteoclastic bone resorption [194]. The imbalance between high RANKL expression and low OPG expression favors bone loss in inflammatory arthritis. Osteoclasts at the bone-pannus interface were found to express thrombin receptor-activating peptides (TRAPs), cathepsin K and calcitonin receptor, all of which are suspected to contribute to bone matrix degradation [194]. These findings demonstrate that osteoclasts are the main cell type mediating bone loss.

1.2.2 Cytokines in RA

Cytokines are small proteins playing important roles in the pathogenesis of RA by regulating cell-cell interactions and communication during the initiation/onset or persistence phase of inflammation in an autocrine, paracrine or even endocrine mode. The action of cytokines could be systemic in blood circulation, or local in a specific inflammatory site. They can be produced by immune cells such as neutrophils, T cells,

B cells or macrophages recruited into the inflamed synovium, or by synoviocytes and even chondrocytes. Different cytokines act by pleiotropy, redundancy, and duality of action, forming a network in the inflammatory milieu [196]. The effects cytokines can elicit in inflammatory arthritis include leukocyte activation, migration, and infiltration to inflammatory sites, and activation of effector cells in the synovium to produce enzymes that cause cartilage and bone damage.

1.2.2.1 Tumor Necrosis Factor α

TNFα is a pleiotropic pro-inflammatory cytokine secreted mainly by macrophage [197], but also produced by other cells, such as adipocytes [198], lymphoid cells [199], mast cells [200], endothelial cells and fibroblasts. TNFα exists in two forms; a type II transmembrane protein arranged in stable homotrimers, and a mature soluble homotrimeric protein (sTNFα) (Figure 13). The soluble, bioactive, 17kDa sTNFα is released from the TNFα transmembrane protein through proteolytic cleavage by TNF alpha converting enzyme (TACE, also named ADAM17). The recombinant human TNFα used in this study is a soluble 157 amino acid protein (17.4 kDa) which corresponds to the C-terminal extracellular domain of the transmembrane protein. TNFα and TNFβ (also called lymphotoxin-α) share close structural homology, with 28% amino acid sequence identity. They both activate the same TNF receptors. Human and murine TNFα share 79% amino acid sequence identity and demonstrate significant cross-species reactivity. As the name suggests, TNFα is able to kill tumor cells. It is necessary for immune responses against bacterial infections, as well as for inducing septic shock, and is involved in autoimmune diseases and diabetes. TNFα signals through two receptors, TNFR1 and TNFR2. Both membrane-bound trimers and soluble TNFα activate TNFR1, but only membrane TNFα activates TNFR2 [201]. TNFR1 is widely expressed in tissues while TNFR2 is limited to endothelial cells and leukocytes. TNFα binds to TNFR1 and, via NF-κB and AP-1 activation, maintains cell survival and initiates proinflammatory responses (Figure 13). TNFR1 contains a death domain (DD),

which participates in protein-protein interaction leading to downstream caspase activation and apoptosis. TNFR2 has no DD motif and regulates cell migration, proliferation, antigen presentation, tissue repair and angiogenesis [201].

TNFα plays a critical role in the pathogenesis of RA. It can be detected in the early phase of RA at the systemic level [202, 203] and it is also highly expressed in rheumatoid joint cell culture [179]. Researchers have also detected high levels of TNFα in synovial fluid [202]. Transgenic mice overexpressing human TNFα spontaneously develop chronic inflammatory polyarthritis, a disease similar to RA, which suggests significant involvement of TNFα in the pathogenesis of RA [204].

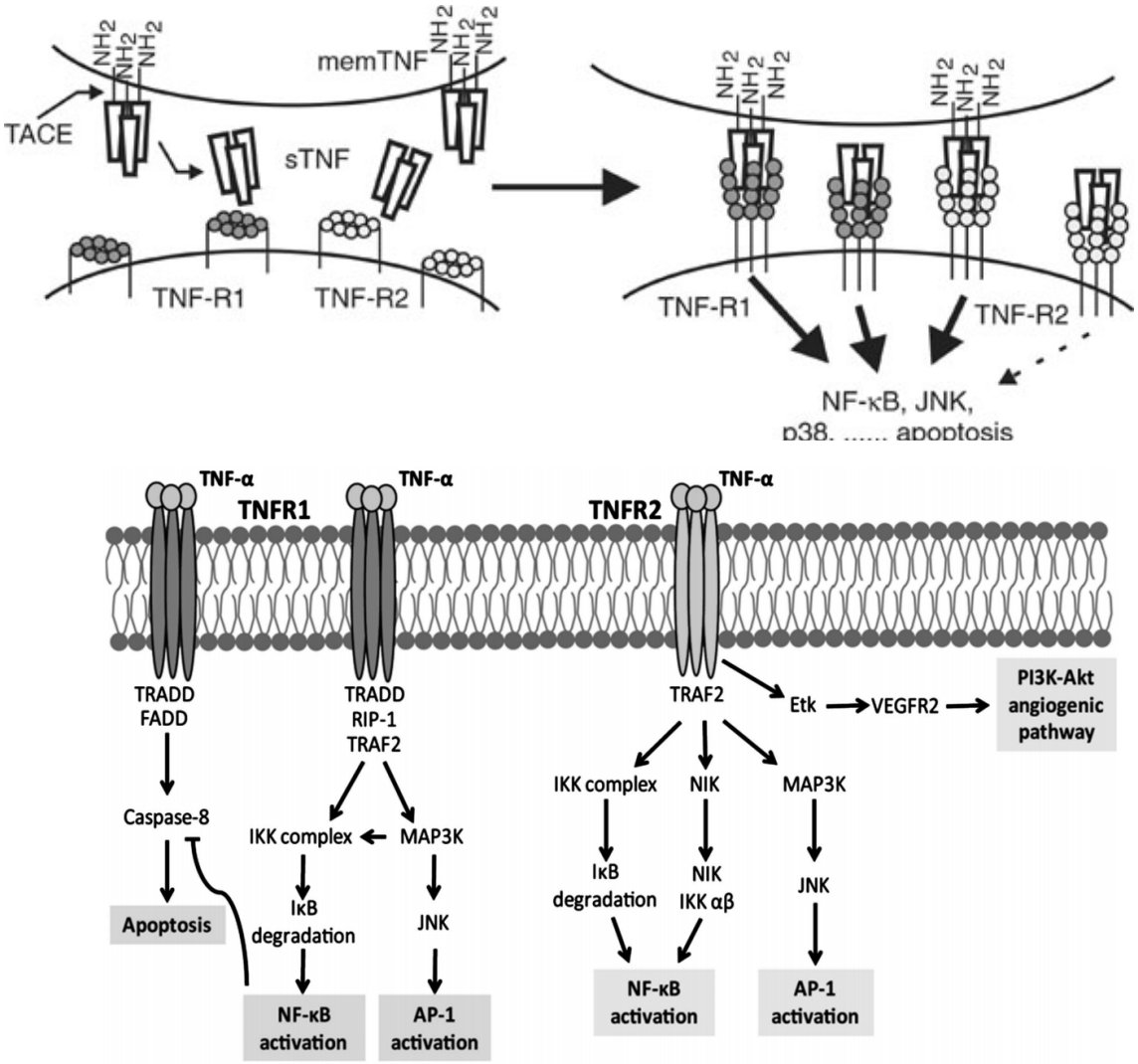


Figure 13. Two forms of TNFα and TNFα mediated signaling (From [201, 205])

1.2.2.2 Interleukin-1

The IL-1 family includes three members IL-1α, IL-1β and IL-1Ra (naturally occurring IL-1 receptor antagonist—trade name anakinra). IL-1α and IL-1β are encoded by two distinct genes *IL1A* and *IL1B* respectively. Cellular proteases cleave the inactive intracellular precursor proteins (31 kDa, pro-IL-1α and pro-IL-1β) into the mature, secreted forms (17 kDa, mIL-1α and mIL-1β). They can bind and activate the same receptor, IL-1 receptor type 1 (*IL1R1*), to produce biological effects. Similar to TNFα, IL-1 is mainly produced by activated monocytes/macrophages [206]. Both IL-1α and IL-1β are detected abundantly at all phases of RA [206], even in the plasma of RA patients [207, 208]. Levels of IL-1 increase ten-fold in RA synovial fluid compared to OA or non-inflammatory joint disease [209]. IL-1Ra is a decoy receptor that limits IL-1α and IL-1β signaling. IL-Ra-deficient mice of a certain genetic background spontaneously develop autoimmune arthritis [210]. The levels of IL-1Ra in RA synovial fluids are also elevated significantly in RA patients compared to those from OA patients, but the lower IL-1Ra to IL-1 ratio implies a role for IL-1Ra and IL-1 imbalance in RA [211].

TNFα was long considered the major regulator of IL-1 [179]. However, other researchers have argued that IL-1 production can be controlled at various levels, some of which may be TNFα-independent, since direct contact between activated T lymphocytes and monocytes/macrophages strongly induced IL-1 secretion without the presence of TNFα [212]. Though TNFα and IL-1 have some overlapping effects, differences between the roles of TNFα and IL-1 were also highlighted [213]: TNFα seems to play an outstanding role in regulating inflammation systemically and locally, while IL-1 is suggested to be more involved locally in cartilage and bone damage [214]. IL-1β was found to be a human osteoclast-activating factor [215], suggesting that inhibition of IL-1β could reduce the bone damaging activity of osteoclasts. Even though

there is debate on the relative importance of these two pro-inflammatory cytokines, we cannot deny that both of them are playing essential roles in RA pathogenesis [216].

1.2.2.3 Interleukin-6

IL-6 is a 26-kDa glycopeptide inducing various cellular responses, orginally known to stimulate B lymphocyte and monocyte differentiation, as well as hybridoma growth [217]. Unlike other cytokines, IL-6 can bind and signal through membrane-bound and soluble receptors (sIL-6R) [217]. The membrane bound receptor IL-6R is a non-signaling α-receptor subunit that can associate with two different gp130 signal-transducing subunits. IL-6 signals primarily through IL-6R in what is called classical signaling. Furthermore, there is another signaling pathway named "trans-signaling pathway": IL-6 can also signal via sIL-6R activation; then sIL-6R binds to membrane-bound gp130 subunits to initiate the cellular response.

In RA, IL-6 activates lymphocytes for proliferation, initiates the acute-phase response and induces osteoclast differentiation. As mentioned above, high levels IL-6 were reported both in serum and synovial fluid from RA patients. Besides IL-6, high sIL-6R concentrations were also detected in synovial fluid from RA patients [218], which was suggested to correlate with osteoclastogenesis involved in bone destruction, as well as with chronic synovitis [219]. IL-6 gene knockout mice were resistant to antigen-induced arthritis [220] and did not develop collagen-induced arthritis (CIA) [221]. High circulating IL-6 levels and detrimental effects on bone formation were reported in IL-6 transgenic mice overexpressing IL-6 [222]. The role of IL-6 has been summarized in the pathophysiology of RA [223]: as a proinflammatory cytokine, IL-6 induces the synthesis of acute phase proteins (such as CRP) and the iron regulatory peptide hepcidin (causing anaemia), acts on the hypothalamic–pituitary–adrenal axis (HPA, causing fatigue), affects bone metabolism (causing osteoporosis), and regulates B cell and T cell differentiation. Reduction of systemic arthritis through blocking IL-6 functional responses in mouse model [224], and restoration of arthritis following

injection of an sIL-6R–IL-6 fusion protein to IL-6 knock-out mice [225], highlighted the essential role of IL-6 trans-signaling in RA.

1.2.2.4 Other cytokines

Other cytokines are also involved in the pathogenesis of RA, such as IL-17, IL-15, IL-21, IL-22, IL-23, *etc*.[196]. They play different roles in altering immune cell functions and other effector responses in arthritis (Figure 14 and Table 5). IL-17, IL-21 and IL-22 can be produced by Th17 cells. IL-17 represents a family of six major isoforms, IL-17A-F. IL-17 signals through the IL-17 receptor. IL-17A and IL-17F are found to be increased in arthritic joints [196]. IL-17A is a proinflammatory cytokine that is up-regulated in the circulation of RA patients [226]. The pathogenic role of IL-17A in murine models of inflammatory arthritis is predominantly to promote disease (see section 1.2.1.1 “The role of T cells in RA”). IL-17A-deficient mice are resistant to CIA [227] and to spontaneous arthritis in mice also lacking IL-1Ra [228]. Blocking IL-17A or its receptor with antibodies reduces disease in mice (reviewed in [229]). Similar to IL-17, IL-15 is also proinflammatory and its serum level also correlates with disease severity (reviewed in [196]). IL-23 knockout mice (*Il23p19*-/-) are resistant to collagen-induced arthritis (CIA) [230], and mice transgenic for p19, the α-chain subunit of IL-23, display systemic inflammation [231], indicating a role for IL-23 in inflammatory arthritis.

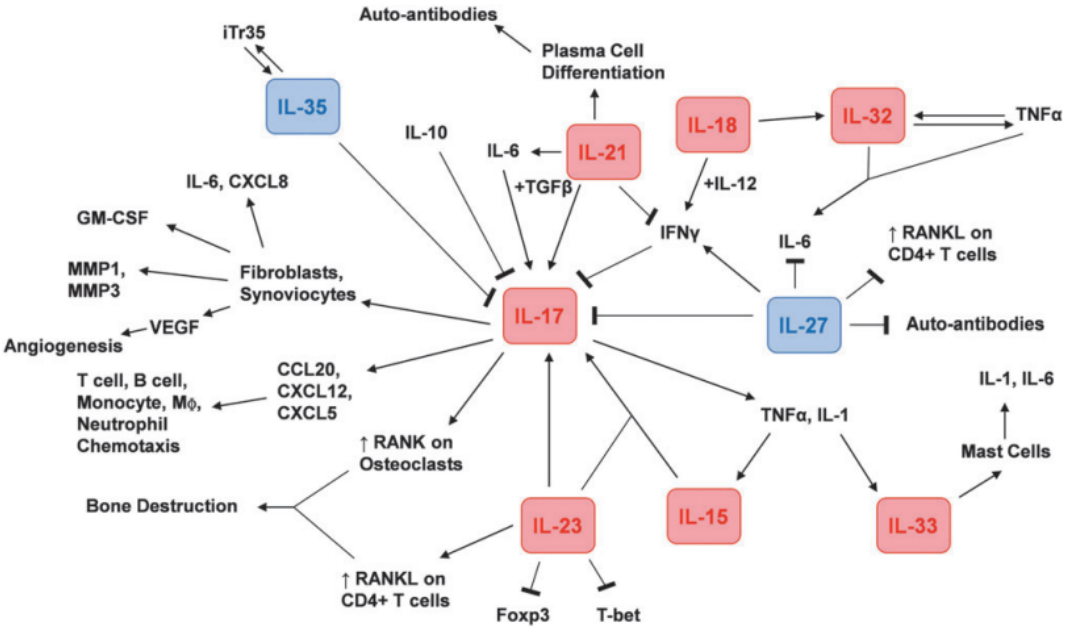


Figure 14. The cytokine network in lymphoid tissues and inflamed joints in autoimmune arthritis (pathogenic, pro-inflammatory fashion is in red; a protective, anti-inflammatory fashion is in blue, from [196])

Cytokine	*Cell source*	*Receptors*	*Cells affected*	*Pathways influenced*	*Reference*
IL-15	Fibroblast-like synoviocytes, macrophages	IL-15Rα, IL-15Rβ, common γ-chain (γC)	T cells, NK cells, B cells, monocytes, macrophages, neutrophils, mast cells, dendritic cells, fibroblasts	Induces IL-17 production	Giri and others 1994; Dubois and others 2000; Ziolkowska and others 2000 Yoshihara and others 2007
IL-17	Th17, γδ T cells, CD8+ T cells, NKT cells	IL-17RA, IL-17RB, IL-17RC, IL-17RD, IL-17RE	T cells, NK cells, B cells, monocytes, macrophages,, neutrophils, mast cells, dendritic cells, fibroblasts	Induces angiogenesis, proinflammatory cytokine production, cell infiltration, innate immune response, and RANK expression on osteoclasts	Albanesi and others 2000 Bush and others 2001 Toy and others 2006 Rong and others 2009 Shahrara and others 2009 Pickens and others 2010 Sawa and others 2011
IL-18	Macrophages, dendritic cells, fibroblasts, chondrocytes	IL-18Rα IL-18Rβ	T cells, NK cells, macrophages, chondrocytes	Facilitates IFN-γ production, monocyte recruitment into the synovium, and angiogenesis	Gracie and others 1999 Yamamura and others 2001 Ruth and others 2010
IL-21	T cells	IL-21R, common γ-chain (γC)	B cells, T cells, NKT cells, NK cells	Induces Th17 differentiation and plasma cell differentiation, inhibits IFN-γ	Habib and others 2002 Korn and others 2007 Nurieva and others 2007 Ettinger and others 2008 Yang and others 2008
IL-23	Dendritic cells, monocytes, macrophages	IL-12Rβ1, IL-23R	T cells	Helps maintain Th17 cells, induces RANKL on CD4+ T cells	Parham and others 2002 Murphy and others 2003 Yago and others 2007 Lemos and others 2009
IL-27	Dendritic cells, monocytes, epithelial cells	WSX1, gp130	T cells	Stimulates IFN-γ production, blocks IL-6, inhibits IL-17, downregulates RANKL	Pflanz and others 2004 Awasthi and others 2007 Niedbala and others 2008 Rajaiah and others 2010 Kamiya and others 2011
IL-32	NK cells, T cells, epithelial cells, monocytes, fibroblasts	PR3	Monocytes, macrophages, epithelial cells	Stimulates the production of TNF-α, IL-1β, IL-18	Dinarello and Kim 2006 Joosten and others 2006 Novick and others 2006 Shoda and others 2006 Heinhuis and others 2011
IL-33	Fibroblasts	T1/ST2	Mast cells	Activates mast cell release of proinflammatory cytokines	Leung and others 2004 Schmitz and others 2005 Xu and others 2008 Palmer and others 2009
IL-35	T regulatory cells, iT_R35	IL-12Rβ2, gp130	T cells	Suppresses Th17 differentiation, induces IL-35+Foxp3- T cells	Devergne and others 1997 Collison and others 2007 Niedbala and others 2007 Collison and others 2010

Foxp3, Forkhead box 3; IFN, interferon; IL, interleukin; iT_R35, inducible T regulatory cell 35; NK, natural killer; NKT, natural killer T; PRT3, proteinase 3; RANK, receptor activator of nuclear factor κ B (RANK); RANKL, RANK ligand; Th, T helper cell; WSX, tryptophan-serine-X.

Table 5. Roles of some cytokines in the pathogenesis of autoimmune arthritis (From [166])

1.2.3 Chemokines in RA

Chemokines are a superfamily of small molecular weight cytokines initially found to play crucial roles in leukocyte migration into the inflamed synovium during inflammation (Figure 15). There are four subfamilies of chemokines (CC, CXC, XC and CX3C) divided by structural criteria according to the number and arrangement of conserved cysteine residues. The first two well-defined chemokines were CXCL-8 (IL-8) and CCL2 (MCP-1) [232]. Chemokines bind to and activate chemokine receptors, which constitute a large subfamily of G-protein coupled receptors. According to the context in which they function, chemokines can be characterized into several types: inflammatory chemokines, homeostatic chemokines, and even dual-function chemokines [232]. Inflammatory chemokine receptors have a promiscuous feature: one chemokine can bind to several chemokine receptors, and one chemokine receptor can have multiple chemokine ligands. Recently there was another subfamily of receptors called atypical chemokine receptors added to the chemokine receptor nomenclature [233]. Compared with classical signaling chemokine receptors, these atypical chemokine receptors can bind to many chemokine ligands without transducing cell signals.

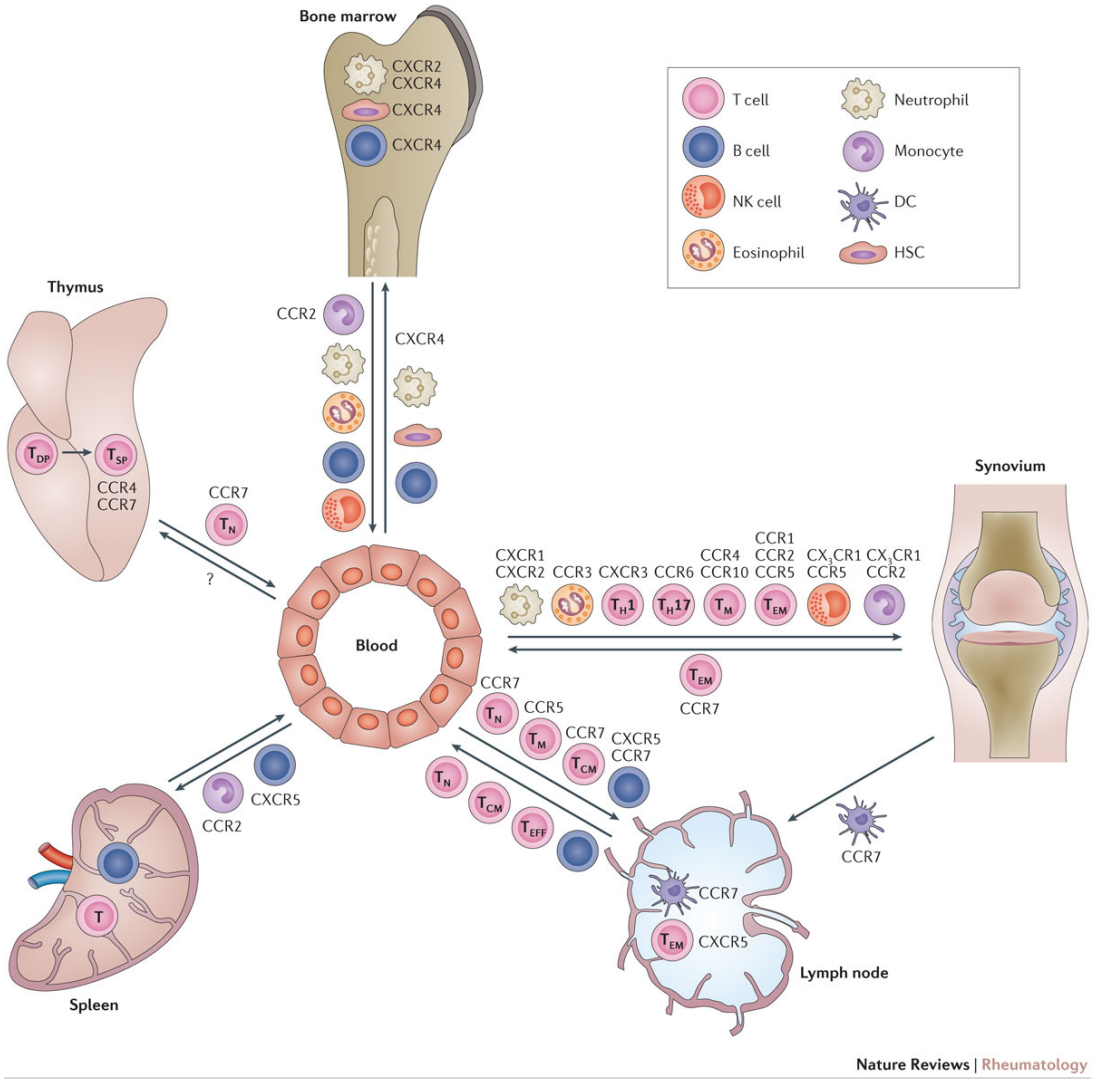


Figure 15. Leukocyte trafficking into the inflamed synovium (from [234])

The function of chemokines in the pathogenesis of RA includes chemoattraction of neutrophils, lymphocytes and monocytes into the synovium. They play a role in endothelial activation, angiogenesis, synovial fibroblast migration and proliferation, pseudoemperipolesis (homing and survival of lymphocytes in the pannus), and the regulation of cartilage and bone metabolism. The following is a summary of known features and functions of chemokines in RA, as reviewed by Szekanecz and Koch [234]:

1) Chemokine detection: "The CXC-chemokines CXCL1 (GROα), CXCL4 (PF4), CXCL5 (ENA78), CXCL6 (GCP2), CXCL7 (NAP2), CXCL8 (IL-8), CXCL9 (MIG), CXCL10 (IP10), CXCL12 (SDF1), CXCL13 (BCA1) and CXCL16 (SR-PSOX) have been detected in sera, synovial fluids and synovial tissues of patients with RA and are mainly produced by synovial macrophages. The expression of these chemokines varies at different stages of RA".

2) Functions of CXC-chemokines in RA: "CXCL1, CXCL5 and CXCL8 have neutrophil chemoattractant properties. CXCL5, CXCL7, CXCL8 and CXCL12 are pro-angiogenic. CXCL4, CXCL9 and CXCL10 are angiostatic".

3) Functions of CC-chemokines in RA: "CCL2 (MCP-1), CCL3 (MIP1α), CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CCL13 (MC-P4), CCL14 (HCC1), CCL15 (HCC2), CCL16 (HCC4), CCL17 (TARC), CCL18 (PARC), CCL19 (MIP3β), CCL20 (MIP3α), CCL21 (SLC) and CCL28 (MEC) are all expressed in sera and synovia of RA patients. They exert chemotactic activity mainly toward monocytes and lymphocytes".

4) "XCL1 (lymphotactin) is chemotactic for lymphocytes. … CX3CL1 (fractalkine) is chemoattractant for synovial fibroblasts, and is also a pro-angiogenic factor".

5) "Chemokines such as CXCL5 and CCL2 are potential targets for citrullination in RA. Citrullinated chemokines have reduced or altered chemotactic properties".

6) CXC-chemokine receptor functions: "CXCR1 and CXCR2 are expressed by neutrophils that have been shown to contribute to RA; CXCR3 regulates the homing of

leukocytes into inflammatory sites (including the RA synovium that linked to the presence of Th1 cells). CXCR4 mediates chemotaxis of lymphocytes into the synovium. …CXCR4, CXCR5, CXCR6 and CXCR8 are all involved in inflammatory synovitis."

7) CC-chemokine receptor functions: "they are primarily involved in mononuclear-cell migration, and some are involved in neutrophil or T helper cell migration (The CC-chemokine receptors CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7 and CCR10 are abundantly expressed in the synovium in RA)."

8) "A few atypical chemokine receptors are also described in RA such as ACKR3, ACKR5 and ACKR6".

9) "In animal models of RA, administration of blocking antibodies that bind to CXCL1, CXCL5, CXCL8, CXCL12, CXCL13, CXCL16, CCL2, CCL3, CCL5, CCL24 and CX3CL1 can prevent synovitis. There are many potential difficulties with targeting of the chemokine pathway for treatment of RA, including redundancy of chemokines and chemokine receptors, interference with homeostatic function, etc."

1.2.4 Animal Models of RA

Rodent models are commonly used to study the pathogenesis of inflammatory arthritis and to evaluate potential new therapeutic agents for clinical use, although all animal models have their shortcomings. Numerous animal models of arthritis [235] have been described and classified into two main categories, experimentally induced arthritis models by active or passive immunization, and genetically manipulated spontaneous arthritis models, advances in which are thoroughly reviewed in [236]. These animal models recapitulate the features of human rheumatoid arthritis, such as synovitis and pannus formation [237]. Some of the widely used mouse and rat models for studying arthritis are shown in Table 6 [238]. Trigger induced models include adjuvant-induced arthritis, oil-induced arthritis, pristane-induced arthritis,

collagen-induced arthritis (CIA, induced by injection of type II heterologous collagen in complete Freund's adjuvant), antigen-induced arthritis (AIA, e.g. methylated BSA in complete Freund's adjuvant as an antigen), zymosan-induced arthritis, GPI (Glucose-6-phosphate isomerase)-induced arthritis and streptococcal cell wall-induced arthritis [236, 239, 240] (Table 6). Models of spontaneous development of arthritis in rodents include: IL-1Ra knockout mice, IL-6R knockin, Inducible Jun knockout, K/BxN, SKG, TNFα-transgenic (overexpression of TNFα), Human/SCID chimeric mice, Human DR4-CD4 mice, gp130 mutant mice and TS1xHACII mice [236, 241-243]. Immune complex-mediated models such as Collagen-antibody-induced arthritis and K/BxN serum-transfer arthritis model are induced by serum transfer, which is considered passive immunization. As mentioned before, K/BxN model, gp130 mutant mice and SKG mice are T cell dependent [244], while the K/BxN serum-transfer arthritis model is neutrophil-dependent mediated by autoantibodies [245]. Whether other models are T-cell driven is shown in Table 6.

Model	Abbreviation	Species[a]	Feature	IC	T cell
Trigger-induced models					
Non-specific immune stimuli					
Adjuvant-induced arthritis	AA	Lewis rat	Autoimmune	-	+
Oil-induced arthritis	OIA	DA rat	Autoimmune	-	+
Pristane-induced arthritis	PIA	DA rat	Autoimmune	-	+
Cartilage directed autoimmunity					
Collagen-induced arthritis	CIA	DBA mouse	CII AI	+	+
Proteoglycan-induced arthritis	PGIA	Balb/c mouse	PG AI	+	+
Infectious agents/exogenous triggers					
Streptococcal cell wall arthritis	SCW-A	Lewis rat	Persistent bacteria AI	-	+
Flare	SCW-F	Mouse	Th17	-	+
Antigen-induced arthritis	AIA	Rabbit, mouse	Persistent antigen	+	+
Flare	AIA-F	Mouse	Th17	-	+
Transgenic spontaneous models					
HTLV-induced arthritis	HTLV	Mouse	Viral tax antigen	-	+
KRN arthritis	KRN	K/BxN mouse	GPI AI	+	+
SKG arthritis	SKG	Mouse	ZAP-70 T cell defect	-	+
GP130 arthritis	GP130	Mouse	STAT3, T cell defect	-	+
TNF transgenic arthritis	TNFtg	Mouse	TNF overexpression	-	-
IL-1ra transgenic arthritis	IL-1ra-/-	Balb/c mouse	Autoimmune T cells	±	+
IL-1 transgenic arthritis	IL-1tg	Mouse	IL-1 overexpression	-	-
Immune complex models					
Collagen type II	CAIA	DBA mouse	Mouse CII antibody	+	-
KRN serum	GPI	Balb/c mouse	Mouse GPI antibody	+	-
Poly-L-lysine-lysozyme	PLL-L	DBA mouse	Cationic antigen	+	-

Table 6. Animal models of arthritis ([238])

1.2.5 RA Treatment

The goal of RA treatment is to control pain, stop inflammation, relieve symptoms, prevent or reduce joint and organ damage, improve physical function and reduce long-term complications so as to improve quality of life. The first line of medication is non-steroidal anti-inflammatory drugs (NSAIDs), which tend to control symptoms by blocking cyclooxygenase-2 (Cox-2) and the production of prostaglandins, prostacyclins and thromboxanes. Side effects of NSAIDs are mainly gastrointestinal intolerance, and possible cardiovascular complications. The second line is referred to as conventional disease modifying anti-rheumatic drugs (cDMARDs), such as anti-malarials, methotrexate, D-penicillamine and gold salts, which have been used as the first therapeutic approach in the past few years [246], and have little anti-inflammatory activity but ameliorate the course of the disease in a majority of patients. Methotrexate is considered as the anchor non-biological drug in RA treatment with its established long-term safety and clinical efficacy [246]. Other DMARDs reported in clinical trials include Tacrolimus (TAC, immunesuppresive agent blocking T cell activation by specifically inhibiting the calcineurin pathway), Leflunomide (LEF) and Iguratimod (IGU, also named T-614) [246]. The biological agents (bDMARDs) comprise monoclonal antibodies and cytokine antagonists. There are products available in the clinic against TNFα, IL-1, IL-6, IL-17A, IL-20, RANKL, GM-CSFR, and B cell marker CD20 [246]. Janus kinase (JAK) inhibitor is a new class of drug. Though side effects were also reported for these anti-cytokine products and even for the JAK inhibitors, such as herpes zoster infection, nasopharyngitis, gastrointestinal events and increase in LDL, HDL, creatinine and creatinine phosphokinase (CPK), they still showed higher efficacy and safety profiles when compared with traditional DMARDs, which is meaningful for future research direction.

1.2.5.1 TNFα blockade

Original studies for bDMARDs found that anti-TNFα antibody could decrease the secretion of other cytokines/chemokines, such as IL-1, IL-6, IL-8 and GM-CSF, by synovial cells from RA patients but not OA patients [247], leading to a concept of TNFα as "master regulator" [248]. These TNFα-regulated proinflammatory cytokines and chemokines were highly expressed in RA tissues. Several aspects of the proposed mechanism of action have been summarized for the dominating effect of anti-TNFα, including down-regulation of proinflammatory cytokines, chemokines and adhesion molecules (effect on leukocyte trafficking), VEGF (effect on angiogenesis), MMPs (effect on joint destruction); and also normalization of hematological abnormalities [249].

