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Intracellular signaling mechanisms in the innate immune response to viral infections

Thesis for the degree of Philosophiae Doctor

Trondheim, July 2009

Norwegian University of Science and Technology
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Children`s and Women`s Health

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ISBN 978-82-471-1624-1 (printed ver.)
ISBN 978-82-471-1625-8 (electronic ver.)
ISSN 1503-8181

Doctoral theses at NTNU, 2009:120

Printed by NTNU-trykk

NORGES TEKNISK-NATURVITENSKAPELIGE UNIVERSITET
DET MEDISINSKE FAKULTET

Intracellulære signalmekanismer i den medfødte immunresponsen til virus infeksjoner

Infeksjoner forårsaket av aggressive virus, som enkelte varianter av influensa virus og coronavirus som forårsaker "severe acute respiratory syndrome" (SARS), er forbundet med høy dødelighet og utgjør en betydelig global trussel. Kroppens første møte med mikrobielle patogener er mediert av reseptorer i immuncellene. Disse reseptorene gjenkjenner spesifikke patogene komponenter og har fått fellesbetegnelsen "mønster gjenkjennings reseptorer" (PRR). De best studerte PRRene kalles Toll lignende reseptorer (TLR) og er transmembrane proteiner som finnes enten på celleoverflaten eller intracellulært. I tillegg har man nylig identifisert flere cytoplasmiske reseptorer, delt inn i Nod-lignende reseptorer (NLR) og RIG-I-lignende reseptorer (RLR). Eksempler på patogene komponenter som gjenkjennes er deler av celleveggen til bakterier, som lipopolysakkarid (LPS), og virale nukleinsyrer.

De fleste virus entrer cellen via endocytose, fusjonerer med endosomale membraner og frigir arvematerialet sitt i cytoplasma. Cellen er utstyrt med et repertoire av gjenkjenningsmekanismer på ulike trinn av virus infeksjonen, f.eks. TLR3 som binder viralt dobbeltrådet RNA (dsRNA) i endosomet og RIG-I som binder viralt enkeltrådet RNA (ssRNA) i cytoplasma. Etter at TLR3 eller RIG-I har bundet virale nukleinsyrer settes det i gang signalmekanismer som involverer ulike adapter proteiner, kinaser og transkripsjonsfaktorer. Disse mekanismene regulerer genuttrykket av antivirale cytokiner og interferoner (IFN). TLR3- og RIG-I-avhengige responser resulterer i høy aktivering av transkripsjonsfaktoren interferon regulerende faktor (IRF) 3 og påfølgende produksjon av type I IFN, en viktig faktor i kroppens medfødte forsvar mot virus. Likevel er de molekylære mekanismene bak interaksjonen mellom virus og vertscelle fortsatt i stor grad ukjent. I dette studiet beskriver vi betydningen av c-Src, en tyrosin kinase, i antiviral signalisering mediert av virus-gjenkjennende PRRer.

I første del av dette arbeidet viser vi at c-Src aktiveres av viralt dsRNA og assosierer med TLR3. Ved hjelp av kjemisk og genetisk hemming fant vi at c-Src er viktig for antiviral immunrespons, spesifikt for aktivering av transkripsjonsfaktorene IRF3 og STAT1 i respons til dsRNA, og at kinasen utøver sin funksjon nedstrøms for TLR3 adapterproteinet TRIF. Ved bruk av konfokal mikroskopi så vi at viralt dsRNA blir tatt opp i cellen via endocytose. Vi fant TLR3 i endoplasmatisk retikulum i hvilende celler, og etter tilsats av dsRNA observerte vi en fraksjon av TLR3 i endosomale strukturer. Vi observerte også økt lokalisering av c-Src i endosom/lysosom i dsRNA-behandlede celler. Videre resultater viser at c-Src også forsterker den antivirale responsen til Sendai virus (SV), som gjenkjennes av den cytoplasmiske reseptoren RIG-I. Spesifikt viser vi at hemming eller utslåing av c-Src nedsetter SV-indusert aktivering av transkripsjonsfaktoren IRF3 og induksjon av IFN-β nedstrøms for RIG-I og adapterproteinet MAVS. C-Src assosierer med flere komponenter i RIG-I signalveien, og interagerer spesifikt med RING domenet til et cytoplasmisk adapterprotein kalt TRAF3, som er viktig for både TLR3- og RIG-I-mediert immunrespons til virus. I siste del av arbeidet så vi at utslåing av c-Src hemmer IRF3 aktivering og induksjon av IRF3-regulert genuttrykk (IP10) i tidlig TLR-mediert respons. I en senere fase av TLR responsen observerte vi imidlertid at manglende c-Src funksjon øker IRF5 aktivering og induksjon av IRF5-regulert genuttrykk

(IL-6). Vi fant at c-Src positivt regulerer utrykket av transkripsjonsfaktoren ATF3, en negativ regulator av TLR-indusert IL-6 produksjon.

