LUND UNIVERSITY

Signal transduction in macrophages. Intracellular pathways activated by microbial constituents

Olsson, Sandra

2006

Sektionen för Cellulär och Molekylär Farmakologi
Institutionen för Experimentell Medicinsk Vetenskap
Lunds Universitet

Signal transduction in macrophages
Intracellular pathways activated by microbial constituents

Akademisk avhandling som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligt försvaras i Segerfalksalen, Wallenberg Neurocentrum, Sölvegatan 17, Lund tisdagen den 19:e december 2006, kl. 09.00

av

Sandra Olsson

Fakultetsopponent:
Docent Lillemor Mattsson Hultén
Sahlgrenska akademin
Wallenberglaboratoriet
Göteborgs Universitet
Göteborg

Organization	Document name
LUND UNIVERSITY Dept. of Experimental Medical Science Division of Cellular and Molecular Pharmacology	DOCTORAL DISSERTATION
	Date of issue 2006-12-19
	Sponsoring organization
Author(s)	
Sandra Olsson	

Title and subtitle

Signal transduction in macrophages. Intracellular pathways activated by microbial constituents

Abstract

Macrophages play an essential role in the defense against infection by phagocytosis and by production of inflammatory mediators. Such mediators are TNF-alpha and eicosanoids. This thesis is based on studies performed with the use of microbial constituents which induce arachidonate release and /or TNF-alpha production. Whole Gram-positive bacteria Staphylococcus aureus, the bacterial products LPS and peptidoglycan, the yeast preparation zymosan and particulate beta-glucan were used to stimulate cells. The main focus has been to elucidate the potential role of the non-receptor tyrosine kinases from the Src family (SFK) and the Tec kinase Btk, in signaling pathways induced by microbial constituents.

Many microbial agents elicit arachidonate release in macrophages that lead to the formation of eicosanoids. SFK are important in bacteria- and zymosan-induced arachidonate release and these studies bring forward that SFK has a pivotal role acting proximally of several known members of the signaling like ERK, p38 and PLC-gamma2. Btk is also observed to be a part of the signaling pathway downstream of SFK. Differences were detected between bacteria- and zymosan-induced responses, probably due to use of different receptors.

Zymosan can bind to several receptors and our results suggests that zymosan-induced arachidonate release is mediated by the beta-glucan receptor dectin-1. Furthermore we show that the adaptor protein gab2 is affected by particulate beta-glucan- and zymosan-stimulation, indicating a role in dectin-1 signaling.

SFK are also seen to be involved in TNF-alpha production induced by microbial constituents, but their mode of action is still unknown. We have also studied the role of membrane rafts in LPS-induced TNF-alpha production, but the importance of membrane rafts in this system is still uncertain. In summary this thesis has contributed to an increased understanding of the role of SFK and other signaling components in eicosanoids and TNF-alpha production in macrophages.

Key words: Src, Btk, eicosanoids, dectin-1, macrophage, arachidonic acid, TNF,

Classification system and/or index termes (if any):

Supplementary bibliographical information:		Language English
ISSN and key title:		ISBN 91-85559-58-X
Recipient's notes	Number of pages	Price
	Security classification	

Distribution by (name and address) Sandra Olsson, sandra.olsson@med.lu.se

Signature Sandra Olsson Date 061106

Signal transduction in macrophages
Intracellular pathways activated by microbial constituents

Sandra Olsson

Department of Experimental Medical Science
Lund University

-2006-

Department of Experimental Medical Science
Biomedical Center B12
SE-221 84 Lund
Sweden
Sandra.olsson@med.lu.se

Printed in Sweden
Media-Tryck, Lund University

ISBN 91-85559-58-X

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their roman numeral.

I. Olsson, S. and R. Sundler, *Different roles for non-receptor tyrosine kinases in arachidonate release induced by zymosan and Staphylococcus aureus in macrophages.* J Inflamm (Lond), 2006. **3**: p. 8.

II. Olsson, S. and R. Sundler, *The macrophage β-glucan receptor mediates arachidonate release induced by zymosan: Essential role for Src family kinases.* Mol Immunol, 2006. (In press)

III. Olsson, S. and R. Sundler, *The role of lipid rafts in LPS-induced signaling in a macrophage cell line.* Mol Immunol, 2006. **43**(6): p. 607-12.

ABSTRACT

Macrophages play an essential role in the defense against infection by phagocytosis and by production of inflammatory mediators. Such mediators are TNFα and eicosanoids. This thesis is based on studies performed with the use of microbial constituents which induce arachidonate release and/or TNFα production. Whole Gram-positive bacteria *Staphylococcus aureus,* the bacterial products LPS and peptidoglycan, the yeast preparation zymosan and β-glucan were used to stimulate the cells. The main focus has been to elucidate the potential role of the non-receptor tyrosine kinases from the Src family (SFK) and the Tec kinase Btk, in signaling pathways induced by microbial constituents.

Many microbial agents elicit arachidonate release in macrophages that lead to the formation of eicosanoids. SFK are important in bacteria and yeast induced arachidonate release and these studies bring forward that SFK has a pivotal role in acting proximally of several known members of the signaling like ERK, p38 and PLCγ2. Btk is also observed to be a part of the signaling pathway downstream of SFK. Differences were detected between bacteria- and zymosan-induced responses, probably due to use of different receptors.

Zymosan can bind to several receptors and our results suggest that zymosan-induced arachidonate release is mediated by the β-glucan receptor dectin-1. Furthermore we show that the adaptor protein gab2 is affected by β-glucan and zymosan stimulation, indicating a role in dectin-1 signaling.

SFK are also seen to be involved in TNFα production induced by microbial constituents, but their mode of action is still unknown. We have also studied the role of membrane rafts in LPS-induced TNFα production, but the importance of membrane rafts in this system is still uncertain. In summary this thesis has contributed to an increased understanding of the role of SFK and other signaling components in eicosanoid and TNFα production in macrophages.

CONTENTS

ABBREVIATIONS

Btk	Bruton's tyrosine kinase
COX	Cyclooxygenase
DAG	Diacylglycerol
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
Gab	Grb-associated binder
IL	Interleukin
IFN	Interferon
IP_3	Inositol 1,4,5-trisphosphate
ITAM	Immunoreceptor tyrosine activation motif
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
LT	Leukotriene
LTA	Lipoteichoic acid
MAP kinase	Mitogen-activated protein kinase
MEK	MAP kinase kinase
MEKK	MEK kinase
MK	MAP kinase activated kinase
Mnk	MAPK-interacting kinase
MyD	Myeloid differentiation marker
NK	Natural killer
NF$\varkappa$B	Nuclear factor $\varkappa$B
PDK	Phosphoinositide-dependent kinase
PG	Prostaglandin
PGN	Peptidoglycan
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PI4,5P	Phosphatidylinositol 4,5-bisphosphate
PI3,4,5P	Phosphatidylinositol 3,4,5-trisphosphate
PKB (Akt)	Protein kinase B
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PR	Proline rich
PRRs	Pathogen recognition receptors
SH	Src homology
SFK	Src family kinases
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TX	Thromboxane

PROTEIN DOMAINS

Several signal transduction proteins contain conserved intramolecular domains. The first protein modules identified were SH2 and SH3, found to be specific binding domains in he Rous sarcoma virus oncogen *src* but also in other proteins (Cohen 1995). Later other domains where recognized.

SH2 (Src homology 2) Binds selected phosphotyrosine motifs. The specificity of individual SH2 domain lies in the residues surrounding the phosphotyrosine.

SH3 (Src homology 3) Binds short peptide motifs containing key proline residues.

SH4 (Src homology 4) N-terminal sequence in Src family kinases, probably a site for fatty acylation of myrsitate and palmitate and thus potentially implied in membrane binding [1].

PH (Pleckstrin homology domain) Originally described as a protein motif of approximately 100 amino acid residues in the protein pleckstrin. It binds phospholipids and therefore involved in membrane targeting.

C2 Binds to phospholipids in a Ca^{2+}-dependent manner, mediating binding to membranes [2].

PREFACE

This thesis primarily concerns signaling in macrophages induced by microbial constitutes. The thesis is based on three papers. Two papers concern bacteria- and yeast-induced signaling leading to arachidonic acid release with special interest in Src family kinases, and one deals with the topic of membrane rafts in LPS signaling. To put the papers in a wider context, an overview of different related areas is presented in the background section in this thesis.

GENERAL INTRODUCTION

Background

All through our lives we must defend ourselves against infection by foreign organisms (bacteria, viruses, fungi and parasites). These organisms can cause pathological damage if they multiply uncontrolled in our body. Luckily most infections are short and leave no or little permanent damage due to our immune system. As a first line of defense we have a physical and chemical barrier, the skin is a very important one. On the inside the mucosal membranes of epithelial cells protects us from harmful agents and the cilia in the trachea removes harmful substances by movement. Chemical barriers include different enzymes, acid in the stomach, fatty acids in secretion and defensine, produced by intestinal cells. Another physical barrier is the normal bacterial flora in the gut, throat, mouth, vagina and on the skin. When the barriers are broken the immune system is activated. The immunes system is divided into two major parts, the innate and adaptive immune systems. The innate immune system is an unspecific and fast system while the adaptive is a slower but more specific one. The innate system is also necessary for activation of the adaptive immunity. The cells active in the innate immune system are monocytes/macrophages, granulocytes and natural killer (NK) cells. Lymphocytes (T- and B-lymphocytes) constitute the cells in the adaptive system.

Leukocytes are white blood cells and arise from pluripotent stem cells through two main lineages of differentiation. The lymphoid linage produce lymphocytes and the myeloid linage produce mast cells, macrophages/monocytes, dendritic cells and granulocytes. Granulocytes can be further subdivided into neutrophils, basophils and eosinophils.

Inflammation is the body's reaction to an infection or tissue damage and leads to an increase in blood supply to the infected area, increased capillary permeability and migration of leukocytes to the site of infection. These events result in the signs of inflammation, redness, pain, swelling and heat. Once at the site of infection professional phagocytes (macrophages, circulating monocytes and neutrophils) recognize the infecting agents by receptors on there surface and engulf them through phagocytos [3], leading to formation of a phagosome. The phagosomes fuse with lysosomes containing degrading enzymes resulting in destruction of the agent. Phagosomes also present fragments of engulfed agent on their surface allowing recognition by lymphocytes. Lymphocytes of the adaptive immune system can transform into antibody producing cells (B-lymphocytes) or mediate the cellular immune response (T-lymphocytes). Interaction of pathogens and macrophages also lead to the production of cytokines and other important mediators of inflammation.

Inflammation must be tightly regulated, too little and the body is overwhelmed by infection but too much will lead to severe disease symptoms. Chronic inflammation is a serious threat to us and many diseases have features of chronic inflammation such as diabetes [4], atherosclerosis [5] Alzheimer disease [6], Parkinson's disease [7], psoriasis [8], rheumatoid arthritis [9]. Furthermore is chronic inflammation leading to an increased risk for cancer [10].

Resolution of inflammation is a regulated process including suppression of proinflammatory gene expression, leukocyte migration and activation, followed by inflammatory cell apoptosis and clearance [11]. The regulation of this process is not completely clear but it is known that phagocytosis by macrophages of neutrophils leads to inhibition of production of pro-inflammatory substances and production of anti-inflammatory mediators, presumably involved in resolving inflammation [12]. Also different eicosanoids such as lipoxines, cyclic prostaglandins (cyPGs) [11] and prostaglandins (PGs) may be involved.

Macrophages

Macrophages are leukocytes from the myeloid linage. They originate from the bone marrow, circulate in the blood stream as monocytes and migrate into the surrounding tissue where they mature into macrophages [13]. The migration of monocytes to tissue is enhanced at site of inflammation. Macrophages are long lived cells (months) and together with neutrophils they are the first ones to encounter pathogens in the tissue.

Macrophages are found in the body as Kupffer cells (liver), Langerhans cells (skin), osteoclasts (bone), microglia (brain), alveolar macrophages (lung) and as pleural or peritoneal macrophages in body cavities. They participate in vide array of processes, and they are key cells in inflammation.

Some of the biological functions of macrophages in the immune system include:

- Production of cytokines
- Production of complement components
- Production of eicosanoids
- Antigen presentation
- Phagocytosis and killing of ingested microorganisms
- NO production
- Secretion of lysosomal enzymes
- Wound healing and tissue reorganization
- Killing of tumor cells (ADCC, TNF)

Macrophages can become activated and change their morphology, function and metabolism gaining an enhanced capacity to kill microorganisms and tumor cells [13].

Activated macrophages are larger, have a more ruffled plasma membrane, an increased capacity for adherence and spreading as well as formation of pseudopods (Fig. 1). Furthermore activated macrophages produce cytokines (tumor necrosis factor α (TNFα), interleukin-1 (IL-1) and interleukin-6 (IL-6) central to the initiation and maintainace of inflammation. These cytokines affect surrounding cells and vascular endothelium to attract and recruit more phagocytes and other leukocytes. Activated macrophages are also more efficient in their antigen presentation to T-lymphocytes.

To become activated macrophages need two signals (the classical pathway), priming by interferon-γ (IFN-γ) [14] and tumor necrosis factor-α (TNFα) itself or an inducer of TNFα. IFN-γ can be produced by T-lymphocytes, one example of the interaction between innate and adaptive immunity. The second signal is often a result of Toll-like receptor (TLR)-ligation, resulting in endogenous TNFα production.

Recently other ways of activating macrophages have been reported, leading to "alternatively activated macrophages" [15] and type II activated macrophages [16]. "Alternatively activated macrophages" is activated by IL-4 and IL-13, which lead to a phenotype involved in humoral immunity and repair. They produce IL-10 and IL-1 receptor antagonist (acts to block IL-1-induced responses). IL-10 is an anti-inflammatory cytokine, capable of inhibiting synthesis of pro-inflammatory cytokines like TNFα [17].

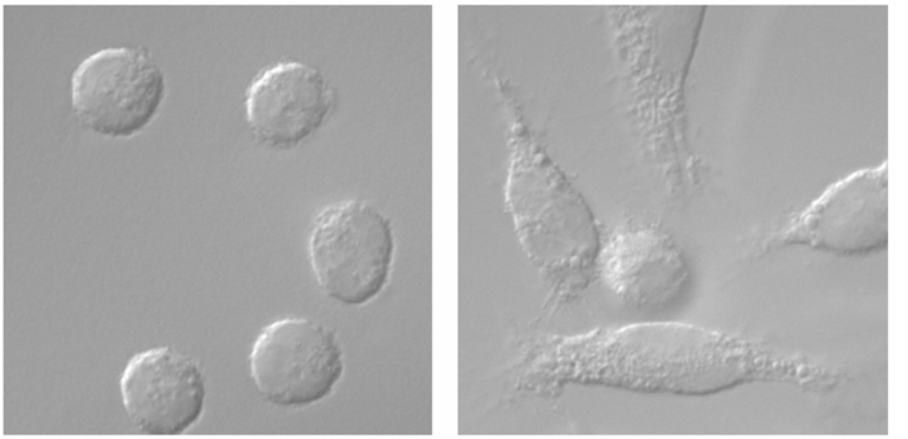

Figure 1. Resident mouse macrophages.
Macrophages cultured over night and thereafter left unstimulated (left) or stimulated for 4h with LPS (right). Images were recorded on an inverted microscope at x100 magnification.

Type II activation phenotype needs two signals, the first being ligation of FcγR and the second is stimuli that signal through TLRs, CD40 or CD44. The stimulation induce a drastic induction of IL-10 (as well as induction of other cytokines), and they are suggested to have anti-inflammatory properties [18]. Alternatively activated macrophages and type II activated macrophages are clearly distinctive from each other as they induce different set of cytokines [18].

The different types of activated macrophages may be of different population of macrophages with different biological functions [18] or they may have the ability to switch between activation phenotypes [19].

Macrophages are extremely effective in destroying pathogens but constant activation would lead to tissue damage. Activated macrophages release antimicrobial mediators such as oxygen radicals, NO and proteases, all toxic for the host. Macrophage activation is therefore highly regulated. Signals that down regulate macrophage activation may come from within the macrophages themselves or from the surrounding cells such as T- lymphocytes. T-lymphocytes regulate macrophage activation by mechanisms that control IFN-γ synthesis, one way is by regulating the half-life of the mRNA encoding IFN-γ. IL-10 also play a role in decreasing macrophage activation [20]. Moreover is apoptosis another regulatory mechanism.

Eicosanoids

The term eicosanoids denotes a large family of oxygenated C20 fatty acids. The precursor for all eicosanoids is arachidonate. The arachidonic acid cascade is a series of complex biosynthetic pathways that produce a number of lipid mediators from arachidonate, including prostaglandines, thromboxanes (TXs), leukotrienes (LTs), lipoxins, hepoxilines, isoprostanes and various hydroxyl, epoxy and hydroperoxy fatty acids (Fig. 2). Arachidonate may be reacylated into phospholipids, which is suggested to be involved in regulation of free arachidonic acid within the cell. Macrophages stimulated with zymosan produce PGE2 and LTC4 [21], but they can also synthesise LTB4 [22] and 6-Keto-prostaglandin-F1 alpha [23].

Metabolism by cyclooxygenase enzymes (COX) gives rise to the PGs and TXs. COX-2 is induced in inflammatory cells as an result of stimulation and is responsible for the elevated PGs production that occur upon cellular activation, whereas COX-1 is expressed in quiescent cells. Metabolism of arachidonic acid by the 5-lipoxygenase (5-LOX) pathway gives rise to hydroxyl and hydroperoxy derivates (5-HETE and 5-HPETE respectively) and to LTs. The 15-LOX pathway results in lipoxines while the 12-LOX pathway results in hepoxilins. Leukotrienes and prostaglandins are generated through intracellular biosythesis while lipoxins are produced by cell-cell interaction, a process known as transcellular biosynthesis [11].

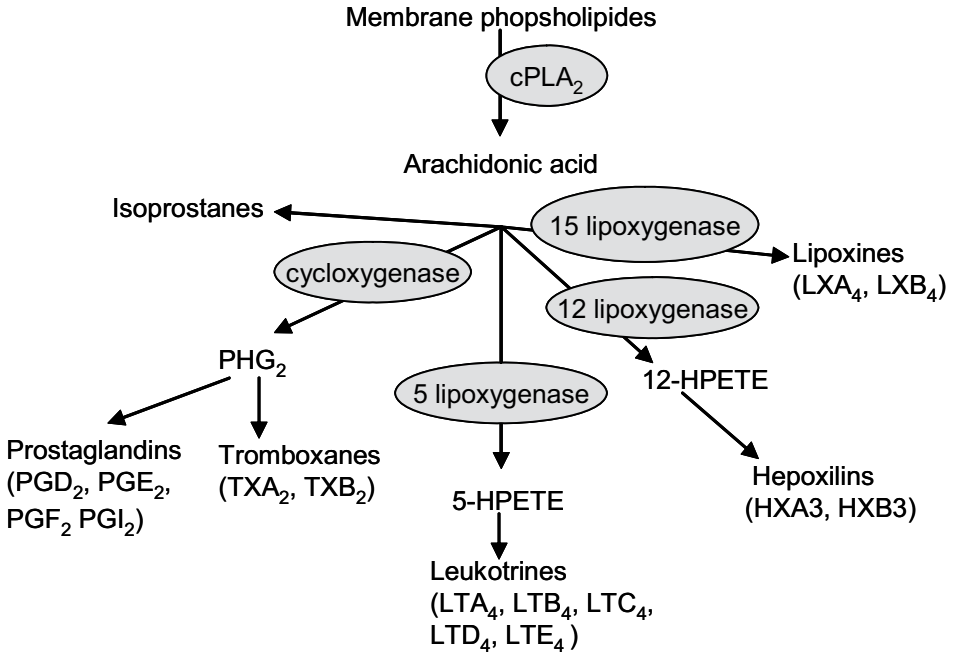

Figure 2. Overview of eicosanoids.
Arachidonate is the precursor of eicosanoids. Prostaglandins and leukotrienes are produced in macrophages after zymosan stimulation.