1.2.5.2 IL-1 blockade

Recombinant IL-1Ra, an IL-1 decoy receptor, is a shorter half-life protein that requires daily injection, which is not considered a better solution than TNF blockade [250]. Blocking IL-1 was anticipated to be a promising therapy for other classic rheumatic diseases like gout or even other autoimmune diseases like systemic juvenile idiopathic arthritis [251]. Antagonists (anakinra) and blocking antibodies (AMG108) towards the IL-1 receptor as well as monoclonal antibodies versus IL-1β (canakinumab) have been the subject of clinical trials for RA [252]. Compared to anakinra's short half-life, canakinumab has a longer half-life. For those RA patients who are insensitive to anti-TNF therapy, IL-1-targeting therapy presents promising benefits. Other IL-1-targeting agents are undergoing clinical trials [252].

1.2.5.3 IL-6 blockade

Results from Phase III clinical trials of interleukin-6 receptor-blocking monoclonal antibody tocilizumab in RA highlighted its positive effect in reducing disease activity [253]. Tocilizumab in combination with or without methotrexate is more effective, with no additional or less severe adverse effects when compared to treatment of early RA

with methotrexate alone [254]. RA patients who are intolerant to TNF-inhibitor may benefit in the future from tocilizumab.

1.2.6 Roles of LPA in RA

The ATX-LPA pathway in the pathogenesis of RA has been reviewed previously [72, 255]. Here is a brief summary:

1) Detection of ATX and LPA *in vivo* and *in vitro*: High levels of ATX mRNA in FLS from RA patients and in arthritic joints from animal models have been reported [256, 257]. ATX [256] and LPA [40] have also been detected in synovial fluid. These experiments point toward a role for the ATX-LPA axis in RA.

2) LPAR-mediated cell responses: Previous studies showed that LPA receptors LPA_1, LPA_2, and LPA_3 but not LPA_4 or LPA_5 were detected in RA FLS, with LPA_1 as the main expressed receptor [40, 257]. The functional responses induced by LPA include stimulation of FLS motility through LPA_1, cytokine/chemokine (IL-6, IL-8) release through LPA_1, LPA_3 and lipid mediator synthesis (COX-2 expression and prostaglandin E_2 (PGE_2) production) [40, 257]. LPA-induced cytokine/chemokine secretion was dependent on ERK1/2, p38MAPK and Rho kinase activation, but LPA-induced FLS migration was only dependent upon p38MAPK and Rho kinase activation [257]. Silencing of the LPA_1 receptor in RA FLS decreased the proliferation induced by TNF and sensitized the cells to TNF-induced apoptosis [258].

3) Mice overexpressing human TNFα, the CIA model and the K/BxN serum transfer arthritis (STA) have been used to investigate the involvement of the ATX-LPA axis in RA. Conditional ablation of ATX in TNFα-transgenic mice and in the CIA model reduced the severity of arthritis, synovial inflammation, and hyperplasia [256]. In the K/BxN serum transfer arthritis model, $LPA_{1/3}$ antagonist treatment also attenuated the severity of arthritis and reduced synovial inflammation, cartilage damage and bone erosion [259]. One report demonstrated that LPA_1 deficient mice were also protected

from collagen-induced arthritis [260]. All studies showing reduced clinical arthritis in experimental models have been summarized in Table 7 [255].

4) Studies on the role of LPA in cartilage and bone damage in RA: LPA can be produced by osteoblasts, serve as an autocrine and paracrine factor to coordinate osteoblast and osteoclast activity, enhance osteoclast differentiation, and may be indirectly involved in bone loss in RA. LPA was also reported to stimulate rat chondrocyte proliferation [261], induce collagen I production in human chondrocyte [262] and inhibit chondrocyte apoptosis [263]. An important role of LPA_1 and ATX in chondrocyte proliferation was reported in both LPA_1 and ATX knockout zebrafish and mice [264]. These reports indicate that ATX-LPA signaling may also be involved in regulating normal cartilage formation and development as well as cartilage and bone destruction in RA indirectly by affecting chondrocyte growth and death. How the other LPA receptors are involved in cartilage and bone damage in RA and whether or not LPA is protective with respect to cartilage and bone damage still requires further investigation.

Studies showing reduced clinical arthritis, synovial inflammation and hyperplasia in experimental models by interfering with the ATX–LPA.

Arthritis model	Treatment	Genetic approach	Additional findings	Refs.
CIA	BrP-LPA (LPA receptor antagonist and ATX inhibitor)	–	Disease incidence reduction	(Nikitopoulou et al., 2013)
K/BxN serum transfer model	Ki16425 (LPA_{1-3} antagonist)	–	Increased synovial apoptosis and osteoblast differentiation; reduction of osteoclast differentiation.	(Orosa et al., 2014)
CIA	LA-01 (LPA_1 antagonist)	–	Reduction of osteoclast formation and suppression of Th17 cells.	(Miyabe et al., 2013)
CIA	–	LPA_1 Knockout mice	Total protection against arthritis. Suppression of differentiation of Th17 cells and osteoclast formation.	(Miyabe et al., 2013)
hTNF/CIA	–	Conditional genetic ATX ablation in mesenchymal cells	–	(Nikitopoulou et al., 2012)

Table 7. Studies showing the involvement of ATX-LPA in arthritis models (From [228])

3 Experimental Models Used in this Study

3.1 The Murine Air Pouch Model

The subcutaneous injection of air into the back of mammals (e.g., rats) to create an air pouch has been used for decades to study inflammation [265, 266]; this model also mechanically reorders the tissues, and forms a closed cavity that structurally resembles that of the inflamed synovium [266]. Moreover, the lining cells inside of the pouch consist primarily of macrophages and fibroblasts, similar to the observation in the synovial cavity (Figure 13) [266]. Although the linings in the subcutaneous air cavities may not be identical to the joint lining tissue, they show all the essential features of a sealed cavity lined with a sheet of fibroblasts. The air pouch model is suitable for studying the early events of local inflammation, particularly the dynamics of inflammatory mediator production (prostaglandins, leukotrienes, and cytokines) and cell migration in exudate collected at specific time-point [267]. The air pouch used in this study is formed in 6 days by subcutaneous injection of air into the intracapsular area of the back of BALB/c mice. Quantification of mediators and cells in the air pouch is convenient for determining the degree and resolution of inflammation and for assessing the anti-inflammatory activity of drugs.

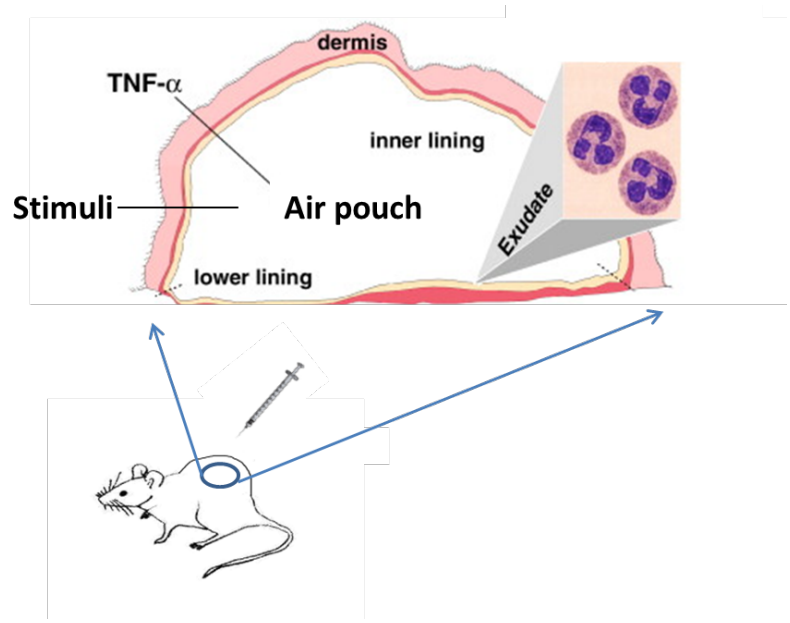


Figure 16. Schematic illustration of the murine dorsal air pouch model

3.2 Fibroblast-like Synoviocytes from Rheumatoid Arthritis Patients

There are two ways to obtain fibroblast-like synoviocytes from RA patients: from synovial fluid (fluid derived, fd-FLS) or from surgical specimens of synovial tissue (tissue derived, td-FLS) [268]. This study focuses on LPA signaling in RAFLS as well as crosstalk with TNFα signaling, to understand the mechanism by which LPA induces cytokine/chemokine secretion with and without the priming effect of TNFα in RAFLS. The synoviocytes mentioned in this study are either from human primary FLS purchased at passage 0 or 1, or from td-FLS.

4 General Objectives

In inflammatory arthritis like RA, one of the key features is the severe and chronic inflammation mediated by cytokines, chemokines, and other lipid mediators such as LPA in the synovium. This study focused on the role of LPA in mediating cytokine/chemokine secretion and other inflammatory responses. The LPA-mediated inflammatory response was investigated using a murine dorsal air pouch model. Since activated synoviocytes play a key role in the inflamed synovium in RA patients, the mechanism by which LPA mediates secretion of several cytokines/chemokines with or without pretreatment by TNFα in the FLS was also investigated.

4.1 Previous studies and unanswered questions (*in vivo* and *in vitro*)

4.1.1 A previous study using an *in vivo* experimental model of inflammation has demonstrated that both LPA_1 and LPA_3 mediate the recruitment of leukocytes into the inflammatory site through a mechanism that is dependent on LPA-mediated chemokine synthesis and exacerbated by the pro-inflammatory cytokine TNFα [269]. That study indicated that LPA contributes to the regulation and maintenance of the inflammatory response in RA, partly through stimulating the production of various cytokines/chemokines by FLS. These cytokines/chemokines may subsequently contribute to the recruitment of immune cells, such as neutrophils, monocytes, T cells and B cells from the peripheral blood into the inflamed synovium. In the qualitative chemokine analysis from the previous study, besides KC, CXCL13 expression triggered by LPA with or without TNFα was also observed, using a semi-quantitative method called Proteome Profiler Mouse Antibody Array Panel [269]. Blocking CXCR2 (KC receptor) and neutralizing KC with anti-KC antibody greatly reduced LPA-mediated recruitment of leukocytes into TNFα-primed air pouches. However, CXCR2 neutralization did not completely block LPA-induced leukocyte recruitment with TNFα priming. Whether CXCL13 secretion mediated by LPA also contributes to leukocyte recruitment into the inflammatory site in the air pouch is unknown. Since CXCL13 is a

chemoattractant for homeostatic B cells, and CXCL13 protein expression and secretion were detected in RA synovial fluid T cells, it is possible that CXCL13 plays a role in recruiting leukocytes, especially lymphocyte recruitment.

4.1.2 A previous *in vitro* study highlighted in RAFLS roles for ERK1/2 (p42/44 MAPK), p38 MAPK and Rho kinase in LPA-mediated IL-8 secretion [257]. In addition, this IL-8 secretion can be enhanced by TNFα. In the same study, TNFα also enhanced LPA_3 receptor expression in RAFLS, a phenomenon which is correlated with LPA-mediated super-production of cytokines/chemokines by TNFα-primed cells. The downstream signaling pathways of those above-mentioned kinases, however, are not clear in cytokine/chemokine production mediated by LPA in human RAFLS. Though there are reports indicating a role for MSK and CREB in LPA-mediated IL-8 expression in various cell lines [155, 270], it is unclear whether in RAFLS MSK and CREB are necessary or important for IL-8 secretion mediated by LPA. It is also not clear after TNFα pretreatment in RAFLS, whether the mechanism whereby LPA-induced IL-8 secretion is the same for the enhanced production of IL-8 or even other chemokines.

4.2 Hypothesis

The general hypothesis of this study is: Signaling through LPA receptors promotes the production of chemokines and recruitment of leukocytes to sites of inflammation. MSKs are master regulators of chemokine/cytokine production induced by LPA in RAFLS.

4.3 Objectives

4.3.1 To study whether LPA-mediated CXCL13 secretion regulates recruitment of leukocyte subsets into the air pouches with TNFα priming in the mouse air pouch model.

The second chapter of this thesis is aimed at understanding the role of cytokines/chemokines other than KC mediated by LPA in the recruitment of leukocyte subsets including lymphocytes under the exacerbation of TNFα *in vivo*. The goal of the

first part of this thesis project was thus to study the role of other chemokines, such as CXCL13/BLC/BCA-1, in LPA-mediated lymphocyte recruitment in the TNFα-pretreated air pouch. Chapter 2 will present the results of these experiments.

4.3.2 To analyze the signaling pathways involved in LPA-induced chemokine secretion in human FLS with and without TNFα priming.

We hypothesize that the MSK-CREB axis is involved in LPA-mediated chemokine secretion, and that the MSK-CREB axis signals downstream of MEK-ERK, p38MAPK and Rho kinase activation. The mechanism by which LPA mediates chemokine (IL-8 and MCP-1) secretion is presented in Chapter 3.

We also hypothesize that TNFα will synergize with LPA, by enhancing the activity of MSKs and CREB, resulting in higher production of chemokines in RAFLS. The mechanism by which TNFα primes FLS for LPA-mediated super-production of cytokines/chemokines is addressed in Chapter 4.

Chapter 2

LPA promotes T cell recruitment through synthesis of CXCL13

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2.1 Abstract

Lysophosphatidic acid (LPA) is a bioactive phospholipid playing an important role in various inflammatory diseases by inducing expression and secretion of many inflammatory cytokines/chemokines. Here we report in a murine air pouch model of inflammation that LPA induced CXCL13 secretion in a time-dependent manner and with exacerbation of the response when LPA was administered after a pretreatment with TNFα, a key inflammatory cytokine. LPA mediates recruitment of leukocytes, including that of CD3+ cells into unprimed and TNFα-primed air pouches. CXCL13 neutralization using a blocking antibody injected into air pouches prior to administration of LPA into TNFα-primed air pouches decreased CD3+ cell influx. Our data highlight that LPA-mediated CXCL13 secretion plays a role in T cell recruitment and participates in regulation of the inflammatory response.

Key words: Lysophosphatidic acid; CXCL13; mouse air pouch model; CD3+ cells; inflammation

2.2 Introduction

Lysophosphatidic acid (LPA) is a bioactive phospholipid with a simple structure containing a three-carbon glycerol backbone and a single acyl side chain that can vary in length and saturation [1]. By binding to and activation of its specific G protein-coupled receptors (GPCRs), LPA has been shown to evoke a great diversity of cellular responses, pointing out its important role in various physiological and pathophysiological situations [2, 3]. Increasing numbers of studies show that LPA plays a role in various inflammatory diseases [4–6]. Elevated LPA levels were detected in several biological fluids collected from different animal models of inflammation or patients with inflammatory diseases [2, 7, 8]. Increased expression of the LPA producing enzyme autotaxin (ATX) has also been reported in synovial tissues from patients with rheumatoid arthritis (RA) [8, 9]. Elevated expression of ATX and/or aberrant expression of LPA receptors were also found in several human malignancies [10]. LPA not only acts as a mediator implicated in cellular migration, growth, and immune cell chemokinesis, but also promotes directed cell motility indirectly by inducing cytokine/chemokine secretion [11]. CXCL8 is one of the leukocyte chemoattractant chemokines reported to be induced by LPA in fibroblast-like synoviocytes from RA patients [9], as well as various other cell types [12–15].

Chemokines play a key role in cellular trafficking of leukocytes during inflammation and immune surveillance [16]. CXCL13 is a CXC chemokine characterized as the sole B cell chemoattractant signal, originally named BLC and BCA-1 [17]. The only known receptor for CXCL13 is CXCR5, which exclusively binds CXCL13 [18]. CXCR5 is expressed by mature B cells [19], a subset of CD4+ and CD8+ T cells in secondary lymphoid tissue follicles [20], immature dendritic cells (DCs) [21], and macrophages [22]. The CXCL13-CXCR5 axis regulates T cell migration to the germinal centers in lymphoid tissues for early T-B cell collaboration and B cell activation [23], induction of migration of immature DCs [21], and maintenance of epithelial cell angiostatic activity [24]. Human CXCL13 is also reported to be an

agonist of human CXCR3 receptor, which plays an important role in recruitment of activated T cells into secondary lymphoid tissues [25]. CXCL13 has been considered a putative diagnostic marker for some acute or chronic infectious and inflammatory diseases [26–30]. Increased CXCL13 production by osteoblasts from osteoarthritis patients in response to stimulation with IL-1β has been reported [31].

In the mouse air pouch model of inflammation activation of LPA1 and LPA3 receptors regulates leukocyte recruitment mainly through CXCL1 chemokine synthesis and its cognate receptor CXCR2 [11]. In this model TNFα, which is a key inflammatory cytokine in autoimmune diseases such as RA, increases the expression of LPA1 and LPA3 in the air pouch lining tissue. By mimicking a proinflammatory environment, priming of air pouches or of human synovial fibroblasts with TNFα exacerbates cytokine/chemokine secretion in response to LPA [9, 11]. Even though LPA induces the secretion of numerous cytokines/chemokines, whether LPA is able to recruit leukocytes including T and B cells to an inflammatory site through synthesis of CXCL13 has not been investigated. We used the murine air pouch model to assess the interaction between LPA, CXCL13, and lymphocyte recruitment after local pretreatment with TNFα, which mimics severe inflammation in vivo. Neutralization of CXCL13 with a blocking antibody was also performed to determine whether LPA-mediated CXCL13 secretion regulates recruitment of leukocyte subsets into the air pouches. We demonstrate that LPA induces the recruitment of leukocytes including T lymphocytes into air pouches through a mechanism that is mostly dependent on CXCL13 synthesis.

2.3 Materials and Methods

2.3.1 Materials

Oleoyl-sn-glycero-3-phosphate (LPA) was purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Murine TNFα was from PeproTech Inc. (Rocky Hill, NJ, USA). CXCL13 ELISA dual kit, rat anti-mouse CXCL13 antibody (rat IgG2A, clone 143614), control rat IgG2A (clone 54447), and Proteome ProfilerTM Mouse Cytokine Array Panel A were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Anti-CD16/CD32, anti-mouse CD11b-eV450, and their matched isotype controls were from eBioscience (San Diego, CA, USA). Anti-mouse CD3e-APC, anti-mouse CD19-PE, and their matched isotype controls were from BD Bioscience (San Diego, CA, USA). All other reagents were obtained from Sigma-Aldrich Canada (Oakville, ON, Canada).

2.3.2 The Air Pouch Model

Female Balb/c (wild type) mice 6–8 weeks old (Charles River, St.-Colomban, Canada) were used to create air pouches. All experimental procedures carried out on mice were approved by the Animal Care Committee at Laval University and conformed to the Canadian Council on Animal Care standards and guidelines.

Air pouches were raised on the dorsum of mice by subcutaneous injection of 3 mL sterile air on days 0 and 3 as previously described [11]. Before the injection of air, mice were briefly anesthetized with isoflurane. On day 7, LPA (3 μg) in 1 mL of phosphate-buffered saline (PBS) supplemented with 0.1% endotoxin-free delipidated bovine serum albumin (BSA) was injected into air pouches. TNFα (50 ng) was injected into air pouches 16 h prior to stimulation with LPA or administration of the CXCL13 neutralizing antibody. To assess the impact of CXCL13 neutralization on LPA-induced leukocyte recruitment, the rat anti-mouse CXCL13 blocking antibody (10 μg) was injected into the air pouch 15 min prior to stimulation with LPA. At specific times, mice were anesthetized with isoflurane and killed by asphyxiation using CO2. Air pouches were washed twice with 1 mL of PBS containing 5 mM EDTA and harvested pouch

fluids were centrifuged 5 min at 3000 rpm. The supernatants were collected and kept at −80°C for later cytokine/chemokine measurements. Cell pellets were suspended in PBS-EDTA and counted using the Moxi mini automated cell counter (ORFLO, Hailey, ID, USA) prior to cell staining for flow cytometry analyses.

2.3.3 Flow Cytometry Analysis

For flow cytometry analysis, the cell pellets from each group of mice were suspended in flow cytometry staining buffer (eBioscience, San Diego, CA, USA) and the cells were pooled into one tube. The samples were then incubated with the anti-CD16/CD32 antibody (0.5 μg/106 cells) for 15 min on ice for FcγR blocking prior to cell staining with 0.1 μg of anti-mouse CD11b-eV450, 0.1 μg of CD3e-APC, and 0.1 μg of anti-mouse CD19-PE for 45 min. Cell suspensions were then processed for FACS analysis using a FACSCalibur (Becton Dickinson, Mississauga, ON, Canada).

To prepare single cell suspension of splenocytes, the spleens from mice with inflamed air pouches were collected, mechanically disrupted, and passed through a strainer according to the manufacturer's instructions (BD Bioscience). FACS analysis used mouse splenocytes as positive controls for titration of anti-CD3 and anti-CD19 antibodies and gating of CD3+ and CD19+ cells.

2.3.4 Assessment of CXCL13 Secretion in the Air Pouch Lavage Fluids

The air pouch exudates from each treatment group (5–10 mice) were pooled and incubated with the Proteome Profiler Mouse Antibody Array Panel A according to the manufacturer's instructions for qualitative and semiquantitative analysis of cytokine/chemokine production by densitometry. Each pair of duplicate spots on the film represents a specific cytokine/chemokine. For accurate quantification of the levels of CXCL13 in air pouch lavage fluids from each mouse, a CXCL13 ELISA was performed according to the manufacturer's instructions (R&D Systems). Each sample was tested in duplicate and the results were compared with a standard curve that was

generated using known concentrations of CXCL13. The dynamic range of the CXCL13 ELISA is 15.6 pg/mL–1000 pg/mL.

2.3.5 Statistical Analysis

Experiments were performed with 5–10 mice/group and results are expressed as mean ± SE of representative studies. All statistical analysis was performed using Prism 5.0 software. Statistical significance of the difference between samples of two different treatments was determined by -test (two-tailed value). For the time course studies, statistical significance between nontreated (NT) samples or samples treated at 0 h and those treated for the indicated time points was determined by one-way ANOVA, Dunnett's multiple comparison test. Multiple comparisons in the same experiment were made using one-way ANOVA, Bonferroni multiple comparison test. values less than 0.05 were considered statistically significant.

2.4 Results

2.4.1 LPA-Mediated Release of CXCL13

LPA injected into air pouches has been reported to induce the synthesis of multiple cytokines/chemokines including IL-6, IL-1β, IL-16, KC, IP-10, and MIP-2 [4, 11]. Whereas the chemokine CXCL1 (also named KC or Gro-α) plays a role in LPA-mediated leukocyte recruitment into the mouse air pouch, blocking CXCL1 or its receptor CXCR2 does not completely reduce leukocyte influx suggesting the involvement of other chemokines or inflammatory mediators [4, 11]. In this series of experiments we focused on LPA-induced CXCL13 secretion into the air pouches. As previously reported [11], injection of 3 μg LPA into the air pouch for 2 hours increases the secretion of CXCL13 as assessed using a qualitative mouse Cytokine/Chemokine Antibody Array assay (Figure 1(a)). Pretreatment of the air pouch tissues with TNFα (50 ng) for 16 hours also increased the levels of CXCL13 in the air pouch exudates relative to mice injected with vehicle alone. The combined effect of TNFα pretreatment prior to LPA stimulation enhances CXCL13 synthesis as estimated by densitometry (Figure 1(b)).

ELISA was then used to accurately quantify the kinetics of CXCL13 secretion (Figure 2(a)). The release of CXCL13 was significantly increased at 30 min after LPA stimulation and remained elevated up to 4 hours, the last time tested. TNFα injected into the air pouches also induced CXCL13 secretion in a time-dependent manner (Figure 2(b)). A significant increase in CXCL13 secretion was observed at 4 hours and reached a maximum at 12 hours after TNFα treatment, after which it declined. Although not statistically significant, a trend for higher levels of CXCL13 in air pouch lavage fluids at 16 hours following TNFα treatment was observed compared to mice injected with vehicle alone (Figures 2(b) and 2(c)). When air pouches were pretreated with TNFα for 16 hours, LPA induced robust secretion of CXCL13, which peaked at 2–4 hours after LPA stimulation (Figure 2(c)). TNFα injected into the air pouches prior to LPA

stimulation for 2 hours greatly potentiated CXCL13 secretion compared to mice injected with TNFα alone or LPA alone (Figure 2(d)).

2.4.2 LPA Recruits Various Leukocyte Subtypes into the Air Pouch

Since CXCL13 is a ligand for CXCR5, a chemokine receptor expressed by mature B cells [19], and a subset of CD4+ and CD8+ T cells [20], we next determined whether LPA-mediated CXCL13 secretion contributes to recruitment of leukocyte subsets toward LPA into TNFα-pretreated air pouches. As reported previously for LPA alone [11], LPA injected in TNFα-pretreated air pouches (16 hours) stimulated the recruitment of leukocytes in a time-dependent manner (Figure 3(a)). An increase in the number of migrated leukocytes was detectable 2 h after LPA injection, peaked after 6 h, and declined thereafter. CD11b+ cells were the most prominent population in air pouch lavage fluids (Figure 3(b), left panel). We focused on CD11b− cells and performed CD19-labelling to determine by FACS whether CD19+ B lymphocytes could be detected in air pouch exudates. Even though the CD19-PE antibody labeled B cells isolated from mouse spleens (Figure 3(c)), no CD19+ B lymphocytes were detected in air pouch exudates (Figure 3(b), middle panel). However, CD11b−/CD3+ cells were detected in air pouch lavage fluids (Figure 3(b), right panel). Figure 3(d) shows that stimulation with LPA for 6 hours enhanced significantly the number of CD3+ cells in air pouch exudates (3.07 ± 0.53 × 104 cells, % of total leukocytes,) compared to mice injected with vehicle alone (1.7 ± 0.32 × 104 cells, % of total leukocytes,). The number of CD3+ cells in air pouch lavage fluids collected from TNFα-pretreated air pouches was not different from that of mice injected with the vehicle alone. Furthermore, LPA injected into TNFα-primed air pouches stimulated the recruitment of CD3+ cells in a time-dependent manner (Figure 3(e)). As observed for total leukocytes, recruitment of CD3+ cells peaked at the 6-hour time point following injection of LPA into air pouches (1.21 ± 0.19 × 105 cells, % of total leukocytes,).

2.4.3 Effect of Blocking CXCL13 on LPA-Mediated CD3+ Cell Recruitment

It was reported previously that antibody neutralization of CXCL13 can prevent migration of double-negative regulatory T lymphocytes to cardiac allografts implanted in the abdomen of mice [32]. To examine whether a correlation exists between CXCL13 secretion in response to LPA and CD3+ lymphocyte recruitment in our mouse model, a neutralizing anti-CXCL13 antibody was injected into TNFα-primed air pouches prior to LPA stimulation. Injection of the neutralizing antibody against CXCL13 prior to LPA into the air pouch significantly reduced LPA-induced CD3+ lymphocyte recruitment into TNFα-primed air pouches, whereas the isotype control antibody had no significant effect on LPA-mediated CD3+ lymphocyte influx (Figure 4). Taken together, the data indicate that CXCL13 plays a role in LPA-mediated recruitment of CD3+ lymphocytes into the air pouches.

2.5 Discussion

Extracellular LPA is a bioactive lysophospholipid produced by ATX that mediates its effects through activation of various LPA receptors [2]. Using the mouse air pouch model of inflammation, we previously demonstrated that LPA promotes the influx of neutrophils and other leukocyte subtypes including macrophages/monocytes and lymphocytes through activation of two LPA receptors, LPA1 and LPA3 [11]. Stimulation of these LPA receptors expressed by cells lining the air pouch cavity promotes the synthesis of various chemokines/cytokines (IL-6, IL-1β, IL-16, KC, IP-10, MIP-2, and CXCL13), the synthesis of which is greatly enhanced by TNFα injected into the air pouches 16 hours prior to LPA. Although LPA-mediated KC synthesis was shown to play a predominant role in the recruitment of leukocytes into the air pouches, neutralization of KC or blocking of its cognate receptor CXCR2 was not able to totally abrogate the influx of leukocytes [11]. In the present study, we focused on CXCL13, a key chemoattractant of B cells and of subsets of T lymphocytes [19–21]. We report that administration of LPA or TNFα into air pouches increased the levels of CXCL13 in air pouch lavage fluids in a time-dependent manner. The combination of a pretreatment of the air pouch tissues with TNFα prior to LPA stimulation greatly enhanced LPA-mediated CXCL13 secretion. The release of CXCL13 induced by LPA peaked 2 hours ahead of the time point of maximal leukocyte recruitment, including that of CD3+ immune cells. Consistent with a role for CXCL13 in LPA-mediated CD3+ cell homing, antibody neutralization of CXCL13 prevented the influx of these T cells into TNFα-pretreated air pouches.

Elevated levels of ATX and of LPA have been reported in synovial fluids collected from RA patients [4, 7–9]. The ATX-LPA axis is emerging as a regulator of lymphocyte homing and inflammation [33]. ATX binds to lymphocytes in a α4β1-dependent manner [34]. Through activation of LPA receptors expressed by T cells [33], LPA induces a polarized morphology that is required for transendothelium

migration, a key step for regulation of naive T cell entry into secondary lymphoid organs [34, 35]. However, LPA does not promote directed cell migration in vitro but exerts a chemokinetic effect that increases the chemoattractant effect of chemokines regulating human and mouse T cell homing in various tissues [34–37]. We cannot exclude the possibility that LPA injected into air pouches has a motility-stimulating effect on leukocytes. However, we showed that CXCL13 secretion induced by LPA preceded the peak of leukocyte recruitment, including that of CD3+ cells by several hours. There was a twofold increase in the total number of CD3+ cells in lavage fluids from TNFα-pretreated air pouches after 6 hours of stimulation with LPA, but when expressed as a percentage of total leukocytes no increase in the relative abundance of CD3+ cells was noticed due to the massive recruitment of CD11b+ cells (i.e., neutrophils) at this time point [11]. Antibody neutralization of CXCL13 prior to LPA stimulation suggests that LPA was exerting its effect on CD3+ cell influx into TNFα-pretreated air pouches in a manner that is dependent on CXCL13 synthesis. The lack of small molecule inhibitors of CXCR5 or CXCR5 neutralizing antibodies precluded further analyses of the molecular pathways by which LPA recruits CD3+ cells in this in vivo model of inflammation.

As CXCL13 is a ligand of CXCR5, which is expressed on B cells, macrophages, monocytes, double-negative Treg cells [32], and T helper cells in human and mouse [22, 38–40], it is possible that CXCL13 induced by LPA could play an important role in leukocyte homing in various diseases. CXCL13 was originally identified as a B cell chemoattractant [17]. Furthermore, lysophospholipids such as S1P and LPA have been suggested to regulate splenic B cell homing through CXCL13-mediated integrin-dependent adhesion [41]. Recruitment of B cells into the air pouch wall has been reported following stimulation with oxidized phospholipids and LPS [42]. In our experiments, after blocking Fc receptors with an anti-CD16/CD32 antibody, no B cells were detected in the lavage fluids collected after stimulation of TNFα-pretreated air

pouches with LPA. Whether B cells are recruited by LPA and remain sequestered in the air pouch wall will need further studies.

DCs [43], human monocytes/macrophages, and CD4+ T cell subsets are potent inducible sources of CXCL13 [44, 45]. We report that the basal levels of CXCL13 in air pouch lavage fluids increased quickly in response to LPA and well before an increase in immune cell influx could be monitored. CXCL13 secretion could be mediated through binding of LPA to its cognate receptors, possibly LPA1 and LPA3 [11], expressed by air pouch lining cells or discrete populations of cells recruited early. Although identification of cells that contribute to CXCL13 production awaits further characterization, this study suggests that a role for the CXCL13-CXCR5 axis in LPA-mediated regulation of immune cell trafficking to sites of inflammation cannot be ignored.

CXCL13 has been identified as a serologic marker predictive of disease severity in early RA [28, 29]. High levels of CXCL13 were measured in synovial fluids from RA patients, with RA synovial T helper cells contributing to CXCL13 secretion [46, 47]. Within the RA synovium CXCL13 is expressed in areas of B cell accumulation characteristic of ectopic lymphoid follicles where subtypes of CXCL13-expressing T cells (CD3+ and CD4+) and monocytes/macrophages colocalize [47–49]. The receptor for CXCL13 is upregulated in the RA synovium and associated with the presence of CXCR5 positive B cells and T cells infiltrating the synovia [22]. Of note Zheng et al. [50] reported that neutralization of CXCL13 at the boosting stage reduced the development of ectopic lymphoid follicles and the severity of collagen-induced arthritis in mice.