Samlet viser disse resultatene at c-Src tyrosin kinase medvirker i antivirale immunresponser nedstrøms for ulike PRRs og sannsynligvis i respons til flere virus. TLR3 gjenkjenner viralt dsRNA i endosom/lysosom og rekrutterer c-Src for å sette i gang antiviral signalisering. C-Src assosierer med adapterproteinet TRAF3, hvor signalveiene fra TLR3 og RIG-I møtes, og binder spesifikt til dets RING domene. C-Src utøver en differensiel rolle i medfødt immunaktivering av IRF transkripsjonsfaktorer, og hemmer TLR-trigget IL-6 induksjon ved å kontrollere ATF3 uttrykket i en sen fase av TLR responser.

Vi tror at disse resultatene bidrar til økt forståelse av medfødte responsmekanismer til virus infeksjon. Slik forståelse er essensiell for utvikling av nye antivirale behandlingsformer og vaksiner.

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Finansieringskilde: Samarbeidsorganet NTNU/Helse Midt-Norge

Ovennevnte avhandling er funnet verdig til å forsvares offentlig for graden PhD i molekylær medisin
Disputas finner sted i Auditoriet, Laboratoriesenteret, onsdag 01-07-2009 kl. 12.15

ACKNOWLEDGEMENTS

This work has been carried out at the Department of Laboratory Medicine, Children´s and Women's Health, Faculty of Medicine, The Norwegian University of Science and Technology, Norway, in the laboratory of Professor Marit Walbye Anthonsen. A part of the work was carried out in the laboratory of Professor Terje Espevik (Department of Cancer Research and Molecular Medicine). I am grateful for the financial support from Samarbeidsorganet NTNU/Helse Midt-Norge.

I want to express my gratitude to my supervisor Professor Marit Walbye Anthonsen for excellent guidance along the way. Thank you for teaching me research, sharing your extensive knowledge, pushing and supporting me, and for always finding time to answer questions and suggest directions. I am also grateful to my co-supervisor Professor Terje Espevik for teaching me the tricks of the trade in confocal microscopy, and for sharing tools and expertise on Toll-like receptors.

I thank all the members of the Toll group for scientific discussions, technical help and clever advices. I especially thank Kjartan Egeberg who has helped me out of various difficulties concerning the confocal microscope. I also thank the members of the Human genetics group, in particular Veslemøy Malm Landsem and Trine Løkken for great technical support. I am thankful to the members of the DNA-repair group for creating the best atmosphere at the lunch-room and on other social venues. I thank my fellow PhD students and friends, Thuy Nguyen, Bjarte Bergstrøm, Mari Sæther, Kathrin Torseth, Dr. Nadra Nilsen and Cathrine Knetter, for creating an inspiring working environment in many ways. Nadra and Cathrine; you made my stay in Worcester much more memorable! I particularly appreciate all the interesting scientific and non-scientific discussions we had on our cosy porch. Kathrin; thanks for being the best room-mate and friend during the time at MTFS. Mari; I truly enjoy all the crazy fun we have had on work-related social events and else. Thuy and Bjarte; thanks for valuable conversations on antiviral signaling pathways and other issues coming up to our minds, and for great collaboration in the lab.

The co-authors who have contributed to the papers in this work are highly acknowledged. A special thank to Dr. Egil Lien, who kindly invited me to his lab at UMass.

Finally, I wish to express my gratitude to my friends and family. Thanks to all my friends for making my off-work life exciting and laughable. I am especially in debt to my parents who gave me the best start of life and who has always encouraged and supported me. Above all I am grateful to my dearest Øyvind and Einar for making my life complete. I thank Einar for distracting me in his own talented way and for always putting a big smile on my face. I thank Øyvind for continuously reminding me of the noble art of biological science, and for sharing his intelligent thoughts morning and night. Your love, care and support mean everything!

Trondheim, March 2009

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Ingvild Bjellmo Johnsen, Thuy Thanh Nguyen, Monika Ringdal, Anne Merete Tryggestad, Egil Lien, Oddmund Bakke, Terje Espevik, Marit W. Anthonsen. 2006. TLR3 associates with double-stranded RNA and c-Src tyrosine kinase on endosomes to initiate antiviral signaling. *EMBO J*. 25: 3335-3346.