Many of the eicosanoids play a substantial role in different phases of inflammation, mostly having pro-inflammatory properties but some are found to have anti-inflammatory properties. Lipoxines have anti-inflammatory properties, whereas PGE2 exhibit both pro- ands anti-inflammatory effects. PGE2 has a number of pro-inflammatory properties including inducing fever, increasing vascular permeability and vasodilatation and enhancing pain and oedema caused by other agents [22]. Both aspirin and ibuprofen, well known non-steroidal anti-inflammatory drugs (NSAID) function as COX inhibitors. LTB4 increases vascular permeability is a chemotactic agent for leukocytes, induces release of lysosomal enzymes and enhances generation of reactive oxygen species and production of pro-inflammatory cytokines like TNFα, IL-1 and IL-6 [22].

Phospholipids and phosholipases

Lipids can not only be used as a source of energy but lipids also form the cell membranes and function in cellular signaling. Phospholipids make up great portions of the cellular membrane and are composed of a glycerol backbone coupled to two

fatty acids chains and a phosphate group coupled to an alcohol, such as ethanolamine, choline, inositol or serine. Phospholipases are enzymes that hydrolyse phospholipids and they have different names depending on their site of action on the phospholipids. Phosholipase $(PLA)_1$ and PLA_2 hydrolyse ester bounds at *sn*-1 and *sn*-2 position respectively.

Phosholipase A (PLA)

Arachidonic acid is most commonly found on the stereospecific nomenclature (*sn*)-2 position of phospholipids. Release of arachidonic acid from membrane phospholipids is the rate limiting step in the formation of eicosanoids and PLA_2 is the responsible enzyme. Cells and tissues from $cPLA_2$ deficient mice are incapable of producing leukotrienes and prostaglandins in macrophages [24].

There are three main groups of PLA_2, secreted PLA_2 ($sPLA_2$), intracellular group VI calcium independent PLA_2 (GVI $iPLA_2$) and group IV cytosolic PLA_2 (GIV $cPLA_2$, here called $cPLA_2$). All mammalian cells contain several of these enzymes. The $cPLA_2$ family includes $cPLA_2$ α, β, γ, δ, ε and ζ [25]. PLA_2 play diverse functional roles and many of them can participate in the release of arachidonic acid. However $cPLA_2\alpha$ is the only PLA_2 with specificity for arachidonic acid and its primary function is to mediate agonist induced release of arachidonic acid for eicosanoid production. $cPLA_2\alpha$ contains two catalytic domains A and B interspaced by a gene unique sequence. $cPLA_2\alpha$ have a N-terminal C2 domain, which is involved in Ca^{2+}-dependent phospholipid binding [26, 27] and contain several phosphorylation sites (Fig. 3).

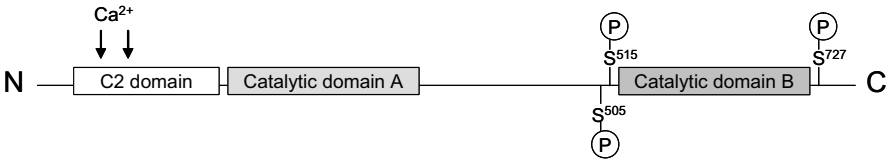

Figure 3. Structure of $cPLA_2$

The production of eicosanoids in response to phagocytosis of zymosan requires formation of arachidonic acid by $cPLA_2\alpha$. But recently it was suggested that another PLA, $sPLA_2$, may also effect eicosanoid generation in macrophages. Peritoneal macrophages from $sPLA_2$ null mice showed a decreased leukotriene and prostaglandin generation in response to zymosan than macrophages from wild type mice, although there were still a significant release of both leucotrineas and prostaglandins [28]. Furthermore is $sPLA_2$ recruited to the phagosomes after stimulation with zymosan, where it colocalize with $cPLA_2\alpha$ [29].

Regulation

In macrophages stimulated with zymosan cPLA$_2\alpha$ translocates to forming phagosomes and to membrane ruffles at the plasma membrane [30]. In other systems, using calcium-mobilizing agonists cPLA$_2$ may translocate to endoplasmic reticulum (ER), Golgi or the perinuclear region [25, 31]. An important step in the regulation of cPLA$_2\alpha$ involves its translocation from cytosol to membrane to access substrate. Normally translocation is induced by increases in intracellular Ca^{2+} concentrations, which bind to the C2 domain [31]. The catalytic domain can also stabilize the binding of cPLA$_2\alpha$ to the membrane in a Ca^{2+}-independent fashion [32]. There have also been reports on cPLA$_2$ activation without detectable changes in intracellular Ca^{2+}concentrations [33, 34].

In most systems activation of cPLA$_2\alpha$ is regulated by phosphorylation in addition to a rise in intracellular Ca^{2+}concentrations, but the process is not clearly understood. The catalytic domain of cPLA$_2\alpha$ contains several important phosphorylation sites, ser505, ser727 and ser515. Phosphorylation on these sites can increase the enzymatic activity of cPLA$_2$ [25, 35]. Depending on cell type and stimulus, cPLA$_2\alpha$ can be phosphorylated by MAP kinases on ser505 [36, 37], by Ca^{2+}/ calmodulin dependent protein kinase II (CamKII) on ser515 [35] or by MAP kinase interacting kinase (Mnk-1) or a closely related isoform on ser727 [38]. Inhibition of Mnk-1 decreases the arachidonate release (paper I). The phosphorylation of the sites may be interactive since Mnk-1 is activated by members of the MAP kinase family [39] and ERK can be activated downstream of calmodulin-dependent protein kinase II (CaMKII) [40].

The mechanism by which phosphorylation of cPLA$_2\alpha$ increases its activity is not fully understood, but perhaps it influences the membrane binding. Supporting this idea is a study demonstrating that phosphorylation on ser505 increases the phospholipide binding affinity at low Ca^{2+} concentration [41]. Other studies have demonstrated that phosphorylation on ser505 and ser727 plays an important role in regulating release of arachidonic acid under transient increases in Ca^{2+}, but seems to be less important under high sustained increase in Ca^{2+} [38]. Phosphorylation can also increase the enzymatic activity of cPLA$_2$.

In addition to calcium and phosphorylation in the regulation of cPLA$_2$ there is evidence that other mechanisms may also play a role. The catalytic domain of cPLA$_2$ can regulate membrane binding through association with phospholipids. PI4,5P binds to cPLA$_2$, facilitating membrane binding and activity and cPLA$_2$ possesses full activity in the absence of Ca^{2+} when PI4,5P or PI3,4,5P were present [42]. Studies also show that PI4,5P bind to cPLA$_2$ and that the specific PI4,5P binding is involved in cellular activation of cPLA$_2$ [43]. It is suggested that binding of polyphosphoinositids (PIPs) may induce conformational changes that optimally position the catalytic domain on the membrane [43]. So even if C2 domain is sufficient for membrane targeting in the presence of high concentrations of Ca^{2+}, the potential ability of specific lipids to bind

to $cPLA_2$ may be the molecular basis for activation while lowering its requirement for Ca^{2+}.

Many questions remain unclear about $cPLA_2$ regulation and PIPs. It is also poorly understood how Ca^{2+}, phosphorylation, and potentially PIPs work in concert to achieve the subcellular targeting and activation. $cPLA_2$ may also interact with proteins, such as vimentin [44], $cPLA_2$-interacting protein (PLIP) [45], p11/calpactin light chain [46], some annexins [47] and caspases [48], which may affect its regulation.

$cPLA_2$ and disease

Eicosanoids and $cPLA_2$ are implied to be involved in various diseases, and $cPLA_2\alpha$, genetically deficient mice have provided evidence for its critical role in regulating physiological processes and various diseases, including allergic responses [24]. Elevated levels of $cPLA_2\alpha$ has been observed in cancers [49] and the downstream PGE2 is known to promote tumor growth [50].

Cytokines

Macrophages produce cytokines, which is a large group of peptides and glycopeptides produced by different types of cells. They are important mediators of immunity, inflammation, growth and differentiation. Cytokines normally mediate their effects on short distances in a paracrine or autocrine fashion. Some cytokines though have a systemic effect. The action of one cytokine is often influenced by other cytokines, so they form a cytokine network, which is rearranged dependent on stimuli. Normally cytokines are secreted at low levels and bind to high affinity receptors. Monocytes/macrophages and T-lymphocytes are the major cytokine producing cells. Members of the cytokine group include interleukins (IL), interferons (IFN), tumor necrosis factor (TNF), colony stimulating factors, growth factors and chemokines.

TNFα

TNFα is a cytokine and member of the TNF superfamily [51], which include TNFα and β, FAS ligands and herpes-virus entry mediator (HVEM) ligands as well as several other proteins.

TNFα is a cytokine, which is synthesized as a membrane bound precursor but is released by protolytic cleavage as a 17kDa soluble molecule. The biologically active form of TNFα is a homotrimer. TNFα is produced by many cell types including macrophages, dendritic cells, T-lymphocytes, B-lymphocytes, NK cells, mast cells, granulocytes and some tumor cells.

TNFβ, also called lymphotoxin, is only produced by some subsets of activated B- and T-lymphocytes. TNFα and β share approximately 30% amino acid homology, and they both bind to the same receptors, inducing similar biological responses. Two

structurally distinctive receptors for TNF is identified, TNFR-I and TNFR-II and these are expressed in all cell types.

TNFα exert diverse biological activities and is associated with disease related to inflammation and autoimmunity. Examples of diseases where TNFα is involved are, septic chock [52], cachexia caused by other diseases [53], insulin tolerance [54], graft-versus host disease [55], rheumatoid arthritis [56], Cohn's disease [57] and several infections including HIV. TNFα is also important in acute and chronic inflammation. The influence on acute inflammation are numerous and include activation of monocytes/macrophages and other cells to produce cytokines and chemokines and stimulation of adhesion molecules on endothelial cells, allowing adhesion of leukocytes

TNFα is the key inflammatory mediator in chronic inflammatory processes. The chronic inflammatory diseases Crohn's disease and rheumatoid arthritis are treated with a combination of anti-TNFα therapy and conventional drugs [58, 59]. There are many cytokines involved in theses diseases but TNFα is a central cytokine able to induce the production of other pro-inflammatory cytokines and chemokines [59]. Anti-TNFα therapy, the treatment with either monoclonal antibodies against TNFα or soluble TNF-receptors [58, 60], has the aim to reduce TNFα activity. These chronic anti-TNFα therapies can have side effects including lymphoma and infections, even if they are very rare [61]. Anti-TNFα therapy was first used in attempts to treat septic shock, which is associated with invasive infections and where TNFα plays an important role [62, 63].

Initially TNFα was considered to be an anti-tumor agent, as it causes necrotic regression of certain forms of cancer. It has since been shown that the cytotoxic properties of TNFα are not restricted to tumor cells, but TNFα also affect normal tissue. Therefore systemic TNFα therapy is proven to be ineffective and with severe side effects. Locally administrated TNFα in combination with vasoactive drugs in isolated limb perfusion has though proven to have an anti-tumor effect [64]. TNFα does not only have anti-tumor properties but also causes progression of cancer, working as tumor promoting factor [65]. TNFα may also function as a link between chronic inflammation and the subsequent development of cancer [65, 66].

SIGNAL TRANSDUCTION

To transfer information from the cell surface to an appropriate cellular response signal transduction within the cell is necessary. This includes receptor binding, transfer of the signal into the cell and involvement of several signaling molecules of which many are kinases. In addition localization of the signal/signaling molecules within the cell is also important for the signal transduction to function properly.

Receptors

Innate immune recognition of invading pathogens is mediated by a set of receptors (pathogen recognition receptors, PRRs) that recognize conserved microbial structures enabling the host to quickly identify a broad range of pathogens. TLRs, dectin-1, Scavenger receptors and Integrins (CR3, CR4) are different types of PRRs. Recognition by the PRRs often leads phagocytosis, followed by destruction and an induction of an inflammatory response, although all PRRs are not phagocytic. Recognition by TLR and by dectin-1 lead to a direct induction of an inflammatory response, whereas many other PRRs can contribute to the response by presentation of microbes to TLR [67].

Dectin-1

Dectin-1 is member of the myeloid-cell-expressed natural killer cell-receptor-like C-type lectin family. Dectin-1 was originally thought to be dendritic cell specific receptor, from where it got its name "dendritic-cell-associated C-type lectin-1" [68]. Now the receptor is known to be expressed in many other cell types, including macrophages, monocytes, neutrophils and a subset of T-lymphocytes [69, 70]. In areas where pathogens enter the body such as intestine [71] and lung [69] dectin-1 is expressed at high levels.

Dectin-1 is a type II transmembrane receptor, containing one lectin-like carbohydrate recognition domain, which recognizes glucans and an immunoreceptor tyrosine activation motif (ITAM) like motif [68] in its cytoplasmic tail, which is involved in cellular activating. Dectin-1 can function as a monomer *in vitro,* and there is no evidence of oligomerization [67].

Dectin-1 recognizes β-1,3- and β-1,6-linked glucans. Whole yeast cells as well as zymosan also bind to the receptor [72, 73]. The form of the fungi may though play a role in receptor recognition as *Candida. albicans* filaments failed to bind to dectin-1, while the yeast forms did [74]. The ability of dectin-1 to recognize β-glucans on yeast indicate that glucan is exposed at the surface (possibly restricted to certain areas) and not as traditionally thought buried under glycosylated mannoproteins. Some bacteria also bind to dectin-1 [75].

ITAM motives are composed of a twice-repeated YxxL sequence flanking seven variable residues. The first repeat in dectin-1 is different from the traditional ITAM, and only the second traditional repeat is necessary for dectin-1 function [67]. Traditional ITAM sequences in other receptors become tyrosine phosphorylated by Src family kinases (SFK) which lead to activation of Syk (or ZAP70) and downstream signaling. SFK is also shown to be involved in dectin-1 signaling [76] (and paper II), but the requirement for Syk is limited and cell type specific. In macrophages Syk seems to be involved in arachidonic acid release [76] and respiratory burst [77].

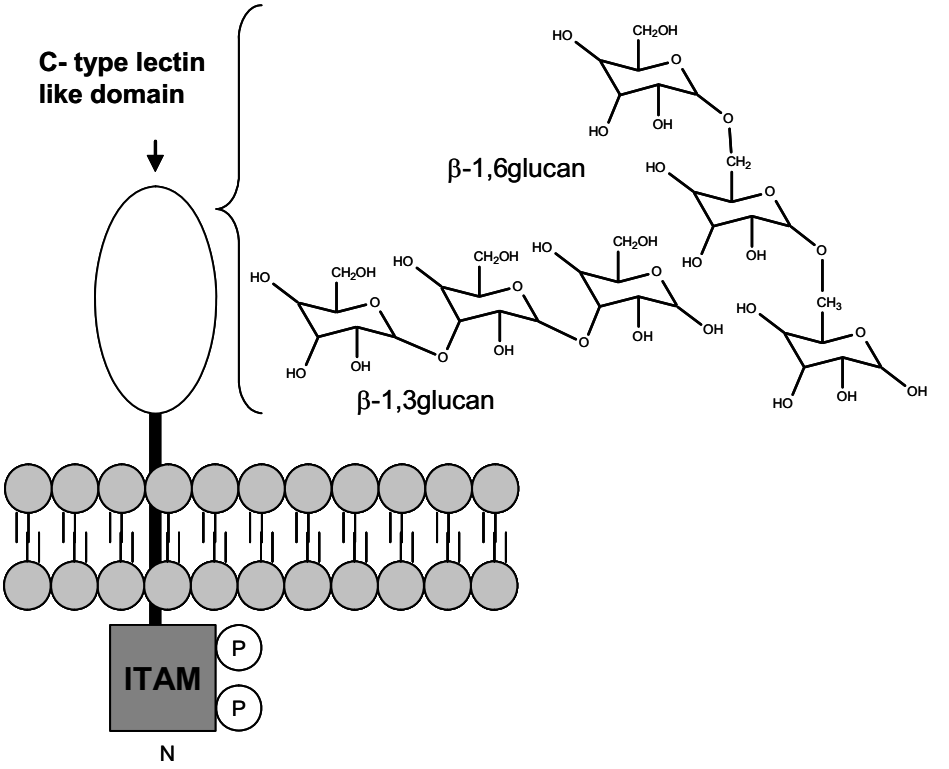

Figure 4. Dectin-1 and its ligands, β-1,3glucan and β-1,6glucan

Dectin-1 induces production of various cytokines [67], arachidonate release [76] (and paper II), respiratory burst and phagocytosis [78]. Induction of certain inflammatory responses, like TNFα production, by dectin-1 also requires a signal from TLR2 and TLR6 [79, 80]. Although TNFα production in the absence of TLR2 signaling is also observed. The germtube of *Aspergillus fumigatus* induce TNFα in the absence of TLR2 signaling [81]. The TLR binding ligand involved is not known since β-glucans do not bind to TLRs, but is possible that other ligands on the yeast particle recognizing

TLRs are involved. It is also possible that TLR activation can prime responses triggered by dectin-1 [79]. Other inflammatory responses like reactive oxygen production are TLR independent [79].

Not much is known about the role of dectin-1 in disease, but there are indications that the immune response triggered via dectin-1 in certain genetic backgrounds can lead to autoimmune disease [82]. Furthermore preliminary evidence indicates that dectin-1 deficient mice are more susceptible to fungal infections [67] than normal mice. Fungal infections are increasing, which is correlated to the increase in AIDS and immunosuppressive medical treatments [67], this makes the β-glucan interaction and signaling in host cells very interesting.

The family of C-type lectins is continuously growing, and several subgroups are found on macrophages. Recently Dectin-2, belonging to the group of "classical" C-type lectins, was found. Dectin-2 recognizes mannose [83].

Toll-like receptors (TLR)

TLR proteins were first found in *Drosophila* where the Toll protein is necessary for resistance to fungal infections [84] and the TLR 18-Wheeler is involved in recognition of Gram-negative bacteria [85]. To date there are eleven known members of the TLR family and they recognize different ligand as presented in table 1.

TLRs are transmembrane proteins containing an extracellular leucine rich repeat domain and a cytoplasmic signaling domain, the Toll/IL-1R (TIR) domain. TLR4 was established as the lipopolysaccharide (LPS) signaling receptor based on genetic evidence from the LPS-insensitive mouse strain, C3H/HeJ, which has a single point mutation in the TIR domain of TLR4 [86]. The ligand binds to the receptor and the signal is transduced across the plasma membrane. The intracellular TIR domain then interacts with an adaptor protein also containing a TIR domain. There are four known different adaptor proteins, myeloid differentiation factor 88 (Myd88), TIR-domain containing adaptor protein (TIRAP), also called MyD88-adaptor like (Mal), TIR domain-containing adaptor inducing interferon-β (TRIF) also called TICAM-1 and TRIF-related adaptor molecule (TRAM) or TICAM-2. MyD88 was the first adaptor found [87] and is most extensively studied one. MyD88 is essential for signaling pathways via all TLRs that lead to the production of inflammatory cytokines [88]. Myd88 recruits IRAK1 and IRAK4, which are activated by phosphorylation, enabling the recruitment and activation of TRAF6. TRAF6 then activates the TAK1 kinase which in turn activates nuclear factor κB (NFκB) via the IKK complex.