2.6 Figures

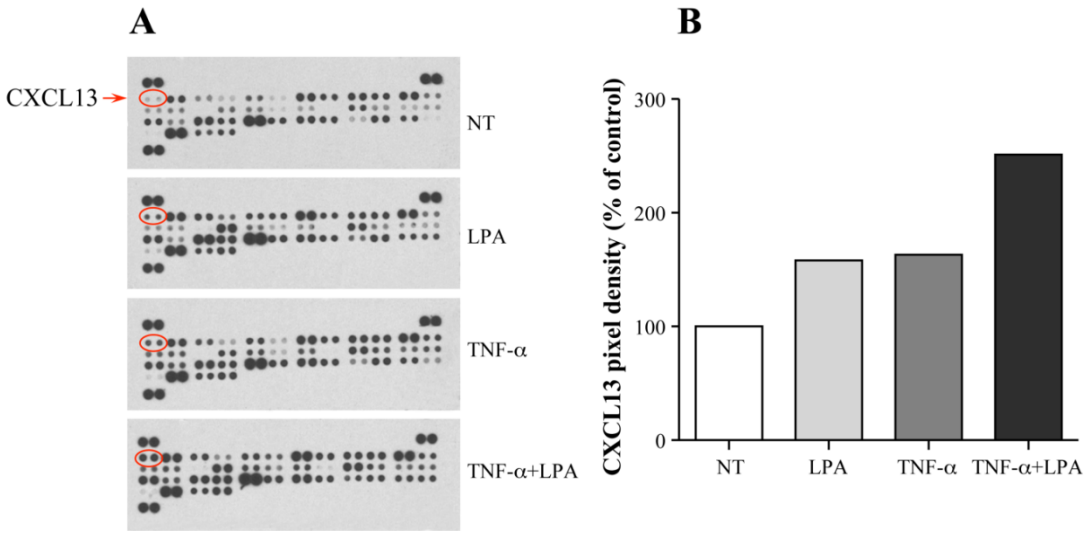

Figure 1 Effect of LPA on CXCL13 secretion in the murine air pouch with or without TNFα pretreatment.

(A) Six-day old air-pouches were produced in the dorsal skin of mice and injected with TNFα or the vehicle for 16 h prior to stimulation with LPA for 2 h. The non-treated group (NT) was injected with vehicle only (PBS-BSA). The air pouch exudates (n=5) were collected and pooled for qualitative analysis of cytokine/chemokine secretion using the Proteome Profiler Mouse Antibody Array Panel A. (B) Data representing CXCL13 pixel density in (A).

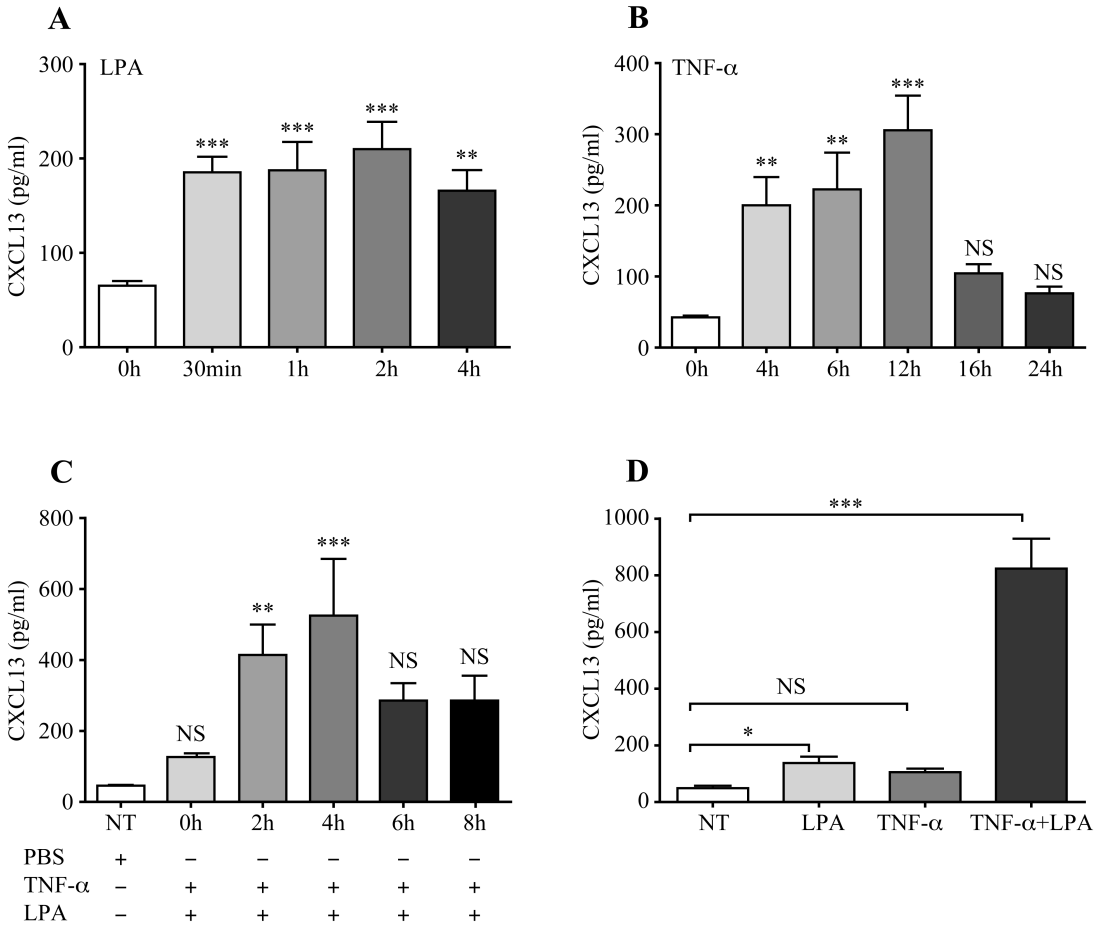

Figure 2 Effect of LPA and TNFα on CXCL13 secretion in the air pouch. (A, B) Kinetics of LPA and of TNFα-mediated CXCL13 secretion

(A) LPA (3 μg) or (B) TNFα (50 ng) was injected into air pouches and air pouch exudates were collected at indicated times. (C) Kinetics of LPA-induced CXCL13 secretion in air pouches pre-treated with TNFα. TNFα was injected 16 h before LPA stimulation. Air pouch exudates were collected at indicated times. (D) Comparison of LPA-mediated CXCL13 secretion in untreated and TNFα-primed air pouches. TNFα or vehicle was injected 16 h prior to administration of LPA for 2 h. Exudates were collected and cytokine/chemokine secretion was measured by ELISA. The non-treated groups (NT) were injected with vehicle only (PBS-BSA). Data are the mean ± SE from three independent experiments performed with at least five mice per group. *P < 0.05, **P < 0.01, ***P < 0.001

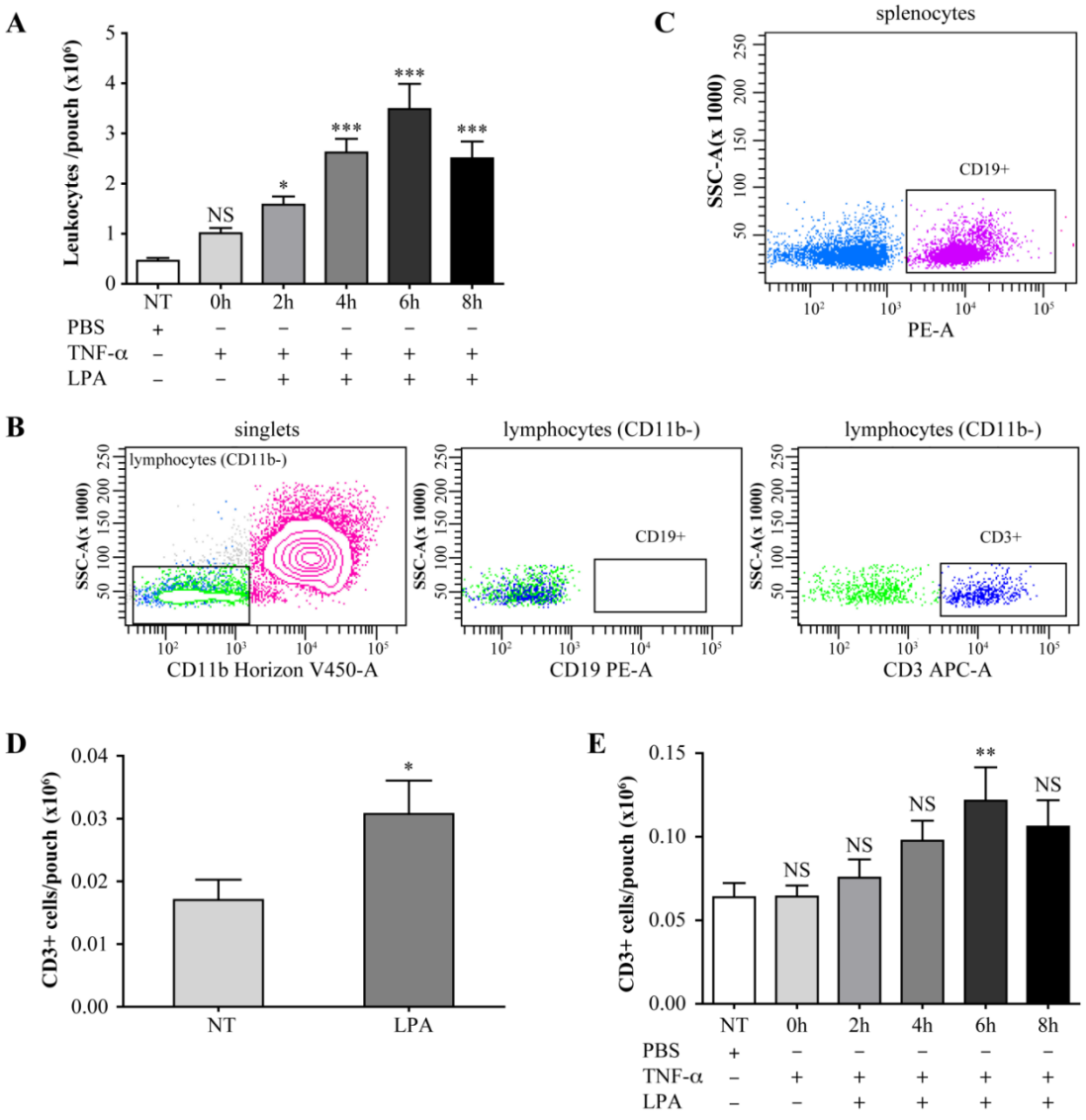

Figure 3 LPA-induced leukocyte recruitment in untreated and TNFα-primed air pouches

(A) Kinetics of LPA-mediated leukocyte recruitment into TNFα-treated air pouches. TNFα (50 ng) was injected into the air pouches 16 h prior to stimulation with 3 μg LPA for the indicated times. Air pouch exudates were collected and the number of leukocytes was determined as described in “Materials and Methods”. (B) Leukocyte populations in lavage fluids collected from air pouches pretreated with TNFα for 16 h and injected with LPA for 6 h. Cells were stained with various leukocyte markers and analyzed by flow cytometry. The CD11b- cells were defined as lymphocytes according to their low granularity (left panel), which stained positive for CD3 (T cells, right panel)

or CD19 (B cells, middle panel). (C) Labelling of B cells isolated from mouse spleen. Splenocytes were prepared as described in “Materials and Methods” and used for titration of anti-CD3e and anti-CD19 antibodies. (D) LPA-induced CD3+ cell recruitment into the air pouches. Air pouch exudates were collected at 6 h post-LPA injection. The total number of leukocytes was measured and that of T cells determined by flow cytometry. (E) The absolute numbers of CD3+ cells recruited by LPA into TNFα-primed air pouches was evaluated as described in (D). Data are the mean ± SE from 6 mice/group. *P < 0.05, **P < 0.01, ***P <0.001 by analysis of variance. SSC= side scatter; FSC = forward scatter.

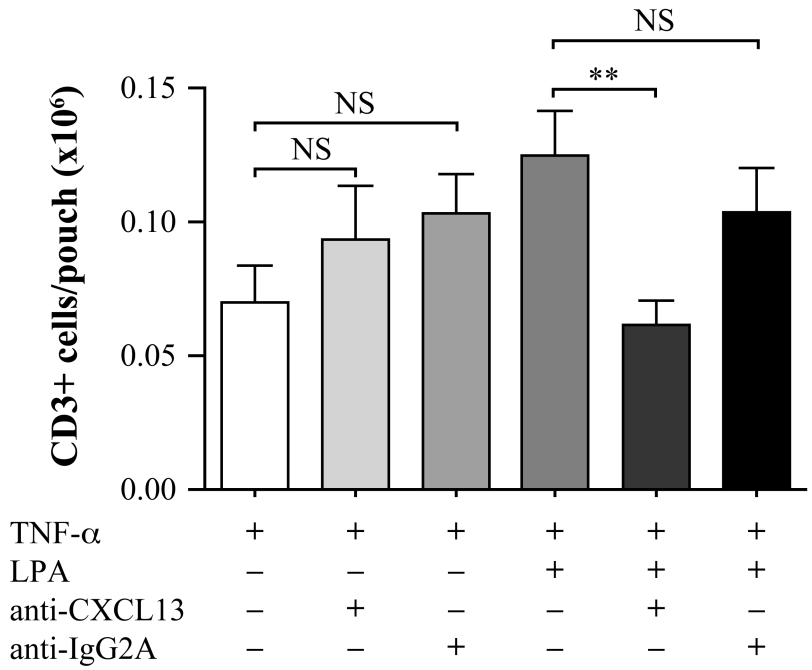

Figure 4 Effect of the CXCL13 neutralizing antibody on LPA-mediated lymphocyte recruitment into TNFα-primed air pouches

Air pouch tissues were pretreated with TNFα (50 ng) for 16 h prior to administration of LPA (3 μg). Where indicated, the anti-CXCL13 neutralizing antibody or the isotype control antibody (IgG2a, 10 μg) was administered into the air pouches 15 min prior to stimulation with LPA for 4 h. The absolute numbers of CD3+ T cells in air pouch lavage fluids was determined by flow cytometry as described in Materials and Methods. Data are the mean ± SE of at least 5 mice/group. **P < 0.01 by analysis of variance.

2.7 Conclusions

In summary, we provide evidence that LPA-induced CXCL13 secretion contributed to the recruitment of CD3+ T cells within the air pouch environment under conditions of inflammation exacerbated by TNFα. This study extends the known role of CXCR2 ligand chemokines to the massive recruitment of leukocytes induced by LPA in this mouse model of inflammation [11]. Given that ATX-derived LPA plays a role in the pathogenesis of RA [7–9, 51], LPA-mediated CXCL13 secretion raises the question whether LPA contributes to the recruitment of lymphocytes and extranodal lymphoid neogenesis during chronic inflammation.

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Chapter 3

Lysophosphatidic acid-induced IL-8 secretion involves MSK1 and MSK2 mediated activation of CREB1 in human fibroblast-like synoviocytes

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3.1 Abstract

Lysophosphatidic acid (LPA) is a pleiotropic lipid mediator that promotes motility, survival, and the synthesis of chemokines/cytokines such as interleukin-8 (IL-8) and interleukin-6 by human fibroblast-like synoviocytes from patients with rheumatoid arthritis (RAFLS). In those cells LPA was reported to induce IL-8 secretion through activation of various signaling pathways including p38 mitogen-activated protein kinase (p38 MAPK), p42/44 MAPK, and Rho kinase. In addition to those pathways we report that mitogen- and stress-activated protein kinases (MSKs) known to be activated downstream of the ERK1/2 and p38 MAPK cascades and CREB are phosphorylated in response to LPA. The silencing of MSKs with small-interfering RNAs and the pharmacological inhibitor of MSKs SB747651A shows a role for both MSK1 and MSK2 in LPA-mediated phosphorylation of CREB at Ser-133 and secretion of IL-8 and MCP-1. Whereas CREB inhibitors have off target effects and increased LPA-mediated IL-8 secretion, the silencing of CREB1 with short hairpin RNA significantly reduced LPA-induced chemokine production in RAFLS. Taken together the data clearly suggest that MSK1 and MSK2 are the major CREB kinases in RAFLS stimulated with LPA and that phosphorylation of CREB1 at Ser-133 downstream of MSKs plays a significant role in chemokine production.

KEYWORDS:

Fibroblast-like synoviocytes; Interleukin-8; Lysophosphatidic acid; Mitogen- and stress-activated protein kinases; cAMP response element-binding protein

3.2 Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by chronic inflammation and severe destruction of joints leading to functional disability. The etiology of RA remains largely unknown, but includes the activation of synoviocytes and the recruitment of polymorphonuclear neutrophils. Once arrived at the inflamed synovial compartment, activated neutrophils release a variety of proteases, myeloperoxidase, lipid mediators such as prostaglandins and leukotrienes [1], and cytokines\ chemokines including IL-1b [2], TNFa [3], and IL-8 [4]. Ultimately, the inflammatory and hyperplastic synovial tissue invades and destroys the adjoining cartilage and bone [5].

Fibroblast-like synoviocytes (FLS) are prominent in RA pathogenesis. In the healthy joint, FLS are responsible for the synthesis of extracellular matrix proteins including collagen, fibronectin, hyaluronic acid, and other molecules that facilitate the lubrication and function of cartilage surfaces [6]. Under long-standing inflammatory conditions, activated FLS produce a large variety of cytokines, chemokines, and other inflammatory mediators that help to recruit and retain leukocytes, leading to ongoing inflammation and tissue destruction [7–9]. One such chemokine produced by FLS is CXC chemokine interleukin-8 (IL-8), a major chemoattractant for the migration of neutrophils into the synovium [10]. The expression of IL-8 is markedly upregulated in RA, and is associated with development of clinical signs of synovial inflammation [11]. In early RA enhanced IL-8 expression is observed in synoviocytes of the lining layer [12] whereas in established disease, both synoviocytes and macrophages at the cartilage-pannus junction show increased expression of IL-8 [13]. In an animal model of arthritis, neutralizing IL-8 antibodies protect against leukocyte infiltration and tissue damage in the early phase of joint inflammation [10]. Moreover, in RA patients, treatments inhibiting IL-8 production resulted in decreased migration of neutrophils into the joint and diminished arthritis activity [14–16].

The bioactive lipid lysophosphatidic acid (LPA) has been reported to induce cell proliferation, migration, cytokine production, survival, and angiogenesis in many cell types [17]. LPA binds to specific G protein-coupled receptors (LPA1-6) to influence cell behaviour [17]. The majority of extracellular LPA is produced from lysophosphatidylcholine by autotaxin (ATX), a secreted lysophospholipase-D initially identified from melanoma cell lines [18]. Elevated levels of both ATX [19] and LPA [20] have been detected in synovial fluids from RA patients. Studies from our laboratory revealed that human RAFLS express three LPA receptors, LPA1-3 [19]. LPA stimulates significant production and secretion of IL-8 from human RAFLS [19]. In the mouse air pouch model, administration of LPA into the air pouch enhances the infiltration of neutrophils due to the production of KC [21], a murine homologue of IL-8. Although the signaling pathways implicated in LPA-induced IL-8 production have been reported in other cells types [22–25], a thorough understanding of the signaling pathways involved in LPA-induced IL-8 secretion in human FLS is still incomplete.

The nuclear transcription factor CREB is widely expressed and known to be activated by signaling events induced by growth factors [26]. LPA also stimulates CREB phosphorylation at Ser-133 in various cells types [27–32]. In addition to protein kinase A many other kinases, including mitogen- and stress-activated kinases (MSKs), can phosphorylate CREB at Ser-133 [33]. MSKs are activated downstream of MAPKs. The structure of MSKs includes two kinase domains connected with a linker region and a Cterminal docking domain which assures binding to the activated extracellular-signal regulated kinase (ERK) and p38 MAPKs. In the case of human MSK1, Ser-360, Thr-581 and Thr-700 are phosphorylated by these MAPKs [34]. Among the residues autophosphorylated by activated MSK Ser-376 is essential for phosphorylation of MSK1 substrates. The activation of MSK2 seems to be very similar to that of MSK1 [35,36]. Because MSKs can be phosphorylated by both ERK and p38 MAPKs, they are activated by many physiological and pathological stimuli. For instance, MSKs are able

to integrate signals induced by growth factors, proinflammatory cytokines, and cellular stresses [37], as well as those induced by LPA [32,38].

We previously highlighted in RAFLS a role for p42/44 MAPK and p38 MAPK in LPA-mediated IL-8 secretion [19]. To gain insight into the mechanism by which LPA-triggered signal transduction cascades in RAFLS lead to IL-8 secretion we monitored the activation of MSKs and CREB and assessed the contribution of MSK and CREB protein isoforms to chemokine synthesis. Here, we provide the first evidence that LPA promotes IL-8 and MCP-1 secretion through both MSK1 and MSK2 mediated phosphorylation of CREB in RAFLS.

3.3 Materials and Methods

3.3.1 Reagents

1-Oleoyl-sn-glycero-3-phosphate (LPA) was purchased from Sigma-Aldrich Canada (Oakville, ON, Canada) and Avanti Polar Lipids (Alabaster, AL, USA). TNFα was from PeproTech Inc. (Rocky Hill, NJ, USA). The Human Cytokine/Chemokine Luminex Multiplex Immunoassay kit was from Millipore Corporation (St. Charles, MO, USA). The Proteome Profiler^{TM} Human Phospho-Kinase Array kit was purchased from R&D Systems Inc. (Minneapolis, MN, USA). Human IL-8 and MCP-1 ELISA kits were purchased from BioSource International Inc. (Camarillo, CA, USA) and R&D Systems Inc. (Minneapolis, MN, USA), respectively. Antibodies to human MSK1, MSK2, and phospho-MSK1 (Ser-376)/MSK2 (Ser-360) were from R&D Systems Inc. Antibodies to ERK, phospho-ERK, p38 MAPK, phospho-p38 MAPK, CREB, phosphoCREB (Ser-133), and phospho-MSK1 (Ser-360) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies to actin and the p85 subunit of PI 3-kinase were from Sigma-Aldrich Canada (Oakville, ON, Canada) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), respectively. MSK inhibitor SB747651A was from Axon Medchem (Groningen, The Netherlands). CREB inhibitors KG-501 and 217505 were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada) and Calbiochem (San Diego, CA, USA), respectively. The jetPRIME siRNA transfection reagent was from Polyplus Transfection Inc. (New York, NY, USA). MSK1/2 siRNAs and CREB1/2 shRNA lentiviral particles were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Cell culture reagents were purchased from Wisent Inc. (St-Bruno, QC, Canada). Propidium iodide (PI) was purchased from Invitrogen Canada (Burlington, ON, Canada). Accutase cell detachment solution was from eBioscience (San Diego, CA, USA). All other chemicals and reagents were obtained from Sigma-Aldrich Canada (Oakville, ON, Canada).

3.3.2 Cell Culture

Human primary FLS at passage 0 or 1 were purchased from Asterand (Detroit, MI, USA). Cells were obtained from RA patients who were diagnosed according to the criteria developed by the American College of Rheumatology (ACR) and were undergoing joint surgery on the knee or hip. Cells were maintained under standard conditions (37°C and 5% CO2) and grown in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin 100 IU), and streptomycin (100 mM). Cells were used up to passage 9.

3.3.3 Cell Treatment and Viability

Semi-confluent cells were starved with serum-free medium for 24 h before treatment. At the moment of cell treatment, the culture medium was replaced with fresh serum-free medium containing various concentrations of the tested compounds as indicated in details below. Propidium iodide (PI) was used to evaluate the viability of RAFLS by flow cytometry. Cells were detached using Accutase cell detachment solution and incubated with PI (5 mg/ml). PI negative RAFLS were considered viable.

3.3.4 Multiplex Immunoassay

After starvation, cells were treated with 5 mM LPA for 24 h.Where indicated starved cells were pre-incubated with 80 ng/ml α for 8 h and washed three times with serum-free medium prior to stimulation with LPA. Cell culture supernatants were collected and human cytokines/chemokines IL-1a, IL-1b, IL-8, IL-15, Eotaxin, GM-CSF, IP-10, MCP-1, MIP-1a, MIP-1b, and RANTES were monitored using a Luminex Multiplex Immunoassay according to the manufacturer's instructions. The dynamic range of the assay is 3.2-10000 pg/ml.

3.3.5 Proteome Profiler™ Human Phospho-Kinase Array

After starvation for 24 h, cells were treated with 5 mM LPA for the indicated times. Cells were then lysed and the cell lysate was applied to the array kit. Changes in the

levels of phosphorylated proteins in response to LPA were assayed using the Proteome Profiler^{TM} Human Phospho-kinase Array following the recommended protocol.

3.3.6 Gene Silencing

To knock down gene expression of MSK and CREB in FLS, cells were either transfected with MSK1 or MSK2 siRNA or transduced with CREB1 or CREB2 shRNA lentiviral particles. For gene silencing of MSK1 and/or MSK2, cells grown to 30-50% confluence in 6-well plates were transfected with jetPRIME transfection reagent with 50 nM MSK1 and/or MSK2 siRNA in serum-free DMEM medium according to the manufacturer's recommendation. For gene silencing of CREB1 or CREB2, cells were transduced with 15 ml of CREB1 or CREB2 shRNA lentiviral particles containing 7.5×10^4 infectious units (IFU) of virus in DMEM containing 10% FBS according to the manufacturer's recommendation. Forty-eight hours post-transfection or post-transduction, cells were lysed and the efficiency of silencing was confirmed by Western blot. For evaluating the effect of gene silencing on chemokine secretion, transfected or transduced cells were cultured for an additional 24 h in the presence of LPA before quantification of IL-8 and MCP-1 in the supernatants by ELISA.

3.3.7 Western Blotting

To examine the gene silencing efficiency, cells were lysed 48 h post siRNA transfection or shRNA transduction as described in the above section. To monitor the levels of phospho-MSK and phospho-CREB, cells were exposed to 5 mM LPA for various times (up to 60 min). Where indicated, cells were pre-treated with SB747651A (up to 5 mM) for 30 min prior to LPA stimulation (5 mM, 5 min). To monitor the effect of SB747651A on the levels of phospho-ERK and phospho-p38 MAPK, cells were stimulated with LPA for 5 min and 15 min, respectively. Cells were lysed in boiling sample buffer [50 mM Tris/HCL (pH6.8), 10% (v/v) glycerol, 50 mM DTT, 4% (v/v) SDS] for 7-10 min. Equal amounts of protein were separated by 12% SDS-polyacrylamide gel electrophoresis.

Proteins were later transferred from polyacrylamide gel to methanol soaked Immobilon PVDF membranes (Pall Canada Ltd, Ville St-Laurent, QC, Canada). Primary antibody incubation was performed overnight at 4 degree. The membranes were then washed three times and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Membranes were washed three times and antibody-antigen complexes were revealed using Western Lightning chemiluminescence reagent according to the manufacturer's instructions (Perkin Elmer Life Sciences, Woodbridge, ON, Canada).

3.3.8 IL-8 and MCP-1 ELISA

FLS (2.5×10^4 cells/well) were plated in 24-well plates and serum starved for 24 h prior to stimulation with 5 mM LPA in serum-free medium for an additional 24 h. Where indicated cells were transfected with siRNAs or transduced with shRNA 48 h before LPA treatment or were pre-treated with the inhibitors of MSK (SB747651A) and CREB (KG-501 and 217505) for 30 min prior to stimulation with LPA. Cell culture supernatants were collected and stored at -80 degree until ELISA was performed. IL-8 and MCP-1 in all samples were monitored in duplicate, according to the manufacturer's protocol. Optical densities were determined using a SoftMaxPro40 plate reader at 450 nm. The results were compared with a standard curve that was generated using known concentrations (pg/ml) of IL-8 or MCP-1.

3.3.9 Statistical Analysis

Unless otherwise stated, the data are from three independent experiments and results presented are expressed as mean values ± SE. All statistical analyses were performed using Prism 4.0 software. Statistical significance of the difference between samples of two different treatments was determined by t-test (two-tailed p value). For studies using samples from more than two different treatments, statistical significance between control and treated cells was determined by one-way ANOVA, Dunnett's

multiple comparison test. P values less than 0.05 were considered statistically significant.

3.4 Results

3.4.1 LPA Induces Secretion of Multiple Inflammatory Cytokines/Chemokines in Human RAFLS

To determine the effect of LPA on the production of a broad range of cytokines/chemokines that are relevant to RA pathogenesis we utilized a Luminex Multiplex Immunoassay kit. Among the 11 cytokines/chemokines monitored, IL-1a, IL-1b, IL-15, Eotaxin, GM-CSF, MIP-1a, and MIP-1b were not secreted by control RAFLS or by cells stimulated with LPA, TNFα, or their combination (not shown). RAFLS produced low basal amounts of IL-8, IP-10, MCP-1, and RANTES, but the secretion of these four chemokines was increased 53-, 3.3-, 97-, and 6.1-fold on average in response to LPA, respectively (Table 1). TNFα pretreatment of RAFLS for 8 h also strongly enhanced the subsequent release of IL-8, IP-10, MCP-1, and RANTES. Except for MCP-1,the combined effect of TNFα pretreatment and LPA stimulation on chemokine synthesis was synergistic. Under those conditions the release of IL-8, IP-10, and RANTES was enhanced 156-, 862-, and 50-fold on average as compared to cells stimulated with LPA in the absence of TNFα pretreatment, respectively.

3.4.2 LPA Stimulates the Phosphorylation of Several Kinases and Transcription Factors in RAFLS

To define the mechanism underlying LPA-induced IL-8 secretion in human FLS, we examined the phosphorylation state of specific proteins in kinase signaling pathways using the Proteome Profiler^{TM} Human Phospho-kinase Array kit. In addition to p38 MAPK and ERK1/2, which have previously been shown to be activated in response to LPA [19], LPA treatment increased the phosphorylation of various signaling proteins including MSK1/2, CREB, MEK1/2, STAT transcription factors, b-catenin, Akt, and

eNOS (Fig. 1). In this study, we focused on the phosphorylation of MSK1/2 and CREB which were both increased ~2-fold in response to stimulation with LPA for 5 and 10 min compared to unstimulated cells.

3.4.3 LPA Promotes the Phosphorylation of MSKs and CREB in RAFLS

Both p38 MAPK and ERK1/2 were reported to phosphorylate MSK1 on multiple residues including Ser-360, Thr-751 and Thr700 [37]. Phosphorylation of Thr-751 in the C-terminal kinase domain is essential for activation of this kinase domain which in turn autophosphorylates three residues including Ser-376 located in the linker region and Ser-212 in the N-terminal kinase domain, which is responsible for phosphorylation of MSK1 substrates [37]. To ascertain the time frame of LPA-mediated activation of MSKs and CREB, FLS were stimulated with LPA for up to 60 min, and cell lysates were subjected to Western blotting with phospho-MSK1 (Ser-376)/MSK2 (Ser-360), phospho-MSK1 (Ser-360) and phopho-CREB (Ser-133) antibodies. As shown in Fig. 2A, treatment of cells with LPA enhanced the phosphorylation of MSK1/MSK2 in a time dependent manner. Compared to unstimulated cells, LPA-induced a transient phosphorylation of MSK1/MSK2 that was maximal at 5 min (~3-fold increase) and decreased thereafter to near basal levels. No phosphorylation of MSK1 at Ser-360 could be detected under those conditions (data not shown). Cell stimulation with LPA induced a more sustained phosphorylation of CREB compared to the kinetics of MSK1/MSK2 phosphorylation in RAFLS. LPA-mediated phosphorylation of CREB at Ser-133 reached a plateau between 5 to 15 min and slowly declined thereafter, but remained significantly above the basal value at 60 min (Fig. 2B). Stimulation with TNFα also induced a transient phosphorylation of MSK1/MSK2 that was maximal at 15 min (~6-fold increase) and decreased thereafter to near basal levels at 4 h. CREB phosphorylation at Ser-133 in response to TNFα reached a plateau between 15 min to 1 h (~4.5-fold increase) and slowly declined to basal levels at 8 h. At the time of LPA

addition to RAFLS pretreated with TNFα for 8 h the levels of MSK1/MSK2 and CREB phosphorylation were similar to those in unstimulated RAFLS (data not shown).