Table 1. TLR ligands

TLR1	Lipoprotein from mycobacterial [89]in complex with TLR2
	Synthetic lipoprotein structure PAM3CYSK4 [90] in complex with TLR2
TLR2	Peptidoglucan (PGN) from Gram-positive bacteria [91] in coorporation with TLR6 [80]
	Lipoteichoic acid (LTA) from Gram-positive bacteria [91]
	Zymosan in cooperation with TLR6 [80]
	Lipoarabinomannan from mycobacteria (LAM) [92]
	Whole Gram-positive bacteria [93] in complex with TLR6[80]
	Endogenous Hsp 70 [94]
	Secreted microbial products derived from Group B Steptococcus [95]
TLR3	Double stranded RNA [96]
TLR4	LPS [97]
	Taxol [98]
	Endogenous Hsp 70 [94]
TLR5	Bacterial flagelllin [99]
TLR6	Mycoplasma lipoproteins (MALP) [90]
TLR7	Single stranded RNA [100]
TLR8	Single stranded RNA [100]
TLR9	Unmethylated DNA [101]
TLR10	Unknown
TLR11	Uropathogenic bacteria [102]
	Protozoan-derived profiling like protein [103]
TLR12	Unknown
TLR13	Unknown

Nearly all TLRs recruits MyD88, but only some recruit TIRAP, TRIF and TRAF, giving rise to specificity in signaling. TIRAP is involved in both TLR2 and TLR4 signaling. In TLR4 signaling TIRAP recruits MyD88 to the plasma membrane [104]. TRAM acts exclusively in the TLR4 pathway [105], where it recruits and activates TRIF in a Myd88-independent pathway resulting in the activation of the transcription factor interferon (IFN) regulatory factor 3 (IRF3) and induction of type-I INFs as well as a delayed NFκB response.

Full activation of inflammatory responses by TLR may require assembly of receptor signaling complexes which may include other transmembrane proteins than TLR. Many microbes are recognized by several TLRs as well as by additional innate recognition systems such as the dectin-1. Some TLRs form homo- or heterodimers. TLR2 needs a partner for induction of TNFα production but formation of TLR2 homodimers are not identified [80]. TLR2 and TLR6 heterodimer seem to be necessary for Gram-positive bacteria-, peptidoglycan- (PGN) and zymosan-induced TNFα production [80]. Furthermore TLR2 can associate with TLR1 [89, 90]. TLR2

also use non-TLR partners. TLR2 and dectin-1 cooperate for β-glucan-induced TNFα production [106].

Before finding TLRs CD14 was thought to be the LPS binding receptor. CD14 is found in two forms, a membrane-bound glycosyl phosphatidylinositol (GPI)-anchored protein and soluble form lacking the GPI anchor [107]. CD14 lack an intracellular domain that could transduce the signal. CD14 together with MD2 (extracellular protein essential for LPS signaling via TLR4 [108]) are found to associate with TLR4 and increase the sensitivity to LPS.

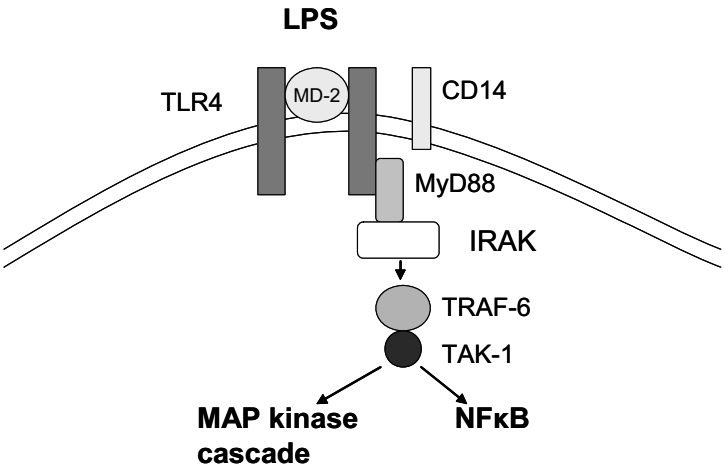

Figure 5. LPS signaling through TLR4.
Schematic overview of the signaling pathway for LPS-induced signaling through TLR4.

S.aureus receptor

Staphylococcu aureus is an opportunistic Gram-positive human pathogen, that can cause a range of diseases [109]. Several receptors may be involved in *S.aureus* signaling.

The cell wall of Gram-positive bacteria contains PGN, lipoteichoic acid (LTA) as well as lipoproteins, all known TLR ligands. Recent data indicate the lipoprotein is the active component in the cell wall of *S.aureus*, signaling via TLR2 [110], although studies have shown that *S.aureus* and PGN signal through TLR2/6 [80, 111]. TLR2 also seem to be essential for PGN recognition in microglia but other receptors are implied to be involved in recognition of whole *S.aureus* [112].

Other receptors that could be involved in *S.aureus*-induced signaling are CD36 and integrin α5β1. CD36 binds LTA and *S.aureus* and brings it to the TLR2 receptor inside the cell. CD36 is implied to play a role in phagocytosis as well as in cytokine induction [113]. Integrinα5β1 by bind to *S.aureus* using fibronectin as a link between integrin and bacteria and may be involved in bacterial adherence and colonization [114].

Tyrosine kinases and phosphatases

The dynamic regulation of protein tyrosine phosphorylation is crucial for a number of cellular processes including cell growth, differentiation, migration, and cell death. It thus represents a powerful control point for integration of environmental signals into cellular responses. Regulation of tyrosine phosphorylation is determined by the balance between protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). In the immune system precise and coordinated regulation of this equilibrium allows for rapid responses to foreign antigens, whereas an imbalance between PTKs and PTPs can have pathologic consequences.

Non-receptor tyrosine kinases

Protein tyrosine kinases can be divided into two families: receptor tyrosine kinases and non-receptor tyrosine kinases. The family of receptor tyrosine kinases are activated by ligand-induced receptor dimerization and tyrosine autophosphorylation, followed by recruitment and activation of signaling molecules that contain Src homology 2 (SH2), SH3, phosphotyrosine binding domains and/or PH domains, resulting in functional responses [115]. The family of non-receptor tyrosine kinases can be divided into eleven subfamilies, including Src and Tec family kinases. They have been shown to be involved in inflammatory cell functions including degranulation, proliferation, differentiation and apoptosis in response to antigens, cytokines, chemokines, and various pro-inflammatory mediators.

Src family kinases

Src was first identified 1977, in cells transformed by Rous sarcoma virus [116]. There are now nine members of the Src family, Src, Lck, Hck, Fyn, Blk, Lyn, Fgr, Yes and Yrk. Src, Fyn, Yrk and Yes are expressed in most tissues [117], while the others are more restricted in the expression. Fgr, Hck, and Lyn are found in myeolid cells, while Blk, Fgr, Lck and Lyn is found in lymphoid cells [117], but both Lck and Lyn have also been found in neurons. Cells are likely to express multiple Src family kinases, which can be found in many different subcellular locations such as, caveolae, focal adhesions, endosomes or nucleus [117].

SFK share a conserved domain structure, consisting of a SH3, SH2 and a kinase domain (SH1). The catalytic domain consists of a N- and a C- terminal lobe. All members of the SFK family also contain a SH4 membrane domain at their N-

terminus, which is myristoylated and/or palmitoylated [118]. Fatty acid acylation on the SH4 domain is involved in targeting SFK to cellular membranes. The SH4 region is followed by a unique domain of 50-70 residues which is divergent among members of the family. They also all contain a short regulatory C-terminal tail with an autoinhibitory phosphorylation site (tyr527 in Src) [118] (Fig. 7). It was in the research about Src that SH domains and the concept of modular protein interactions was born. These and other domains have now been found in many molecules involved in signaling, and are shortly described in the beginning of this thesis.

Activation

The SH2 and SH3 domains play central roles in regulation of SFK catalytic activity. High resolution crystal structures of Src and Hck in their inactive state, have provided an insight in how intramolecular interaction stabilize the inactive conformation of these kinases [119, 120].

The SH2 domain interacts with phosphorylated tyr527 (in Src) and adjacent residues in the negative regulatory tail. Tyr527 is phosphorylated by the cytoplasmic tyrosine kinase Csk and there is studies indicating that loss of tyr527 phosphorylation leads to activation of Src catalytic activity [121]. Phosphorylation of tyr527 may promote an interaction between SH2 and the negative regulatory tail. The SH3 domain on the other hand interacts with sequences in the linker region between the SH2 domain and the catalytic domain. The linker region does not contain proline rich regions but adopts a polyproline II helix conformation in association with the SH3 domain [118]. These interactions result in SH2 and SH3 domain structures that turn inward and make intramolecular interactions that lock the catalytic site in inactive conformation. The SH3 domain packs itself against the N-terminal lobe and the SH2 domain against the C-terminal lobe of the catalytic domain. Autophosphorylation of tyr416 (in Src) may also have a regulatory role. It is not phosphorylated in inactive wild type Src, but is constitutively phosphorylated in activated oncogenic Src mutants.

To conclude it is suggested that there are multiple ways to activate SFK, including; displacement of the intramolecular interaction of the SH2 and SH3, dephosphorylations of tyr527 by a phosphatases or phosphorylation of tyr416. One way the displacement of the intramolecular interaction of the SH2 and SH3 is by ligand binding to the SH3 domain. The adaptor protein SAP can bind to Fyn SH3 domain and this is suggested to disrupt the autoinhibitory interaction between the SH3 domain and the linker region [122] (Fig. 6).

Both the SH2 and SH3 domains are not only involved in intramolecular regulation of SFK but are also involved in intermolecular interactions that regulate Src catalytic activity, Src localization and recruitment of substrates.

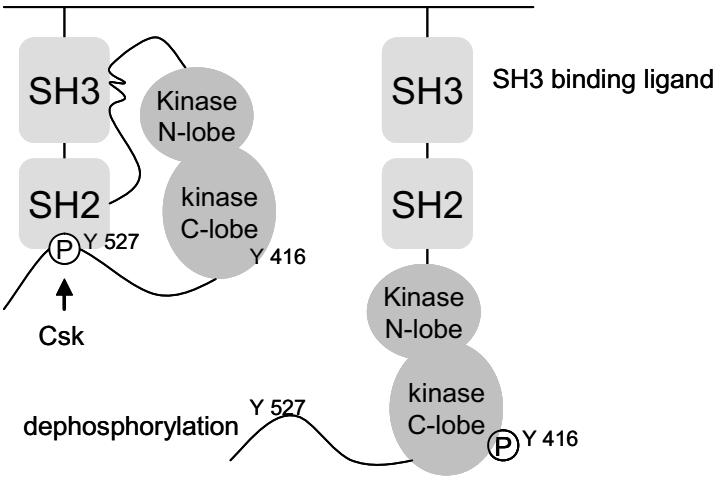

Figure 6. Src activation
Src is in a folded inactive state (left) and in an open active state (right)

Function of SFK
SFK are involved in signaling via immunoreceptors, the most studied receptors being, B-lymphocyte receptors (BCRs), T-lymphocyte receptors (TCRs), receptors for Fc part of Ig (FcεR and FcγR) on macrophages, NK cells and mast cells. In FcR SFK phosphorylate tyrosine residues in ITAMs of the receptor [123]. Even if the knowledge about the role of SFK in other systems is limited, they are implied to be involved in signaling via other macrophage receptors such as dectin-1 (which also contains an ITAM [68]) and TLRs. More than one SFK may be involved in signaling, as the case of FcR signaling in mast cells, where both Lyn and Fyn is involved in different ways [124].

In macrophages SFK are implied to be involve in LPS activation via TLR [125, 126]. LPS treatment of macrophages results in an increase in tyrosine phosphorylation and activation of the SFKs, Lyn, Hck, Fgr and Src [126, 127]. The role of SFK in LPS signaling may be dispensable, since although the level of total cell phosphotyrosine is reduced, bone marrow-derived macrophages from *fgr-, hck- and lyn-* deficient mice have no obvious defects in LPS-induced activation [128].

Inhibitors of SFK
There are several Src family kinase inhibitors with different specificity, the most widely used beeing PP2 [129]. Other inhibitors are Src kinase inhibitor 1 (SKI-1) [130] and SU6656 [131]. As an example of the diverse specificity within the SFK is the specificity for the SFK Lck, with a IC_{50} of approximately 4nM for PP2, 88nM for SKI-1 and 688mM for SU6656.

Tec family kinases

The first Tec family kinse (Tec, tyrosine kinase expressed in hepatocellular carcinoma) was found in 1990 [132]. Now the family comprise five mammalian members, Btk (Bruton's tyrosine kinase), Bmx (also known as Etk), Itk (also known as Emt), Rlk (also known as Txk), and Tec. Tec family kinases have been identified in several other species such as *Drosophila* and zebrafish [133]. The Expression of Tec family kinases is restricted primarily to the hematopoietic system although some have been detected elsewhere [132, 134, 135]. Btk is detected in B-lymphocytes, mast cells, platelets, monocytes/macrophages, neutrophils and dendritic cells.

Characteristic features for Tec family kinases (with the exception of Rlk) is a pleckstrin homology domain (PH domain) located at the N-terminus, followed by a short so-called Tec homology (TH) domain, unique to each family member. PH domains are able to bind phospholipids. TH domains consisting of a Btk homology (BH) motif and one or two proline rich regions have been implicated in the autoregulation of Tec kinases [136]. Furthermore, Tec kinases contain SH2 and SH3 domains. SH2 and SH3 are known to bind to sequences containing phosphorylated tyrosine residues or to interact with proline-rich sequences, respectively [137] (Fig. 7).

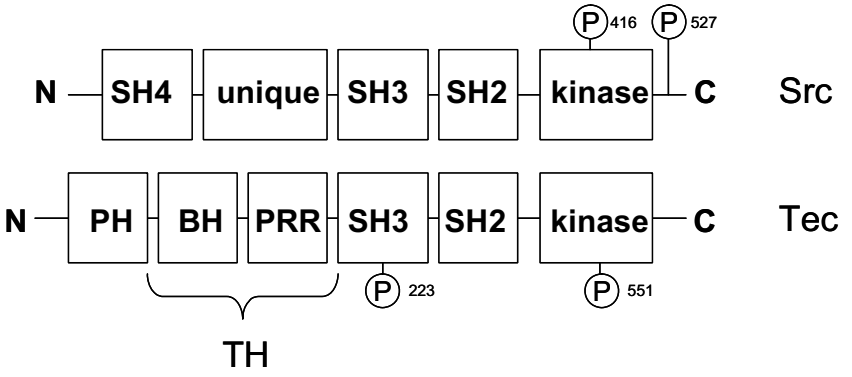

Figure 7. Various domains in Src and Btk

PH; pleckstrin homology, SH; Src homology, BH; Btk homology, TH; Tec homology, PRR; proline rich region, P; phosphorylation sites

Activation

Tec family kinases have been most studied in B-lymphocytes, and studies have shown that activation of Tec kinases upon antigen binding requires two key regulatory steps. For the first step, the Tec kinase has to be localized to the plasma membrane, a process mediated by the interaction of the PH domain with PI3,4,5P

generated by phosphatidylinositol 3-kinase (PI3K) activity [138]. This brings Btk close to other signaling molecules. As a second step of activation tyr551, within the activation loop of the kinase domain, is phosphorylated by SFK, which subsequently, upon autophosphorylation of tyr223 within the SH3 domain result in full activation of the Tec kinase [139, 140]. The tyr223 is located within the substrate binding surface of Btk, this suggests that autophosphorylation at this site may be critical for modulating protein-protein interactions rather than as mechanism to regulate enzymatic activity. Other intermolecular and intramolecular interactions may also be involved the in regulation of Tec family kinases [141].

Function of Tec kinases

Tec family kinase play a role in Ca^{2+} mobilization, regulation of apoptosis, cytoskeleton remodeling, gene expression, proliferation and more specifically in B-lymphocyte development. Mutations in Btk were found to associate with the human disorder X-linked agammaglobulinemia (XLA), an immunodeficiency disorder associated with decreased serum immunoglobulins and impaired B-lymphocyte development. Therefore most studies about Btk is conducted in B-lymphocytes.

The expression of Btk, Bmx and Tec in monocytes/macrophages suggests a functional role. Several studies have implicated a role for Tec family kinases (mostly Btk) in TLR signaling. Immunoprecipitation experiments in the human promonocytic cell line THP1 demonstrated an interaction between Btk and members of the TLR signaling pathway such as Myd88, Mal1 and IRAK4, but not TRAF6 [142]. Btk can also bind to the TIR domain in TLR4, 6, 8 and 9 [142]. Furthermore, the TLR4 ligand LPS induces activation of Btk and Tec in human monocytes [142, 143] and the adaptor Mal is phosphorylated by Btk during TLR2 and TLR4 signaling [144].

But the specific role of Btk in LPS induced signaling leading to induction of TNFα is complex. Human Btk deficient monocytes show reduced LPS-induced TNFα production after 18h stimulation [143]. Macrophages from *xid* macrophages (lacking a functional Btk) show reduced LPS-induced TNFα production after 48h stimulation [145]. Whereas Btk deficient human monocytes stimulated for 4h with LPS showed no alteration in the TNFα production [146]. Neither could we detect any reduction in LPS-stimulated (4h) TNFα production after inhibition of Btk using the Btk inhibitor LFM-A13 in the macrophage-like cell line RAW264.7 or human monocytes (own observation). The differences could depend on the different length of stimulation, longer times resulting in secondary effects.

Btk is also involved in many other inflammatory responses in macrophages such as NO production [147] and arachidonate release (paper I). Tec family kinases are involved in phospholipase γ (PLCγ) activation and Ca^{2+} signaling important in many

signaling events, such as arachidonic acid release in macrophages. But the mode in which Btk works is unclear. In B-lymphocytes Btk activation result in a rise of inositol 1,4,5-trisphosphate (IP_3) and depletion of Ca^{2+} stores [148]. Furthermore Btk can affect the synthesis of PI4,5P, a substrate for both PLCγ2 and PI3K, thereby influencing PLCγ2 activation and Ca^{2+} regulation [149]. Other ways Btk may interact with PLCγ2 is via phosphorylation [150]. Even though Btk deficient B-lymphocytes after stimulation do not shown any alteration of the overall tyrosine phosphorylation of PLCγ2 they have a reduced Ca^{2+} response. In the same study it was demonstrated that the specific phosphorylation of tyr753 and tyr759, but not tyr1197 and tyr1217 in PLCγ2 was Btk-dependent [150].

Inhibitors
To study the role of Btk different inhibitors can be used. LFM-A13 is a potent inhibitor which binds to the catalytic site of Btk [151], while Terrreic acid is more unspecific also inhibiting Emt/Itk [152].

PI3K and AKT

PI3K is involved in a vide array of cellular events such as cytoskeleton rearrangement, migration, differentiation and proliferation. PI3K phosphorylates the inositol phospholipids, phosphatidylinositol (PI), phosphatidylinositol 4-monophosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate (PI4,5P) at the 3'position to generate phosphatidylinositol 3-monophosphate (PI3P), phosphatidylinositol 3,4-bisphosphate (PI3,4P) and phosphatidylinositol 3,4,5-trisphosphate (PI3,4,5P). PI3P is constitutively present and its levels are largely unaltered upon cellular stimulation. PI3,4 P and PI3,4,5P are on the other hand almost absent in resting cells, but their concentrations rise sharply upon stimulation with a variety of ligands.