3.4.4 MSKs are involved in LPA-Induced Chemokine Secretion in RAFLS

In the next series of experiments the contribution of MSK1 and MSK2 to LPA-induced IL-8 and MCP-1 secretion was examined using a gene silencing strategy. As shown in Fig. 3A and B, transfection of FLS with small interfering RNAs of MSK1 and MSK2 resulted in diminished expression of these proteins 48 h post-transfection compared to control RAFLS or cells treated with a non-targeting control siRNA sequence (37% decrease for MSK1 and 69% decrease for MSK2). Furthermore, silencing of MSK1 and MSK2 had no effect on the levels of total CREB (Fig. 3A and B). On the one hand, silencing of MSK1 significantly reduced LPAmediated IL-8 secretion by ~44% ($p < 0.001$) compared to RAFLS treated with the non-silencing siRNA control (Fig. 3C). On the other hand, silencing of MSK2 decreased LPA-mediated IL-8 secretion by 37% ($p < 0.001$) compared to cells treated with control siRNA (Fig. 3C). The effect of silencing MSK1 and MSK2 simultaneously was additive and reduced LPA-mediated IL-8 secretion by 83% compared to RAFLS treated with non-silencing control siRNA, thereby suggesting that signaling through both MSK1 and MSK2 regulates IL-8 production. As an alternative approach to assess the role of MSK activation in LPA-mediated IL-8 and MCP-1 secretion we used the selective MSK inhibitor SB747651A [39]. Pre-treatment of RAFLS with SB747651A for 30 min prior to LPA stimulation attenuated LPA-induced IL-8 and MCP-1 production in a dose-dependent manner. IL-8 secretion was significantly reduced with 0.1 Mm SB747651A and the highest dose tested (5 mM) inhibited LPAinduced IL-8 by 83% (Fig. 3D). LPA-induced MCP-1 secretion was also reduced by 25% and 59% in RAFLS treated with 0.5 mM and 5 mM SB747651A, respectively (Fig. 3E). The percentage of PI positive cells treated with 5 mM SB747651A for 24 h was identical to that of

untreated RAFLS (2.7 ± 0.5% vs 2.8 ± 0.5%), indicating that inhibition of chemokine synthesis was not mediated by a cytotoxic effect of the MSK inhibitor.

3.4.5 CREB is involved in LPA-Induced Chemokine Secretion in RAFLS

To assess the involvement of CREB in LPA-mediated signaling pathways, we silenced CREB1 and CREB2 in RAFLS. Transduction of cells with CREB1 shRNA lentiviral particles reduced the expression of total CREB by 61% ($p < 0.001$) 48 h post-transfection, compared to cells transduced with control shRNA lentiviral particles and LPA-mediated activation of CREB is dependent on MSKs in RAFLS non-transduced RAFLS (Fig. 4A). Silencing of CREB2 was without significant effect on the levels of total CREB compared to non-treated cells or RAFLS transduced with control shRNA lentiviral particles (Fig. 4B). Silencing of CREB1 significantly reduced LPA-mediated IL-8 and MCP-1 secretion (Fig. 4C and E). Although a role for CREB2 cannot be totally excluded (Fig. 4D), the results suggest that CREB1 regulates the production of chemokines in response to LPA.

3.4.6 LPA-mediated Activation of CREB is Dependent on MSKs in RAFLS

Having shown that LPA induced the phosphorylation of MSKsand CREB, we hypothesized that CREB was phosphorylated by MSKs. To test this hypothesis we investigated a potential role for MSKs in LPA-induced CREB activation in MSK1- and/or MSK2-silenced synovial fibroblasts. As shown in Fig. 5A, the silencing of MSK1 significantly reduced LPA-induced CREB phosphorylation at Ser-133 by 48% ($p < 0.01$). LPA-induced CREB phosphorylation at Ser-133 was also reduced in RAFLS silenced for MSK2 but the decrease (32%) was not significant compared to cells treated with control siRNA (Fig. 5A). However, the concomitant silencing of MSK1 and MSK2 reduced LPA-mediated CREB phosphorylation to near basal levels. Next, we evaluated the effect of the inhibitor of MSKs SB747651A [39]. At 0.1 mM SB747651A reduced LPA-induced CREB phosphorylation to near basal levels, while higher concentrations of the inhibitor reduced the levels of phosphorylated CREB at Ser-133 to below that

seen in unstimulated RAFLS (Fig. 5B). The effect of SB747651A was specific since LPA-induced activation of the MSK upstream kinases p38 MAPK and ERK was not inhibited (Fig. 5C and D). Taken together the data clearly suggest that MSK1 and MSK2 are the major CREB kinases in RAFLS and that phosphorylation of CREB at Ser-133 downstream of MSKs plays a significant role in LPA-mediated IL-8 and MCP-1 production.

3.4.7 The Impact of CREB Inhibitors on LPA-Induced Signaling and IL-8 Production by RAFLS

The next series of experiments evaluated the impact of two structurally related pharmacological inhibitors (217505 and KG-501) that disrupt the interaction of the CREB-binding protein (CBP) with CREB and inhibit CREB-mediated gene transcription [40]. The CREB inhibitor 217505 failed to inhibit LPA-mediated IL-8 production but instead increased IL-8 secretion by RAFLS in a dose dependent-manner. At 10 mM, the highest concentration tested, 217505 enhanced IL-8 secretion by about 2.5-fold (Fig. 6A). The related phosphorylated compound KG-501 also increased LPA induced IL-8 secretion up to 4.1-fold at 5 mM. Increasing the concentration of KG-501 up to 25 mM reduced its stimulatory effect on LPA-mediated IL-8 production (Fig. 7A). Because CREB inhibitors were unable to reduce IL-8 secretion, we assessed the impact of those compounds on LPA-mediated MSK1/MSK2 and CREB phosphorylation. At 5 mM, 217505 alone increased the phosphorylation of MSKs by ~105% and that of CREB by ~700%, and their levels of phosphorylation were enhanced further in response to LPA (Fig. 6B). In contrast, the same concentration of KG-501 had no effect on basal or LPA-mediated phosphorylation of MSKs and CREB in RAFLS (Fig. 7B).

3.5 Discussion

LPA is a potent bioactive lipid that stimulates the gene expression of inflammatory mediators such as chemokines, cytokines, mitogens and pro-angiogenic factors [41]. LPA has been suggested as an important molecule stimulating the inflammatory responses in RA. The presence of LPA and elevated levels of ATX, the enzyme that produces extracellular LPA, have been reported in synovial fluids from RA patients [19,20,42,43]. Stimulation of RAFLS with LPA induces the release of IL-8, a potent chemoattractant for neutrophils [19]. The functional responses to LPA are induced by several LPA receptors, with LPA1 being more strongly expressed by RAFLS [19,20]. A role for LPA1 in the production of IL-8 and IL-6 was highlighted using selective LPA receptor agonists and antagonists [19]. LPA3 may also contribute to IL-8 production, since the expression of this receptor by RAFLS is increased by TNFα, and LPA3 receptor-deficient mice showed decreased recruitment of leukocytes into air pouches in response to LPA due to reduced secretion of KC, a murine homolog of human IL-8 [21]. In the present study we showed that LPA induces the synthesis of a broader range of cytokines/chemokines in FLS, including IL-8, IP-10, MCP-1, and RANTES. Furthermore, in TNFα pretreated FLS, LPA-induced secretion of chemokines was strongly enhanced. These data suggest that LPA may commit fibroblasts to exacerbate an inflammatory microenvironment to enhance leukocyte accumulation as well as FLS migration, proliferation and matrix metalloproteinase production [44].

The signaling pathways regulating the expression of IL-8 have been investigated in RAFLS. Both p38 MAPK and ERK1/2 are activated by LPA and the use of cell-permeant inhibitors of MAPKs has demonstrated a role for p38 MAPK and ERK1/2 in production of IL-8 and IL-6 as well [19]. Using a phospho-kinase array we confirmed that both p38 MAPK and ERK1/2 were phosphorylated and we identified new proteins that were phosphorylated in response to LPA, including MSKs and CREB. Since little information was available on the role of MSK and CREB isoforms in

LPAmediated signaling in synovial fibroblasts we focused on these two phosphorylated proteins. Using several approaches we provide new information suggesting that LPA-mediated production of IL-8 and MCP-1 is dependent on MSK activation. The major finding of this study in RAFLS is that MSK-mediated phosphorylation of CREB at Ser-133 plays a significant role in the regulation of IL-8 and MCP-1 secretion induced by LPA.

Our data show that LPA mediates the activation of MSK in RAFLS. MSK1 is a downstream target of ERK1/2 and p38 MAPKs that phosphorylate the kinase at Ser-360, Thr-581 and Thr-700 [37]. In addition MSK1 can autophosphorylate several residues including Ser-212, Ser-376, Ser-381, Ser-750, Ser-752 and Ser-758 [45]. MSK1 phosphorylation at Ser-360 was not detected in RAFLS, thereby suggesting phosphorylation of other residues by ERK1/2 and p38 MAPKs. In contrast, the autophosphorylation site Ser-376 required for MSK1 activity was phosphorylated in response to LPA. Both MSK1 and MSK2 are expressed by RAFLS. Though our experiments could not discriminate between phosphorylation of MSK1 at Ser-376, MSK2 at Ser-360 or a combination of the two, we observed that MSK1 or MSK2 silencing reduced LPA-mediated IL-8 secretion, and that combined silencing of MSK1 and MSK2 further reduced IL-8 production. The activation of MSK1 has been reported in various cell types [29,31,32,46] and a role for MSKs in LPA-induced responses was evaluated using H89, a potent inhibitor of MSK1 and of protein kinase A [39]. Here we show that the MSK inhibitor SB-747651A inhibited LPA-mediated IL-8 and MCP-1 production. Taken together the results suggest that both MSK1 and MSK2 isoforms are activated in response to LPA and contribute to chemokine production in RAFLS.

MSKs were initially identified as CREB kinases leading to CREB transcriptional activity. MSKs are predominantly found in cell nuclei [47]. In addition to CREB, several nuclear proteins and transcription factors are known targets of MSKs including histone H3, ATF1, p65Rel and STAT3 to name a few [37]. Our screen to identify proteins in RAFLS that were phosphorylated in response to LPA revealed that CREB as

well as Akt, eNOS, and several STATs (including STAT3) were phosphorylated in response to LPA. Phosphorylation of CREB is triggered by diverse extracellular stimuli, including several growth factors [48,49], and stress signals including hypoxia [50] and ultraviolet light [47]. These stimuli activate CREB through multiple signaling pathways that eventually converge on the phosphorylation of a critical residue, Ser-133. When phosphorylated at Ser-133, CREB binds to CREB binding proteins to activate transcription of target genes. There are a number of cellular kinases known to phosphorylate CREB, including p90RSK [50], MSK1 [47], AKT [51], MAPKAP-2 [52], PKC [53], and Ca2+/calmodulin-dependent protein kinases [54]. Among these CREB kinases, p90RSK and MSK1, which are serine/threonine kinases, are known to promote CREB phosphorylation in response to growth factor stimulation. MSK1 is activated by both ERK1/2 and p38 MAPK [47]. The inhibitors of ERK1/2 PD98059 and of p38 MAPKs SB203580 have been reported to decrease LPA-induced CREB phosphorylation in rat-2 fibroblast cells [31]. Because inhibition of ERK1/2 and p38 MAPK reduced LPA-mediated IL-8 secretion in RAFLS [19], and phosphorylation of MSKs reached a peak prior to that of CREB, we suspected that MSKs were the CREB kinases. In the present study we show that silencing of MSK1 or MSK2 partially reduced LPA-induced phosphorylation of CREB at Ser-133 and that combined silencing of MSK1 and MSK2 abolished CREB phosphorylation. CREB phosphorylation induced by LPA was also abolished by the MSK inhibitor SB747651A [39]. Taken together the data suggest that CREB is a target of both MSK1 and MSK2 in RAFLS stimulated with LPA.

In this study we show that the inhibition or the silencing of MSKs inhibits CREB phosphorylation at Ser-133 and the production of IL-8 by LPA, thereby suggesting a functional link. Silencing of CREB with shRNA directly links CREB1 to LPA-induced IL-8 and MCP-1 secretion downstream of MSKs. In contrast to the MSK inhibitor, treatment with CREB inhibitors (KG-501 and 217505) did not decrease but increased LPA-induced IL-8 secretion. In addition, we discovered that 217505 alone or in

combination with LPA enhanced the levels of phosphorylated MSKs and CREB. The effect was specific to 217505 since the levels of phospho-MSK and phospho-CREB were not altered by KG-501, which is a phosphorylated form of 217505. Treatment of non-small cell lung cancer cells with low mM concentrations of KG-501 has also been reported to enhance IL-1b-induced IL-8 expression [55]. The two inhibitors KG-501 and 217505 are small molecules that bind to the KIX domain of CBP and disrupt its interaction with phosphorylated CREB [40,56]. Because KG-501 and 217505 disrupt CBP-CREB interaction we cannot exclude that the transcriptional activity of CREB on the IL-8 promoter is independent of CREB and/or favors the recruitment of another transcription co-activator involved in CREB-dependent transcription machinery [55,57]. Our findings established that CREB phosphorylation by MSKs is a crucial event in LPA-induced IL-8 and MCP-1 secretion. Because LPA-induced chemokine secretion was not completely lost by inhibition or silencing of MSKs and CREB, it is likely that there could be additional pathways that are involved in LPA-mediated chemokine production in RAFLS. Thus, understanding the mechanism of how LPA regulates MSK signaling and transcription factor activity in nuclei requires further analyses.

In summary, we have demonstrated for the first time that LPA-induced IL-8 secretion in RAFLS is dependent on MSK-mediated activation of CREB. A summary of the results from this study is presented in Fig. 8. We have further shown that RAFLS express the two MSK isoforms MSK1 and MSK2 and that they both contribute to phosphorylate CREB at Ser-133, which is crucial for its transcriptional activity. The results imply that elevated levels of LPA in synovial fluids, which promote the secretion of various chemokines including IL-8 and MCP-1 by FLS, may worsen inflammation in arthritis through activation of the MSK-CREB signaling axis. Therefore, targeting the activation of MSKs with pharmacological inhibitors might be a promising strategy in the treatment of RA and other inflammatory diseases.

3.6 References

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3.7 Table

Table 1 Effect of LPA on cytokine/chemokine secretion in human RAFLS with or without TNFα pre-treatment.

Table 1
Cytokine/Chemokine secretion in cell culture supernatants.

Cytokine/Chemokine			Non-treated	Treatment					
				LPA		TNF-α-Priming		TNF-α-Priming ± LPA	
			Mean ± SE	Mean ± SE	*p* vs. NT	Mean ± SE	*p* vs. NT	Mean ± SE	*p* vs. NT
			pg/ml	pg/ml		pg/ml		pg/ml	
IL-8	Donor 1	Exp. 1	0.43 ± 0.13	21.6 ± 1.0	d	283.3 ± 17.0	d	3180 ± 591.9	c
	Donor 1	Exp. 2	0.25 ± 0.02	21.7 ± 1.4	d	247.6 ± 29.3	d	3277 ± 760.4	c
	Donor 2	Exp. 1	0.85 ± 0.17	18.9 ± 1.9	d	169.6 ± 37.1	d	3093 ± 749.0	c
IP-10	Donor 1	Exp. 1	1.48 ± 0.11	2.1 ± 0.2	d	244.7 ± 23.3	d	3161 ± 1063.0	b
	Donor 1	Exp. 2	1.31 ± 0.03	5.1 ± 2.1	d	238.1 ± 19.2	d	3167 ± 587.5	c
	Donor 2	Exp. 1	1.77 ± 0.26	8.6 ± 1.1	d	248.6 ± 40.3	d	3990 ± 570.0	c
MCP-1	Donor 1	Exp. 1	10.4 ± 1.0	1158 ± 79.0	c	940.9 ± 151.9	c	1171 ± 153.0	c
	Donor 1	Exp. 2	8.2 ± 1.2	930.3 ± 167.5	c	953.1 ± 37.5	c	1417 ± 41.0	c
	Donor 2	Exp. 1	11.8 ± 3.0	784.3 ± 103.4	c	828.9 ± 56.1	c	1331 ± 97.5	c
RANTES	Donor 1	Exp. 1	9.6 ± 0.9	21.0 ± 0.6	d	1142 ± 228.3	b	2208 ± 251.9	c
	Donor 1	Exp. 2	10.4 ± 2.7	98.2 ± 45.9	d	1232 ± 232.1	a	3248 ± 452.4	c
	Donor 2	Exp. 1	24.4 ± 1.5	164.8 ± 20.5	d	1346 ± 168.1	b	2406 ± 299.0	c

a: $p < 0.05$, b: $p < 0.01$, c: $p < 0.001$, d: $p > 0.05$

Human FLS from RA patients were treated with 5 mM LPA for 24 h with or without TNFα pre-treatment (80 ng/ml) for 8 h. Cell culture supernatants were harvested to monitor cytokine/chemokine secretion as described in Materials andmethods. Results are presented as means ± SE. The data from three distinct experiments performed in quadruplicate on RAFLS from two different donors are shown. For statistical comparative analyses we compared non-treated cells to LPA-, TNFα-, and TNFα/LPA-treated cells. a $p < 0.05$; b $p < 0.01$, c $p < 0.001$ and d $p > 0.05$.

3.8 Figures

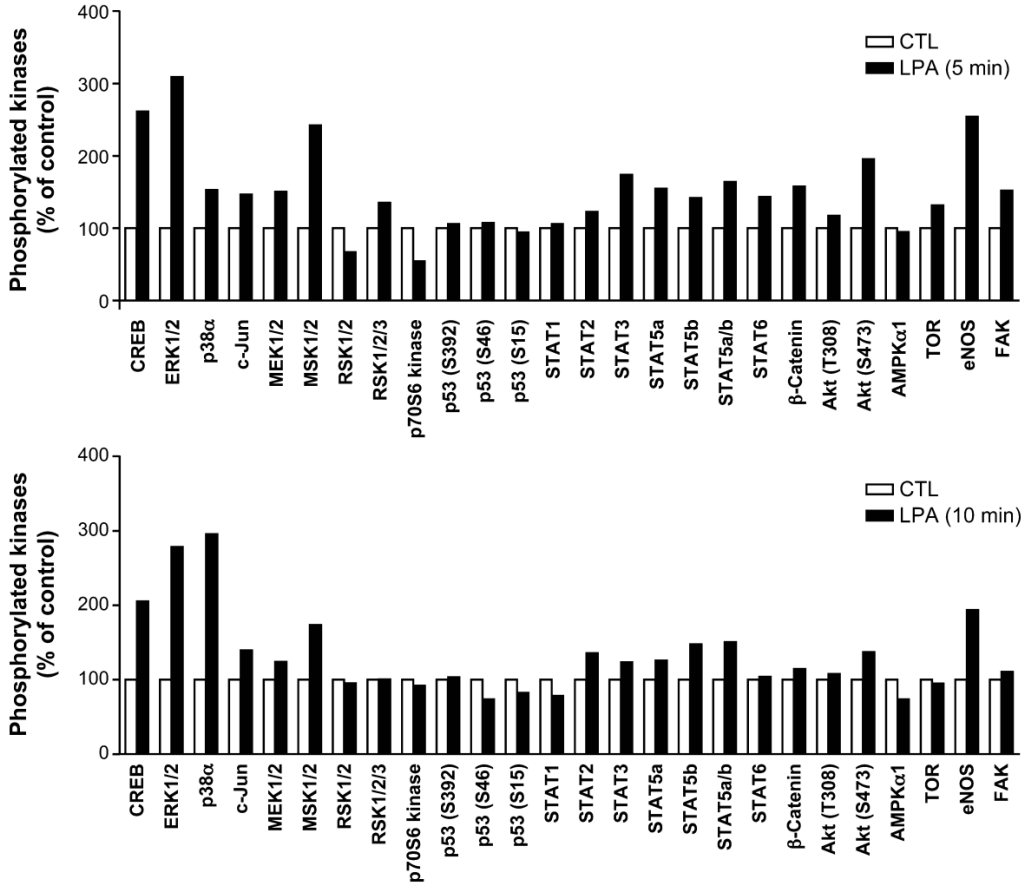

Figure 1 Effect of LPA on kinases and kinase substrate phosphorylation in human RAFLS.

Cells were treated with 5 mM LPA for up to 10 min. Phosphorylated proteins in cell lysates were monitored using the Proteome Profiler^{TM} Human Phospho-Kinase Array kit. Data were quantified as pixel density and are presented as percentage of control (CTL) non-stimulated cells.

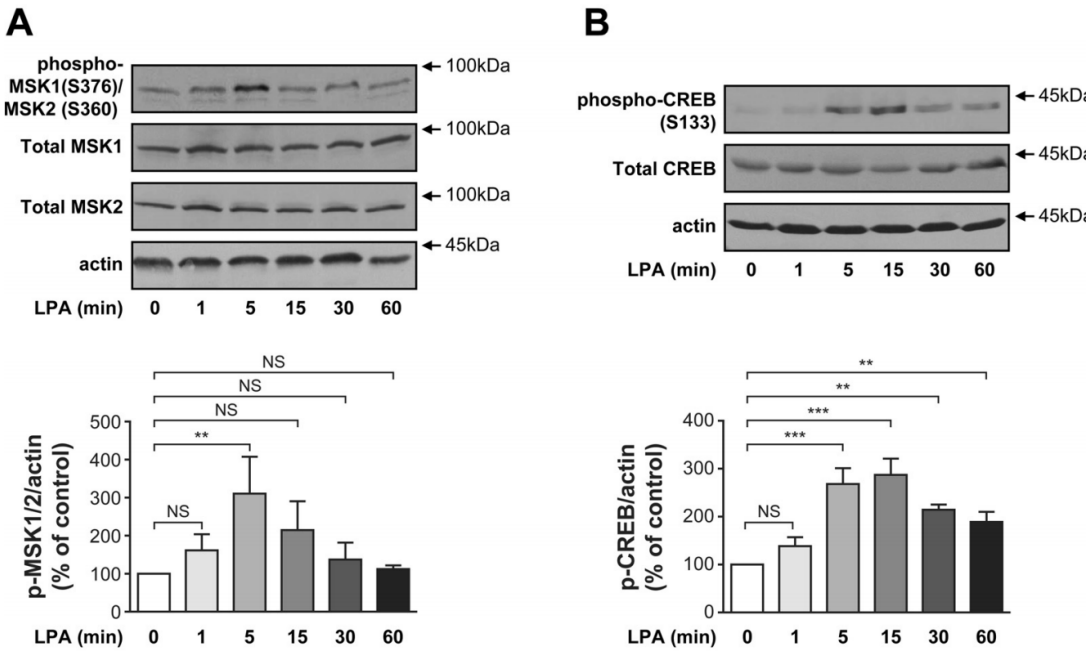

Figure 2 Effect of LPA on MSKs and CREB phosphorylation in human RAFLS.

Cells were stimulated with 5 mM LPA for up to 60 min and cell lysates were subjected to Western blot analyses. The blots shown are from one experiment representative of at least three independent experiments with similar results. Phosphorylated MSKs (A) and CREB (B) were quantified densitometrically. Data were normalized using actin as control for protein loading. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

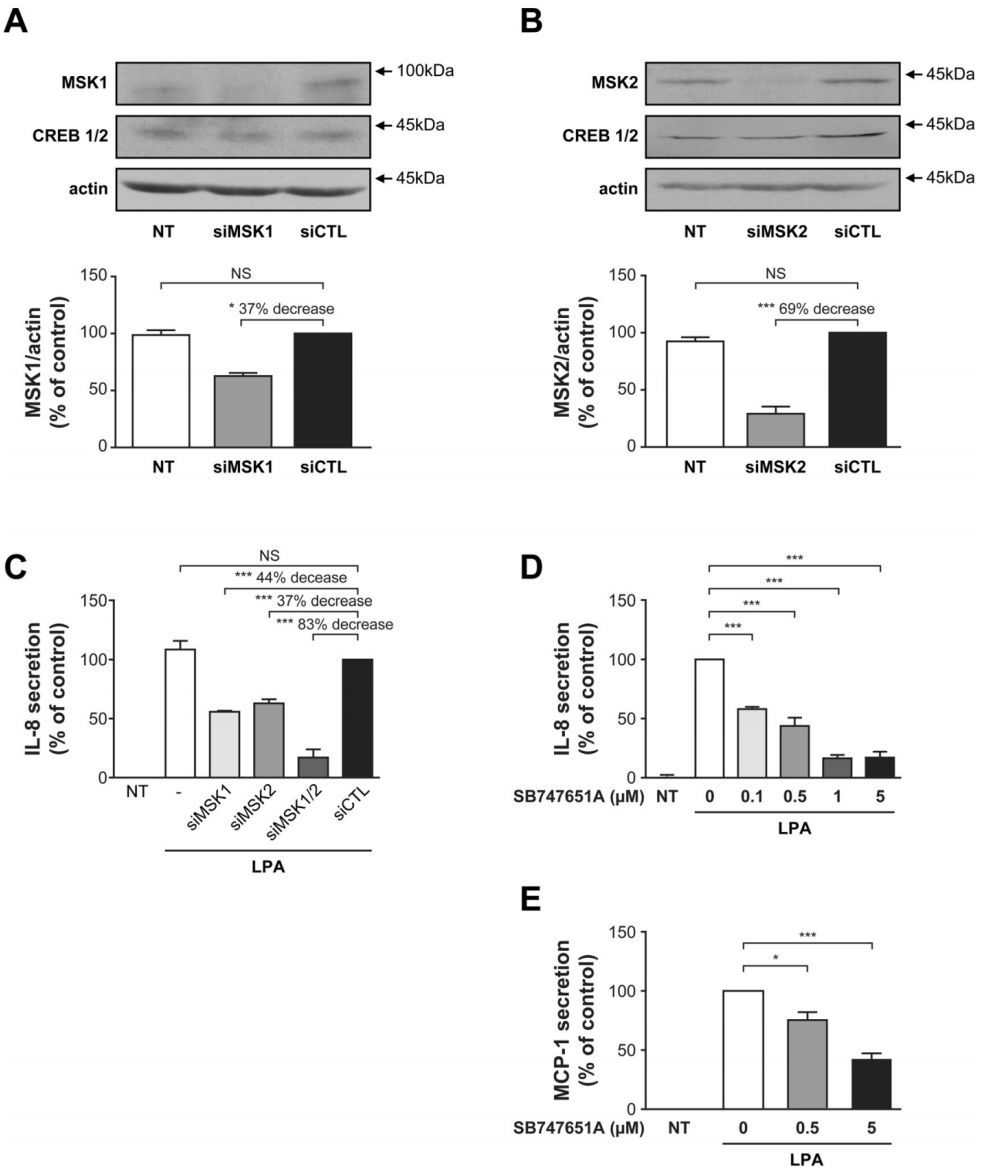

Figure 3 Effect of inhibition of MSKs on LPA-induced IL-8 secretion in human FLS.

(A, B) Cells were transfected with siMSK1, siMSK2 or control siRNA as described in materials and methods. The blots shown are from one experiment representative of at least three independent experiments with similar results. MSK1 (A) and MSK2 (B) were quantified densitometrically and data were normalized using actin as control for protein loading. For comparison the ratio MSK/actin in cells transfected with non-silencing control siRNA (siCTL) was set at 100%. (C) LPA-induced IL-8 release by cells silenced for MSK1, MSK2, or MSK1 and MSK2. For statistical comparative analyses, the level of IL-8 produced in response to LPA (5 mM, 24 h) by

cells transfected with control siRNA (siCTL) was set at 100%. (D, E) Cells were treated with the inhibitor of MSKs SB747651A prior to stimulation with 5 mM LPA for 24 h. For statistical comparative analyses, the level of IL-8 (D) and MCP-1 (E) secreted by cells stimulated with LPA was compared to that of stimulated cells treated with the inhibitor. The data are the means ± SE from three experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

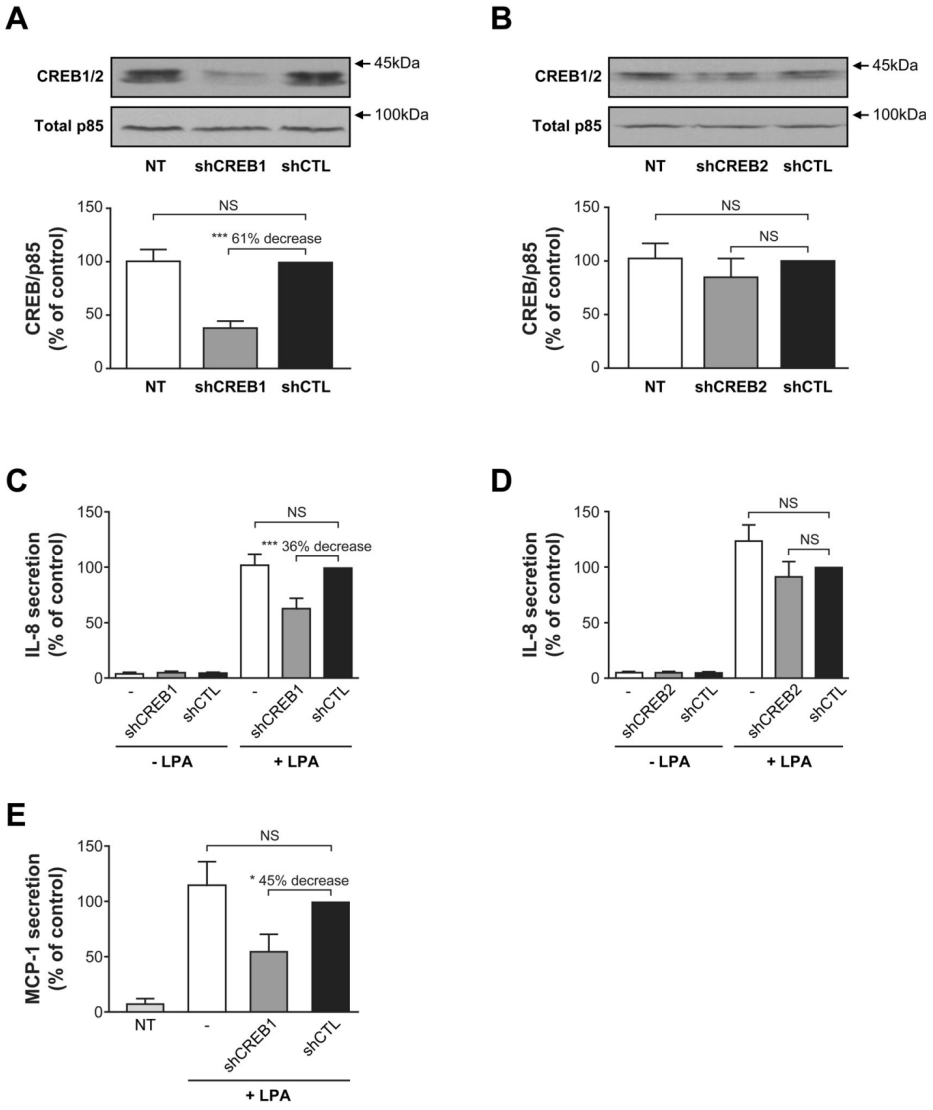

Figure 4 Effect of silencing CREB on LPA-induced IL-8 secretion in human RAFLS.

(A, B) Cells were transduced with shCREB1, shCREB2 or control shRNA lentiviral particles as described in Materials and methods. The blots shown are from one experiment representative of at least three independent experiments with similar results. Total CREB was quantified densitometrically and was normalized with respect to the p85 subunit of PI 3-kinase as control for protein loading using the same membrane. For comparison the ratio CREB/p85 in cells transduced with non-silencing control shRNA (shCTL) was set at 100%. (C, D, E) Forty-eight hours post-transduction with shRNA, cells were stimulated with 5 mM LPA for 24 h and the amounts of IL-8 (C, D) and

MCP-1 (E) released into the supernatants were monitored. For statistical comparative analyses, the level of chemokine secreted by cells transduced with non-silencing control shRNA (shCTL) was set at 100%. The data are the means ± SE from three experiments. * $p < 0.05$; *** $p < 0.001$.

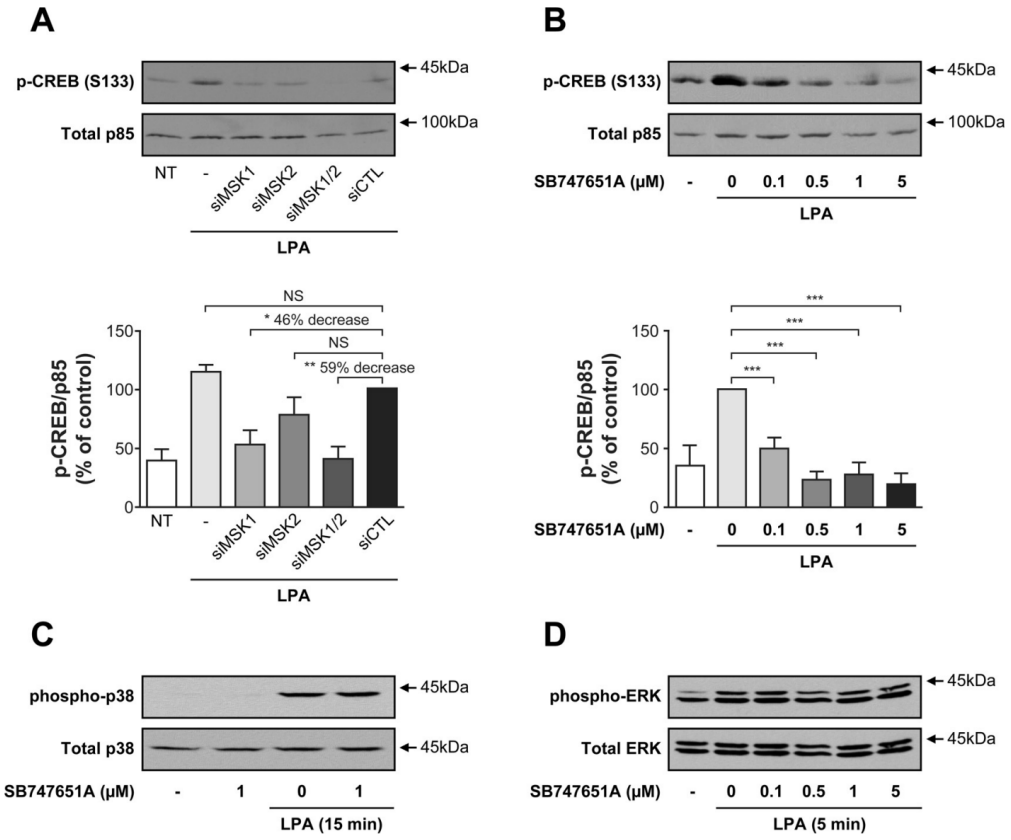

Figure 5 Effect of silencing MSKs and of a MSK inhibitor on LPA-induced CREB phosphorylation.