PI3K is divided into three classes based on substrate specificity, structure and likely mode of regulation [153]. The preferred substrate for class I PI3Ks is PI4,5P, which the other two classes do not have preference for. Class II PI3Ks phosphorylate *in vitro* PI and PI4P, and they are insensitive to the widely used PI3K inhibitor wortmannin. Wortmannin is a cell permeable inhibitor of PI3K class I and III [154]. Class III PI3Ks only accept PI, and is suggested to fulfill a housekeeping role in membrane trafficking and vesicle morphogenesis [155].

Class I PI3Ks can be further subdivided according to their association with either receptor tyrosine kinases (class IA) or G-protein coupled receptor (class IB) signaling pathways. The class IA PI3Ks are heterodimers consisting of one catalytic (p110α, p110β or p110δ) and one regulatory subunit (p85α, p85β, p55γ or their spliced variants). The activity of class 1A PI3K increase after zymosan stimulation [156].

The regulatory unit of class 1A PI3Ks contain two SH2 domains, and the p85 isoforms also have an SH3 domain, proline rich domains and a breakpoint cluster

homology domain. The SH2 domains bind tyrosine phosphorylated motifs on upstream molecules, bringing the catalytic subunit to signaling complexes adjacent to the membranes and its substrate [153]. The SH3 domain can interact *in vitro* with proline rich domains in other proteins and the proline rich regions in the regulatory subunit of PI3K can also associate in vitro with SH3 domains of other proteins including SFK, but not much is known about the *in vivo* roles of SH3, proline rich and breakpoint cluster homology domain of p85 subunit of PI3K. The catalytic subunits of class 1A PI3Ks, contains a C-terminal kinase domain, a N-terminally located p85-binding domain and a binding domain for GTP-bound Ras and a phosphoinositide kinase homology domain with unknown function [153].

Class IB PI3Ks consist of a catalytic subunit (p110γ), which associates to a distinct family of adapter subunits (p101) unrelated to class IA adaptor subunits. Class 1B PI3Ks are activated by binding of G-protein βγ-subunits to specific sites in both adapter and catalytic subunit [157].

The mayor product of class 1A PI3K is PI3,4,5P which functions as a second messenger and can interact with molecules in downstream transduction pathways such as phosphoinositide-dependent kinase-1 (PDK-1), Akt, PLCγ and Btk via their PH domains.

Akt, a downstream target of PI3K

A down stream target of PI3K is Akt originally discovered as an oncogen [158], it was later found to encode a serine/threonine kinase [159]. Other names of Akt are PKB and RAC-PK. There are three isoforms of Akt (Akt1 Akt2 and Akt3) [160]. All are widely expressed and share similar activation mechanisms [160].

Akt is thought to be activated by recruitment to the plasma membrane and subsequent phosphorylation thr308 and ser473 (in Akt1) by the upstream kinases PDK1 and PDK2. The Akt PH-domain mediates binding of Akt to PI3,4,5P, generated by PI3K, resulting in membrane translocation and a conformational change making thr308 accessible for phosphorylation by PDK1 [161]. The kinase responsible for phosphorylation of ser473 is poorly characterized but is termed PDK2 and is also PI3K dependent [161]. High activation of Akt1 is associated with phosphorylation of ser473 [162] and thr308. The phosphorylation of Akt is PI3K dependent. PI3,4,5P binding of both PDK1 and Akt is also likely to promote colocalization thereby facilitating phosphorylation of Akt. Akt is often activated in a PI3K dependent fashion but also PI3K independent activation is also observed.

The SFK can also influence Akt; PI3K can interact with Src through both the SH2 and SH3 domain in Src [117], and Akt is found downstream of Src affecting Akt via PI3K [163].

PLC

Phospholipases can generate second messengers implicated in signal transduction. PLC converts phosphatidylinositol 4,5-bisphosphate (PI4,5P) into the Ca^{2+} mobilizing second messenger, IP_3 and the PKC-activating second messenger, diacylglycerol (DAG). IP_3 is critical for modulation of Ca^{2+} responses within the cell, mainly through binding to the ER-resident ligand-gated IP_3 receptor, IP_3R (Fig. 8).

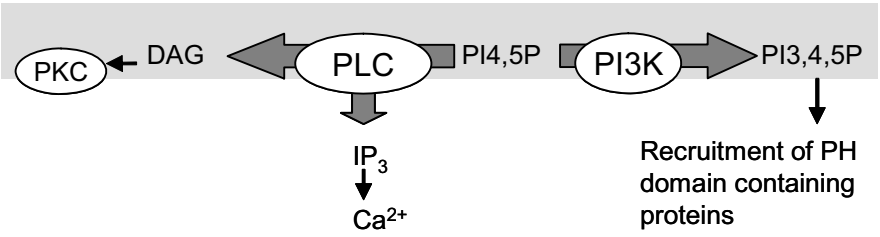

Figure 8. PI3K and PLC signaling is connected.
Phosphatidylinositol 4,5-bisphophate (PI4,5 P) is a substrate for both PLC and PI3K.

The PLC family includes six subgroups (β, γ, δ, ε, ζ and η) consisting of at least 13 different isozymes [164]. All PLC isoforms are subjected to strict regulation and are inactive in quiescent cells. PLCγ is mainly regulated through receptors with intrinsic tyrosine kinase activity or receptors linked to the activation of non-receptor tyrosine kinases. PLCγ1 is widely expressed, but PLCγ2 is primarily restricted to the haematopoietic lineage. PLCγ1 is not detected in peritoneal mouse macrophages [165].

PLCγ contains an N-terminal PH domain, a number of EF hands, a catalytic region divided into two (X and Y) and a C2 domain in the C-terminal. The C2 domain in PLC has not been found to have a role in Ca^{2+} dependent lipid membrane binding. Between the X and Y catalytic regions, a PH domain, split into two, two SH2 domains and a SH3 domain are situated. The EF-hands in PLC are not involved in Ca^{2+} binding, and their function in PLC remains unidentified.

Activation of PLCγ2

To be activated PLC need to be translocated to the plasma membrane. In some systems PI3K indirectly or directly supports translocation, possibly by binding of PI3,4,5P to the PH domain in PLCγ [166]. Translocation of PLCγ2 in macrophages stimulated with zymosan is inhibited by PI3K inhibitors [165]. It has also been suggested that binding of a SH2 domain to an adaptor molecule may lead to translocation [167, 168].

To stimulate PLCγ, phosphorylation on specific tyrosine residues is important [169, 170], but phosphorylation-independent stimulation have also been suggested [171]. PLCγ is phosphorylated at a number of positions. The role of tyrosine phosphorylation in PLCγ activation is still unclear. In some systems phosphorylation of PLCγ is essential for activation. Tyrosine phosphorylation of PLCγ2 is required for B-lymphocyte signaling [169, 170]. Furthermore both zymosan and bacteria induce phosphorylation of PLCγ2 [165] in macrophages.

It is suggested that PLCγ is regulated by intramolecular inhibitory constraints imposed by regions within PLCγ and that phosphorylation of tyrosine residues in this region and/or other interactions with the SH2 and SH3 domains could lead to conformational changes overcoming the intramolecular inhibition. In PLCγ1 tyrosine kinases bind through the most N-terminal SH2 domain, leading to tyrosine phosphorylation, intermolecular interaction of the phosphorylated residues with the other SH2 domain and activation of the phospholipase [172]. In B-lymphocytes tyrosine kinases Lyn, Syk and Btk, PI3K and an adaptor protein BLNK are involved in regulation of PLCγ2 [173], but the involved signaling molecules in zymosan induced PLCγ2 activation in macrophages are much less known. Our studies indicate a role for SFK, but not Btk, in the overall tyrosine phosphorylation of PLCγ2 (paper I).

Mitogen activated protein kinases

Protein phosphorylation is the most common mechanism of protein regulation and protein kinases represent approximately 2% of the proteins encoded by eukaryotic genomes. Mitogen activated protein kinases (MAP kinases) are serine/threonine kinases, activated by various stimuli, leading to phosphorylation of downstream components, including other kinases (MAPK activated kinases MKs), transcription factors and cytosolic proteins. The MAP kinases comprise different groups of kinases, the classical MAP kinase (ERK1 and ERK2, referred to as ERK1/2), p38 isoforms, c-jun N-terminal Kinases (JNKs), ERK3, ERK4, ERK5, ERK7 and ERK8. ERK5, also known as big MAP kinase 1 (BMK1), is twice as big as other MAP kinases. Not much is known about ERK3, ERK4, ERK5, ERK7 and ERK8. The MAP kinase p38 is composed of a group of four enzymes, p38α, β, β2, γ and δ (The p38γ isoform is also known as ERK-6). The p38 isoforms α, β and δ are also named

stress activated protein kinase (SAPK) 2-4. The JNK group consists of JNK1, JNK2 and JNK3, also referred to as SAPK γ, α and-β).

The MAP Kinases are activated by phosphorylation by MAP kinase kinases (MAPKKs), which themselves are phosphorylated by MAP kinase kinase kinases (MAPKKKs). These kinases form parallel yet interwoven signaling cascade(s), called the mitogen activated protein cascade (Fig. 9). Generalizing one can say the ERK1/2 and ERK5 are activated by mitogens and p38 and JNK are primarily activated by cellular stress and inflammatory cytokines. ERK1/2 is involved in proliferation, differentiation, migration survival and other cellular processes. MAP kinase p38 has been associated with stress responses and apoptotic processes but also plays an important role in inflammation, proliferation and differentiation and stabilization of mRNA. JNK are implied in proliferation, differentiation, and apoptosis, while ERK5 is involved in proliferation and differentiation.

ERK1/2 activate transcriptional factors (NF-AT, ELK-1, c-fos), kinases (Mnk, Msk, Rsk) and cytoskeleton proteins. Downstream of p38 transcription factors (ATF1, ATF2, Elk-1, NFκB, Ets-1, MEF2C, SAP-1A) and kinases (Msk, Mnk, MK2,-3 and -5) can be found. JNK phosphorylates transcription factors (c-jun, ATF-2, NF-ATc1, HSF-1 and STAT3). Some cytosolic targets of JNK exist but there is no known MKs as downstream targets. The amino terminal half of ERK5 contains the kinase domain which is similar to that of ERK1/2. ERK5 can induce activation of genes, activate kinases (SGK) and phosphorylate other substrates (MEF2) [174]. ERK1/2, p38 and JNK are all able to activate transcription factors and are recognized mainly for their ability to mediate signaling events regulating transcription, but they have also been implicated in translation [175, 176].

The interwoven signal pathways in the MAP kinase cascade can be illustrated by the MK, Mnk-1, which can be activated by both ERK1/2 and p38 [39] and the necessary inhibition of both p38 and ERK (via MEK1/2) for a nearly complete inhibition of TNFα production [176].

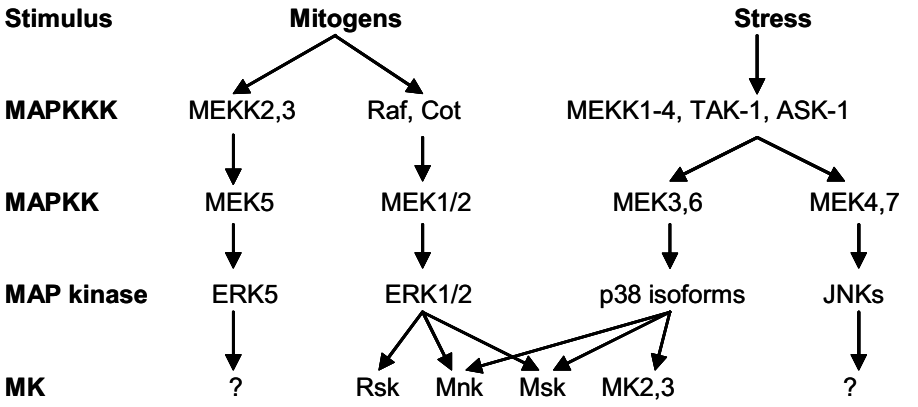

Figure 9. Mitogen activated kinase signaling cascade

MAP kinase activation

For activation of ERK1/2, p38 and JNK dual phosphorylation of both residues in the, thr-X-tyr motif, (X is different between the kinases) by MEKs are required. The activation of MAP kinases may also be affected by scaffolding proteins. Known scaffolds of the ERK cascade are; Kinase suppressor of Ras (KSR) [177], MEK partner1 (MP1) [178], β-arrestin [179], Sef-1 [180], paxilline [181] and Grb-associated binder (Gab) [182]. Gab1 and Gab2 mediates EGF-stimulated ERK activation pathway in breast carcinoma cells through binding and activation of SHP2 [182]. Activation of SHP2 can then lead to conversion of Ras to the GTP form via Ras specific guanine nucleotide exchange factor son of sevenless (Sos). Other scaffolding molecules act in a more direct way [183]. Signal specificity can also be obtained through subcellular localization and binding to substrate and regulators via non-catalytic sites [184, 185]. The MAP kinase pathways are terminated by dephosphorylation of their components by phophatases.

MAP kinases and cPLA$_2$ activation

MAP kinases are involved in arachidonic acid release and in the activation of cPLA$_2$ [186]. cPLA$_2$ is the major enzyme responsible for release of arachidonate and is regulated by phosphorylation and an increase in intracellular Ca^{2+}. Both ERK1/2 and p38 are able phosphorylated cPLA$_2$ [36, 37]. JNK and the MK, Mnk-1 are also implied in the activation of cPLA$_2$ [38] (and paper I).

MAP kinases and TLR signaling
Stimulation of macrophages with whole bacteria, bacterial cell wall components (LPS, PGN and LTA) and yeast cell walls components (zymosan) lead to activation of MAP kinases. All of these stimuli can signal through TLRs and induce TNFα. LPS signals through TLR4 and induces phosphorylation of ERK1/2, p38 and JNK. The transforming growth factor beta-activated kinase-1 (TAK1) is activated by LPS [187]. TAK1 is a member of the MAP kinase cascade upstream of JNK and p38 and is involved in TLR3 and TLR4 mediated NF-κB and AP-1 activation [188]. TAK1 is therefore a signaling component coupling the MAP kinase cascade to the TLR signaling pathway. MAP kinases are also implied in the translational regulation of LPS-induced TNFα production in macrophages [176].

SFK and MAP kinases
Non-receptor tyrosine kinases are involved in many signaling systems and SFK are found to affect MAP kinases. In macrophages activated by bacteria, zymosan or LPS, MAP kinases are found to be downstream of SFK [189] (and paper I). Src is found upstream of MEK-1 in transfected cells [190] and it is suggested that Src influence the Ras-Raf-MEK pathway is executed upstream of Ras [191]. SFK can also influence MAP kinases via the adaptor molecule Gab. On the other hand MAP kinases are also found to have an effect on Gab [192].

Inhibitors of MAP kinases
U0126 is a MEK1/2 inhibitor [193], acting by suppressing the activation, but it is also found to affect the ERK5 pathway [194]. The p38 specific inhibitor SB 203580 [195] is widely used in studies regarding the role of p38. SP600125 is a JNK inhibitor [196], but we observed an inhibition of the phosphorylation in ERK, p38 as well as JNK using it (paper I). Thus in our system the inhibitor may affect several MAP kinases.

SIGNALING LOCALISATION

To obtain specificity in cellular signal transduction the localization of signaling complexes/reactions is of importance. This can be achieved in numerous ways, by localization in different cellular compartments, assembly of membrane domains or by using different scaffolding/adaptor proteins.

Adaptor proteins

Several families of scaffolding adaptors exists, one of them is the Gab family. When scaffold adaptors bind to signal molecules they can assemble multimeric signal complexes. Little is so far known about the function of scaffolding adaptor proteins, but in general they appear to serve as signal amplifiers and to contribute to the specificity of the signal.

Gab2

Gab proteins comprise a distinct group of scaffolding adaptors (Gab1, Gab2, Gab3, Dos and Soc1), well conserved from worms to mammals. Soc1 is expressed in *Caenorhabditis elegans*, Dos in *Drosophila* and Gab1, Gab2 and Gab3 in expressed in mammals. Gab2 is ubiquitously expressed.

Gab is located in the cytosol of unstimulated cells and upon stimulation becomes recruited to the plasma membrane where it is phosphorylated. Gab2 contains an N-terminal PH domain, which may bind to PI3,4,5P on the inner surface of the plasma membrane, facilitating membrane localization. The membrane recruitment of Gab2 was dependent on the PH domain of Gab2 as well as PI3K in FcR-initiated phagocytosis [197].

Gab2 contains tyrosine as well as serine and threonine phosphorylation sites and undergoes phosphorylation in response to diverse stimuli. The kinases responsible for Gab phosphorylation differ but SFK often seems to be involved. The FcR evoked tyrosine phosphorylation of Gab2 is impaired in Lyn deficient cells [197]. Furthermore IL-6-induced tyrosine phosphorylation of Gab2 was SFK-dependent and the SFK Hck was associated with Gab2 in the same study [198]. It is not clear whether SFK directly phosphorylate Gab or if a kinase downstream of SFK mediates the phosphorylation. Gab can also be phosphorylated on serine and threonine residues, which may have a regulatory function [199, 200].

After the N-terminal PH domain Gab2 contains multiple tyrosine- containing motifs and two proline rich domains. Once phosphorylated the tyrosine-containing motifs provide binding sites for SH2 domain-containing signaling molecules including p85 subunit of PI3K, PLC and SHP-2 thus creating a platform for further signaling molecules to join to the complex. The proline rich domains are potential binding sites for SH3 domain-containing molecules such as Grb2 and SFK.

Function of Gab2

Based on knockout experiments the most important role of Gab2 appears to be in mast cells and allergic responses, although Gab2 also may play a part in rheumatoid arthritis [201] and cancer [202]. Very little is known of the potential role of Gab in macrophages and their role in TLR or dectin-1 signaling is not known; although Gab2 could be involved in inflammatory responses in macrophages as it is phosphorylated in response to both zymosan and β-glucan stimulation (paper II). The possibility that Gab2 with multiple potential binding sites might physically integrate several proximal signal components and possibly also may recruit cytosolic enzymes to the plasma membrane is very interesting.

Membrane rafts

The fluid mosaic model of Singer and Nicholson from 1972 [203] implying a random organization of proteins and lipids, where both are free to diffuse in the lipid bilayer, has recently been modified. The idea of lipid domains was suggested about two decades ago when it was recognized that lipids may have heterogeneities in the lateral distribution and be organized in domains [204].

Later Brown and Rose observed that certain fractions of the plasma membrane are resistant to solublization by cold non-ionic detergents, and these fractions were found to be enriched in glycolipids, and therefore named detergent insoluble glycolipid rich complexes (DIGs). These domains are also called detergent resistant membranes (DRM) and glycolipid enriched membrane (GEM). It was shown later that these domains are enriched in sphingolipids and cholesterol. In 1997 Simons and Ikonen formulated the "raft hypothesis" where small membrane domains function as potential signaling platform by selectively excluding and including proteins [205].

Originally lipid rafts were seen as the detergent (1% Triton X- 100) -insoluble material that floats at the interface of a 5%/30% sucrose step gradient after centrifugation but this has recently changed with the development of other detection methods and more studies around the methods used for isolation. At the Keystone symposium on Lipid Rafts and Cell Function 2006 a definition of membrane rafts was formulated; Membrane rafts are small (10-200nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes [206]. Membrane rafts is used instead of the older name lipid raft, to point out that both lipids and proteins contribute to the generation of theses membrane microdomains.

"Membrane rafts are small (10-200nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes"

A special member of the membrane rafts family is caveolae, small plasma membrane invaginations, stabilized by caveolin-1, a cholesterol-binding protein [207, 208]. Caveolae are a morphologically identified domain which (size of approximately 50-100nm diameter [209]) can be demonstrated by electron microscopy. Membrane rafts on the other hand can not be measured directly because they cannot be distinguished from the surrounding membranes; therefore several indirect methods have been used to determine their size. These studies have resulted in variable estimates of the domain size and they are know thought to be 10-200nm [206]. Larger complexes may be the result of coalescence of multiple smaller rafts [206].

It is now widely accepted that plasma membrane in mammalian cells contains heterogeneities in terms of lipid and protein composition and that compartmentalization of signaling molecules probably is of importance for rapid transmembrane signaling. Isolation of rafts with different detergents leads to isolation of rafts of different composition; this could reflex the pre-existing heterogeneity among rafts or be induced by the different detergents. Using several non-detergent methods it has become clear that there is heterogeneity among membrane rafts [210]. The heterogeneity is not limited to three dimensional space but also involve time. They may be relatively stable or transient but are all viewed as dynamic structures. Furthermore there are indications that membrane rafts not only exist in the plasma membrane but also in intracellular membranes such as ER and mitochondria membranes [206]. With improvement of raft isolation methods it might be possible to differentiate between different subclasses of membrane rafts, with different protein and lipid composition.

Function of membrane rafts

The physiological significance of membrane rafts is not yet clear, but one postulated role is in the recruitment and concentration of molecules involved in signaling [211], leading to compartmentalization of this cellular process. Little is known about the lipid and protein composition of rafts in macrophages and what is having an effect on the formation of these domains. Membrane rafts have though been implicated in a range of macrophage functions such as LPS mediated activation [212-214], major histocompatibility complex (MHC) class II antigen presentation [215] and phagocytosis [216].

Disruption of membrane rafts

Cholesterol has an important role in stabilizing membrane rafts by stabilizing sphingolipids, and different ways of changing the cholesterol content of the rafts are used to study the role of rafts. One well used raft-disrupter is methyl-β-cyclodextrin (MβCD). MβCD form complexes with cholesterol and subsequently extracts cholesterol from the membrane, leading to disruption of protein association with membrane rafts. One obstacle with MβCD is that it is able to activate MAP kinases [217] (and paper III). Nystatin a fungal metabolite that binds membrane cholesterol can also be used to disrupt membrane rafts [209]. Other ways of modulating the cholesterol content is by using an agent blocking cholesterol biosythesis such as lovestatin/mevinolin [218].

Isolation/ visualization of membrane rafts

Different techniques can be used to study membrane rafts. The traditional way is to use a detergent resistance analysis. And the most commonly used one is the sucrose density gradient ultracentrifugation method to separate detergent -soluble and insoluble fractions and subsequent Western blotting allows identification of proteins included in detergent resistant membranes (DRM). This method however my cause alteration of the rafts, leaving behind "rafts" that do not resemble the original

membrane domain [210, 219]. There have also more recently been developed microscopic techniques for studying membrane rafts, as well as non-detergent isolation of membrane rafts and separation of raft proteins by immunoaffinity chromatography [210, 220].

PRESENT INVESTIGATION

Background and aim

The overall aim of this thesis was to investigate signaling pathways induced by bacteria and yeast which leads to release of proinflammatory mediators such as TNFα and eicosanoids in macrophages. Dysregulation of proinflammatory mediators is a cause of chronic inflammation and the regulation of their formation is therefore of great interest.

Phosphorylations/dephosphorylations are important in many signaling events and kinases are the enzymes mediating phosphorylation. We focused our interest in this study on the potential role of non-receptor tyrosine kinases such as SFK and the Tec family kinase Btk.

In the present studies both yeast and bacterial stimuli were used. Zymosan, a cell wall preparation from the yeast *Saccharomyces cerevisea*, composed principally of β-glucans, α-mannan and proteins [221], but also pure particulate β-glucan was used. The cell wall of Gram-negative and Gram-positive bacteria differ considerably, including the molecules exposed on their surface. LPS is found in Gram-negative bacteria, and PGN is a component of the cell wall of Gram-positive bacteria. LPS, PGN as well as whole *S.aureus*, a Gram-positive bacteria, were also used to stimulate the macrophages.

Paper I and II

The precursor of eicosanoids is almost exclusively arachidonic acid and the rate limiting enzyme for its formation is $cPLA_2$. $cPLA_2$ is regulated by both phosphorylation and elevated intracellular Ca^{2+} levels, and several of the upstream components are known. SFK can be activated in macrophages, but the role of SFK and Btk in bacteria- and yeast-induced $cPLA_2$ activation and subsequent eicosanoid production is not known.

The specific aims were to:

- Delineate signaling leading to arachidonate release in primary macrophages. The Study was focused on SFK and Btk (I)
- Investigate the role of the kinase Mnk-1 in arachidonate release. (I)
- Investigate whether PI3K is involved in *S.aureus*-induced arachidonate release
- Investigate which zymosan receptor(s) that initiates signaling to arachidonic acid release (II).
- Study the potential role of the adaptor protein Gab2 in yeast- induced activation of macrophages (II).

Paper III and additional information

TNFα is a powerful pro-inflammatory cytokine implied not only in our immune defense but also in several chronic inflammatory diseases, autoimmunity and possibly also in the progression of cancer. The regulation of its formation is therefore of interest. Several microbial constituents including LPS are known to stimulate TNFα production.

The specific aims were:

- To explore the possible role of membrane rafts in LPS-induced signaling leading to TNFα production (III)
- To investigate the potential role of SFK and Btk in the signaling to TNFα production induced by microbial constituents.

Results and discussions

Different roles for non-receptor tyrosine kinases in arachidonate release induced by zymosan and *Staph. aureus* in macrophages (paper I)

In this study primary mouse macrophages were used; they were stimulated with bacteria and yeast (zymosan), to trigger eicosanoid production. The precursor of eicosanoids is arachidonic acid and a key enzyme for its formation is $cPLA_2$, regulated by both phosphorylation and elevated intracellular Ca^{2+} levels. Several molecules, such as MAP kinases and PI3K as well as Ca^{2+} elevation, probably via PLCγ2, are thought to be involved in the signaling pathways increasing $cPLA_2$ activation and the subsequent eicosanoid production [156, 186].

SFK is a large family of non-receptor tyrosine kinases and many members of SFK exist in macrophages, but knowledge about their role in arachidonic acid releases is scarce. A redundancy [222] between members is seen as well as the involvement of more than one SFK in the same pathway [124]. Therefore two broad SFK inhibitors (PP2 and SKI-1) with different specificity [129, 130] were used in this study. Zymosan is a stronger inducer of eicosanoid production (measured as release of arachidonic acid) than bacteria. We found that both SFK inhibitors decreased arachidonate release in a concentration dependent fashion induced by zymosan and *S.aureus*. Furthermore, inhibition of SFK also decresed the activation/ phosphorylation of MAP kinases, Akt and PLCγ2. These findings demonstrate that SFK have an important role in arachidonate release and may be involved in both branches of the pathway leading to activation of $cPLA_2$; involvement of the phosphorylation of $cPLA_2$ via MAP kinases and involvement in Ca^{2+} elevation via PLCγ2.

Btk is a tyrosine kinase involved in B-lymphocyte activation and development, but its potential involvement and role in arachidonic acid release is unknown. Activation of Btk can be induced by its phosphorylation by SFK [139]. SFK phosphorylate Btk at tyr551 , which results in an autophosphorylation on tyr223 [140]. Our findings demonstrate a SFK dependent increase in phosphorylation of tyr223 in Btk after stimulation of macrophages with bacteria and zymosan. Furthermore, inhibition of Btk using a specific Btk inhibitor [151] decreased the arachidonate release, which was accompanied by an partial inhibition of Akt phosphorylation. The Btk-inhibition did not affect the phosphorylation of MAP kinases or PLCγ2. Btk is involved in Ca^{2+} regulation in many cells, but its mode of action in macrophages is unrevealed. Btk can affect Ca^{2+} levels by; phosphorylation of PLCγ2 [148, 223], enhancement of the IP_3 production [148] and by affecting the level of the PLCγ2 and PI3K substrate PI4,5P [149]. Our results demonstrate that Btk is involved in *S.aureus*- and zymosan-induced eicosanoid production. Zymosan stimulation also results in PLCγ2 phosphorylation, but this was not Btk-dependent. Therefore it is likely that Btk has an effect on

eicosanoid production via Ca^{2+} elevation independent of PLCγ2 phosphorylation. Further studies are needed to elucidate the role of Btk in macrophages stimulated by microbial constituents.

Both the MAP kinases ERK and p38 can cause phosphorylation of $cPLA_2$ at ser505, and their downstream substrate Mnk-1 is also implied in this process, by phosphorylation of $cPLA_2$ at ser727 [38]. We could confirm the role of Mnk-1 in zymosan-induced arachidonate release in macrophages using CGP57380, a low molecular weight inhibitor of Mnk-1 [224]. CGP57380 was able to inhibit arachidonate release by approximately 50%.

Additional information
PI3K plays a major role in zymosan-induced arachidonate release probably by involvement of either MAP kinase activation or Ca^{2+} elevation via PLCγ2. Previous studies show that the Gram-negative bacteria *Prevotella intermedia* induce arachidonate release in a PI3K dependent manner [156], but the role of PI3K in arachidonic acid release induced by Gram-positive bacteria is less known.

We show that Gram-positive, *S.aureus*-induced arachidonate release in a PI3K-dependent fashion (Fig.10). However our data indicates that the MAP kinase phosphorylation is PI3K-independent (Fig. 11). This is in contrast to zymosan-induced phosphorylation of ERK and p38, which is PI3K-dependent [156]. And when a Gram-negative bacteria is used, where phosphorylation of ERK but not p38 is PI3K-dependent [156]. Our results indicate that PI3K plays a role in *S.aureus*-induced arachidonate release by exerting an effect on Ca^{2+} levels via Btk or possibly PLCγ2.

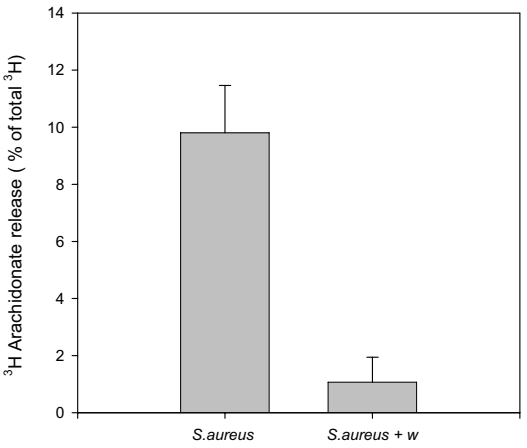

Figure 10. S.aureus-induced arachidonate release is PI3K dependent.
Resident macrophages were incubated overnight with [^{3}H]arachidonic acid and then stimulated with S.aureus for 45min with or without wortmannin. The release of radiolabel arachidonate is expressed as % of total cellular [^{3}H], corrected for the release in control cultures. (w=wortmannin)

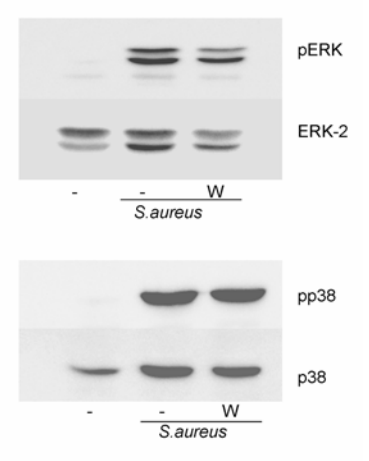

Figure 11. PI3K inhibition does not alter S.aureus- induced MAP kinase activation.
Residential macrophages were stimulated with S.aureus for 20min with or without wortmannin. Cell lysate were prepared and subjected to SDS-PAGE, followed by Western blot analysis using phosphospecific antibodies against ERK and p38. (w=wortmannin)

Differences in the signaling pathways utilized by bacteria and zymosan in macrophages.
Both zymosan and *S.aureus* can signal through TLR2/TLR6 [80] but both stimuli can also bind to other receptors. We have found differences in the signaling pathways induced by zymosan and *S.aureus* leading to arachidonate release. It is therefore likely that the two stimuli signal through different receptors in macrophages. This hypothesis is based on the following observations:

- Phosphorylation of Akt and PLCγ2 is detected after zymosan but not *S.aureus* stimulation.
- Zymosan- but not bacteria-induced ERK activation is PI3K-dependent
- Zymosan- but not bacteria-induced Btk phosphorylation is PI3K-dependent

The macrophage β-glucan receptor mediates arachidonate release induced by zymosan. Essential role for Src family kinases (paper II)

The discrepancies in bacteria- and zymosan-induced signaling (paper I), lead to interest in identification of the zymosan receptor(s) that triggered arachidonate release in peritoneal macrophages.

Zymosan, is composed principally of β-glucans, α-mannan and protein [221]. It may signal through several receptors and among the postulated receptors for non-opsonized zymosan are the mannose receptor, TLR2/TLR6, the β-glucan receptor dectin-1 and the C-type lectin and mannan receptor SIGNR1 [73, 80, 225]. The β-glucan receptor dectin-1 may contribute to phagocytosis [78] as well as the induction of proinflammatory mediators [67] and is therefore a candidate receptor for zymosan-induced arachidonic acid release.

The receptor used by zymosan to induce arachidonic acid release was studied using soluble β-glucan (or the soluble β-glucan laminarin) and mannan to block dectin-1 [70] or mannose receptor respectively. Pretreatment with soluble β-glucan and laminarin but not mannan decreased the arachidonate release induced by zymosan considerably. Furthermore, zymosan-induced activation/phosphorylation of all studied molecules was decreased by inhibition of dectin-1 by soluble β-glucan. Addition of mannan to inhibit the mannose receptor did not affect the activation/phosphorylation of studied molecules. To further convince us that the main receptor for zymosan induced arachidonate release is the β-glucan receptor, dectin-1 we compared the signaling pathways induced by zymosan with that induced by particulate β-glucan, a known dectin-1 specific ligand. Both stimuli induced similar phosphorylation pattern of ERK, PLCγ2, Akt and Btk, all known to be involved in the signaling pathway resulting in arachidonate release.

Zymosan can signal through TLR2/TLR6, as can PGN [80]. Opposite to zymosan, PGN is a very weak eicosanoid inducer. This indicates that TLR2/TLR6 are probably not the major receptors for zymosan-induced arachidonate release.

Adaptor proteins can be used to localize and/or amplify signals in cells. Gab2 is such an adaptor protein. Its role in dectin-1 signaling is unknown but our present results indicate that it is involved in dectin-1 mediated signaling. Gab2 contains tyrosine as well as serine and threonine phosphorylation sites and undergoes phosphorylation in response to diverse stimuli, in some systems via SFK [198, 226]. β-glucan and zymosan induce phosphorylation of Gab2 in a SFK-, but not Btk-dependent manner, demonstrating the involvement of Gab2 in dectin-1 signaling in macrophages.

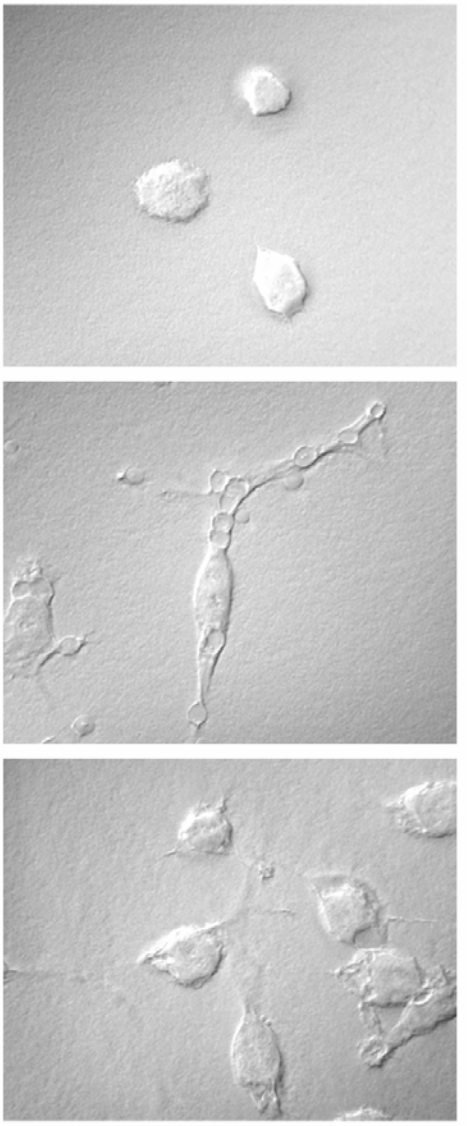

Figure 12. Soluble β-glucan inhibits the uptake of particulate β-glucan.
RAW264.7 cells stably transfected with dectin-1 cultured over night and thereafter left unstimulated (top) or stimulated with particulate β-glucan (middle). Addition of soluble β-glucan inhibits spreading of macrophages as well as phagocytosis of particulate β-glucan (bottom). Images were recorded on an inverted microscope at×100 magnification.

Additional information
Particulate β-glucan activates macrophages resulting in spreading and phagocytosis. Soluble β-glucan is used as an inhibitor of dectin-1. In fig. 12 we shown that soluble β-glucan inhibit spreading of macrophages as well as phagocytosis of particulate β-glucan in RAW264.7 cells stably transfected with dectin-1 [106]. This confirms that soluble β-glucan is a good inhibitor of dectin-1.

Akt is known to be phosphorylated in macrophages after zymosan and particulate β-glucan stimulation [156] (and paper II), and this is inhibited by addition of soluble β-glucan but not mannan (paper II). To verify that this response is mediated via dectin-1, we used RAW264.7 cell stably transfected with dectin-1. In dectin-1 transfected cells Akt is phosphorylated by both zymosan and particulate β-glucan, this could not be detected in untransfected cells (Neo) (Fig. 13). Furthermore we found that particulate β-glucan-induced phosphorylation of Akt is PI3K- as well as SFK-dependent in dectin-1 transfected cells. A finding which confirm that Akt is activated via dectin-1 and is situated downstream of SFK and PI3K.

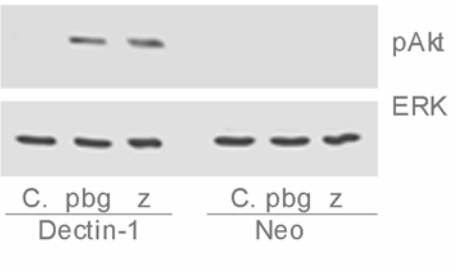

Figure 13. *Akt phosphorylation in RAW264.7 cells stably transfected with dectin-1.*
RAW264.7 cells stably transfected with dectin-1 (Dectin-1) or untransfected cells (Neo) were stimulated 30min with particulate β-glucan (pbg) or zymosan (z). Cell lysate were prepared and subjected to SDS-PAGE, followed by Western blot analysis using a phosphospecific antibody against Akt(ser473). Membrane was reprobed with an antibody against ERK to verify equal loading.