(A) Cells were silenced for MSK1 and MSK2 alone or in combination and stimulated with 5 mM LPA for 5 min 48 h post-silencing as described in Materials and methods. (B) Cells were treated with the MSK inhibitor SB747651A for 30 min prior to stimulation with 5 mM LPA for 5 min. The blots shown are from one experiment representative of at least three independent experiments with similar results. Phosphorylated CREB (p-CREB) was quantified densitometrically and normalized with respect to the p85 subunit of PI 3-kinase as control for protein loading using the same membrane. For comparison the ratio CREB/p85 in LPA-stimulated cells treated with non-silencing control siRNA (siCTL) was set at 100%. The data are the means ± SE from three experiments. (C, D) Cells were treated with the MSK inhibitor SB747651A for 30 min prior to stimulation with 5 mM LPA for 15 min (C) and 5 min (D). The blots

for phospho-p38 MAPK (phosphop38) and phospho-ERK are representative of two independent experiments with similar results. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

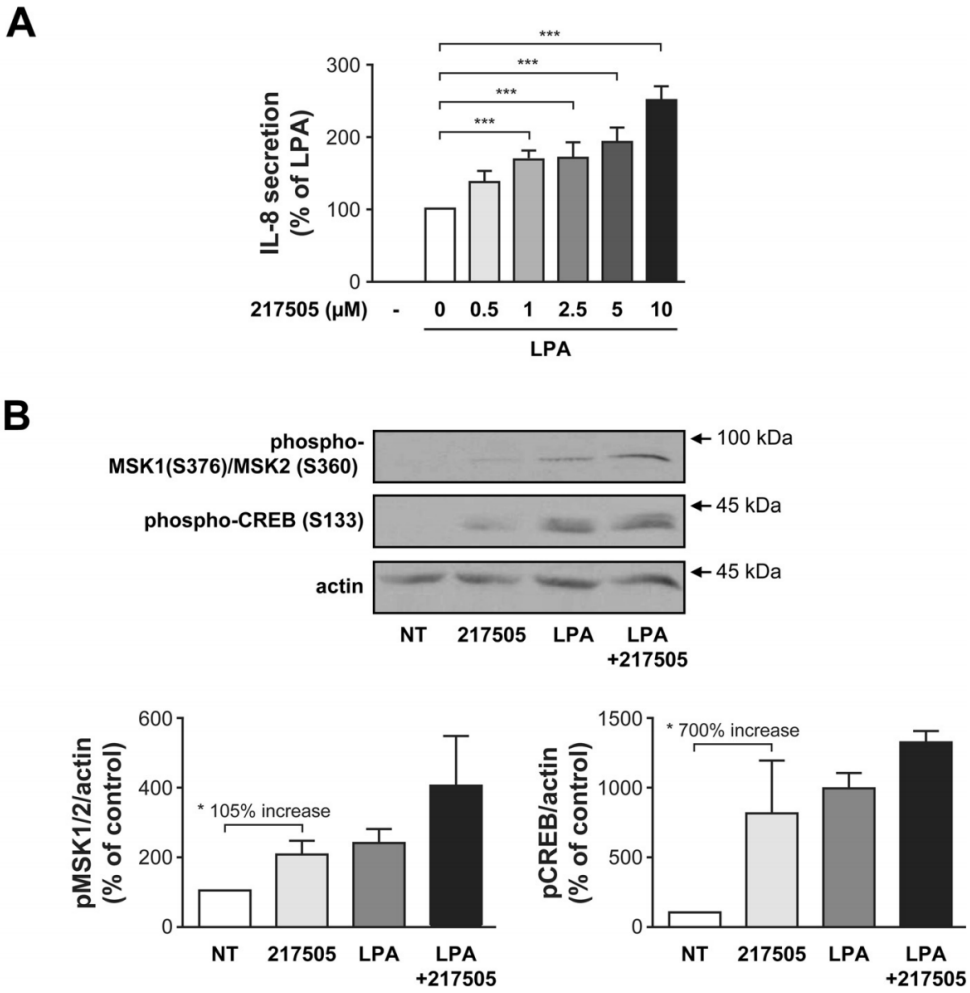

Figure 6 Effect of CREB inhibitor 217505 on LPA-induced IL-8 secretion and phosphorylation of MSKs and CREB

(A) Cells were treated with 5 mM LPA in the absence or presence of CREB inhibitor 217505 for 24 h. For statistical comparative analysis, the amount of IL-8 secreted by cells stimulated with LPA was compared to that of cells treated with the inhibitor prior to stimulation with LPA. The data are the means ± SE from three experiments. ** $p < 0.01$; *** $p < 0.001$. (B) Cells were treated with the CREB inhibitor 217505 (5 mM) for 30 min prior to stimulation with 5 mM LPA for 5 min. The blots shown are from one experiment representative of at least three independent experiments with similar results. Phosphorylated MSKs and CREB were quantified densitometrically and were normalized with respect to total actin. * $p < 0.05$.

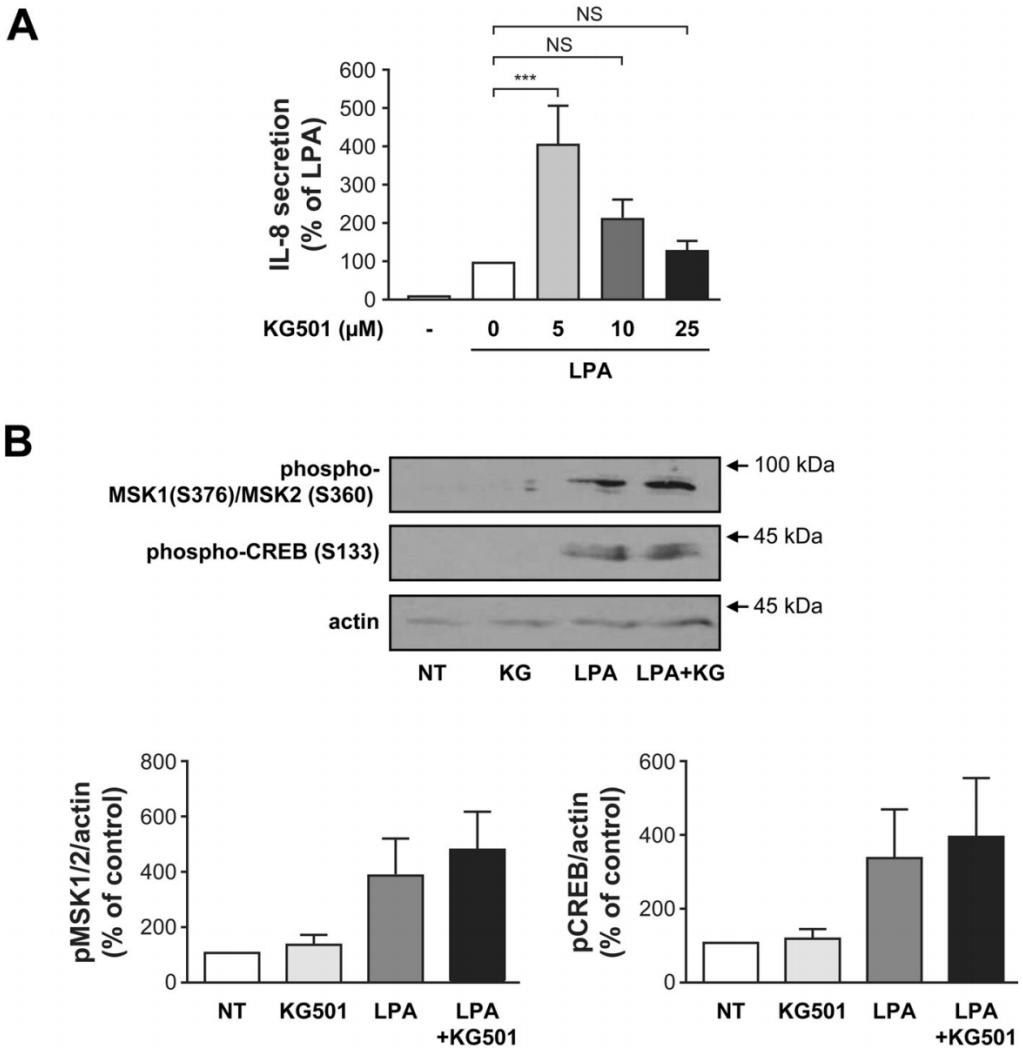

Figure 7 Effect of CREB inhibitor KG-501 on LPA-induced IL-8 secretion and phosphorylation of MSKs and CREB.

(A) Cells were treated with 5 mM LPA in the absence or presence of CREB inhibitor KG-501 for 24 h. For statistical comparative analysis, IL-8 secreted by cells stimulated with LPA was compared to that of cells stimulated in the presence of the inhibitor. The data are the means ± SE from three experiments. *** $p < 0.001$. (B) Cells were treated with the CREB inhibitor KG-501 (5 mM) for 30 min prior to stimulation with 5 mM LPA for 5 min. The blots shown are representative of three independent experiments with similar results. Phosphorylated MSKs and CREB were quantified densitometrically and were normalized with respect to total actin. The data are the means ± SE from three experiments.

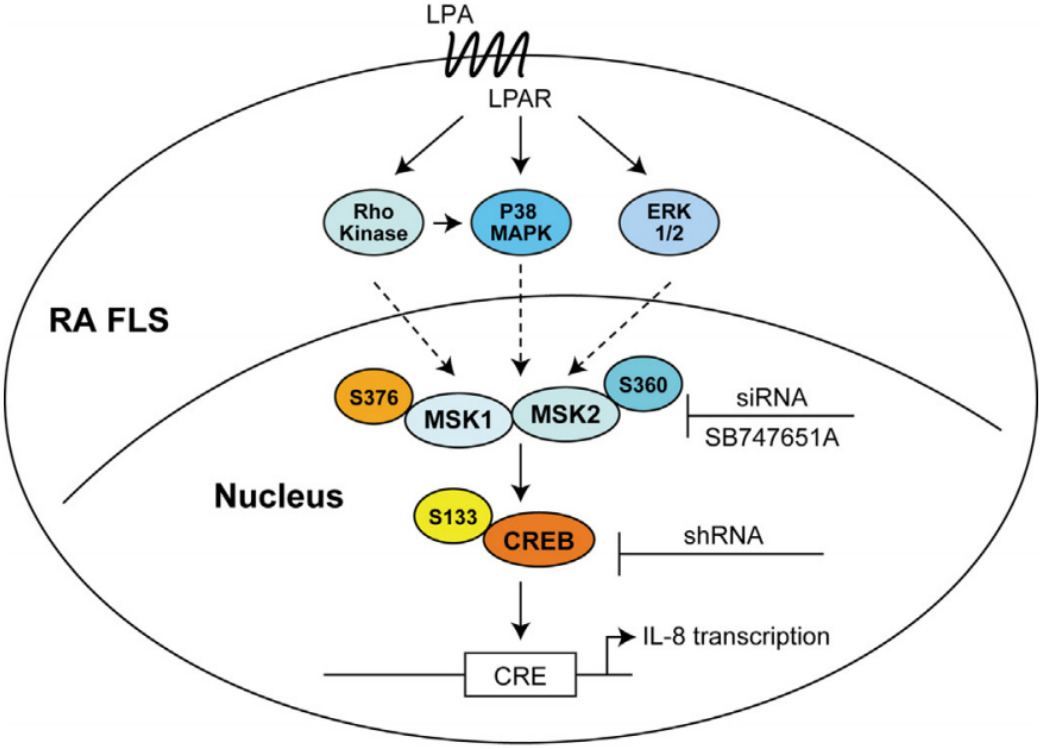

Figure 8 Schematic illustration of signal transduction pathways involved in LPA-induced IL-8 expression in human RAFLS.

LPA binds to LPA receptors on the cell surface and induces IL-8 secretion through activation of the Rho kinase, p38MAPK, and ERK pathways (Zhao et al. Mol. Pharmacol., 2008, 73 (2): p. 587-600). In response to LPA MSK1 and MSK2, the substrates of p38 MAPK and ERK, are activated and phosphorylate CREB at Ser-133, thereby regulating IL-8 gene expression.

Chapter 4

Tumor Necrosis Factor α governs the sensitivity of lysophosphatidic acid-induced cytokine/chemokine secretion to signaling inhibitors in synovial fibroblasts

Manuscript in preparation

Running title: TNFα priming alters LPA signaling in synoviocytes

Key words: Lysophosphatidic acid; Tumor necrosis factor-α; cytokine/chemokine; p38MAPK; ERK1/2; Kinases, Fibroblast-like synoviocytes

4.1 Abstract

Lysophosphatidic acid (LPA) is a pleiotropic bioactive lysophospholipid involved in inflammatory mediator synthesis. Signaling through p38MAPK, ERK, Rho kinase, and MSK-CREB contributes to LPA-mediated IL-8 production in fibroblast-like synoviocytes (FLS) from rheumatoid arthritis (RA) patients. The study was undertaken to investigate how LPA activates MSKs and how signaling crosstalk between TNFα and LPA contributes to the super-production of cytokines/chemokines. RAFLS pretreated or not with TNFα for 8 h were stimulated with LPA. Immunoblotting with phospho-antibodies monitored MSK activation. Cytokine/chemokine production was measured using ELISA and multiplex immunoassays. LPA induced MSK activation by signaling through ERK whereas p38MAPK, Rho kinase, NF-κB or PI3K contributes to IL-8 synthesis via MSK-independent pathways. Priming with TNFα enhanced LPA-mediated MSK phosphorylation and cytokine/chemokine production. After priming with TNFα, inhibition of ERK, MSK, or other signaling pathways alone (except PI3K) failed to attenuate LPA-mediated IL-8 synthesis even if MSK was completely or partially inhibited. In TNFα-primed cells, inhibition of LPA-mediated cytokine/chemokine synthesis required a specific combination of inhibitors such as p38MAPK and ERK for IL-8 and IL-6, and Rho kinase and NF-κB for MCP-1. LPA induces IL-8 synthesis through ERK1/2-MSK-dependent and MSK-independent pathways. In TNFα-primed RAFLS the super-production of IL-8 and IL-6 induced by LPA occurs mainly via MSK-independent pathways and simultaneous inhibition of at least two signaling pathways was required to block their synthesis. This may have important clinical implications for the development of drugs targeting these signaling pathways for the treatment of RA.

4.2 Introduction

LPA is a monoacyl phospholipid acting as an extracellular molecule involved in many physiological and pathophysiological conditions [1]. LPA signals via binding to its G-protein-coupled receptors, which in turn trigger various downstream signaling cascades through activation of associated heterotrimeric G proteins, including Gi/o, G12/13, Gq, and Gs [1]. LPA receptors identified until now have been named LPA1-LPA6 [2]. Studies using cloned receptors and genetic knockout mice have uncovered the biological significance of the LPA signaling cascades [3].

Rheumatoid arthritis (RA) is a systemic, severe autoimmune disease associated with chronic inflammation of peripheral joints and adjacent tissues, as well as hyperplasia of synovial lining cells (synovial fibroblasts) along with infiltration of immune cells into the synovial cavity, forming a pannus, leading to joint deformation and pain [4]. Synovial fibroblasts contribute to inflammation by secreting various cytokines/chemokines and matrix metalloproteases in response to inflammatory mediators such as TNFα and LPA [5, 6]. LPA can be produced from lysophosphatidylcholine (LPC) by autotaxin (ATX) [7]. The presence of ATX, LPC and LPA were detected in synovial fluids from RA patients [8-12]. Synovial fibroblasts express LPA1, LPA2 and LPA3, of which LPA1 is the most abundant [6, 9]. Genetic deletion of LPA1 in mice conferred resistance to type II collagen-induced arthritis [12]. Treatment of mice with an LPA1 antagonist [12, 13] and conditional genetic ablation of ATX in synovial fibroblasts [8] both reduced the severity of experimental arthritis. LPA1 and LPA3 are both involved in LPA-mediated cytokine/chemokine release by RAFLS in vitro [9], and in vivo using the murine air pouch model [14]. LPA1 also mediates synovial fibroblast migration [6] and confers resistance to TNFα-induced apoptosis [15]. LPA induces these cellular responses by activating downstream signaling kinases including extracellular-signal-regulated kinase (ERK), mitogen activated protein kinase (p38MAPK), and Rho kinase (ROCK) [9].

Mitogen- and stress-activated protein kinases 1 and 2 (MSKs, formerly called ribosomal protein S6 kinases A5 and A4) can be activated by either ERK or p38MAPK [16, 17]. The kinase domains composing MSK1/2 include a C-terminal and N-terminal kinase domain harboring several phosphorylation sites [18]. Human MSK1 is phosphorylated by MAPK at Ser-360, Thr-581 and Thr-700 located in the C-terminal domain [19]. Phosphorylation of the MSK C-terminal domain subsequently induces a conformational change in MSK1, which permits autophosphorylation on Ser-212, Ser-376 and Ser-381 by the C-terminal kinase domain and phosphorylation of MSK substrates by the N-terminal kinase domain [19]. MSK was reported to phosphorylate various transcription factors involved in gene expression including CREB, ATF-1, p65 and STAT3, as well as chromatin components such as histone H3 and HMGN1 [16, 17]. The E3 ubiquitin ligase TRIM7 is also a MSK target [20]. We previously reported in RAFLS that the MSK-CREB signaling axis was activated in response to LPA [21].

TNFα and IL-6 are key components in the cytokine/chemokine network of RA [22, 23]. IL-8, MCP-1/CCL2, RANTES/CCL5 and IP-10 also contribute to the pathogenesis of RA as chemotactic factors of neutrophils [24], monocytes [25] or T cells [26, 27]. Previous studies showed that induction of a pro-inflammatory environment by TNFα upregulates LPA1 and/or LPA3 expression and strongly enhances cytokine/chemokine release induced by LPA [9, 14]. Transient phosphorylation of MSK1/2 in response to TNFα was also reported [21], but it remains unclear whether this pathway contributes to LPA-induced super-production of cytokines in TNFα-primed RAFLS. This prompted us to determine whether signaling crosstalk between LPA and TNFα points to MSK hyper-phosphorylation resulting in synergistic induction of LPA-mediated cytokine/chemokine secretion.

4.3 Materials and Methods

4.3.1 Reagents

TNFα was purchased from PeproTech Inc (Rocky Hill, NJ, USA). 1-Oleoyl-sn-glycerol 3-phosphate sodium salt (LPA, 18:1) was purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Antibodies against human phospho-MSK1 (Ser-376)/MSK2 (Ser-360), phospho-MSK1 (Ser-360), phospho-MSK1 (Thr-581), and human phospho-MSK1 (Ser-212) were from R&D Systems Inc (Minneapolis, MN, USA). Antibody against total actin was from Sigma-Aldrich Canada (Oakville, ON, Canada). Inhibitors of p38MAPK (SB203580), ERK (PD98059), Rho kinase (Y27632), and NF-κB (Bay11-7082) were purchased from Calbiochem (San Diego, CA, USA). The PI3K inhibitor wortmannin was from Millipore Corporation (St. Charles, MO, USA). MSK inhibitor SB-747651A was obtained from Axon Medchem (Groningen, The Netherlands). Human IL-8 ELISA kit was purchased from BioSource International Inc. (Camarillo, CA, USA). The human cytokine/chemokine Luminex multiplex immunoassay kit (Milliplex® MAP Kit, detecting MCP-1, IL-6, IL-8, IP-10, and RANTES) was from Millipore Corporation (St. Charles, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) was from wisent Inc. (St-Bruno, QC, Canada). Propidium iodide and Annexin V-eFluor450 were from BD Pharmingen (Oakville, ON, Canada).

4.3.2 Cell Culture and Treatment

Human primary FLS were obtained from RA patients who were diagnosed according to the criteria developed by the American College of Rheumatology (ACR) and were undergoing joint surgery. Human primary FLS at passage 0 or 1 were purchased from Asterand (Detroit, MI, USA) or isolated from RA synovial specimens obtained from consenting patients with the approval of the CHU de Québec-Laval University ethics committee (B14-04-1946). Cells were cultured under standard conditions (37°C and 5% CO2) and grown in DMEM supplemented with 10% fetal

bovine serum (FBS) as described previously [9, 21]. Cells were used at passages 2 to 7. For the experiments, semi-confluent RAFLS were starved in FBS-free DMEM for 24 h. To evaluate the effect of TNFα, starved cells were pre-incubated with TNFα (80 ng/ml) for 8 h prior to LPA treatment. Where indicated, cells were pre-treated with the inhibitors of p38MAPK (SB203580), ERK (PD98059), Rho kinase (Y27632), PI3K (wortmannin), NF-κB (Bay11-7082), and MSK (SB-747651A) at indicated concentrations for 30 min prior to LPA stimulation. In some experiments combinations of signaling inhibitors were also tested. Cells were then washed with serum-free DMEM and stimulated with 5 μM LPA in fresh serum-free DMEM containing testing compounds (Fig. 1). For measurement of cytokine/chemokine secretion cells were stimulated for 24 hr as described previously [9, 21]. Cell culture supernatants were collected and stored at -80°C until the ELISA and the multiplex immunoassay for human cytokines/chemokines were performed. Data points in each of the independent experiments were monitored in duplicates. To monitor the levels of MSK phosphorylation RALFS were stimulated with 5 μM LPA for 5 min, a time at which phosphorylation of MSK1 at Ser-376 or of MSK2 at Ser-360 is maximal [21].

4.3.3 Analyses of Cytokine/Chemokine Production

IL-8 was monitored according to the manufacturer's protocol. Optical densities were determined using a SoftMaxPro5 plate reader at 450 nm. The detection range of the IL-8 kit is 15.6-1000 pg⁄ml.

The human cytokines/chemokines IL-6, IL-8, IP-10, MCP-1, and RANTES were monitored using a Luminex multiplex immunoassay (Milliplex® MAP Kit) according to the manufacturer's instructions. The dynamic range of the assay is 3.2-10000 pg/ml. The assay sensitivities (minimum detectable concentrations, mean ± SD, pg/ml) were 0.9 ± 1.3, 0.4 ± 0.7, 8.6 ± 14, 1.9 ± 3.4, and 1.2 ± 1.9 for IL-6, IL-8, IP-10, MCP-1, and RANTES, respectively.

4.3.4 Analyses of MSK Phosphorylation and CREB Phosphorylation

RAFLS stimulated with LPA for 5 min were lysed in boiling sample buffer [50 mM Tris-HCl (pH6.8), 10% (v/v) glycerol, 50 mM DTT, 4% (v/v) SDS] for 7-10 min. Equal amounts of protein were loaded into the wells and separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were later transferred from polyacrylamide gel to methanol soaked PVDF membranes (Pall Canada Ltd, Ville St-Laurent, QC, Canada). Primary antibody incubation was performed overnight at 4°C, 1 h at 37°C, or 2 h at room temperature according to optimization tests. The membranes were washed three times and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Membranes were washed three times and antibody-antigen complexes were revealed using Western Lightning chemiluminescence reagent according to the manufacturer's instructions (Perkin Elmer Life Sciences, Woodbridge, ON, Canada).

4.3.5 Statistical Analysis

Unless otherwise stated, the data are from at least three independent experiments presented as mean values ± SEM. Prism 5.0 software was used for all statistical analyses. Statistical significance of the difference between samples of two different treatments was determined by t-test (two-tailed p value). For studies using samples from more than one treatments, statistical significance between control and treated cells was determined by one-way ANOVA, Dunnett's multiple comparison test or Bonferroni multiple comparison test according to the context. P values less than 0.05 were considered statistically significant. Two-way ANOVA was used for comparing the mean difference between groups with more than two or three treatments.

4.4 Results

4.4.1 TNFα Up-Regulates MSK Phosphorylation Induced by LPA in RAFLS

Our previous study has shown the key role of the MSK-CREB signaling axis in LPA-mediated IL-8 secretion in RAFLS [21]. Since cell preconditioning with TNFα synergistically enhances LPA-mediated cytokine/chemokine production [21], we first examined the effect of cell priming with TNFα on LPA-induced MSK phosphorylation using various phospho-MSK antibodies. LPA and TNFα have been reported to induce a transient phosphorylation of MSK1 at Ser-376 and/or MSK2 at Ser-360 (henceforth "p-MSK1/2") [21]. However, further efforts to detect MSK1 phosphorylation at other sites (Ser-360, Thr-581) were fruitless (data not shown).

TNFα does not cause, by itself, a phosphorylation of MSK1/2 (Fig. 2A) in RAFLS [21]. Treatment of RAFLS with LPA at a physiological concentration induces a two-fold increase in p-MSK1/2 (Fig. 2A). Incubation of RAFLS with TNFα for 8 h, followed by addition of LPA leads to synergistically increased p-MSK1/2 (30 ± 16 %, $p < 0.001$) (Fig. 2A).

In contrast, TNFα by itself does induce phosphorylation of MSK1 at Ser-212 (henceforth "p-MSK1") (40 ± 12 %, $p < 0.05$) (Fig. 2B). TNFα priming plus LPA addition, as for p-MSK1/2, likewise increases p-MSK1 (56 ± 16 %, $p < 0.001$) (Fig. 2B).

Autophosphorylation of both MSK1 Ser-376 and Ser-212 is required for MSK1 function [19]. Since (Fig. 2A, B) TNFα priming enhanced LPA-stimulated accumulation of both p-MSK1 and p-MSK1/2, this enhancement pathway likely fully activates and signals via MSK1.

4.4.2 Impact of Signaling on LPA-Mediated MSK Phosphorylation in Control- and TNFα-Primed RAFLS.

Previous studies showed that signaling through ERK, p38MAPK, Rho kinase [9] and PI3K [28-31], as well as the MSK-CREB system [21] mediate LPA-induced cytokine/chemokine secretion [21]. In addition to Rho kinase, ERK and p38MAPK, MSKs have binding sites for, and are phosphorylated by, either ERK or p38MAPK [16, 17]. To test whether ERK and p38MAPK signal via MSKs in our model, we used kinase-specific inhibitors and read out p-MSK1/2 and p-MSK1.

Incubation of RAFLS with inhibitors of p38MAPK, Rho kinase, or PI3K did not affect the level of LPA-induced p-MSK1/2 or p-MSK1 without TNFα (Figs. 3A, 3B, 3D and 4A, 4B, 4D). In contrast, the ERK inhibitor significantly decreased LPA-induced p-MSK1/2 and p-MSK1 by 50 ± 20% (Fig. 3C) and 42 ± 7% (Fig. 4C), respectively.

Under TNFα-primed circumstances, inhibition of ERK significantly reduced the levels of p-MSK1/2 and p-MSK1 induced by LPA by 50 ± 20% (Fig. 3C) and 66 ± 8% (Fig. 4C), respectively. The elevated level of p-MSK1 at Ser-212 observed after 8 h preconditioning with TNFα was also reduced when the ERK pathway was inhibited (Fig. 4C). Inhibition of p38MAPK significantly attenuated p-MSK1/2 by 31 ± 7% (Fig. 3A). Although statistical significance was not reached, p-MSK1 at Ser-212 in cells treated with the inhibitor of p38MAPK was constantly reduced to the levels measured in unprimed RAFLS stimulated with LPA (Fig. 4A). Inhibition of Rho kinase and PI3K in TNFα-primed RAFLS stimulated with LPA was also found to significantly decrease the level of p-MSK1/2 (Figs. 3B and 3D) but not p-MSK1 (Figs. 4B and 4D).

The next series of experiments evaluated the effects of inhibiting ERK in combination with one or more signaling pathways. Inhibition of both ERK and p38MAPK did not further reduce MSK1/2 phosphorylation levels compared to cells treated with ERK inhibitor alone (Figs. 5A and 5C). Simultaneous inhibition of ERK signaling together with Rho kinase, or p38MAPK and Rho kinase, or p38MAPK and

PI3K, or all of the signaling pathways further reduced p-MSK1/2 (Fig. 5A). In contrast, the same combinations of inhibitors slightly attenuated p-MSK1 at Ser-212 phosphorylation when compared to cells treated with the ERK inhibitor alone (Fig. 5C). The most potent combinations were inhibition of ERK, p38MAPK, and Rho kinase, and that of ERK, Rho kinase, and PI3K signaling (Fig. 5C).

Since inhibition of p38MAPK, Rho kinase, or PI3K alone partially inhibited LPA-induced p-MSK1/2 in TNFα primed RAFLS (Figs. 3A, 3B, 3D, and 5B) we performed experiments to determine whether simultaneous inhibition of at least two pathways could further diminish MSK phosphorylation. As shown in Fig. 5B, all combinations of inhibitors moderately decreased the levels of p-MSK1/2. However, inhibition of Rho kinase and p38MAPK or Rho kinase and PI3K did not decrease the levels of p-MSK1/2 compared to cells treated with the inhibitor of Rho kinase alone. Combination of p38MAPK and PI3K, or of p38MAPK, PI3K, and Rho kinase inhibitors reduced p-MSK1/2 to an extent greater than inhibition of either signaling pathway alone (Fig. 5B). In contrast, these combinations of inhibitors did not reduce p-MSK1 at Ser-212 (Fig. 5D). Taken together the data indicate that in both control and TNFα-primed RAFLS stimulation with LPA results in the phosphorylation of MSK1 at Ser-376 and Ser-212 and/or of MSK2 at Ser-360 via ERK. In TNFα-primed inhibition of p38MAPK, Rho kinase, and PI3K alone or in combination selectively reduced the LPA-mediated phosphorylation events at Ser-376 of MSK1 (or Ser-360 of MSK2).

4.4.3 Inhibition of p38MAPK, ERK, Rho kinase, or MSK Activation Alone Does Not Regulate LPA-Mediated IL-8 Secretion in TNFα-Primed RAFLS.

We previously reported that priming of RAFLS with TNFα for 8 h strongly enhances the subsequent release of IL-8 as well as of other cytokines/chemokines in response to stimulation with LPA [9]. Early reports also highlighted that this super-production of cytokines/chemokines by TNFα conditioned cells was dependent on LPA1 and LPA3 receptor activation [9, 14]. Though LPA was reported to induce

cytokine/chemokine secretion through activation of multiple signaling pathways including MSK1, MSK2, ERK, and p38MAPK, the contribution of MSKs and other kinase cascades to LPA-mediated cytokine/chemokine production after priming with TNFα has not been investigated. In the next series of experiments we investigated in primed and unprimed cells the impact of inhibiting p38MAPK, ERK, Rho kinase, PI3K, MSKs, or the transcription factor NF-κB on LPA-mediated IL-8 secretion. Consistent with previous studies, inhibition of p38MAPK, ERK, Rho kinase, or MSKs significantly reduced LPA-mediated IL-8 secretion (Figs. 6A-6D). Inhibition of PI3K and NF-κB also decreased LPA-induced IL-8 secretion (Figs. 6E and 6F).

IL-8 secretion due to TNFα priming was sensitive to inhibition by SB-747651A (Fig. 6D), wortmannin (Fig. 6E), and the highest dose of Bay11-7082 (Fig. 6F), whereas the other signaling inhibitors were without any effect. However, control experiments revealed that the effect of the NF-κB inhibitor (Bay11-7082) at 10 μM was highly cytotoxic for RAFLS (Figs. 7A and 7B). Surprisingly, LPA-induced IL-8 secretion became insensitive to the inhibitors of p38MAPK, ERK, Rho Kinase, MSKs, and NF-κB (Figs. 6A-6D and 6F). Only LPA-induced IL-8 secretion remained sensitive to inhibition by wortmannin after priming with TNFα (Fig. 6E).