To summarize the signaling pathway leading to arachidonate release in macrophages via dectin-1 it is likely that SFK are positioned proximally, affecting the downstream targets (MAP kinases, Akt, PLC and Btk) in a direct or indirect fashion. PI3K also plays a central role, with the ability to influence MAP kinases, PLCγ2, Akt and Btk activation. PLCγ2 also has the ability to regulate both Ca^{2+} levels as well as the phosphorylation of $cPLA_2$, and is in turn probably influenced by Btk. This results in a somewhat complex signaling network which is illustrated in fig. 14. The non-receptor tyrosine kinase syk is also implied to be involved in this pathway [76, 77], but is not required for phagocytosis in macrophages [78]. In other ITAM containing receptors SFK is found upstream of Syk [227]. The possible position and role of Syk

in the signaling network induced by dectin-1 is far from clear. Gab2 may function as a scaffold, bringing the signaling molecules together to increase the transduction efficiency.

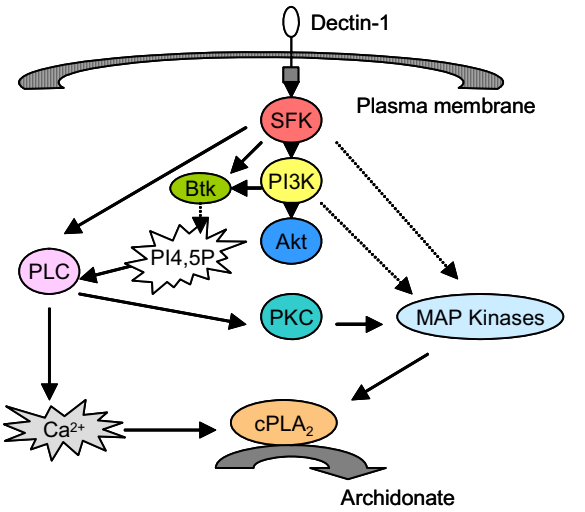

Figure 14. Dectin-1 signaling leading to arachidonate release.
Proposed signaling pathway for particulate β-glucan-induced arachidonic acid via dectin-1.

The role of lipid rafts in LPS-induced signaling in a macrophage cell line (paper III)

Not only the specific signaling molecules but also their localization within the cell is of importance to obtain a response. One way of organizing signaling molecules and events within the cells is through membrane rafts. Membrane rafts are highly dynamic cholesterol and sphingolipid-enriched domains involved in the 2-dimensional compartmentalization of molecules within membranes. The murine macrophage cell line RAW264.7 was used to obtain sufficient amount of cells needed for isolation of lipid rafts using sucrose gradient centrifugation. LPS activates macrophages by binding to TLR4, leading to production of cytokines (e.g TNFα). LPS can also bind to CD14, GPI-linked protein, lacking an intracellular domain and is therefore unable to transducer signals. Instead it works as a co-receptor to TLR [228]. MAP kinases are found downstream of TLR, and are important in TNFα production [176] in macrophages. We detected CD14, and the MAP kinases ERK and p38 in detergent-insensitive domains positively stained with the membrane raft marker, flotillin-1, after LPS treatment. Other molecules involved in TLR signaling were not detected in the "lipid raft fractions". A redistribution of CD14 in membrane rafts is also seen in macrophages by Dai et al. [229] confirming that CD14 can changes its localization upon LPS stimulation. Other results point out that GPI-anchored proteins may be constrictively resident in membrane rafts [214, 230].

To further investigate the role of membrane rafts in LPS-induced TNFα production we tried to disrupt the membrane raft and thereby affect the signaling interaction within them. Cells were preincubated with different disruptors of membrane rafts such as nystatin, MβCD, or lovestatin, before stimulation with LPS. The treatments did not alter the distribution of flotillin-1. Furthermore the disruptors did not inhibit TNFα production, which would be expected if membrane rafts play a significant role in TNFα production. Instead nystatin surprisingly induced TNFα production, as well as ERK and MEK-1 phosphorylation by itself.

To conclude not much is known about the role of specific membrane domains in TLR signaling, but our and other studies [214] indicate that localization of molecules in specific membrane domains can be of importance.

Non-receptor tyrosine kinase involvement in TNFα production in macrophages (additional information)

TNFα is an important pro-inflammatory mediator, induced in macrophages by different microbial stimuli. Yeast (zymosan) as well as whole bacteria and other microbial constitutes can signal through different TLRs (table 1). In this study we used zymosan, the Gram-positive bacteria *S.aureus*, LPS and PGN all known to be able to signal through TLRs to induce TNFα production. It is known that tyrosine kinase inhibitors are potent suppressors of LPS-induced TNFα production [126] and that activation of tyrosine kinases is detected after LPS stimulation in monocytic cells [126, 231]. However, the specific roles of the tyrosine kinases in LPS-induced TNFα are not elucidated. SFK such as Hck, Lyn and Fgr are activated by LPS [126], but macrophages from mice deficient in these kinases do not show any impairment in LPS-induced TNFα production; neither is MAP kinase nor NFκB activation affected [128]. Using the macrophage like cell line RAW 264.7, we observed that inhibition of SFK, using the inhibitor PP2, reduced TNFα production induced by different microbial constituents, including whole *S.aureus* (Fig.15). At mRNA level the effect of the SFK inhibitors was smaller (Fig. 16).

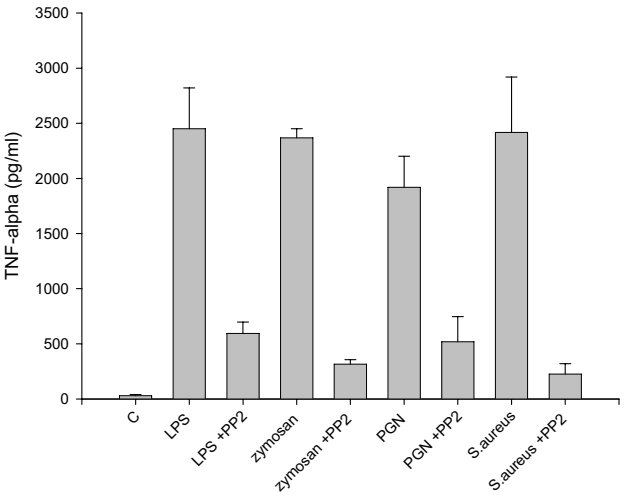

Figure 15. Inhibition of SFK inhibit TNFα production induced by microbial constituents.

RAW264.7 cells were left untreated or pre-treated with PP2 (5μM)for 15 min before addition of microbial constituents. After 4 hours the culture media was collected and analyzed for TNFa using a commercial ELISA kit. (n=3)

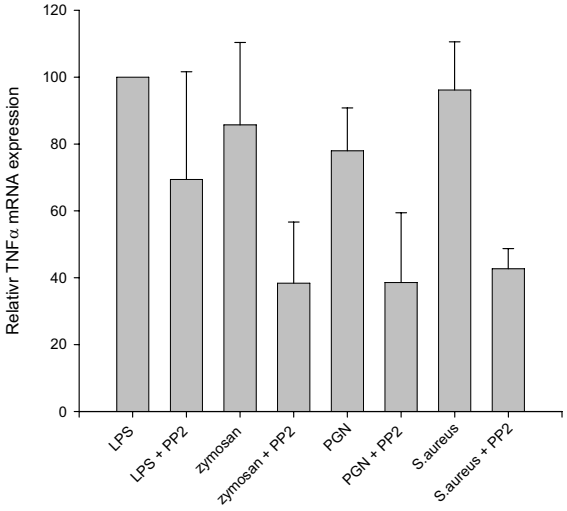

Figure 16. Northern blot analysis of TNFα mRNA induced by microbial constituents.

RAW264.7 cells were pretreated with PP2(5μM) for 15 min before stimulation with microbial constituents for 90 min. Total RNA was extracted and subjected to electrophoresis and transferred to nylon membrane. TNFa mRNA levels were normalized to those fore β-actin and the LPS-induced level of TNFa mRNA is set to 100. (n=3)

MAP kinases are involved in TNFα production via TLR [176] and we have previously found that inhibition of SFK affect MAP kinases (paper I) in primary macrophages. Therefore we investigated the result of SFK inhibition on ERK and p38 phosphorylation. The SFK inhibitor SKI-1 did not alter the ERK phosphorylation induced by microbial constituent, while p38 phosphorylation induced by zymosan, but not by the other stimuli, was somewhat inhibited. p38 but not ERK (Fig. 17) phosphorylation was decreased by PP2 treatment. Inhibition of both ERK and p38 is needed for effective inhibition of LPS-induced TNFα production in primary macrophages [176].

eIF4E, situated downstream of ERK and p38, is a key protein in translational regulation and a correlation of its phosphorylation state and protein synthesis has been detected in some systems [176]. We found that the phosphorylation state of eIF4E was not affected by SFK inhibition and phosphorylation of this molecule could also be found in resting cells.

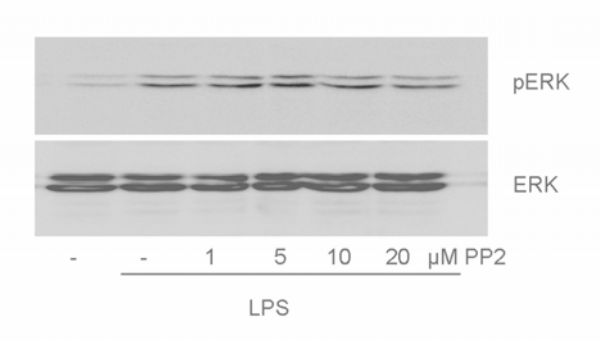

Figure 17. Inhibition of SFK do not affect ERK phosphorylation.

Different concentrations of PP2 was added 15 min prior to LPS (10ng/ml) fore 20 min. Cell lysate were prepared and subjected to SDS-PAGE, followed by Western blot analysis using phosphospecific antibodies against ERK.

Activation of Btk can be induced through phosphorylation by SFK, and Btk is implied to play a role in TLR signaling [142, 143, 232], but there are controversy concerning its role. We were unable to detect a Btk-dependence in microbial constituent-induced TNFα production using a specific Btk inhibitor, LFM-A13.

To conclude; SFK play a role in TNFα production via TLR, but the level of regulation is still unclear even if some members of the pathway are ruled out as potential targets of SFK.

Concluding remarks

Gram-positive bacteria (*S.aureus*) and yeast (zymosan) both induce release of arachidonic acid, the precursor of eicosanoids, in a SFK- and Btk-dependent fashion. However they activate different signaling molecules, and are therefore implied to signal via different receptors. Both zymosan and particulate β-glucan were fund to signal through the β-glucan receptor dectin-1. They activate several signaling molecules such as SFK and Btk as well as PI3K, PLCγ2, Akt and MAP kinases. SFK act proximally while Btk is found downstream of SFK. SFK, in contrast to Btk, also channels signaling via other signaling molecules mentioned and are implied in TNFα production. Tyrosine kinases such as SFK therefore seem to be central in bacteria- and yeast-induced responses in macrophages. Not only the specific signaling molecules and their activation are of importance in creating a precise signaling response but also the localization of the signaling components within the cell. Dectin-1 mediated signaling have an effect on the adaptor protein Gab2, implying that it is involved downstream of the receptor. The localization of signaling molecules was further studied in membrane rafts in LPS-induced signaling via TLR4.

POPULÄRVETENSKAPLIG SAMMANFATTNING

(swedish summery)

Vårt immunförsvar är nödvändigt skydd mot de patogener (bakterier, virus, jäst och parasiter) som finns runtomkring oss. Patogener som kommer in i kroppen och infekterar den, gör att en inflammation bildas. En inflammation är kroppens svar på en infektion (eller skada). Inflammation är en process som gör att immunförsvarets celler ökar sin förmåga att oskadliggöra inkräktarna och att immunförsvarets celler kommer till de delar av kroppen där patogenerna eller skadan är. Inflammationen kan dock bli kronisk och då skada kroppen, det kan man se i flera olika sjukdomar. Det är därför viktigt att dess reglering fungerar.

Makrofager är en viktig typ av celler i den del i immunförsvaret som snabbt men ospecifikt reagerar på en infektion. När makrofagen möter inkräktaren ”äter” den upp den (fagocyterar) och börjar avge olika molekyler som är med och skapar en inflammation och oskadliggör patogenen. Makrofager avger också andra ämnen som motverkar inflammation. I projektet har vi studerat molekyler i makrofager som är involverade i att förmedla signaler. De signalkedjor som studerats startar när en patogen (eller del av patogen) binder till makrofagens yta. Signalkedjorna leder sedan till produktion av olika molekyler (så som eikosanoider och TNFα) involverade i inflammation.

För att makrofagen ska känna igen olika patogener har cellen mottagare (receptorer) på sin yta som binder patogenen till sig och för in signalen i cellen. Kunskapen om vad de olika mottagarna binder till är ännu till stora delar känt. Våra resultat visar att en jästväggsdel, zymosan, binder till en receptor som heter dectin-1, och därmed startar en signal som resulterar i frigörandet av arakidonsyra (delarbete II). Frigörandet av arakidonsyra är första steget vid produktion av en grupp inflammatoriska molekyler som kallas eikosanoider. Gruppen eikosanoider består bl.a. av prostaglandiner och leukotriner. Prostaglandiner har både pro- och anti-inflammatoriska egenskaper och dess produktion kan hindras av både aspirin- och ibuprofenpreparat.

Nedströms receptorn i signalkedjan behövs flera olika molekyler för att förmedla signalerna vidare på ett specifikt sätt så att cellen ger ”rätt” svar. Ett vanligt sätt att förmedla signaler i celler är att sätta dit eller ta bort fosfatgrupper på molekyler involverade i signaleringen. Detta görs av speciella molekyler som kallas kinaser (sätter dit fosfatgrupper) och fosfataser (tar bort fosfatgrupper). En grupp kinaser som sätter dit fosfatgrupper på särskilda tyrosin aminosyror i proteiner, kallas tyrosinkinaser. Det finns flera olika grupper av dessa kinaser och vi har studerat Src kinas familjen och en medlem ur Tec kinas familjen, Btk.

I arbete I visas att Src kinaser förmedlar signalen startad av jästväggsdelen zymosan vidare in i cellen och att Src finns nära mottagaren/receptorn tidigt i signalkedjan. Genom att befinna sig tidigt i signalkedjan påverkar den flera andra signalförmedlare. Btk är en sådan förmedlare som påverkas av Src. Btk påverkar i sin tur flera andra signaleringsprocesser i cellen, vilket resulterar i frigörandet arakidonsyra.

Det är inte bara de enskilda molekylerna som är viktiga i en signalväg, utan även deras lokalisation och möjligheter till kontakt med varandra. Det finns särskilda molekyler som inte själva förmedlar signaler utan sammanför andra förmedlare till komplex, där de lättare kan ”prata” med varandra. En sådan molekyl är Gab. Gab påverkas i makrofager när den kommer i kontakt med jästväggsdelar (delarbete II). Detta tyder på att de är involverade på något sätt i den signalväg vi studerat.

Det finns även andra sätt på vilket signaleringsförmedlare kan samlas för att sprida signalen vidare. Detta kan ske i det fettlager som omger cellen (plasmamembranet). Plasmamembranet fungerar som cellens yttre gräns och håller ihop den. Där finns specifika väldigt små områden som kallas ”membran flottar” i vilka signaleringsmolekyler kan samlas och föra information vidare.

En bakterie del som kallas LPS, binder till en mottagare/receptor (TLR) på makrofager och startar en signalkedja som resulterar i produktion av den inflammatoriska molekylen TNFα. Vi har även visat att vissa signalförmedlare (men inte andra) involverade i den signalvägen kan hittas i ”membran flottar” (delarbete III).

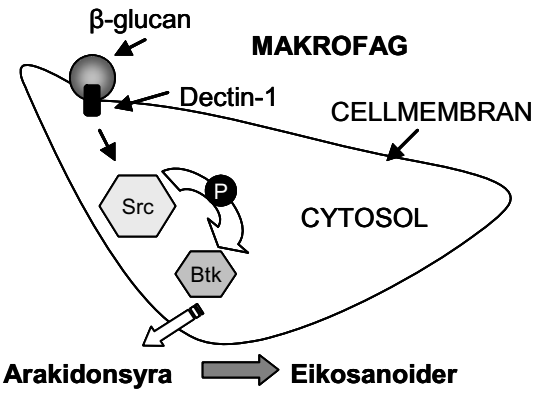

Figure 18. Dectin-1 signalering.
En mycket förenklad bild av hur β-glucan binder till mottagaren dectin-1 och förmedlar signalen vidare via Src kinaser och Btk så att den resulterar i frisättandet av arakidonsyra.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to colleagues and friends, who supported me during these years at the Faculty of Medicine in Lund.

I would like to specially thank;

My supervisor professors *Roger Sundler*, for scientific discussions and for letting me work independently in the lab.

Professor *Anna Hultgårdh*, my co-supervisor, for supporting me during the writing of the thesis. I look forward to be a part of your lab next year.

Special thanks to my lab partner *Karolina Andersson* how thought me a lot about macrophages and lab methods and with whom I have had numerous discussions about science and other matters.

To former members of the research group *Cleas Nauclér* and *Pia Lundqvist*, who both contributed to a nice atmosphere in the lab. Thank you Pia for technical help. *Katarina Bruzelius Salomonsson* for good company in the lab.

A collective thanks to all past and present lab neighbors at BMC B11 and B12 for nice coffee and lunch times with discussion concerning important and less important issues. Special thanks to *Karin Berger* for nice lunch companionship.

Ingegerd Persson for good administrative support, like helping me keeping track of traveling grants and expenses.

Thank you, *Anders Olin* and *Jenny Westin*, for being good partners at LFN.

Nisse, thank you for your love, support and understanding during these past years. Your proof reading skills has always been very helpful. Last but not least, thank you *Melker* for all your hugs and smiles.

REFERENCES

1. Liang, X., et al., *The N-terminal SH4 region of the Src family kinase Fyn is modified by methylation and heterogeneous fatty acylation: role in membrane targeting, cell adhesion, and spreading.* J Biol Chem, 2004. **279**(9): p. 8133-9.

2. Rizo, J. and T.C. Sudhof, *C2-domains, structure and function of a universal Ca2+-binding domain.* J Biol Chem, 1998. **273**(26): p. 15879-82.

3. Underhill, D.M. and A. Ozinsky, *Phagocytosis of microbes: complexity in action.* Annu Rev Immunol, 2002. **20**: p. 825-52.

4. Dandona, P., A. Aljada, and A. Bandyopadhyay, *Inflammation: the link between insulin resistance, obesity and diabetes.* Trends Immunol, 2004. **25**(1): p. 4-7.

5. Laberge, M.A., K.J. Moore, and M.W. Freeman, *Atherosclerosis and innate immune signaling.* Ann Med, 2005. **37**(2): p. 130-40.

6. McGeer, E.G. and P.L. McGeer, *Innate immunity in Alzheimer's disease: a model for local inflammatory reactions.* Mol Interv, 2001. **1**(1): p. 22-9.

7. McGeer, P.L. and E.G. McGeer, *Inflammation and neurodegeneration in Parkinson's disease.* Parkinsonism Relat Disord, 2004. **10 Suppl 1**: p. S3-7.

8. Bos, J.D., et al., *Psoriasis: dysregulation of innate immunity.* Br J Dermatol, 2005. **152**(6): p. 1098-107.

9. Goronzy, J.J. and C.M. Weyand, *Rheumatoid arthritis.* Immunol Rev, 2005. **204**: p. 55-73.

10. Moss, S.F. and M.J. Blaser, *Mechanisms of disease: Inflammation and the origins of cancer.* Nat Clin Pract Oncol, 2005. **2**(2): p. 90-7; quiz 1 p following 113.

11. Lawrence, T., D.A. Willoughby, and D.W. Gilroy, *Anti-inflammatory lipid mediators and insights into the resolution of inflammation.* Nat Rev Immunol, 2002. **2**(10): p. 787-95.

12. Fadok, V.A., et al., *Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF.* J Clin Invest, 1998. **101**(4): p. 890-8.

13. Johnston, R.B., Jr., *Current concepts: immunology. Monocytes and macrophages.* N Engl J Med, 1988. **318**(12): p. 747-52.

14. Nathan, C., *Mechanisms and modulation of macrophage activation.* Behring Inst Mitt, 1991(88): p. 200-7.

15. Stein, M., et al., *Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation.* J Exp Med, 1992. **176**(1): p. 287-92.

16. Anderson, C.F. and D.M. Mosser, *A novel phenotype for an activated macrophage: the type 2 activated macrophage.* J Leukoc Biol, 2002. **72**(1): p. 101-6.

17. Bogdan, C., Y. Vodovotz, and C. Nathan, *Macrophage deactivation by interleukin 10.* J Exp Med, 1991. **174**(6): p. 1549-55.

18. Mosser, D.M., *The many faces of macrophage activation.* J Leukoc Biol, 2003. **73**(2): p. 209-12.

19. Porcheray, F., et al., *Macrophage activation switching: an asset for the resolution of inflammation.* Clin Exp Immunol, 2005. **142**(3): p. 481-9.

20. Mege, J.L., et al., *The two faces of interleukin 10 in human infectious diseases.* Lancet Infect Dis, 2006. **6**(9): p. 557-69.

21. Humes, J.L., et al., *Pharmacological effects of non-steroidal antiinflammatory agents on prostaglandin and leukotriene synthesis in mouse peritoneal macrophages.* Biochem Pharmacol, 1983. **32**(15): p. 2319-22.

22. Calder, P.C., *Polyunsaturated fatty acids and inflammation.* Prostaglandins Leukot Essent Fatty Acids, 2006. **75**(3): p. 197-202.

23. Rao, T.S., et al., *In vivo characterization of zymosan-induced mouse peritoneal inflammation.* J Pharmacol Exp Ther, 1994. **269**(3): p. 917-25.

24. Uozumi, N., et al., *Role of cytosolic phospholipase A2 in allergic response and parturition.* Nature, 1997. **390**(6660): p. 618-22.

25. Ghosh, M., et al., *Properties of the Group IV phospholipase A(2) family.* Prog Lipid Res, 2006. **45**(6): p. 487-510.

26. Clark, J.D., et al., *A novel arachidonic acid-selective cytosolic PLA2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP.* Cell, 1991. **65**(6): p. 1043-51.

27. Nalefski, E.A., et al., *Delineation of two functionally distinct domains of cytosolic phospholipase A2, a regulatory Ca(2+)-dependent lipid-binding domain and a Ca(2+)-independent catalytic domain.* J Biol Chem, 1994. **269**(27): p. 18239-49.

28. Satake, Y., et al., *Role of group V phospholipase A2 in zymosan-induced eicosanoid generation and vascular permeability revealed by targeted gene disruption.* J Biol Chem, 2004. **279**(16): p. 16488-94.

29. Balestrieri, B., et al., *Group V secretory phospholipase A2 translocates to the phagosome after zymosan stimulation of mouse peritoneal macrophages and regulates phagocytosis.* J Biol Chem, 2006. **281**(10): p. 6691-8.

30. Girotti, M., et al., *Cytosolic phospholipase A2 translocates to forming phagosomes during phagocytosis of zymosan in macrophages.* J Biol Chem, 2004. **279**(18): p. 19113-21.

31. Evans, J.H., et al., *Intracellular calcium signals regulating cytosolic phospholipase A2 translocation to internal membranes.* J Biol Chem, 2001. **276**(32): p. 30150-60.

32. Evans, J.H. and C.C. Leslie, *The cytosolic phospholipase A2 catalytic domain modulates association and residence time at Golgi membranes.* J Biol Chem, 2004. **279**(7): p. 6005-16.

33. Wijkander, J. and R. Sundler, *Macrophage arachidonate-mobilizing phospholipase A2: role of Ca2+ for membrane binding but not for catalytic activity.* Biochem Biophys Res Commun, 1992. **184**(1): p. 118-24.

34. Balsinde, J., et al., *Cellular regulation of cytosolic group IV phospholipase A2 by phosphatidylinositol bisphosphate levels.* J Immunol, 2000. **164**(10): p. 5398-402.

35. Muthalif, M.M., et al., *Functional interaction of calcium-/calmodulin-dependent protein kinase II and cytosolic phospholipase A(2).* J Biol Chem, 2001. **276**(43): p. 39653-60.

36. Lin, L.L., et al., *cPLA2 is phosphorylated and activated by MAP kinase.* Cell, 1993. **72**(2): p. 269-78.

37. Borsch-Haubold, A.G., et al., *Phosphorylation of cytosolic phospholipase A2 in platelets is mediated by multiple stress-activated protein kinase pathways.* Eur J Biochem, 1999. **265**(1): p. 195-203.

38. Hefner, Y., et al., *Serine 727 phosphorylation and activation of cytosolic phospholipase A2 by MNK1-related protein kinases.* J Biol Chem, 2000. **275**(48): p. 37542-51.

39. Waskiewicz, A.J., et al., *Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2.* Embo J, 1997. **16**(8): p. 1909-20.

40. Illario, M., et al., *Calcium/calmodulin-dependent protein kinase II binds to Raf-1 and modulates integrin-stimulated ERK activation.* J Biol Chem, 2003. **278**(46): p. 45101-8.

41. Das, S., et al., *Mechanism of group IVA cytosolic phospholipase A(2) activation by phosphorylation.* J Biol Chem, 2003. **278**(42): p. 41431-42.

42. Six, D.A. and E.A. Dennis, *Essential Ca(2+)-independent role of the group IVA cytosolic phospholipase A(2) C2 domain for interfacial activity.* J Biol Chem, 2003. **278**(26): p. 23842-50.

43. Das, S. and W. Cho, *Roles of catalytic domain residues in interfacial binding and activation of group IV cytosolic phospholipase A2.* J Biol Chem, 2002. **277**(26): p. 23838-46.

44. Nakatani, Y., et al., *Identification of a cellular protein that functionally interacts with the C2 domain of cytosolic phospholipase A(2)alpha.* J Biol Chem, 2000. **275**(2): p. 1161-8.

45. Sheridan, A.M., et al., *PLIP, a novel splice variant of Tip60, interacts with group IV cytosolic phospholipase A(2), induces apoptosis, and potentiates prostaglandin production.* Mol Cell Biol, 2001. **21**(14): p. 4470-81.

46. Wu, T., et al., *P11, a unique member of the S100 family of calcium-binding proteins, interacts with and inhibits the activity of the 85-kDa cytosolic phospholipase A2.* J Biol Chem, 1997. **272**(27): p. 17145-53.

47. Kim, S., et al., *Differential effects of annexins I, II, III, and V on cytosolic phospholipase A2 activity: specific interaction model.* FEBS Lett, 2001. **489**(2-3): p. 243-8.

48. Kronke, M. and S. Adam-Klages, *Role of caspases in TNF-mediated regulation of cPLA(2).* FEBS Lett, 2002. **531**(1): p. 18-22.

49. Laye, J.P. and J.H. Gill, *Phospholipase A2 expression in tumours: a target for therapeutic intervention?* Drug Discov Today, 2003. **8**(15): p. 710-6.

50. Wang, D. and R.N. Dubois, *Prostaglandins and cancer.* Gut, 2006. **55**(1): p. 115-22.

51. Aggarwal, B.B., *Signalling pathways of the TNF superfamily: a double-edged sword.* Nat Rev Immunol, 2003. **3**(9): p. 745-56.

52. Tracey, K.J., et al., *Shock and tissue injury induced by recombinant human cachectin.* Science, 1986. **234**(4775): p. 470-4.

53. Beutler, B. and A. Cerami, *Tumor necrosis, cachexia, shock, and inflammation: a common mediator.* Annu Rev Biochem, 1988. **57**: p. 505-18.

54. Rabinovitch, A. and W.L. Suarez-Pinzon, *Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus.* Biochem Pharmacol, 1998. **55**(8): p. 1139-49.

55. Reddy, P. and J.L. Ferrara, *Immunobiology of acute graft-versus-host disease.* Blood Rev, 2003. **17**(4): p. 187-94.

56. Feldmann, M., *The cytokine network in rheumatoid arthritis: definition of TNF alpha as a therapeutic target.* J R Coll Physicians Lond, 1996. **30**(6): p. 560-70.

57. Targan, S.R. and L.K. Murphy, *Clarifying the causes of Crohn's.* Nat Med, 1995. **1**(12): p. 1241-3.

58. Haraoui, B., *The anti-tumor necrosis factor agents are a major advance in the treatment of rheumatoid arthritis.* J Rheumatol Suppl, 2005. **72**: p. 46-7.

59. Sands, B.E., *Why do anti-tumor necrosis factor antibodies work in Crohn's disease?* Rev Gastroenterol Disord, 2004. **4 Suppl 3**: p. S10-7.

60. Feldmann, M., et al., *The transfer of a laboratory based hypothesis to a clinically useful therapy: the development of anti-TNF therapy of rheumatoid arthritis.* Best Pract Res Clin Rheumatol, 2004. **18**(1): p. 59-80.

61. Scheinfeld, N., *A comprehensive review and evaluation of the side effects of the tumor necrosis factor alpha blockers etanercept, infliximab and adalimumab.* J Dermatolog Treat, 2004. **15**(5): p. 280-94.

62. Tracey, K.J., et al., *Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia.* Nature, 1987. **330**(6149): p. 662-4.

63. Dinarello, C.A., *Anti-cytokine therapeutics and infections.* Vaccine, 2003. **21 Suppl 2**: p. S24-34.

64. Eggermont, A.M., J.H. de Wilt, and T.L. ten Hagen, *Current uses of isolated limb perfusion in the clinic and a model system for new strategies.* Lancet Oncol, 2003. **4**(7): p. 429-37.

65. Szlosarek, P.W. and F.R. Balkwill, *Tumour necrosis factor alpha: a potential target for the therapy of solid tumours.* Lancet Oncol, 2003. **4**(9): p. 565-73.

66. Coussens, L.M. and Z. Werb, *Inflammation and cancer.* Nature, 2002. **420**(6917): p. 860-7.

67. Brown, G.D., *Dectin-1: a signalling non-TLR pattern-recognition receptor.* Nat Rev Immunol, 2006. **6**(1): p. 33-43.

68. Ariizumi, K., et al., *Identification of a novel, dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning.* J Biol Chem, 2000. **275**(26): p. 20157-67.

69. Taylor, P.R., et al., *The beta-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages.* J Immunol, 2002. **169**(7): p. 3876-82.

70. Herre, J., S. Gordon, and G.D. Brown, *Dectin-1 and its role in the recognition of beta-glucans by macrophages.* Mol Immunol, 2004. **40**(12): p. 869-76.

71. Reid, D.M., et al., *Expression of the beta-glucan receptor, Dectin-1, on murine leukocytes in situ correlates with its function in pathogen recognition and reveals potential roles in leukocyte interactions.* J Leukoc Biol, 2004. **76**(1): p. 86-94.

72. Adachi, Y., et al., *Characterization of beta-glucan recognition site on C-type lectin, dectin 1.* Infect Immun, 2004. **72**(7): p. 4159-71.

73. Brown, G.D. and S. Gordon, *Immune recognition. A new receptor for beta-glucans.* Nature, 2001. **413**(6851): p. 36-7.

74. Gantner, B.N., R.M. Simmons, and D.M. Underhill, *Dectin-1 mediates macrophage recognition of Candida albicans yeast but not filaments.* Embo J, 2005. **24**(6): p. 1277-86.

75. Yadav, M. and J.S. Schorey, *The {beta}-glucan receptor Dectin-1 functions together with TLR2 to mediated macrophage activation by mycobacteria.* Blood, 2006.

76. Suram, S., et al., *Regulation of cytosolic phospholipase A2 activation and cyclooxygenase 2 expression in macrophages by the beta-glucan receptor.* J Biol Chem, 2006. **281**(9): p. 5506-14.

77. Underhill, D.M., et al., *Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production.* Blood, 2005. **106**(7): p. 2543-50.

78. Herre, J., et al., *Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages.* Blood, 2004. **104**(13): p. 4038-45.

79. Gantner, B.N., et al., *Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2.* J Exp Med, 2003. **197**(9): p. 1107-17.

80. Ozinsky, A., et al., *The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors.* Proc Natl Acad Sci U S A, 2000. **97**(25): p. 13766-71.

81. Gersuk, G.M., et al., *Dectin-1 and TLRs permit macrophages to distinguish between different Aspergillus fumigatus cellular states.* J Immunol, 2006. **176**(6): p. 3717-24.

82. Yoshitomi, H., et al., *A role for fungal {beta}-glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice.* J Exp Med, 2005. **201**(6): p. 949-60.

83. McGreal, E.P., et al., *The carbohydrate-recognition domain of Dectin-2 is a C-type lectin with specificity for high mannose.* Glycobiology, 2006. **16**(5): p. 422-30.

84. Lemaitre, B., et al., *The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults.* Cell, 1996. **86**(6): p. 973-83.

85. Williams, M.J., et al., *The 18-wheeler mutation reveals complex antibacterial gene regulation in Drosophila host defense.* Embo J, 1997. **16**(20): p. 6120-30.

86. Qureshi, S.T., et al., *Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4).* J Exp Med, 1999. **189**(4): p. 615-25.

87. Medzhitov, R., et al., *MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways.* Mol Cell, 1998. **2**(2): p. 253-8.

88. Akira, S., K. Takeda, and T. Kaisho, *Toll-like receptors: critical proteins linking innate and acquired immunity.* Nat Immunol, 2001. **2**(8): p. 675-80.

89. Takeuchi, O., et al., *Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins.* J Immunol, 2002. **169**(1): p. 10-4.

90. Takeuchi, O., et al., *Discrimination of bacterial lipoproteins by Toll-like receptor 6.* Int Immunol, 2001. **13**(7): p. 933-40.

91. Schwandner, R., et al., *Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2.* J Biol Chem, 1999. **274**(25): p. 17406-9.

92. Means, T.K., et al., *The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors.* J Immunol, 1999. **163**(12): p. 6748-55.

93. Yoshimura, A., et al., *Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2.* J Immunol, 1999. **163**(1): p. 1-5.

94. Vabulas, R.M., et al., *HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway.* J Biol Chem, 2002. **277**(17): p. 15107-12.

95. Henneke, P., et al., *Novel engagement of CD14 and multiple toll-like receptors by group B streptococci.* J Immunol, 2001. **167**(12): p. 7069-76.

96. Alexopoulou, L., et al., *Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3.* Nature, 2001. **413**(6857): p. 732-8.

97. Hoshino, K., et al., *Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product.* J Immunol, 1999. **162**(7): p. 3749-52.

98. Kawasaki, K., et al., *Mouse toll-like receptor 4.MD-2 complex mediates lipopolysaccharide-mimetic signal transduction by Taxol.* J Biol Chem, 2000. **275**(4): p. 2251-4.

99. Hayashi, F., et al., *The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5.* Nature, 2001. **410**(6832): p. 1099-103.

100. Heil, F., et al., *Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8.* Science, 2004. **303**(5663): p. 1526-9.

101. Hemmi, H., et al., *A Toll-like receptor recognizes bacterial DNA.* Nature, 2000. **408**(6813): p. 740-5.

102. Zhang, D., et al., *A toll-like receptor that prevents infection by uropathogenic bacteria.* Science, 2004. **303**(5663): p. 1522-6.

103. Yarovinsky, F., et al., *TLR11 activation of dendritic cells by a protozoan profilin-like protein.* Science, 2005. **308**(5728): p. 1626-9.

104. Kagan, J.C. and R. Medzhitov, *Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling.* Cell, 2006. **125**(5): p. 943-55.

105. Yamamoto, M., et al., *TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway.* Nat Immunol, 2003. **4**(11): p. 1144-50.

106. Brown, G.D., et al., *Dectin-1 mediates the biological effects of beta-glucans.* J Exp Med, 2003. **197**(9): p. 1119-24.

107. Wright, S.D., *CD14 and innate recognition of bacteria.* J Immunol, 1995. **155**(1): p. 6-8.

108. Shimazu, R., et al., *MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4.* J Exp Med, 1999. **189**(11): p. 1777-82.

109. Lowy, F.D., *Staphylococcus aureus infections.* N Engl J Med, 1998. **339**(8): p. 520-32.

110. Hashimoto, M., et al., *Not lipoteichoic acid but lipoproteins appear to be the dominant immunobiologically active compounds in Staphylococcus aureus.* J Immunol, 2006. **177**(5): p. 3162-9.

111. Takeuchi, O., K. Hoshino, and S. Akira, *Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection.* J Immunol, 2000. **165**(10): p. 5392-6.

112. Kielian, T., N. Esen, and E.D. Bearden, *Toll-like receptor 2 (TLR2) is pivotal for recognition of S. aureus peptidoglycan but not intact bacteria by microglia.* Glia, 2005. **49**(4): p. 567-76.

113. Stuart, L.M., et al., *Response to Staphylococcus aureus requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain.* J Cell Biol, 2005. **170**(3): p. 477-85.

114. Hauck, C.R. and K. Ohlsen, *Sticky connections: extracellular matrix protein recognition and integrin-mediated cellular invasion by Staphylococcus aureus.* Curr Opin Microbiol, 2006. **9**(1): p. 5-11.

115. Schlessinger, J., *Cell signaling by receptor tyrosine kinases.* Cell, 2000. **103**(2): p. 211-25.

116. Brugge, J.S. and R.L. Erikson, *Identification of a transformation-specific antigen induced by an avian sarcoma virus.* Nature, 1977. **269**(5626): p. 346-8.

117. Thomas, S.M. and J.S. Brugge, *Cellular functions regulated by Src family kinases.* Annu Rev Cell Dev Biol, 1997. **13**: p. 513-609.

118. Boggon, T.J. and M.J. Eck, *Structure and regulation of Src family kinases.* Oncogene, 2004. **23**(48): p. 7918-27.

119. Sicheri, F., I. Moarefi, and J. Kuriyan, *Crystal structure of the Src family tyrosine kinase Hck.* Nature, 1997. **385**(6617): p. 602-9.

120. Xu, W., S.C. Harrison, and M.J. Eck, *Three-dimensional structure of the tyrosine kinase c-Src.* Nature, 1997. **385**(6617): p. 595-602.

121. Cooper, J.A. and C.S. King, *Dephosphorylation or antibody binding to the carboxy terminus stimulates pp60c-src.* Mol Cell Biol, 1986. **6**(12): p. 4467-77.

122. Latour, S., et al., *Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signalling in immune regulation.* Nat Cell Biol, 2003. **5**(2): p. 149-54.

123. Geahlen, R.L., M.D. Handley, and M.L. Harrison, *Molecular interdiction of Src-family kinase signaling in hematopoietic cells.* Oncogene, 2004. **23**(48): p. 8024-32.

124. Parravicini, V., et al., *Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation.* Nat Immunol, 2002. **3**(8): p. 741-8.

125. Aki, D., et al., *Modulation of TLR signalling by the C-terminal Src kinase (Csk) in macrophages.* Genes Cells, 2005. **10**(4): p. 357-68.

126. Stefanova, I., et al., *Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase p53/56lyn.* J Biol Chem, 1993. **268**(28): p. 20725-8.