4.4.4 Combination of Signaling Inhibitors Can Reduce LPA-Induced IL-8 Secretion after TNFα Priming

As neither the p38MAPK, ERK, Rho kinase, nor of the NF-κB inhibitor (1 μM) acting independently could inhibit IL-8 in response to LPA with TNFα priming, we next tested various combinations of signaling inhibitors. The combined inhibition of p38MAPK and ERK significantly decreased the production of IL-8 by 61.06 ± 3% (Fig. 8A) compared to the control TNFα-primed RAFLS stimulated with LPA. Though not statistically significant, combinations of the p38MAPK inhibitor with that of Rho kinase, PI3K, or NF-κB showed a tendency to more strongly decrease IL-8 secretion compared to other combinations of inhibitors (Fig. 8A), thereby suggesting a role for p38MAPK

in exacerbating LPA-induced IL-8 release in TNFα-primed RAFLS. In contrast, combined inhibition of signaling through Rho kinase and PI3K or of Rho kinase and NF-κB increased the release of IL-8 by 72.46% ($p < 0.05$) and 40 ± 30%, respectively (Fig. 8A).

The pattern of IL-6 secretion by cells treated with the same combinations of signaling inhibitors was similar to that of IL-8. LPA-induced IL-6 secretion after TNFα priming, which was not inhibited by a pre-incubation with either the inhibitor of p38MAPK (Fig. 6A) or of ERK (Fig. 6B), was reduced by 41 ± 3% when these two signaling pathways were inhibited (Fig. 8B). Simultaneous inhibition of Rho kinase and PI3K also significantly enhanced (260%, $p <0.01$) the release of IL-6 compared to the control TNFα-primed cells stimulated with LPA. MCP-1, IP-10, and RANTES exhibited different inhibition patterns with combination of inhibitors. The release of IP-10 and of RANTES was not affected by any of the combinations of inhibitors (Figs. 8C and 8E) whereas combinations of Rho kinase and PI3K, Rho kinase and NF-κB, or PI3K and NF-κB inhibitors decreased MCP-1 secretion to 50 ± 20 %, 30 ± 20%, and 50 ± 20 % of control LPA-stimulated cells, respectively (Fig. 8D).

4.5 Discussion and Conclusion

MSK1 and MSK2 are nuclear proteins that regulate the expression of several immediate-early genes through phosphorylation of various nuclear proteins involved in regulation of gene transcription [16, 17, 32]. MSK1 is phosphorylated on multiple sites including Ser-360, Thr-581, Thr-700, Ser-212, Ser-376, Ser-381, Thr-630, Ser-647, Ser-657 and Ser-695 in response to various agonists [19]. MSK1 is initially phosphorylated by ERK and p38MAPK at Ser-360, Thr-581, and Thr-700 [19, 33]. This causes activation of the C-terminal kinase of MSK1 that leads to autophosphorylation of Ser-212, Ser-376 and Ser-381 [18, 19]. Phosphorylation of Ser-212 and Ser-376 are essential for activation of the MSK1 N-terminal kinase domain [18, 19]. LPA is a pleiotropic lipid growth factor that regulates the various functional responses of RAFLS including secretion of IL-8 and IL-6 by signaling through ERK, p38MAPK, and Rho kinase [9]. Using pharmacological inhibitors or siRNA we demonstrated in RAFLS that LPA induces MSK1 and MSK2 activation and MSK-mediated CREB phosphorylation at Ser-133, thereby promoting the synthesis IL-8 and MCP-1 [21].

In this study we show that p-MSK1/2 and p-MSK1 at Ser-212 is blocked by an inhibitor of ERK activation. Though LPA induces activation of p38MAPK [9], our data indicate that p38MAPK does not contribute to MSK1 or MSK2 activation in LPA stimulated RAFLS. There are four differentially expressed p38MAPK isoforms (α, β, γ, and δ) in tissues, with only p38MAPKα and p38MAPKβ being sensitive to inhibition by SB203580 [34]. Using fibroblasts from knockout mice, it has been shown that p38MAPKα, but not p38MAPKβ, phosphorylates MSKs [35, 36]. Though all p38MAPK isoforms are expressed within the RA synovium, α and γ isoforms were preferentially activated in those inflamed tissues [37]. Furthermore expression of p38MAPK isoforms was cell specific with p38MAPKαaand p38MAPKγ being preferentially expressed by RAFLS [37]. The fact that RAFLS do not express the p38MAPK isoform involved in MSK activation may explain why SB203580 does not

prevent LPA-induced MSK phosphorylation. In this study we established that LPA activates ERK1/2-MSK1/2 signaling, which in turn phosphorylates CREB [9], and ultimately induces IL-8 secretion by RAFLS. Activation of p38MAPK, Rho kinase, or PI3K contributes to LPA-induced IL-8 secretion but through MSK-independent signaling pathways (Fig. 9).

RAFLS are characterized by the ability to synthesize various inflammatory chemokines/cytokines with immune regulatory properties [38]. Blocking the functions of cytokines such as TNFα or IL-6 reduces joint damage progression in RA patients [39]. TNFa induces a transient activation of MSK1 and MSK2 in keratinocytes [40] and in RALFS as well [9]. As described previously [9] MSK1 Ser-376/MSK2 Ser-360 phosphorylation returns to basal levels after addition of TNFα for 8 hours. Surprisingly ERK1/2-dependent phosphorylation of MSK1 at Ser-212 remained elevated compared to control RAFLS. Stimulation of TNFα-primed RALFS with LPA consistently enhanced p-MSK1/2 and p-MSK1 at Ser-212 mainly via ERK1/2 activation. Whether the ERK1/2 phosphorylation sites were similarly affected could not be elucidated since MSK1 phosphorylation at Ser-360 and Thr-581 could not be detected. After priming with TNFα, inhibition of p38MAPK, Rho kinase, and PI3K slightly reduced LPA-mediated MSK1 Ser-376/MSK2 Ser-360 phosphorylation without reducing MSK1 Ser-212 phosphorylation (Fig. 10). Combinations of inhibitors that further decreased p-MSK1/2 but had no impact on p-MSK1 at Ser-212. Studies using human keratinocytes have reported the activation of MSK1 by TNFα- and IL-1β and that the MSK1 Ser-376 autophosphorylation but not the ERK1/2 and p38MAPK phosphorylation sites Ser-360 and Thr-581 were reduced by a non-specific inhibitor of NF-κB [40, 41]. The mechanisms by which p38MAPK, Rho kinase, and PI3K specifically modulate p-MSK1/2 remains to be investigated. Reduction of MSK1 autophosphorylation at Ser-376 is unlikely to be secondary to inhibition of C-terminal kinase domain of MSK1 by p38MAPK, Rho kinase, or PI3K inhibitors, since autophosphorylation of Ser-212 is not reduced [18, 19, 33]. It remains to be determined

whether MSK1 and MSK2 activation in RAFLS involves distinct kinase cascades. Whereas activation of MSKs relies on ERK1/2, it cannot be excluded that dephosphophorylation of MSK1 at Ser-212 by unknown phosphatases is delayed compared to Ser-376. More careful analysis of the kinetics of MSK dephosphorylation and identification of the phosphatases involved would be required.

Previous studies have shown that dual LPA1/3 receptor antagonists block LPA-mediated super-production of cytokines/chemokines after preconditioning of cells with TNFα [9, 14, 21]. Except for the PI3K inhibitor, we show that after cell priming with TNFα LPA-mediated IL-8 secretion becomes insensitive to inhibition of ERK1/2, Rho kinase, and NF-κB as well. MSK activation via ERK1/2 signaling contributes to cytokine/chemokine production as illustrated by inhibition of LPA and TNFα-mediated IL-8 secretion by SB-747651A, a selective inhibitor of MSKs [42]. It is noteworthy that while MSK is inhibited by SB-747651A LPA-mediated IL-8 production in TNFα-primed RAFLS is not reduced. The data indicate that the ERK1/2-MSK signaling axis is a major pathway involved in IL-8 production in control RAFLS and that priming with TNFα strongly enhances LPA-mediated IL-8 secretion through MSK-independent pathways. Data obtained with SB-747651A should nevertheless be interpreted with caution since the compound inhibits other AGC group kinases (S6K, RSK, PKR2, Rho kinase, Akt and PKA) [42], some of which were identified in RAFLS in a human phospho-kinase array (unpublished data).

Class I PI3Kδ expressed in the RA synovial intimal lining was reported to regulate FLS growth and TNFα signaling [43]. The severity of inflammation in animal models of RA is reduced by PI3Kγ inhibitors or in mice knockout for PI3Kγ [44, 45]. TNFα and LPA activate PI3K signaling [31, 46]. Consistent with this, inhibition of PI3K signaling using the pan-PI3K inhibitor wortmannin, a small fungal metabolite targeting the p110 subunit of PI3K [47], partially reduced LPA-induced IL-8 secretion in unprimed and TNFα-primed RAFLS. The mechanisms by which wortmannin reduces IL-8 production were not investigated. We cannot totally exclude that inhibition of PI3K could interfere

indirectly with Rho kinase, ERK1/2 and/or p38MAPK activation [44], thereby reducing the MSK-dependent and independent pathways involved in chemokine/cytokine production.

Regulation of IL-8 expression involves multiple kinase cascades and transcriptional factors such as AP-1, CREB, C/EBP-1, and NF-κB that bind to the IL-8 promoter [21, 48-50]. Interestingly, inhibition of ERK and p38MAPK signaling was the only combination that significantly reduced LPA-induced IL-8 secretion in TNFα-primed RAFLS. The production IL-6 but not that of other chemokines (MCP-1, RANTES, IP-10) was also reduced by this combination of inhibitors, thereby suggesting the involvement of similar signaling networks and transcription factors in IL-8 and IL-6 gene transcription. Targeting p38MAPK as a potential therapeutic target for treatment of RA has been considered [51]. Though potent and p38MAPK isoform specific inhibitors have been generated, most phase 2 clinical trials have failed due to very limited improvement of RA symptoms or because the drug was not well tolerated [52, 53]. Our study suggests that in chronic inflammatory diseases inhibition of signaling through both p38MAPK and ERK1/2 would be required to dampen the vicious cycle of inflammation.

LPA and TNFα are well known to activate NF-κB signaling in various cell types including synovial fibroblasts [54, 55]. Upon activation with TNFα NF-κB becomes phosphorylated on Ser-536 by the IKK kinase complex [55]. NF-κB is also a MSK substrate [56-58]. Inhibition or silencing of MSK1 was reported to decrease NF-κB Ser-276 phosphorylation and was linked to expression of stem cell factor in human lung fibroblasts [56] and to IL-8 in epithelial cells [57]. LPA-induced NF-κB signaling may contribute to cytokine/chemokine synthesis by RAFLS, since inhibition of NF-κB significantly decreased IL-8 secretion induced by LPA alone, whereas combined inhibition of NF-κB and p38MAPK, and of NF-κB and Rho kinase attenuated LPA-induced IL-8 and MCP-1 production in TNFα-primed RAFLS. However in contrast to RAFLS preconditioned with TNFα, we did not observe degradation of IkB

or phosphorylation of NF-κB at Ser-536 in cells stimulated with LPA alone, whereas stimulation of TNFα-primed RAFLS with LPA attenuated NF-κB Ser-536 phosphorylation (unpublished data). A drawback is that the NF-κB inhibitor (Bay11-7082) does not inhibit the IKKs, but suppresses their activation by targeting components of the ubiquitin system [59], and has possible off-target effects (Fig. 7). Complementary approaches using the MSK inhibitor SB-747651A or silencing of MSKs in RAFLS will be required to determine whether MSK phosphorylates NF-κB at Ser-276 in control and TNFα-primed RAFLS stimulated with LPA.

In conclusion we show that LPA-induced IL-8 production is mediated through the ERK1/2-MSK signaling axis and MSK-independent pathways. Priming of RAFLS with TNFα for 8 h enhances LPA-mediated cell functional responses including MSK activation and cytokine/chemokine production. However, after inflammatory conditioning with TNFα, LPA-mediated cytokine/chemokine secretion was mainly MSK-independent and insensitive to inhibition by Rho-kinase, p38MAPK, or NF-κB inhibitor. In TNFα preconditioned RAFLS simultaneous inhibition of at least two signaling pathways such as ERK1/2 and p38MAPK was required to inhibit IL-8 and IL-6 production. This may have important clinical implications for the development of drugs targeting these signaling pathways for the treatment of RA.

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4.7 Figures

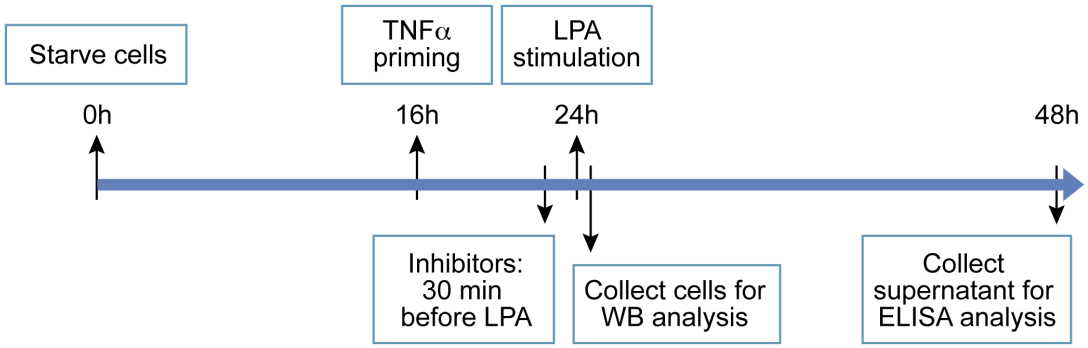

Figure 1 Schematic illustration of the experiment design.

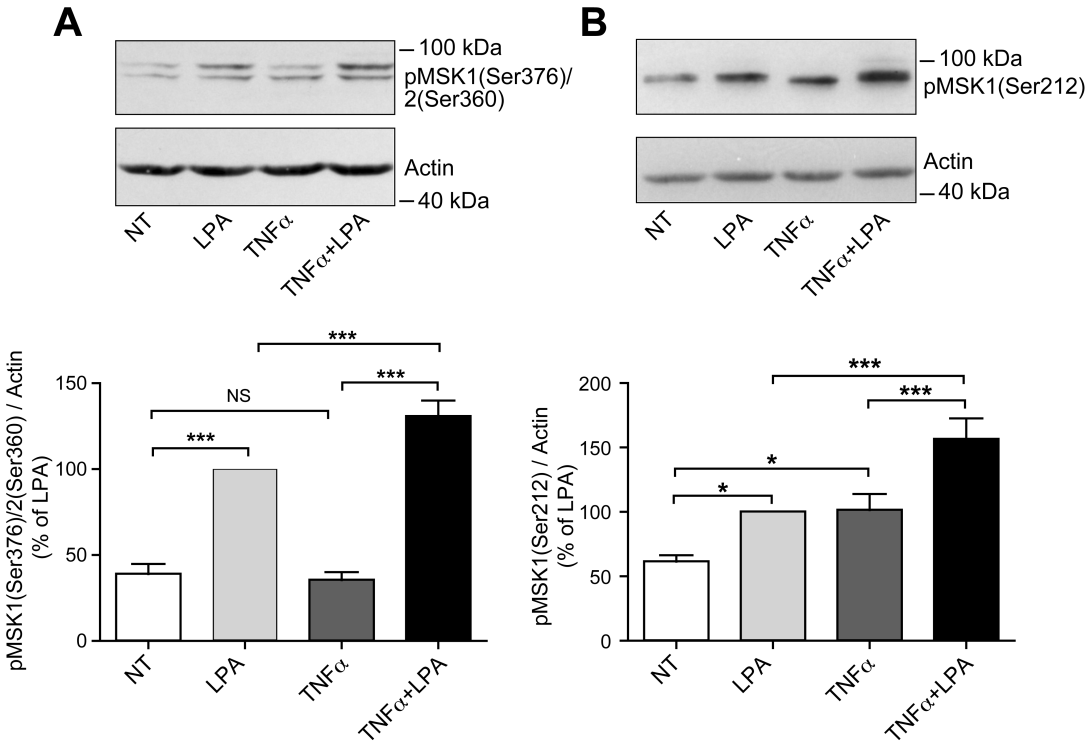

Figure 2 Priming of RAFLS with TNFα enhances LPA-mediated MSK phosphorylation.

RAFLS were treated with or without TNFα (80 ng/ml) for 8 h before stimulation with 5 μM LPA for 5 min. Cell lysates were subjected to Western blot analyses with the indicated antibodies. The upper panels of A and B are Western blots representative of various independent experiments with similar results, respectively. The lower panels A and B are the densitometry quantification of phospho-MSK1 Ser376/MSK2 Ser-360 (n = 22) and of phospho-MSK1 Ser-212 (n = 17), respectively. Data were normalized with respect to actin as a loading control. The LPA-stimulated sample was set to 100% for comparison between experiments. Data are expressed as mean ± SEM. The values were subjected to a one-way ANOVA, Bonferroni multiple comparison test * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

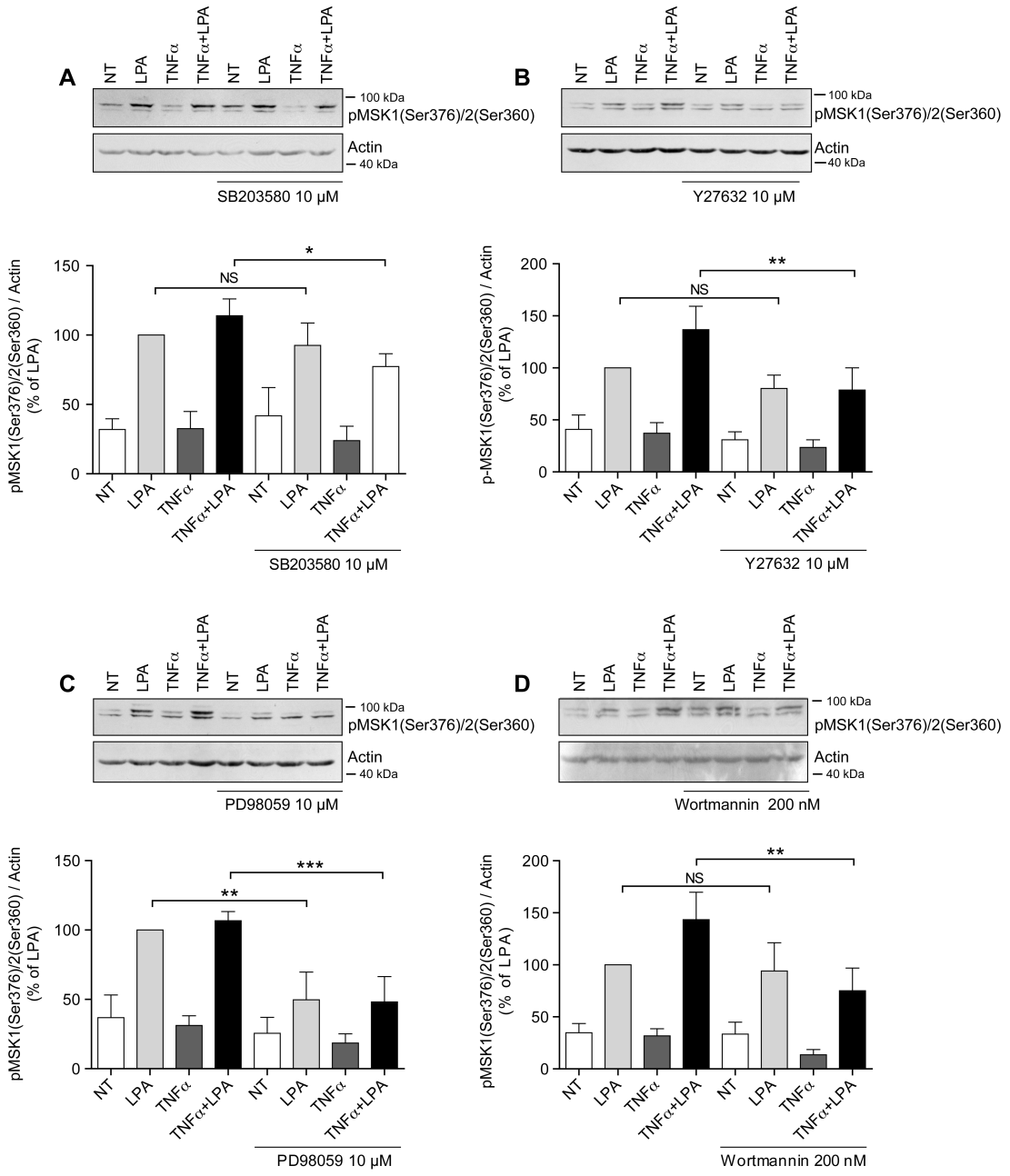

Figure 3 Inhibition of ERK1/2 blocks LPA-mediated MSK1 Ser-376/MSK2 Ser-360 phosphorylation.

RAFLS were treated with or without TNFα (80 ng/ml) for 8 h before stimulation with 5 μM LPA for 5 min. Where indicated the cells were pre-treated for 30 min with the kinase inhibitors prior to stimulation with LPA. The levels of phospho-MSK1 Ser-376/MSK2 Ser-360 were monitored as described in Materials and Methods. The upper panels in A, B, C, and D, are Western blots representative of various independent experiments with similar results. The lower panels are the densitometry quantification

of phospho-MSK1 Ser376/MSK2 Ser-360 from 4 (A, C) or 6 (B, D) independent experiments. Data were normalized with respect to actin as a loading control. The LPA-stimulated sample was set to 100% for comparison between experiments. Data are expressed as mean ± SEM. The values were subjected to a one-way ANOVA, Bonferroni multiple comparison test for selected groups. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

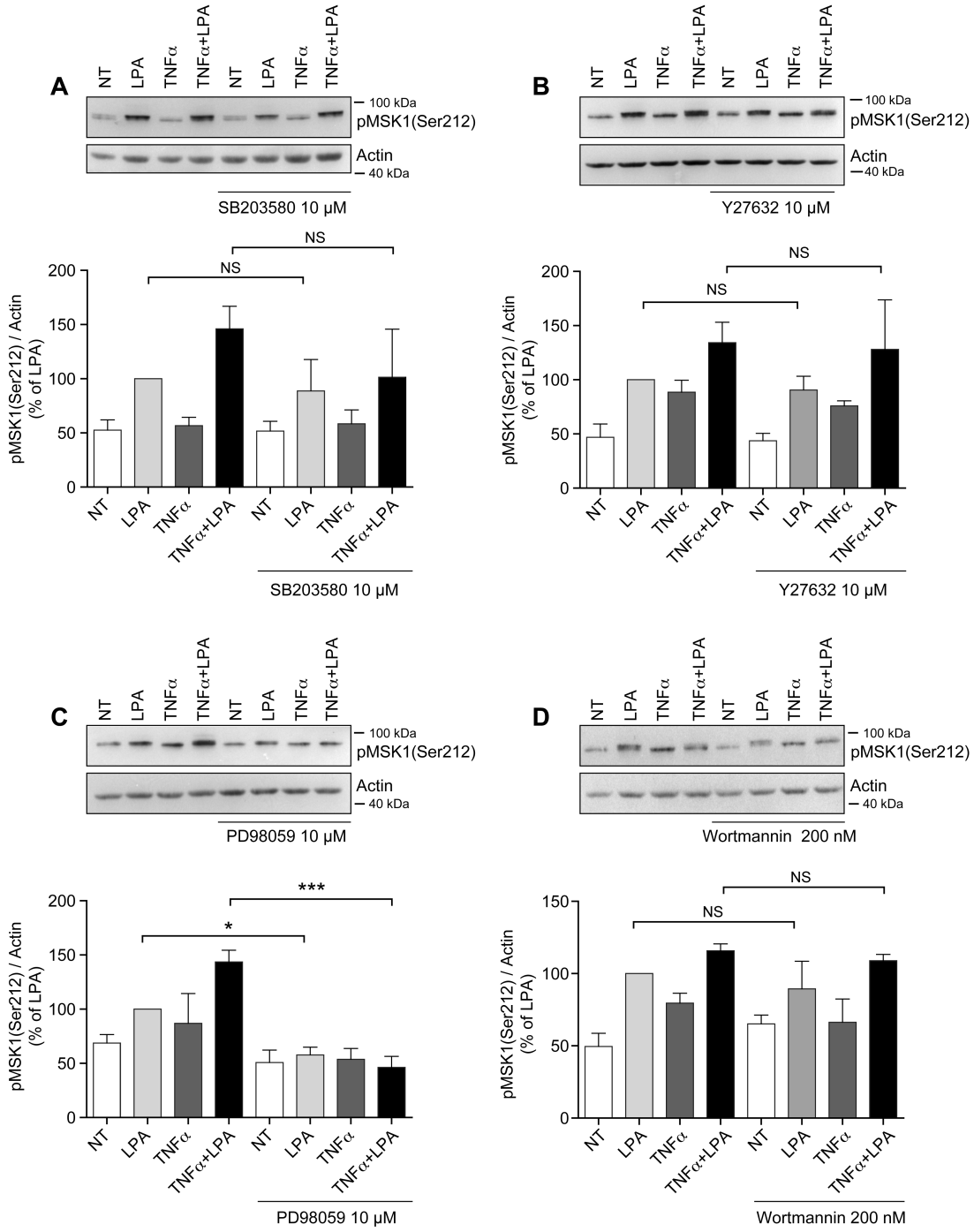

Figure 4 Inhibition of ERK1/2 blocks LPA-mediated MSK1 Ser-212 phosphorylation.

The cells were treated and the samples were analyzed exactly as described in Figure 3, except that phospho-MSK1 Ser-212 was monitored.

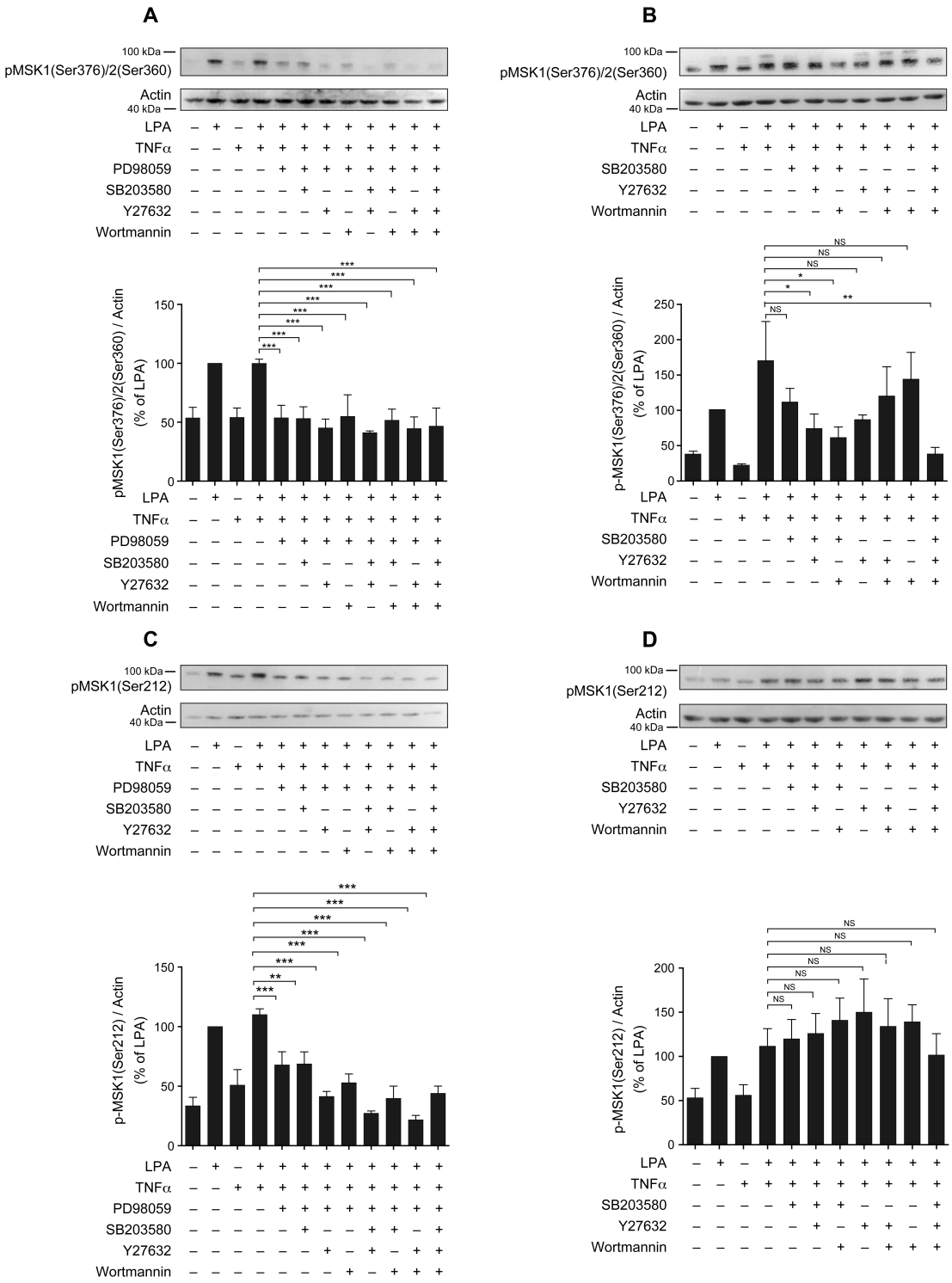

Figure 5 Combinations of signaling inhibitors attenuate LPA-mediated MSK phosphorylation.

RAFLS were treated with or without TNFα (80 ng/ml) for 8 h before stimulation with 5 μM LPA for 5 min. Where indicated, cells were pre-treated for 30 min with the indicated combinations of inhibitors prior to stimulation with LPA. Inhibitor

concentrations were 10 μM for PD98059, SB203580, and Y27632, and 200 nM for wortmannin. The levels of phospho-MSK1/MSK2 were monitored as described in Materials and Methods. The upper panels (A, B, C, D) are Western blots representative of various experiments with similar results. The lower panels are the densitometry quantification of phospho-MSK1 Ser376/MSK2 Ser-360 (A, B) and phospho-MSK2 Ser-212 (C, D) from 3 (A, B, C) or 6 (D) independent experiments. Data were normalized with respect to actin as a loading control. The LPA-stimulated sample was set to 100% for comparison between experiments. Data are expressed as mean ± SEM. The values were subjected to a one-way ANOVA, Dunnett's multiple comparison test for selected groups. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

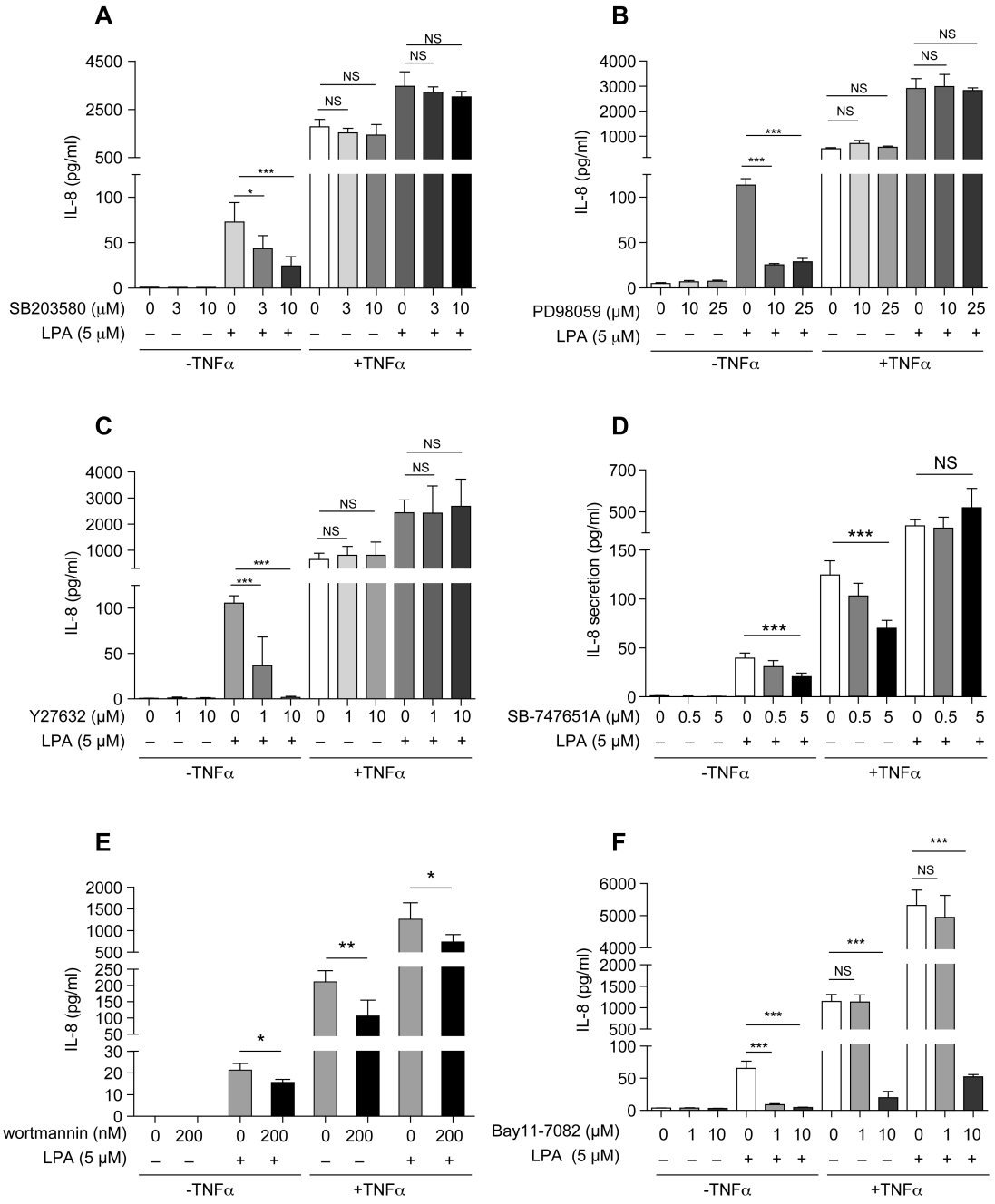

Figure 6 The sensitivity pattern of LPA-mediated IL-8 secretion to signaling inhibitors is altered after priming of RAFLS with TNFα.

RAFLS were treated with or without TNFα (80 ng/ml) for 8 h before stimulation with 5 μM LPA for 24 h. Where indicated, cells were pre-treated for 30 min with the indicated concentrations of the p38MAPK (A), ERK1/2 (B), Rho kinase (C), MSK (D), PI3K (E), or NF-kB (F) inhibitor prior to stimulation with LPA. The supernatants were collected and the IL-8 ELISA was performed. Data are expressed as mean ± SEM

(pg/ml) from 3 (B, C, D, F) or 6 (E) independent experiments. The values were subjected to Student's t-test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

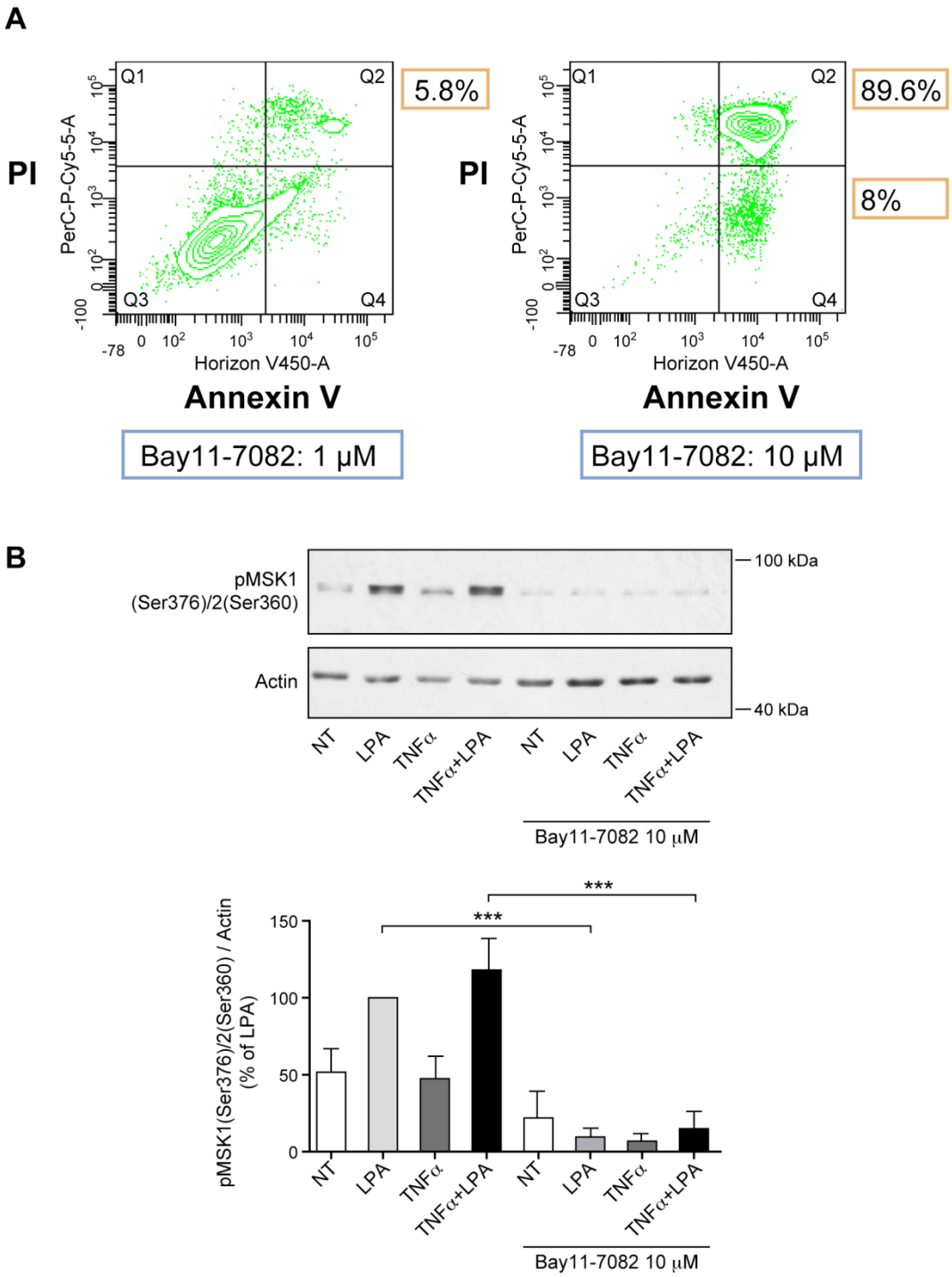

Figure 7 High concentration of Bay11-7082 (10 μM) totally blocks LPA-induced MSK phosphorylation but is highly cytotoxic for RAFLS.

(A) RAFLS cultured in 24-well plates were treated with the indicated concentrations of Bay11-7082 for 24 h. Cells were treated accutase and resuspended in PBS for staining with propidium iodide (PI) and Annexin V-eFluor450 for 20 min. Labeled cells were then analyzed by flow cytometry (Q1: PI+ Annexin V-; Q2: PI+

Annexin V+; Q3: PI- Annexin V-; Q4, PI- Annexin V+). (B) Cells were treated with or without TNFα (80 ng/ml) for 8 h before stimulation with 5 μM LPA for 5 min. Where indicated the cells were pre-treated for 30 min with 10 μM Bay11-7082 prior to stimulation with LPA. The levels of phospho-MSK1 Ser-376/MSK2 Ser-360 were monitored as described in Materials and Methods. The upper panel is a blot representative of three independent experiments with similar results. The lower panel is the densitometry quantification of phospho-MSK1 Ser376/MSK2 Ser-360. Data were normalized with respect to actin as a loading control. The LPA-stimulated sample was set to 100% for comparison between experiments. Data are expressed as mean ± SEM. The values were subjected to a one-way ANOVA, with Bonferroni multiple comparison post test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

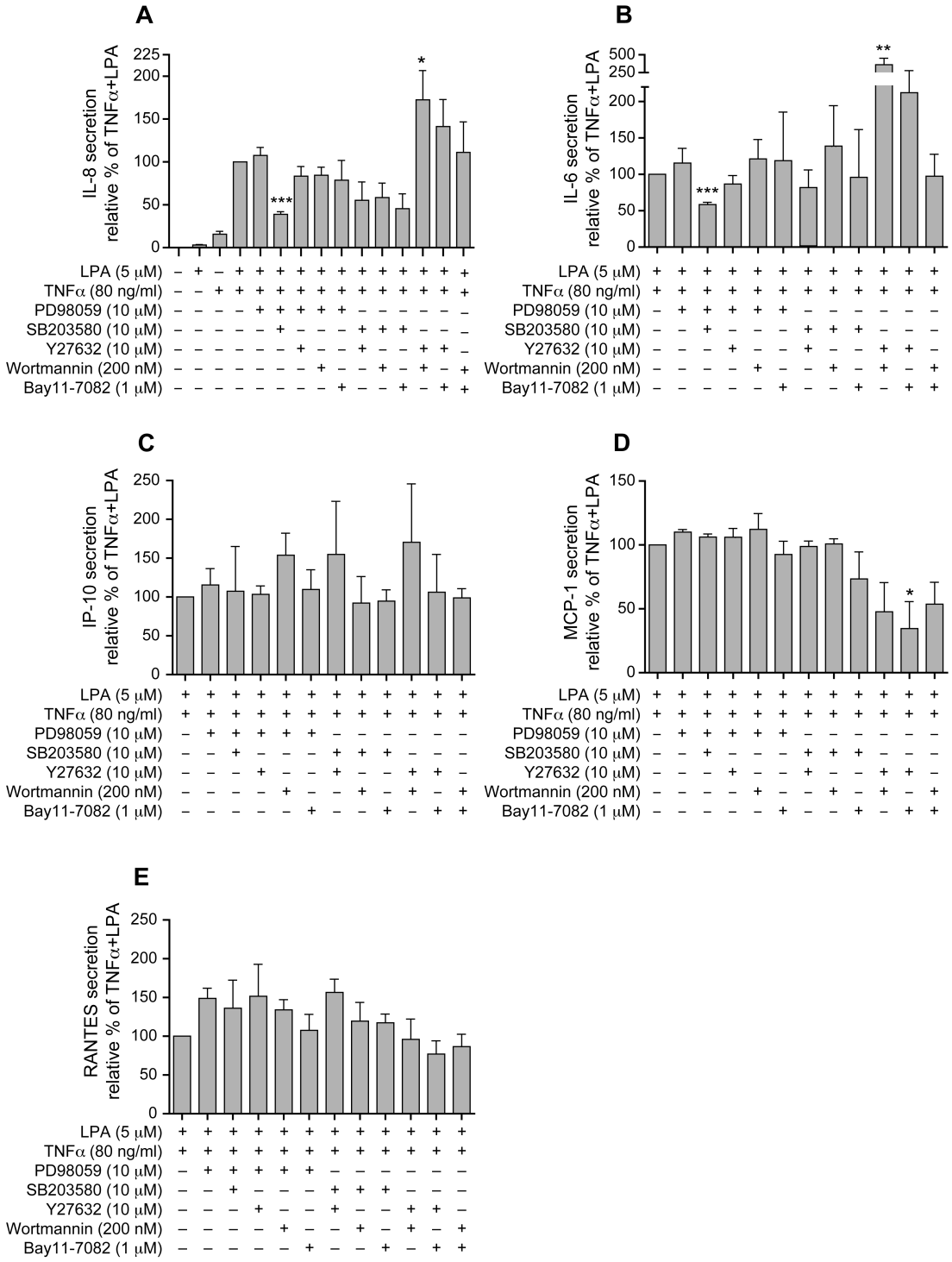

Figure 8 Combinations of signaling inhibitors differentially impact LPA-mediated IL-8, IL-6, IP-10, MCP-1 and RANTES secretion in TNFα-primed cells.

RAFLS were treated with or without TNFα (80 ng/ml) for 8 h before stimulation with 5 μM LPA for 24 h. Where indicated, cells were pre-treated for 30 min with the indicated concentrations of the p38MAPK (A), ERK1/2 (B), Rho kinase (C), MSK (D),

PI3K (E), or NF-kB (F) inhibitor prior to stimulation with LPA. The supernatants were collected and the levels of IL-8 (A), IL-6 (B), IP-10 (C), MCP-1 (D), and RANTES (E) were monitored using a Luminex immunoassay as described in Materials and Methods. The level of chemokine/cytokine produced by TNFα-primed RAFLS stimulated with LPA was set to 100% for comparison between experiments. Data are expressed as mean ± SEM from 4 (A, B, E) or 3 (C, D) independent experiments. The values were subjected to a one-way ANOVA, Dunnett's multiple comparison test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

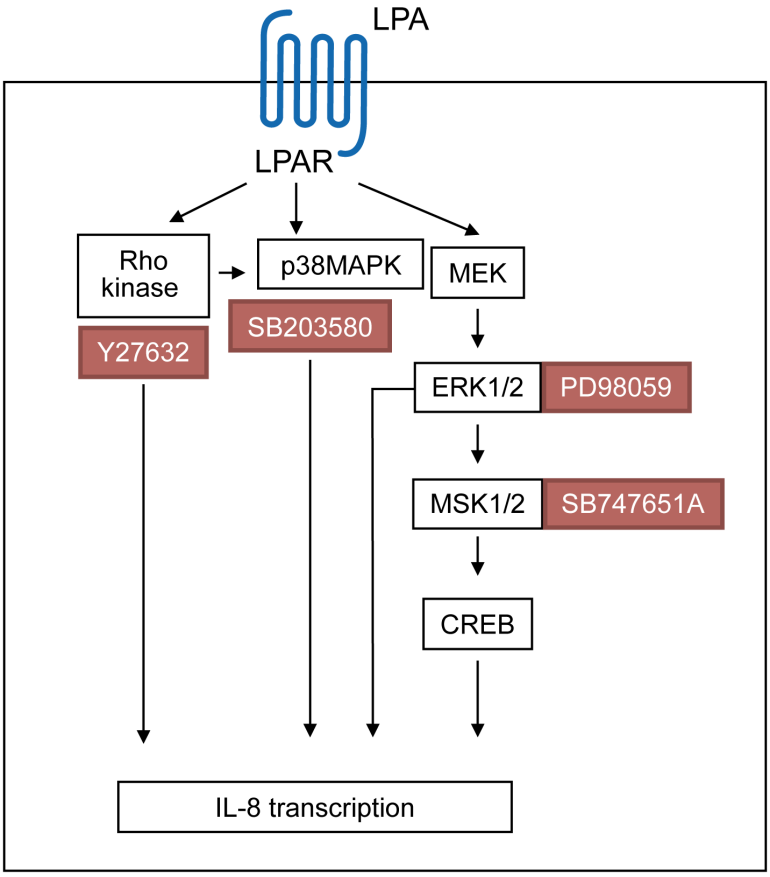

Figure 9 Proposed signaling pathways involved in LPA-induced IL-8 secretion.

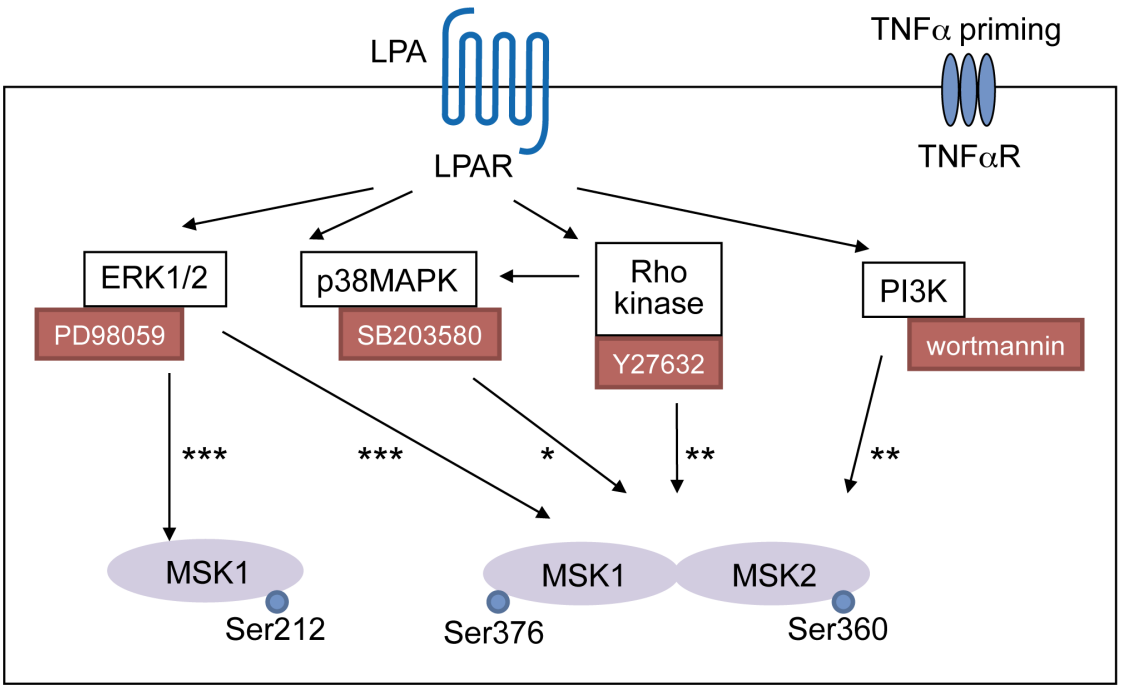

Figure 10 Proposed signaling pathways involved in LPA-induced MSK phosphorylation after priming with TNFα

Chapter 5

General discussion and future directions

The imbalance between proliferation and death of FLS is one of the features of RA as mentioned in the introduction [187], and activation of FLS also contributes to the pathogenesis by damaging synovial membranes and producing inflammatory cytokines that recruit immune cells to the joint [271]. For example, a massive recruitment of neutrophils was detected in the synovial fluid of RA patients [272]. Inflammation is driven by various cytokines and chemokines. The key mediators of RA include TNFα, IL-6 and IL-1β in synovial fluid and plasma of patients with RA [217, 273], the majority of which is derived from macrophages and other activated leukocytes. These factors act in both an autocrine and paracrine fashion in the joints and mediate matrix degradation and tissue remodeling, resulting in joint dysfunction. In RA joints, FLS are the major targets of TNFα. In the synovial fluid of RA patients, significant amounts of bioactive lipids, such as LPA and S1P, and of autotaxin are also detected [72]. The ATX-LPA axis is also involved in the pathogenesis of RA. Since we hypothesized that signaling through LPA receptors promotes the production of chemokines and recruitment of leukocytes to sites of inflammation, contributing to the severe inflammation of RA, we studied *in vivo* and *in vitro* the role of LPA in chemokine and cytokine secretion.

We employed an air pouch model to study the inflammatory response induced by LPA. We followed this up with experiments in *in vitro* RA FLS models to study the precise mechanism whereby LPA can induce the secretion of cytokines and chemokines. In these experiments, we first studied the signaling pathways necessary for mediating the effects of LPA alone. To obtain a more physiologically-relevant picture, we then studied the signaling pathways necessary for LPA-mediated cytokine superproduction following a pretreatment with TNFα. Combined, these experiments will provide insight

into the intertwined mechanisms by which TNFα and LPA contribute to the regulation and the maintenance of inflammatory responses.

5.1 Our principal conclusions based on this study

1. We provided evidence that LPA-induced CXCL13 secretion contributes to the recruitment of CD3+ T cells, part of the leukocyte recruitment mediated by LPA within the air pouch under conditions of inflammation exacerbated by TNFα: 1) administration of LPA or TNFα into air pouches increased the levels of CXCL13 in air pouch lavage fluids in a time-dependent manner; 2) the combination of a pretreatment of the air pouch tissues with TNFα prior to LPA stimulation greatly enhanced LPA-mediated CXCL13 secretion; 3) the release of CXCL13 induced by LPA peaked two hours ahead of the time point of maximal leukocyte recruitment, including that of CD3+ immune cells; 4) antibody neutralization of CXCL13 prevented the influx of these T cells into TNFα-pretreated air pouches.

2. LPA induces the production of a broader range of chemokines in FLS, including IL-8, IP-10, MCP-1 and RANTES. LPA-induced IL-8 secretion in RAFLS is dependent on MSK-mediated activation of CREB. Both MSK1 and MSK2 isoforms are activated in response to LPA and contribute to chemokine production in RAFLS.

3. TNFα pretreatment in FLS enhanced LPA-induced secretion of cytokines and chemokines. Various signaling pathways including p38MAPK, ERK1/2-MSK1/2-CREB axis, Rho kinase, NF-κB and PI3K are involved in the superproduction of IL-8 mediated by LPA and TNFα. TNFα down-regulates the sensitivity of RAFLS to inhibitors. The synergistic effect of TNFα and LPA on cytokine and chemokine secretion required a specific combination of inhibitors, such as those of p38MAPK and ERK for IL-8 and IL-6, but those of Rho kinase and NF-κB for MCP-1.

This study gave deeper understanding of the role of LPA in cytokine/chemokine production and lymphocyte recruitment *in vivo*, the *mechanism* whereby LPA induces

cytokine/chemokine production, as well as the mechanism by which LPA induction of cytokine/chemokine production is enhanced by prior exposure of the cells to TNFα.

These data support a "re-wiring" of the LPA signaling networking controlling IL-8 and possibly IL-6 production after exposure to TNFα. This has clear implications for the development of signaling inhibitors aimed at reducing proinflammatory cytokines and chemokines for use in the clinic.

5.2 Discussion

5.2.1 Targeting LPA receptor mediated cell response

In vivo, a previous study, in the air pouch model, demonstrated that both LPA_1 and LPA_3 mediate the recruitment of leukocytes to the inflammatory site through a mechanism that is dependent on LPA-mediated chemokine secretion and enhanced by the pro-inflammatory cytokine TNFα [269]. In that study, although LPA-mediated KC secretion was shown to play a predominant role in the recruitment of leukocytes into the air pouches, neutralization of KC or blocking of its cognate receptor CXCR2 was not able to totally abrogate the influx of leukocytes. In this study, we have provided evidence that LPA-induced CXCL13 secretion contributes to the recruitment of CD3+ T cells, part of the aforementioned leukocyte recruitment mediated by LPA and TNFα (Chapter 2), which answered some remaining questions from previous studies. It is very possible that blocking LPA_1 and LPA_3-mediated signaling pathways will provide new strategies for the treatment of RA, since both receptors mediate inflammatory responses resulting in higher secretion of proinflammatory cytokines and chemokines to recruit immune cells *in vivo* and *in vitro* (Chapter 2 and Chapter 3). In addition, TNFα pretreatment enhanced LPA_3 expression in RAFLS [257], indicating that the activated phenotype of RAFLS through TNFα stimulation may include LPA receptor up-regulation. Blocking LPA_1 and LPA_3-mediated signaling pathways may also inhibit the activation of synovial fibroblasts; eventually inhibiting the progressive inflammation of the synovium in RA. One report using LPA receptor antagonist Ki16425 [259]

showed a promising treatment for RA. Autotaxin is the main enzyme producing extracellular LPA, involved in many severe pathological conditions. Autotaxin inhibitors could be another strategy for the treatment of RA, since inhibition of ATX could reduce the excessive production of LPA and ATX inhibitors were reported to decrease the synovial fluid-induced motility of synovial fibroblasts [257]. The identified ATX inhibitors have been reviewed in [72] and there are more potent and selective ATX inhibitors are under development [274-276].

5.2.2 Indirect chemoattractant effect of LPA on leukocyte migration

In Chapter 2, we provide evidence that LPA-induced CXCL13 secretion contributed to the recruitment of CD3+ T cells within the air pouch environment. As mentioned in the introduction, the ATX-LPA axis is emerging as a regulator of lymphocyte homing and inflammation [277]. ATX binds to lymphocytes in a $\alpha 4\beta 1$-dependent manner [53]. Through activation of LPA receptors expressed by lymphocytes, LPA could enhance lymphocyte motility in an indirect manner [277, 278]. Consistent with what we observed in Chapter 2, an indirect effect of LPA on lymphocyte migration through chemokine secretion, LPA-mediated CXCL13 secretion may also contribute to the recruitment of lymphocytes and extranodal lymphoid neogenesis during chronic inflammation. In the air pouch model, CXCL13 secretion could be mediated through binding of LPA to its cognate receptors, possibly LPA1 and LPA3 [269], expressed by air pouch lining cells or discrete populations of cells recruited early. Given the reports showing that 1) RA synovial T helper cells are a source of high levels of CXCL13 detected in synovial fluids [279]; 2) within the RA synovium, CXCL13 is expressed in areas of B cell accumulation in ectopic lymphoid follicles, where subtypes of CXCL13-expressing T cells and monocytes/macrophages colocalize [280]; 3) CXCL13 receptor CXCR5 is up-regulated in the RA synovium [281]; 4) CXCR5 is known to be expressed by mature B cells, a subset of CD4+ and CD8+ T cells, immature dendritic cells, and macrophages [282, 283]; it is easy to infer

that LPA-induced CXCL13 secretion may also recruit cells other than CD3+ T cells into the air pouch, such as B cells or monocytes/macrophages. However, the observation of cell influx into the air pouch in Chapter 2 for 8hrs after LPA stimulation may not be long enough to detect certain immune cell recruitment, such as B cells. Monocytes may also be recruited partially by CXCL13 through CXCR5 binding [281]. However, other chemokines such as MCP-1 mediated by LPA may play more dominant roles in monocyte migration [284].

5.2.3 Targeting intracellular signaling pathways in RA

RAFLS activation is strongly associated with cytokine secretion profiles, which are regulated by various intracellular signaling pathways. Dysfunctional intracellular signaling involving JAK/STAT, MAPK, PI3K/AKT/mTOR pathways, which are targets for RA treatment, or even "cross-talk" between them was reported to play an important role in RA [285]. Elevated LPA production in synovial fluids [286, 287] and abundant LPA receptor expression in the synovium [40] may lead to dysfunctional intracellular signaling in RA. Besides targeting LPA receptors or LPA producing enzymes in RA, our investigation of signaling pathways involved in LPA-mediated cytokine and chemokine secretion will also provide new ideas for targeting the specific signaling pathways involved in the development of RA therapies.

In this study, we observed LPA-induced ERK phosphorylation, also enhanced by TNFα pretreatment (Chapter 3 and Chapter 4), which could be one of the activated phenotypes of RAFLS. Levels of ERK phosphorylation in the synovium were found to be higher in RA patients than in normal individuals [288]. Specific inhibitors that target ERK by inhibiting the activation of the upstream regulators MEK1/2 are available with only limited information concerning their therapeutic potential in RA. Some researchers have proposed targeting MEK-ERK as a treatment for RA patients, especially those who are nonresponsive to the anti-cytokine therapies [289]. Though ERK inhibitors FR180204 and PD184352 have been shown to be effective against collagen-induced

arthritis [288, 290], a representative animal model of RA, researchers have expressed concerns about using MEK/ERK pathway inhibitors as a treatment for inflammatory arthritis. As T cells from Lupus patients have defective MEK/ERK pathway signaling and CD4+ T cells treated with MEK/ERK pathway inhibitors become autoreactive *in vitro* [291], using MEK/ERK pathway inhibitors as a treatment in RA might result in the development of a Lupus-like disease. In this study, we observed that inhibition of MEK-ERK abolished LPA-mediated IL-8 secretion but did not decrease IL-8 super-production mediated by LPA and TNFα, which consistently indicated that targeting ERK alone may not be efficient.

Other MAPKs involved in LPA signaling include p38MAPK. Our data showed that inhibition of the p38MAPK pathway did not abolish IL-8 secretion mediated by LPA in TNFα-primed cells. An orally-administered small molecular inhibitor of p38α also failed to show efficacy in RA clinical trials [292, 293], which shifts the focus of inhibitor research towards other signaling pathways. Recent research showed that a drug targeting TAK1 (transforming growth factor-β-activated kinase 1, a MAP3K) named (5z)-7-oxozeaenol (5ZO) is a potential drug for use as a RA therapeutic [271]. TAK1, as a MAP3K, lies upstream of NF-κB, p38 and JNK [294]. 5ZO selectively inhibits NF-κB, p38 and JNK [271]. But 5ZO also has nonselective targets, such as MEK1/2 (regulate ERK) and MKK6 (regulates p38) [271]. Our data in Chapter 4 showed that blocking two signaling pathways of ERK and p38MAPK is necessary to decrease LPA-induced IL-8 and IL-6 secretion, and blocking other signaling pathways (such as Rho kinase and NF-κB) is necessary for MCP-1. This is consistent with the data that 5ZO decreases cytokine and chemokine secretion by blocking several signaling pathways in activated synovial fibroblasts [271]. There are two ways to block both ERK and p38. One is to block an upstream kinase such as TAK1 or MEK; another is to block a shared downstream kinase hub, such as MSK, which leads us to focus on the downstream signaling pathway of p38 and ERK. However, p38MAPK and ERK have multiple substrates [144, 295], which partially explained the insufficiency of blocking MSK

alone for inhibiting cytokine and chemokine secretion mediated by LPA in TNFα-primed cells. However, we cannot deny the important role of MSK-CREB in LPA induced IL-8 secretion.

5.2.4 The role of MSK and CREB in LPA-mediated cytokine and chemokine secretion

MSKs activation is one of the downstream signaling cascades of p38MAPK and ERK [149]. In Chapter 3 and Chapter 4 we hypothesize that MSK activation is the key signaling cascade lying downstream of p38MAPK and ERK for LPA-mediated IL-8 secretion. Data in Chapter 3 showed the important role of both MSK1 and MSK2 in LPA-mediated IL-8 secretion. Though inhibition of MSKs alone did not affect the super-production IL-8 mediated by LPA and TNFα, inhibition simultaneously of MSK upstream kinases ERK and p38MAPK decreased IL-8 and IL-6 secretion significantly (in Chapter 4), which indicates the involvement of MSK-dependent and MSK-independent pathways. In Chapter 3, silencing MSK1 and MSK2 did not completely abolished IL-8 secretion mediated by LPA. Silencing of the CREB gene partially decreased IL-8 and MCP1 secretion mediated by LPA. We cannot exclude the involvement of other MSK substrates and even signaling pathways other than MSK-CREB in LPA-mediated cytokine and chemokine secretion. In Chapter 3, we also observed that LPA stimulation for 5 minutes induced phosphorylation of other kinases or transcription factors in RAFLS, such as RSKs, Akt, eNOS, and STATs, *etc*. P90RSK and Akt are known to phosphorylate CREB [296, 297], and could be the upstream kinases of LPA-mediated CREB activation. STATs may be involved in LPA-mediated MCP-1 secretion, since the MCP-1 gene promoter contains potential STAT binding sites [298, 299]. Similar to IL-8 [300], MCP-1 gene transcription also involves NF-κB, AP-1 and CREB activation [301]. For IL-6, depending on the stimuli and cell type, the DNA binding activity of CREB, C/EBP, AP-1, and NF-κB may differ [302, 303]. CREB has been shown to induce transcription of immune-related genes that possess a CRE element, including IL-2, IL-6, IL-10, TNFα, COX-2 [304], IL-1β [305], IL-8,

CCL3 and CCL4 [306, 307]. The signaling pathways and transcription factors regulating LPA-mediated IL-8, IL-6, MCP-1, IP-10, and RANTES might differ or overlap, resulting in different effects from the same combination of specific signaling pathway inhibitors (data in Chapter 4).

MSK phosphorylation enhancement mediated by LPA and TNFα is observed in this study. Whether MSK activity was enhanced is under investigation by detecting CREB phosphorylation at Ser133. MSK1 was identified to associate with NF-κB p65 and phosphorylate p65 at Ser276 [308, 309]. NF-κB p65 phosphorylation at Ser276 was reported to be required for TNFα-induced IL-8 secretion [310]. LPA-induced MSK phosphorylation may phosphorylate either CREB or NF-κB p65. Our data showed that inhibition of NF-κB decreased LPA-mediated IL-8 secretion with TNFα priming, suggesting the significant involvement of NF-κB. But how NF-κB is involved in LPA signaling still needs further investigation. Since the p65 component of NF-κB interacts with CREB coactivator CBP/p300 at the same region as phosphorylated CREB, it has been proposed that NF-κB activity is inhibited by activated CREB through competition for limiting amounts of CBP/p300 [304]. It is not clear how the balance between CREB activity and NF-κB activity regulates gene expression. Interestingly, we have data showing that NF-κB inhibitor Bay11-7082 decreased MSK phosphorylation at even 1μM without cytotoxicity (data not shown), neither IL-8 superproduction. One report mentioned that Bay11-7082 could partially inhibit TAK1 activity *in vitro* [311], which may partially explain the side effect of Bay11-7082 on MSK phosphorylation.

The role of MSK in inflammatory arthritis has been reviewed in the Appendix. Targeting MSK would be cautious, since MSK inhibitor SB747651A did not decrease LPA-mediated IL-8 secretion while there was TNFα pretreatment. In addition, in LPS-stimulated macrophages, MSK was involved in anti-inflammatory cytokine IL-10 secretion and MSK1/2 double knockout mice present increased inflammation induced by PMA [152]. MSK may mediate distinct cellular responses depending on the cell type and stimuli.

Chapter 6

Perspectives

There are still limitations associated with using the air pouch model to study the role of LPA in inflammatory arthritis. Other arthritis models such as the K/BxN model or the CIA model have been used to study the role of LPA receptors in inflammatory arthritis like RA. Based on the experiments performed in this thesis, I will mention some limitations of these studies and other concerns which may be worth future research to address.