127. Su, S.C., et al., *LTA and LPS mediated activation of protein kinases in the regulation of inflammatory cytokines expression in macrophages.* Clin Chim Acta, 2006.

128. Meng, F. and C.A. Lowell, *Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn.* J Exp Med, 1997. **185**(9): p. 1661-70.

129. Hanke, J.H., et al., *Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation.* J Biol Chem, 1996. **271**(2): p. 695-701.

130. Tian, G., et al., *Structural determinants for potent, selective dual site inhibition of human pp60c-src by 4-anilinoquinazolines.* Biochemistry, 2001. **40**(24): p. 7084-91.

131. Blake, R.A., et al., *SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling.* Mol Cell Biol, 2000. **20**(23): p. 9018-27.

132. Mano, H., et al., *A novel protein-tyrosine kinase, tec, is preferentially expressed in liver.* Oncogene, 1990. **5**(12): p. 1781-6.

133. Smith, C.I., et al., *The Tec family of cytoplasmic tyrosine kinases: mammalian Btk, Bmx, Itk, Tec, Txk and homologs in other species.* Bioessays, 2001. **23**(5): p. 436-46.

134. Hu, Q., et al., *Identification of Rlk, a novel protein tyrosine kinase with predominant expression in the T cell lineage.* J Biol Chem, 1995. **270**(4): p. 1928-34.

135. Yang, E.J., J.H. Yoon, and K.C. Chung, *Bruton's tyrosine kinase phosphorylates cAMP-responsive element-binding protein at serine 133 during neuronal differentiation in immortalized hippocampal progenitor cells.* J Biol Chem, 2004. **279**(3): p. 1827-37.

136. Andreotti, A.H., et al., *Regulatory intramolecular association in a tyrosine kinase of the Tec family.* Nature, 1997. **385**(6611): p. 93-7.

137. Schlessinger, J., *SH2/SH3 signaling proteins.* Curr Opin Genet Dev, 1994. **4**(1): p. 25-30.

138. Salim, K., et al., *Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase.* Embo J, 1996. **15**(22): p. 6241-50.

139. Rawlings, D.J., et al., *Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases.* Science, 1996. **271**(5250): p. 822-5.

140. Park, H., et al., *Regulation of Btk function by a major autophosphorylation site within the SH3 domain.* Immunity, 1996. **4**(5): p. 515-25.

141. Miller, A.T. and L.J. Berg, *New insights into the regulation and functions of Tec family tyrosine kinases in the immune system.* Curr Opin Immunol, 2002. **14**(3): p. 331-40.

142. Jefferies, C.A., et al., *Bruton's tyrosine kinase is a Toll/interleukin-1 receptor domain-binding protein that participates in nuclear factor kappaB activation by Toll-like receptor 4.* J Biol Chem, 2003. **278**(28): p. 26258-64.

143. Horwood, N.J., et al., *Bruton's tyrosine kinase is required for lipopolysaccharide-induced tumor necrosis factor alpha production.* J Exp Med, 2003. **197**(12): p. 1603-11.

144. Gray, P., et al., *MyD88 adapter-like (Mal) is phosphorylated by Bruton's tyrosine kinase during TLR2 and TLR4 signal transduction.* J Biol Chem, 2006. **281**(15): p. 10489-95.

145. Mukhopadhyay, S., et al., *Macrophage effector functions controlled by Bruton's tyrosine kinase are more crucial than the cytokine balance of T cell responses for microfilarial clearance.* J Immunol, 2002. **168**(6): p. 2914-21.

146. Perez de Diego, R., et al., *Bruton's tyrosine kinase is not essential for LPS-induced activation of human monocytes.* J Allergy Clin Immunol, 2006. **117**(6): p. 1462-9.

147. Mukhopadhyay, S., et al., *Bruton's tyrosine kinase deficiency in macrophages inhibits nitric oxide generation leading to enhancement of IL-12 induction.* J Immunol, 1999. **163**(4): p. 1786-92.

148. Fluckiger, A.C., et al., *Btk/Tec kinases regulate sustained increases in intracellular Ca2+ following B-cell receptor activation.* Embo J, 1998. **17**(7): p. 1973-85.

149. Saito, K., et al., *BTK regulates PtdIns-4,5-P2 synthesis: importance for calcium signaling and PI3K activity.* Immunity, 2003. **19**(5): p. 669-78.

150. Humphries, L.A., et al., *Tec kinases mediate sustained calcium influx via site-specific tyrosine phosphorylation of the phospholipase Cgamma Src homology 2-Src homology 3 linker.* J Biol Chem, 2004. **279**(36): p. 37651-61.

151. Mahajan, S., et al., *Rational design and synthesis of a novel anti-leukemic agent targeting Bruton's tyrosine kinase (BTK), LFM-A13 [alpha-cyano-beta-hydroxy-beta-methyl-N-(2, 5-dibromophenyl)propenamide].* J Biol Chem, 1999. **274**(14): p. 9587-99.

152. Kawakami, Y., et al., *Terreic acid, a quinone epoxide inhibitor of Bruton's tyrosine kinase.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2227-32.

153. Vanhaesebroeck, B., et al., *Phosphoinositide 3-kinases: a conserved family of signal transducers.* Trends Biochem Sci, 1997. **22**(7): p. 267-72.

154. Ui, M., et al., *Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase.* Trends Biochem Sci, 1995. **20**(8): p. 303-7.

155. De Camilli, P., et al., *Phosphoinositides as regulators in membrane traffic.* Science, 1996. **271**(5255): p. 1533-9.

156. Hiller, G., et al., *Phosphatidylinositol 3-kinase in zymosan- and bacteria-induced signalling to mobilisation of arachidonic acid in macrophages.* Biochim Biophys Acta, 2000. **1485**(2-3): p. 163-72.

157. Stoyanov, B., et al., *Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase.* Science, 1995. **269**(5224): p. 690-3.

158. Staal, S.P., *Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma.* Proc Natl Acad Sci U S A, 1987. **84**(14): p. 5034-7.

159. Bellacosa, A., et al., *A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region.* Science, 1991. **254**(5029): p. 274-7.

160. Masure, S., et al., *Molecular cloning, expression and characterization of the human serine/threonine kinase Akt-3.* Eur J Biochem, 1999. **265**(1): p. 353-60.

161. Alessi, D.R., et al., *Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha.* Curr Biol, 1997. **7**(4): p. 261-9.

162. Alessi, D.R., et al., *Mechanism of activation of protein kinase B by insulin and IGF-1.* Embo J, 1996. **15**(23): p. 6541-51.

163. Datta, K., et al., *Akt is a direct target of the phosphatidylinositol 3-kinase. Activation by growth factors, v-src and v-Ha-ras, in Sf9 and mammalian cells.* J Biol Chem, 1996. **271**(48): p. 30835-9.

164. Harden, T.K. and J. Sondek, *Regulation of phospholipase C isozymes by ras superfamily GTPases.* Annu Rev Pharmacol Toxicol, 2006. **46**: p. 355-79.

165. Hiller, G. and R. Sundler, *Regulation of phospholipase C-gamma 2 via phosphatidylinositol 3-kinase in macrophages.* Cell Signal, 2002. **14**(2): p. 169-73.

166. Falasca, M., et al., *Activation of phospholipase C gamma by PI 3-kinase-induced PH domain-mediated membrane targeting.* Embo J, 1998. **17**(2): p. 414-22.

167. Fu, C., et al., *BLNK: a central linker protein in B cell activation.* Immunity, 1998. **9**(1): p. 93-103.

168. Ishiai, M., et al., *Cutting edge: association of phospholipase C-gamma 2 Src homology 2 domains with BLNK is critical for B cell antigen receptor signaling.* J Immunol, 1999. **163**(4): p. 1746-9.

169. Rodriguez, R., et al., *Tyrosine residues in phospholipase Cgamma 2 essential for the enzyme function in B-cell signaling.* J Biol Chem, 2001. **276**(51): p. 47982-92.

170. Watanabe, D., et al., *Four tyrosine residues in phospholipase C-gamma 2, identified as Btk-dependent phosphorylation sites, are required for B cell antigen receptor-coupled calcium signaling.* J Biol Chem, 2001. **276**(42): p. 38595-601.

171. Sekiya, F., Y.S. Bae, and S.G. Rhee, *Regulation of phospholipase C isozymes: activation of phospholipase C-gamma in the absence of tyrosine-phosphorylation.* Chem Phys Lipids, 1999. **98**(1-2): p. 3-11.

172. Poulin, B., F. Sekiya, and S.G. Rhee, *Intramolecular interaction between phosphorylated tyrosine-783 and the C-terminal Src homology 2 domain activates phospholipase C-gamma1.* Proc Natl Acad Sci U S A, 2005. **102**(12): p. 4276-81.

173. Kurosaki, T., et al., *Regulation of the phospholipase C-gamma2 pathway in B cells.* Immunol Rev, 2000. **176**: p. 19-29.

174. Nishimoto, S. and E. Nishida, *MAPK signalling: ERK5 versus ERK1/2.* EMBO Rep, 2006. **7**(8): p. 782-6.

175. Pyronnet, S., et al., *Human eukaryotic translation initiation factor 4G (eIF4G) recruits mnk1 to phosphorylate eIF4E.* Embo J, 1999. **18**(1): p. 270-9.

176. Andersson, K. and R. Sundler, *Posttranscriptional regulation of TNFalpha expression via eukaryotic initiation factor 4E (eIF4E) phosphorylation in mouse macrophages.* Cytokine, 2006. **33**(1): p. 52-7.

177. Morrison, D.K., *KSR: a MAPK scaffold of the Ras pathway?* J Cell Sci, 2001. **114**(Pt 9): p. 1609-12.

178. Schaeffer, H.J., et al., *MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade.* Science, 1998. **281**(5383): p. 1668-71.

179. Tohgo, A., et al., *beta-Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation.* J Biol Chem, 2002. **277**(11): p. 9429-36.

180. Torii, S., et al., *Sef is a spatial regulator for Ras/MAP kinase signaling.* Dev Cell, 2004. **7**(1): p. 33-44.

181. Ishibe, S., et al., *Phosphorylation-dependent paxillin-ERK association mediates hepatocyte growth factor-stimulated epithelial morphogenesis.* Mol Cell, 2003. **12**(5): p. 1275-85.

182. Meng, S., et al., *Participation of both Gab1 and Gab2 in the activation of the ERK/MAPK pathway by epidermal growth factor.* Biochem J, 2005. **391**(Pt 1): p. 143-51.

183. Dard, N. and M. Peter, *Scaffold proteins in MAP kinase signaling: more than simple passive activating platforms.* Bioessays, 2006. **28**(2): p. 146-56.

184. Biondi, R.M. and A.R. Nebreda, *Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions.* Biochem J, 2003. **372**(Pt 1): p. 1-13.

185. Imajo, M., Y. Tsuchiya, and E. Nishida, *Regulatory mechanisms and functions of MAP kinase signaling pathways.* IUBMB Life, 2006. **58**(5-6): p. 312-7.

186. Hiller, G. and R. Sundler, *Activation of arachidonate release and cytosolic phospholipase A2 via extracellular signal-regulated kinase and p38 mitogen-activated protein kinase in macrophages stimulated by bacteria or zymosan.* Cell Signal, 1999. **11**(12): p. 863-9.

187. Lee, A.W. and D.J. States, *Both src-dependent and -independent mechanisms mediate phosphatidylinositol 3-kinase regulation of colony-stimulating factor 1-activated mitogen-activated protein kinases in myeloid progenitors.* Mol Cell Biol, 2000. **20**(18): p. 6779-98.

188. Shim, J.H., et al., *TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo.* Genes Dev, 2005. **19**(22): p. 2668-81.

189. David, M.D., et al., *Pure lipopolysaccharide or synthetic lipid A induces activation of p21Ras in primary macrophages through a pathway dependent on Src family kinases and PI3K.* J Immunol, 2005. **175**(12): p. 8236-41.

190. Karni, R., et al., *Active Src elevates the expression of beta-catenin by enhancement of cap-dependent translation.* Mol Cell Biol, 2005. **25**(12): p. 5031-9.

191. Szekeres, C.K., et al., *Eicosanoid activation of extracellular signal-regulated kinase1/2 in human epidermoid carcinoma cells.* J Biol Chem, 2000. **275**(49): p. 38831-41.

192. Arnaud, M., et al., *Phosphorylation of Grb2-associated binder 2 on serine 623 by ERK MAPK regulates its association with the phosphatase SHP-2 and decreases STAT5 activation.* J Immunol, 2004. **173**(6): p. 3962-71.

193. Favata, M.F., et al., *Identification of a novel inhibitor of mitogen-activated protein kinase kinase.* J Biol Chem, 1998. **273**(29): p. 18623-32.

194. Kamakura, S., T. Moriguchi, and E. Nishida, *Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus.* J Biol Chem, 1999. **274**(37): p. 26563-71.

195. Cuenda, A., et al., *SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1.* FEBS Lett, 1995. **364**(2): p. 229-33.

196. Bennett, B.L., et al., *SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase.* Proc Natl Acad Sci U S A, 2001. **98**(24): p. 13681-6.

197. Gu, H., et al., *Critical role for scaffolding adapter Gab2 in Fc gamma R-mediated phagocytosis.* J Cell Biol, 2003. **161**(6): p. 1151-61.

198. Podar, K., et al., *Critical role for hematopoietic cell kinase (Hck)-mediated phosphorylation of Gab1 and Gab2 docking proteins in interleukin 6-induced proliferation and survival of multiple myeloma cells.* J Biol Chem, 2004. **279**(20): p. 21658-65.

199. Lynch, D.K. and R.J. Daly, *PKB-mediated negative feedback tightly regulates mitogenic signalling via Gab2.* Embo J, 2002. **21**(1-2): p. 72-82.

200. Yu, C.F., et al., *ERK regulates the hepatocyte growth factor-mediated interaction of Gab1 and the phosphatidylinositol 3-kinase.* J Biol Chem, 2001. **276**(35): p. 32552-8.

201. Batliwalla, F.M., et al., *Peripheral blood gene expression profiling in rheumatoid arthritis.* Genes Immun, 2005. **6**(5): p. 388-97.

202. Gu, H. and B.G. Neel, *The "Gab" in signal transduction.* Trends Cell Biol, 2003. **13**(3): p. 122-30.

203. Singer, S.J. and G.L. Nicolson, *The fluid mosaic model of the structure of cell membranes.* Science, 1972. **175**(23): p. 720-31.

204. Karnovsky, M.J., et al., *The concept of lipid domains in membranes.* J Cell Biol, 1982. **94**(1): p. 1-6.

205. Simons, K. and E. Ikonen, *Functional rafts in cell membranes.* Nature, 1997. **387**(6633): p. 569-72.

206. Pike, L.J., *Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function.* J Lipid Res, 2006. **47**(7): p. 1597-8.

207. Parton, R.G., *Caveolae and caveolins.* Curr Opin Cell Biol, 1996. **8**(4): p. 542-8.

208. Le, P.U., et al., *Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum.* J Biol Chem, 2002. **277**(5): p. 3371-9.

209. Rothberg, K.G., et al., *Caveolin, a protein component of caveolae membrane coats.* Cell, 1992. **68**(4): p. 673-82.

210. Pike, L.J., *Lipid rafts: heterogeneity on the high seas.* Biochem J, 2004. **378**(Pt 2): p. 281-92.

211. Pralle, A., et al., *Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells.* J Cell Biol, 2000. **148**(5): p. 997-1008.

212. Lei, M.G. and D.C. Morrison, *Differential expression of caveolin-1 in lipopolysaccharide-activated murine macrophages.* Infect Immun, 2000. **68**(9): p. 5084-9.

213. Hornef, M.W., et al., *Intracellular recognition of lipopolysaccharide by toll-like receptor 4 in intestinal epithelial cells.* J Exp Med, 2003. **198**(8): p. 1225-35.

214. Triantafilou, M., et al., *Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation.* J Cell Sci, 2002. **115**(Pt 12): p. 2603-11.

215. Korzeniowski, M., K. Kwiatkowska, and A. Sobota, *Insights into the association of FcgammaRII and TCR with detergent-resistant membrane domains: isolation of the domains in detergent-free density gradients facilitates membrane fragment reconstitution.* Biochemistry, 2003. **42**(18): p. 5358-67.

216. Kwiatkowska, K., J. Frey, and A. Sobota, *Phosphorylation of FcgammaRIIA is required for the receptor-induced actin rearrangement and capping: the role of membrane rafts.* J Cell Sci, 2003. **116**(Pt 3): p. 537-50.

217. Chen, X. and M.D. Resh, *Cholesterol depletion from the plasma membrane triggers ligand-independent activation of the epidermal growth factor receptor.* J Biol Chem, 2002. **277**(51): p. 49631-7.

218. Alberts, A.W., et al., *Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent.* Proc Natl Acad Sci U S A, 1980. **77**(7): p. 3957-61.

219. Shogomori, H. and D.A. Brown, *Use of detergents to study membrane rafts: the good, the bad, and the ugly.* Biol Chem, 2003. **384**(9): p. 1259-63.

220. Matko, J. and J. Szollosi, *Landing of immune receptors and signal proteins on lipid rafts: a safe way to be spatio-temporally coordinated?* Immunol Lett, 2002. **82**(1-2): p. 3-15.

221. Di Carlo, F.J. and J.V. Fiore, *On the composition of zymosan.* Science, 1958. **127**(3301): p. 756-7.

222. Majeed, M., et al., *Role of Src kinases and Syk in Fcgamma receptor-mediated phagocytosis and phagosome-lysosome fusion.* J Leukoc Biol, 2001. **70**(5): p. 801-11.

223. Kim, Y.J., et al., *Mechanism of B-cell receptor-induced phosphorylation and activation of phospholipase C-gamma2.* Mol Cell Biol, 2004. **24**(22): p. 9986-99.

224. Knauf, U., C. Tschopp, and H. Gram, *Negative regulation of protein translation by mitogen-activated protein kinase-interacting kinases 1 and 2.* Mol Cell Biol, 2001. **21**(16): p. 5500-11.

225. Taylor, P.R., et al., *The role of SIGNR1 and the beta-glucan receptor (Dectin-1) in the nonopsonic recognition of yeast by specific macrophages.* J Immunol, 2004. **172**(2): p. 1157-62.

226. Yu, M., et al., *Scaffolding adapter Grb2-associated binder 2 requires Syk to transmit signals from FcepsilonRI.* J Immunol, 2006. **176**(4): p. 2421-9.

227. Fodor, S., Z. Jakus, and A. Mocsai, *ITAM-based signaling beyond the adaptive immune response.* Immunol Lett, 2006. **104**(1-2): p. 29-37.

228. Takeda, K., T. Kaisho, and S. Akira, *Toll-like receptors.* Annu Rev Immunol, 2003. **21**: p. 335-76.

229. Dai, Q., J. Zhang, and S.B. Pruett, *Ethanol alters cellular activation and CD14 partitioning in lipid rafts.* Biochem Biophys Res Commun, 2005. **332**(1): p. 37-42.

230. Lucero, H.A. and P.W. Robbins, *Lipid rafts-protein association and the regulation of protein activity.* Arch Biochem Biophys, 2004. **426**(2): p. 208-24.

231. Weinstein, S.L., et al., *Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of mitogen-activated protein kinases in macrophages.* J Biol Chem, 1992. **267**(21): p. 14955-62.

232. Reid, R.R., et al., *Endotoxin shock in antibody-deficient mice: unraveling the role of natural antibody and complement in the clearance of lipopolysaccharide.* J Immunol, 1997. **159**(2): p. 970-5.