6.1 The role of LPA in lymphocyte function: limitations of the mouse air pouch model and other solutions

LPA has been reported to induce actin polymerization, motility, transendothelial migration [312] and chemokinesis in human naïve T cells [53] and T cell lymphoma cell lines [313]. In addition, the chemokinetic effect of LPA was reported [53] to be additive with the chemoattractant effects of CXCL12 and CCL21 on human naïve T cells, suggesting an interaction between LPA and chemokine signaling in migrating lymphocytes. In this study, we have proposed an indirect role of LPA in inducing leukocyte recruitment by mediating chemokine secretion in the murine dorsal air pouch model.

The air pouch model is successfully used in worm infection research [314] and in research towards testing anti-inflammatory drugs [315]. CD4+ T cells were reported to be recruited, following immunization with T cell dependent antigen TNF-KLH in the air pouch [316]. However, the technical aspects of establishing and maintaining the air pouch, as well as harvesting infiltrating cells mentioned in that study pose an obstacle. Since maintaining the air pouch for an extended period and recovering cells is difficult, combined with the fact that no more than 3% of the cellular infiltration was T cells, the lack of efficient T cell response is the limitation of the air pouch model to study T cell

mediated responses. In this thesis, using stimulation with LPA and priming with TNFα, only around 1% of air pouch-infiltrating cells were CD3+, which is a limitation to study clearly the specific T cell subset. In addition, we also observed that CD3-Ly6g-CD11b+ mononuclear cells were decreased after anti-CXCL13 was employed. Since CXCR5 or CXCR3 is also expressed in monocytes and macrophages, we cannot exclude a role for CXCL13 mediated by LPA in recruiting other cell types besides CD3+ cells.

6.2 How is NF-κB involved in LPA-induced cytokine/chemokine secretion with and without TNFα priming?

During the investigation of signaling pathways involved in TNFα-enhanced IL-8 secretion in response to LPA, we discovered that LPA-induced IL-8 is partially dependent on PI3K activation (Chapter 4), and NF-κB activation was reported to be involved in PI3K signaling [317]. We hypothesize that after TNFα priming, LPA may activate NF-κB, either through indirect activation from PI3K or MSK signaling, or even direct activation, to affect IL-8 secretion. Our data (Chapter 4) showed that the NF-κB inhibitor Bay11-7082 inhibits LPA-induced IL-8 secretion, which is cytotoxic at 10μM. Of notice, Bay11-7082 1μM decreased LPA-mediated IL-8 secretion, but did not affect the superproduction in TNFα-primed cells. Alternative methods using other more specific NF-κB inhibitors or silencing of NF-κB in RAFLS will still be required to understand the involvement of NF-κB activation. In addition, the mechanism of NF-κB activation induced by LPA with or without TNFα is not clear. Whether PI3K or MSK is the factor upstream of NF-κB signaling, and which phosphorylation site of NF-κB is involved, are still pending questions.

6.3 Where is MSK activation located, in the cytosol or inside the nucleus?

Another interesting question is raised since we conclude that IL-8 secretion induced by LPA alone is mainly through MSK-CREB axis activation: Does MSK relocalize from the cytoplasm to the nucleus upon activation, or could upstream kinase ERK activate MSK inside the nucleus? Other reports have mentioned that MSKs are

constitutively localized to the nucleus, which is different from RSK1-4, who exhibits a predominantly cytosolic localization in resting cells [318, 319]. But in RA FLS, which represent a pathological state compared to normal cells, we did not investigate whether MSK localization is nuclear or cytosolic.

6.4 What do the LPA receptor knockout mice look like in arthritis models?

To study the role of LPA in the development of inflammatory arthritis, LPA receptor knockout mice have been used with several methods of inducing arthritis. One study found that LPA_1-deficient mice did not develop arthritis following immunization with type II collagen (CII) and that an LPA_1 antagonist also ameliorated murine CIA [260]. Other research also showed that mice treated with the LPA receptor antagonist Ki16425 showed attenuated arthritis in the K/BxN serum-transfer model by reducing inflammatory mediators and bone-remodeling proteins in the joint, as well as reducing osteoclast differentiation and bone resorption [259]. A recent report demonstrated that loss of ATX-LPA_1 signaling leads to disorganization and proliferation of chondrocytes, causing severe defects in cartilage formation [264], implying an important role for ATX-LPA_1 in the development of arthritis. These observations raised two questions: 1) since LPA has six identified receptors and some of them have overlapping functions, will other LPA receptor knockout mice or even double knockout mice show a similar defect to that observed in LPA_1-deficient mice in those arthritis models? 2) Since chondrocytes and fibroblasts both derive from mesenchyme stem cells, do RAFLS also utilize the ATX-LPA_1 system for their proliferative activity? Though in the previous studies, we did not observe LPA-induced proliferation of RAFLS [257], it is still possible that LPA species other than 18:1 LPA could have an effect on the proliferation of RAFLS in a similar context. Further studies are still required to address these points to understand the mechanism of how LPA is involved in inflammatory arthritis.

6.5 What do the MSK1/2 KO mice look like in arthritis models?

Mice with knockouts of MSK1, MSK2, or both MSK1 and MSK2 are viable and fertile and present no obvious health defects [151]. However, CREB mutants show more severe phenotypes (defects in brain development and memory function [320, 321], dwarfism [322]). CREB may have a wider role in mediating effects via different signal transduction cascades. MSKs were reported to be necessary for anti-inflammatory IL-10 production and DUSP1 production (Dual Specificity Phosphatase 1, a mitogen-activated protein kinase phosphatase) in LPS-stimulated primary macrophages [152], indicating an anti-inflammatory role. In the same report, MSK1/MSK2 double knockouts are hypersensitive to LPS- and PMA-induced inflammation, suggesting MSKs are negative regulators of inflammation. In contrast, our data indicate that MSKs are necessary, in part, for LPA-induced pro-inflammatory cytokine/chemokine secretion in RAFLS. Thus, it would be interesting to study the role of MSKs in an arthritis model, such as the CIA or K/BxN serum transfer models, using MSK1/2 knockout mice.

6.6 What is the mechanism of RAFLS proliferation? Are signaling pathways other than MAPK such as Hippo-YAP signaling involved?

As mentioned in the introduction for synoviocytes from RA patients, one of the most important features of RAFLS is the imbalance of coordination between cell proliferation and cell death which is essential to maintain homeostasis in the function of the joint. This is a similar feature with cancer cells. Original research in Drosophila revealed a signaling pathway named Hippo, which is a conserved regulator of organ size [323, 324]. Hippo signaling has been intensively studied in the past decade, especially in cancer [325, 326]. The mammalian Hippo pathway has also been reported to be a potent regulator of organ size, and its dysregulation leads to tumorigenesis [327]. Interestingly, LPA and S1P were identified in the HEK293A cell line, as well as many other cancer cell lines, as critical regulators of the Hippo-YAP pathway, which controls GPCR-mediated gene expression, cell migration, and cell proliferation [328]. In the

same study, very low concentrations of LPA (0.1μM) could effectively induce YAP/TAZ dephosphorylation. (Exactly which LPA species used was not mentioned). In addition, YAP and/or TAZ are involved in LPA-induced gene expression, cell migration, and cell proliferation. The Hippo-YAP pathway as a downstream branch of GPCR signaling has been suggested too in the above-mentioned article. Another study focusing on the expression and function of YAP in RAFLS has reported that the expression of active nuclear YAP in RAFLS is significantly more than that in osteoarthritis FLS, and TNFα induced YAP dephosphorylation in RAFLS, surprisingly [329]. In addition, YAP also plays a role in regulating RAFLS invasion by modulating focal adhesion formation and MMP production [330]. LPA has at least 6 receptors, which can be coupled to different G-proteins. Therefore, it is possible that one ligand may increase YAP phosphorylation in one cell type but decrease YAP phosphorylation in another cell type depending on which receptor is dominantly expressed and which Gα is coupled to the receptor is activated in that particular cell type. Those observations mentioned above in cancer cells and RAFLS prompted us to study the role of LPA in the Hippo-YAP pathway in RAFLS. However, we did not see YAP dephosphorylation induced by LPA 18:1 (5μM in PBS-0.1% BSA), S1P (5μM in PBS-0.1% BSA) or TNFα (10ng/ml) 15 min after cell starvation for 16 h, but we did observe complete YAP dephosphorylation induced by FBS 10% (data not shown). It will be interesting to study which LPA species or other serum component activates the Hippo-YAP signaling pathway and the mechanism involved, since understanding the role of Hippo signaling pathway in synoviocytes will give a new insight of the mechanism of synoviocyte activation and proliferation, helping for developing novel therapeutic approaches for RA.

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Annexes

Role of mitogen- and stress-activated kinases in inflammatory arthritis

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1. Summary

Lysophosphatidic acid (LPA) is a pleiotropic lipid mediator that promotes motility, survival, and the synthesis of chemokines/cytokines human fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis (RA). The nuclear transcription factor CREB is widely expressed and phosphorylated by signaling events induced by growth factors including LPA. In addition to protein kinase A (PKA) that directly phosphorylates CREB, mitogen- and stress-activated kinases (MSKs) activated downstream of Mitogen Activated Protein Kinase (MAPK) also phosphorylate CREB at Ser133. Phosphorylation of CREB by MSKs is essential for the production of pro-inflammatory and anti-inflammatory cytokines. However, other downstream effectors of MSK1/2 such as NF-kB, histone H3, and HMGN1 may also regulate gene expression in immune cells involved in disease pathogenesis. MSKs are master regulator of cell function that integrate signals induced by growth factors, pro-inflammatory cytokines, and cellular stresses, as well as those induced by LPA.

2. Introduction

Mitogen and Stress Activated Protein Kinases (MSKs) were first identified as an efficient CREB kinase in 1998 [1]. For the past 17 years, MSKs have been investigated thoroughly as regulators of gene expression at multiple levels [2]. The function of MSKs discovered until now is mainly phosphorylation of transcription factors, chromatin-associated proteins and ubiquitin ligase. MSKs are activated in response to mitogenic signals ((e.g. serum, epidermal growth factor and fibroblast growth factor, lysophosphatidic acid (LPA)), neurotransmitters, progesterone, cellular stresses (e.g. UV-irradiation, oxidative stress, arsenite, metals and retinoic acid), and other signals from pro-inflammatory cytokines (e.g. TNFα), as well as PAMP [3]. Through binding to G protein-coupled receptors (GPCRs), cytokine/chemokine and growth factor receptors, or activation of stress sensors, all these stimuli activate various Mitogen Activated Protein Kinase (MAPK) signaling pathways (p38 MAPK and ERK).

Activation ERK1/2 and p38 MAPK directly or indirectly through the phosphorylation of MSKs regulates the function of transcription factors and nuclear proteins involved in gene transcription. The substrates of MSKs including CREB, ATF-1, NF-κB p65, Histone H3, and HMGN1 have been extensively studied and validated in cells silenced for the expression of MSK1 and/or MSK2 [2]. Another protein, E3 ubiquitin ligase TRIM7 was recently reported to be a target of MSK1 using cell silencing approaches, thereby highlighting the crosstalk between different post-translational protein modifications [4]. The roles of MSKs downstream of ERK1/2 and p38 MAPK and their important functions in immunity and disease states, including arthritis, have been highlighted in the book edited by Arthur J. Simon C [3].

Rheumatoid arthtitis (RA) is a severe, chronic and systemic inflammatory disease. Infiltration of multiple blood-derived cells in inflamed joints (macrophages, dendritic cells, T cells, B cells, neutrophils, platelets [5]), high level of cytokines/chemokines, production of lipid mediators and matrix metalloproteinases (MMPs) in synovial joints, synovial cell proliferation leading to synovium thickening, and pannus formation are the hallmarks of RA [6]. All these features eventually lead to cartilage dysfunction of cartilage, damage of adjacent tissues, and deformation of joints associated with chronic pain. At the present time, medical therapy for RA use conventional disease-modifying anti-rheumatic drugs such as corticosteroids, methotrexate, anti-malarials, and TNF inhibitors alone or in combination with methotrexate. New strategies targeting other cytokines, like IL-1 [7, 8] and IL-6 [9, 10], or B cell marker CD20 [11] are also approved for RA treatment. Inhibitors of specific protein kinase pathways also hold potential in the treatment chronic autoimmune diseases. More recently the JAK1/3 inhibitor tofacitinib, which suppresses inflammation driven by immune cells through inhibition of JAK/STAT may be necessary to suppress immune-cell mediated inflammation, was approved by the Food & Drug Administration for the treatment of RA [12]. Although anti-cytokine therapies exert significant benefits for RA patients, but there is still a substantial subset of nonresponsive patients as well as patients who cannot tolerate the current therapy [13]. Presently, researchers focus more on the cellular pathways of inflammation to search for new therapeutic targets for the treatment of autoimmune diseases such as RA [14]. MAPK are potential targets to treat RA because of their important role in regulating cell proliferation, apoptosis, cytokine and MMP expression [15]. The functions of MSKs as important nuclear signaling kinases phosphorylated by MAPKs and regulator of inflammatory gene transcription were investigated extensively during the past decade. More extensive reviews are found elsewhere [16, 17]. In this article, we will summarize current knowledge on MSK signaling in inflammatory arthritis and describe its potential roles in amplification and perpetuation of inflammation.

3. MSKs structure and activation mode

The kinase domains composing MSK1/2 includes a C-terminal and N-terminal kinase domain, harboring several phosphorylation sites [16, 17]. Human MSK1 can be activated by MAPK at Ser360, Thr581 and Thr700 located in C-terminal domain [18]. Phosphorylation of the C-terminal domain induces a conformational change in MSK1, which permits autophosphorylation on Ser212, Ser376 and Ser381 by the C-terminal kinase domain, finally resulting in phosphorylation of MSK substrates by the N-terminal kinase domain [2, 18] (Figure1). Compared with RSKs, the main difference is that RSK is activated by ERK whereas MSK can be activated by both ERK and p38 MAPK through a closely related mechanism that reflects the common domain structure of MSKs and RSKs [19]. The molecular docking interaction between p38 MAPK or ERK1/2 and MSKs has been clearly highlighted previously and will not be further discussed in this review [19, 20].

4. MSKs in immune and synovial cells

4.1 T cells

The key evidence for the aberrant pathway of T cell activation in the initiation and perpetuation of RA is the association between disease pathogenesis and HLA-DRB1 [21, 22]. Th1 cells expressing IFNγ and TNFα are present in RA synovial tissues [21, 23]. Data from animal models of arthritis suggests that IL-17-producing CD4+ T cells (Th17 cells), also contribute to the inflammatory processes [24-26]. The p38α MAPK-MSK1/2 axis was reported to IL-17 synthesis by CD4+ T cells in experimental models of autoimmune diseases [27]. The absence of the msk1/2 gene resulted in the failure of producing IL-17 by murine lymphocytes isolated from the lymph node and the blood [27]. The potential role of MSK1/2 in the regulation of gene transcription downstream of p38α MAPK signaling in T cells is illustrated by the LAT (linker for activation of T cells) signalosome that propagates signals through branching several signaling pathways including that of MAPK [28]. In T cells, MSK is the major kinase responsible for CREB phosphorylation in response to TCR activation, and T cells from MSK1/2 knockout mice showed reduced T cell proliferation in presence of IL-2 [29]. In this study, the authors pointed out that MSK1/2 are highly expressed in the thymus and the spleen and that spleen from MSK1/2 knockout mice contains fewer T cells. Data of tissue-specific pattern of mRNA expression available at the Scripps Research Institute BioGPS Website and Database indicate that MSK1 (gene symbol: RPS6KA5) but not MSK2 (gene symbol: RPS6KA4) is highly expressed in CD19+ B cells, CD4+ T cells, CD8+ T cells, CD56+ NK cells compared with other tissues and cell types. Hence, it is possible that MSK1 and MSK2 have different functions (i.e. substrates specificity) in those cells.

4.2 B cells

CD20+B cells are enriched in the RA synovium and their function mainly includes autoantibody production, T cell interaction and cytokine production [30]. MSK1/2 deficiency has no significant effect on T cells and B cell development [29]. At present, we do not know what impact MSK1 and/or MSK2 deficiency has on T/B cell interaction, cytokine/chemokine production by B cells, and mature B cell proliferation. Mn2+ induced apoptosis of human lymphoma B cells through the activation of caspase-8 [31]. This study using specific pharmacological inhibitors and dominant-negative mutants of p38α MAPK and MSK1 showed the p38α MAPK-MSK1 signaling pathway but not Fas-associated death domain protein drives B cell apoptosis. Nevertheless, the mechanism of how p38α MAPK-MSK1 axis regulates B cell apoptosis is not clear given that caspase-8 does not associate MSK1 and is not of substrate of MSK1 [31]. Another study showed that TGFβ-mediated apoptosis of human Burkitt lymphoma B cells through caspase-8 activation downstream of p38α MAPK, but the possible contribution of MSKs to this effect is not yet known [32].

4.3 Neutrophils

Neutrophils constitute 90% of the cells in RA synovial fluids [33]. The crucial roles of neutrophils in inflammation, inflammatory diseases, systemic autoimmune diseases have been thoroughly reviewed [34-38]. The main function of neutrophil includes phagocytosis, degranulation, production of antimicrobial peptides and proteins, production of reactive oxygen species (ROS), and NETosis (release of neutrophil extracellular traps) [35, 39]. Khandpur et al. showed enhanced NETosis of circulating and synovial fluid neutrophils from RA patients, compared to those from osteoarthritis (OA) patients or healthy individuals [40]. In neutrophils, a role for p38 MAPK has also been reported in chemotaxis [41], regulation of apoptosis [42], cytokine/chemokine and MMP production [43, 44]. The p38 MAPK-MSK1 axis contributes to chemokine production through CREB activation in LPS-stimulated human neutrophils [44]. In this study CREB was presumably phosphorylated by MSK1, but the data require further validation since the authors used Ro-31-8220, a non-selective inhibitor of MSK1 [45] (Table 1). There is another report showing that neutrophils stimulation with sphingosine-1-phosphate (S1P) induces p38MAPK and ERK-dependent phosphorylation of MSK1 to control the secretion of IL-8 [46].

Activation of the ERK and p38 MAPK pathways have been reported in human neutrophils stimulated with chemoattractants, pro-inflammatory cytokines, and Fcγ receptor ligation [47], and their activation is also required for the respiratory burst in TNFα and GM-CSF primed cells [48]. As MSK1 is phosphorylated by ERK1/2 and p38 MAPK in neutrophils under certain conditions, we cannot deny a role for MSKs in the signaling pathway leading to a coordinated pattern of cytokine/chemokine gene expression induced by various stimuli.

4.4 Fibroblast-like synoviocytes (FLS)

FLS plays a substantial role in many pathologic events in inflammatory arthritis. As a key component of the hyperplastic rheumatoid pannus, combined with its invasive phenotype, FLS have a major role in the initiation and perpetuation of destructive joint inflammation [49]. As passive responders, FLS in RA secrete cytokines/chemokines, lipid mediators of inflammation, a subset of extracellular matrix remodeling enzymes and express adhesion molecules. Somatic mutations and epigenetic alterations associated with signaling anomalies may also contribute the invasiveness behavior, resistance to apoptosis, and production of inflammatory cytokines (reviewed in [49]). We demonstrated that Ser376 on MSK1 and possibly Ser360 on MSK2 were transiently phosphorylated in RAFLS shortly after the stimulation of LPA [50] and TNFα as well (unpublished data). A specific inhibitor of MSKs (SB747651A) or silencing of MSK1 and/or MSK2 with siRNAs significantly reduced lPA-induced chemokine secretion (IL-8 and MCP-1) and CREB phosphorylation at Ser133 [50]. FLS priming with TNFα for 8 hours prior stimulation constantly increases the phosphorylation of MSK1/2 at Ser376/Ser360 (unpublished data), as well as IL-8, IL-6, and MCP-1 secretion [50, 51]. These data suggest an important role for MSKs in LPA signaling which leads to inflammatory cytokine/chemokine secretion by FLS in RA. A possible explanation for transient MSK1/2 phosphorylation could be due to the activity of a protein phosphatase such as protein phosphatase 2Cδ, which has been reported to be phosphorylated by ERK and to associate with MSKs [52], or due to dephosphorylation of ERK1/2 and p38MAPK by dual-specific phosphatase 1 (DUSP1) [53]. Further work is needed to pinpoint the phosphatases that regulate the p38α MAPK-MSK1/2 signaling axis in FLS.

To date little is known about the mechanism of how TNFαadrastically enhance the secretion of chemokines in response to bioactive lipids such as LPA and sphingosine-1-phosphate (S1P). Early studies showed correlation between chemokine synthesis and increased expression of a subset of LPA and S1P receptors by cultured FLS or the lining tissue of mouse air pouch when stimulated with TNFα [54]. Both S1P and LPA promote chemokine secretion p38MAPK, ERK1/2 and Rho kinase activation in FLS [55]. Hence, increased expression of certain LPA (LPA1 and LPA3) and of S1P receptors by cells exposed to an inflammatory environment may contribute, at least in part, to enhance intracellular signals that converge to MSK and activation of the transcription factor CREB.

4.5 Macrophages

By producing various pro-inflammatory cytokines/chemokines macrophages play a critical role to cartilage and bone destruction in inflammatory arthritis [56, 57]. There is an imbalance between inflammatory and anti-inflammatory macrophages in the RA synovium [58], and more information on how macrophages contributes to RA disease

activity at both the local and the systemic levels can be obtained by reference to other reviews [59-61]. LPS-mediated activation of MSK1 and MSK2 was associated to COX-2 expression and IL-1β secretion in macrophages [62]. MSK activation is not restricted to TLR4 signaling, as Pam3CSK4 (TLR1/2 agonist), lipoteichoic acid (TLR2 agonist), CpG-DNA (TLR9 agonist) and dectin-1 agonist stimulation all activated MSK1 at Th581 [63]. Inhibition of early expression of COX2 in MSK1/2 knockout macrophages was confirmed by another study, but induction of COX-2 protein and prostaglandin secretion was detected at later time points due to reduced LPS-mediated production of IL-10 and increased COX-2 mRNA stability in the absence of IL-10 [64]. By regulating the CREB/ATF-1 dependent transcription of DUSP1 and of IL-10 MSK1 and MSK2 are also part of negative feedback loop that limits TLR4-driven inflammation. Hence, the absence of this feedback loop may explain why LPS-mediated expression of TNFα, IL-6, IL-12, and late expression of COX-2 is increased in MSK1/2 deficient macrophages [63]. In line with those studies, it was reported that stimulation of MSK1/2 knockout macrophages with zymozan particles reduced the secretion of IL-10 and increased that of IL-12 [65]. So far there is little information on the role of MSK1/2 in mouse models of arthritis by it would be interesting to evaluate the impact MSK knockdown on disease onset, severity and duration as TNFα, IL-1β, IL-6 and chemokines are produced by various cell types including macrophages and other immune cells [66].

5. Roles of MSKs in cytokine/chemokine production

MSKs play a versatile role through the phosphorylation of transcription factors and nuclear proteins that up-regulate the expression of pro-inflammatory and anti-inflammatory gene including chemokines/cytokines and signaling proteins [16, 17]. TNFα, IL-6, IL-2, IL-10 genes share in common a CRE element in the core promoter region that is required for CREB binding and gene transcription [67, 68]. However, phosphorylation of CREB on Ser133 by MSKs has a greater effect on the induction of CREB-dependent immediate-early genes than that induced by PKA possibly due to differential recruitment of CREB co-activator proteins [69]. Activation of ERK1/2 also leads to histone phosphorylation and Sp1 transcription factor binding to the IL-10 promoter [70]. As validated substrates of MSKs histone H3 and HMGN1 may also contribute to immediate-early gene expression through various mechanisms [2].

The expression of IL-8 (CXCL8) is controlled by three different mechanisms: derepression of the gene promoter; transcriptional activation of the gene by NF-κB and JUN-N-terminal protein kinase pathways; and stabilization of the mRNA by the p38 MAPK pathway [71]. In FLS from RA patients the production of IL-8 is upregulated by ~100-fold in response to TNFα or IL-1β [5]. Inhibitors of p38 MAPK inhibit the functional responses to these cytokines including the production of IL-8 [72]. This study suggests positive feedback loop mechanisms that lead to activation of p38 MAPK

pathway and long term IL-8 secretion, which recruit neutrophils to the inflammatory sites. We demonstrated LPA-induced production of IL-8 is inhibited by inhibitors of p38 MAPK and MSK, as well as silencing of MSK1/2 and CREB in FLS [50, 51]. The mechanism by MSK regulates the transcription of CREB-dependent genes such as IL-8 and MCP-1 as well remains is not clear[69]. In RAFLS, NF-κB p65 subunit, but not C/EBP-β or AP-1, regulates IL-8 gene expression under IL-1β stimulation [73]. Whether phosphorylated CREB needs to recruit co-activation proteins (CBP/p300) [74] or synergizes with other transcription factors such as NF-κB p65 subunit [73, 75], C/EBP-β [76, 77], or AP-1 [78-80] to regulate IL-8 expression in FLS requires further investigation.

6. Role of MSK in animal model of arthritis

There is barely article studying the role of MSK in specific animal models of Rheumatoid arthritis such as CIA model, K/BxN model. As mice that lack MSK1 and MSK2 produce elevated levels of IL-10 and IL-1 receptor antagonist protein in response to LPS, MSKs seem to limit inflammation. Whether MSK knockout mice have an effect on arthritis severity induced by stimuli such as collagen or K/BxN serum is still unknown. A study investigating SNPs of p38 signaling network in RA patients demonstrated that, SNPs encoding MSK1 and MSK2 had a significant association with the response to anti-TNF therapy. They also demonstrated that SNP of MSK2 had a recessive effect, which is different from some other SNPs with a dominant effect in p38 signaling pathway.

7. The inhibitors of MSK

The two MSK inhibitors have been used to study various MSK functions including the secretion of cytokine [1, 81-88]. However, these inhibitors are not selective for MSKs and inhibit many other kinases including protein kinase A (PKA) [45]. This is a major limitation since these compounds show better selective for PKA which targets CREB, ATF1, RARα and NF-kB p65 subunit shown to be phosphorylated by MSKs [2, 17, 89]. The selectivity of new MSK inhibitor SB-747651A was evaluated in vitro and shown to superior selectivity for MSKs than that of H89 and Ro31-8220 [45]. In this study, the authors showed that Ro31-8220 reduced LPS-induced TNFα secretion that cannot be attributed to MSK inhibition. In contrast, inhibition of MSK in LPS-stimulated macrophage reduced IL-10 secretion and enhanced that of IL-12 as previously reported in MSK1/2 knockout cells [63, 64]. In oral squamous cell carcinoma, SB-747651A was found to inhibit the phosphorylation of NF-kB p65 subunit [90]. We demonstrated that SB747651A inhibits LPA-mediated chemokine synthesis through inhibition CREB phosphorylation [50]. SB-747651A inhibits CREB phosphorylation without affecting MSK1 on Th581 site (a critical site for MSK1 activation) [64]. Autophosphorylation of Ser212 and Ser376 in MSK1 are poorly affected by SB-747651 thereby suggesting that

the inhibitor targets the N-terminal kinase domain of MSKs [45]. Although SB747651A showed improved selectivity, off target effects cannot be totally excluded since other kinases like ribosomal S6 kinase 1 (RSK1), p70RSK and Rho-associated protein kinase 2 (ROCK-II) are inhibited by compound [45].

Inhibition of p38α MAPK showed efficacy in animal models of arthritis but failed in clinical trials [91-94]. Inhibition of p38 MAPK initiates an imbalance between anti-inflammatory and pro-inflammatory process which exclude this kinase for drug targeting in autoimmune diseases [95]. This is illustrated by the fact that MSKs, the downstream targets of p38 MAPK, differentially regulates the synthesis of two important anti-inflammatory cytokines IL-10 and IL-12 production as a negative feedback in inflammation [63]. From the experience of cancer therapy, monotherapy using signaling inhibitors such as MEK-ERK is not permanently effective, as cells may become resistant to the inhibitor by different mechanism [96]. As the aggressive characteristics of RA synovium was viewed as reminiscent of neoplastic tissue [97], and MAPK signaling plays important role in cell proliferation and cytokine production, it will be necessary to study well the downstream of MAPK signaling pathway, including the role of MSKs, as well as the crosstalk mechanism to better understand their dual roles in inflammatory arthritis.

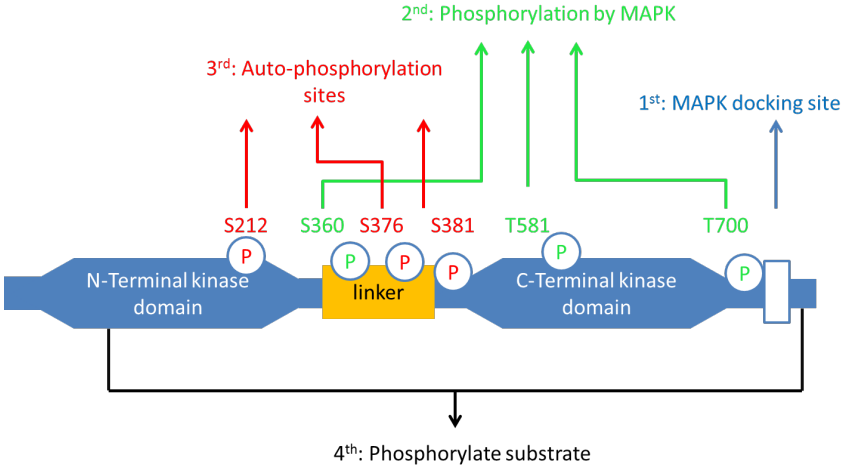
8. Conclusions

Several inflammatory cytokines and lipid mediators of inflammation activate MSK1/2 signaling downstream of p38 MAPK and ERK1/2. Inhibitors of MSK1/2 have effects on different cell types involved in the pathogenesis of RA, which could provide an important advantage in treatment. Inhibitors of MSK inhibit the production of cytokines/chemokines and as well as the responses induced by these pro-inflammatory mediators thereby limiting the activation and/or limiting the recruitment of immune cells to sites of inflammation. However, as reported for other inhibitors of MAPK pathway, targeting MSK in arthritis may have undesirable effects due to inhibition of other kinase pathways or regulation of complex positive and negative feedback loops that could induce imbalance in the production of pro-inflammatory and anti-inflammatory mediators. Screening for more selective inhibitors or developing isoform specific inhibitors of MSK1 and MSK2 is required to establish the applicability as a drug in the future. Furthermore, more research will be necessary to identify the targets downstream of MSK1/2. Understanding how CREB interplays with other transcription factors such as NF-kB or other MSK-dependent pathways regulating protein stability through ubiquitinylation may enable the development of drugs that have less adverse effects for treatment of chronic inflammatory diseases.

Table 1: Specificity and selectivity of inhibitors currently being used to inhibit MSK1/2

MSK inhibitors	IC50	Mechanism of action	Reference
SB747651A	0.5 nM	Selectively targets MSK1/2; Inhibits the N-terminal kinase domain of MSKs; >300-fold selectivity over RSK1 and >3000-fold selectivity over GSK-3	[45, 98, 99]
Ro-31–8220	8 nM	Inhibitor of PKC, MSK1, RSK, S6K1, GSK3	[83, 100]
H89	120 nM	Inhibitor of MSK1, S6K1 and ROCK-II, PKA	[83]

Figure 1. Schematic of human MSK1 activation.



Abbreviation:
AP-1: Activator protein 1
ATF-1: Activating transcription factor
C/EBP-β: CCAAT/enhancer binding protein beta
CREB: cAMP response element-binding protein
DUSP-1: Dual-specific phosphatase-1
GM-CSF: Granulocyte macrophage colony-stimulating factor
GSK3: Glycogen synthase kinase 3
HMGN1: High Mobility Group Nucleosome Binding Domain 1
JAK: Janus kinase
LPA: Lysophosphatidic acid
LPS: Lipopolysaccharide
MCP-1: Monocyte Chemoattractant Protein-1
MSK1: Mitogen and Stress Activated Protein Kinase 1 (RPS6KA5)
MSK2: Mitogen and Stress Activated Protein Kinase 2 (RPS6KA4)
Pam3CSK4: Synthetic triacylated lipoprotein
PAMPs: Pathogen associated molecular patterns
PKC: Protein kinase C
PP2Cδ: Protein phosphatase 2Cδ
RAR: Retinoic acid receptor
RK2: Double-stranded-RNA-dependent protein kinase 2
RSK1: 90kDa ribosomal S6 kinase (p90rsk), also known as MAPKAP-K1 (RPS6KA1)
ROCK-II: Rho-associated protein kinase 2
S6K1/p70S6K1: Ribosomal protein S6 kinase beta-1(RPS6KB1)
STAT: Signal Transducer and Activator of Transcription
S1P: sphingosine-1-phosphate
TGFβ: Transforming growth factor beta
TLR: Toll-like receptor
TNFα: Tumor necrosis factor alpha
TRIM7: Tripartite motif containing 7

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