Paper II

Ingvild Bjellmo Johnsen, Thuy Thanh Nguyen, Bjarte Bergstrøm, Katherine A. Fitzgerald, Marit W. Anthonsen. 2009. The tyrosine kinase c-Src enhances retinoic acid inducible gene-I (RIG-I)-elicited antiviral signaling. *J. Biol. Chem. In press.*

Paper III

Thuy Than Nguyen, **Ingvild Bjellmo Johnsen**, Cathrine F. Knetter, Egil Lien, Katherine A. Fitzgerald, Finn Drabløs and Marit Walbye Anthonsen. 2009. Activating transcription factor 3 is regulated by c-Src tyrosine kinase and confers differential gene expression downstream Toll-like receptors. *Manuscript.*

ABBREVIATIONS

AIM2	absent in melanoma 2
AP-1	activator protein-1
ASC	apoptosis-associated speck-like protein containing a CARD
ATF	activating transcription factor
BAF	brahma associated factor
Btk	bruton`s tyrosine kinase
CARD	caspase activation recruitment domain
CARDINAL	caspase recruitment domain-containing protein 8
CBP	CREB binding protein
CREB	cAMP response element binding
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	danger associated molecular pattern
DBD	DNA binding domain
DC	dendritic cell
pDC	plasmacytoid dendritic cell
mDC	myeloid dendritic cell
DDX3	dead box protein 3
DNA	deoxyribonucleic acid
Ds	double-stranded
DUBA	deubiquitinating enzyme A
ECD	ectodomain
EGFR	epidermal growth factor receptor
EIF2α	eukaryotic translation initiation factor 2α
EMCV	encephalomyocarditis virus
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FADD	fas-associated via death domain
GCN5	general-control-of-amino-acid synthesis 5
HAT	histone acetyltransferase
HMG	high mobility group
HPV	human papilloma virus
HSV	herpes simplex virus
IAD	IRF association domain
IFN	interferon
IFNAR	interferon receptor
IκB	inhibitor of NFkappa B
IKK	IkappaB kinase

IL	interleukin
IL-1R	interleukin-1 receptor
IRAK	interleukin-1 receptor associated kinase
IRF	interferon regulatory factor
ISG	interferon stimulated genes
ISGF3	interferon stimulated gene factor 3
ISRE	interferon sensitive response element
JAK	janus kinase
JEV	japanese encephalitis virus
JNK	jun N-terminal kinase
LBP	lipopolysaccharide binding protein
LCMV	lymphocytic choriomeningitis virus
LLR	leucine rich repeat
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAL	MyD88 adaptor-like
MAPK	mitogen-activated protein kinase
MAPKKK	MAP kinase kinase kinase
MAVS	mitochondrial antiviral signaling protein
MCMV	mouse cytomegalovirus
Mda5	melanoma differentiation-associated gene-5
MH2	mad-homology 2
MHC	major hitocompatibility complex
MITA	mediator of IRF3 activation
MxA	myxovirus resistance A
MyD88	myeloid differentiation primary response gene 88
NALP	nacht domain-, leucine-rich repeat- and PYD-containing protein
NDV	newcastle disease virus
NF-κB	nuclear factor-kappa B
NIK	NF-κB-inducing kinase
NK	natural killer
NLR	nod-like receptors
NLRX1	NLR family member X1
NOD	nucleotide oligomerization domain
OAS	oligodentylate synthetase
PAMP	pathogen associated molecular pattern
PKC	protein kinase C
PKR	protein kinase R
poly IC	polyinosinic acid:cytidylic acid

PRD	positive regulatory domains
PRR	pattern recognition receptor
PYHIN	pyrin and HIN domain-containing protein
RHIM	RIP homotypic interaction motif
RIG-I	retinoic inducible gene-i
RIP	receptor interacting protein
RNA	ribonucleic acid
ROS	reactive oxygen spesies
RSV	repiratory syncytial virus
SARM	sterile-alpha and armadillo motif containing protein
SARS	severe acute respiratory syndrome
SH	src homology
SIKE	suppressor of interferon IKKε
Ss:	single-stranded
STAT	signal transducers and activators of transcription
STING	stimulator of interferon genes
SV	sendai virus
TAK1	transforming growth factor β–activated kinase 1
TBK1	TANK-binding kinase 1
TFIID	transcription factor II D
TIR	toll/interleukin-1 receptor
TNFR	tumor necrosis factor receptor
TNF	tumor necrosis factor
TLR	toll-like receptor
TRAF	TNF receptor associated factor
TRAM	TRIF related adaptor molecule
TRIF	TIR domain-containing adaptor protein inducing interferon beta
VSV	vesicular stomatitis virus

1 INTRODUCTION

A virus is an infectious agent that is unable to grow and reproduce outside a host cell. Various viruses can infect almost any type of body tissue, from the brain to the skin. In contrast to bacterial infections, viral infections cannot be treated with antibiotics. In fact, in most cases human viral infections can be effectively fought by the body's own immune system. Still emerging viruses, such as the H5N1 variant of the influenza virus and the coronavirus causing subacute airway respiratory syndrome (SARS), cause substantial morbidity and mortality. Importantly several cancers are associated with viral infections, *e.g.* cervical cancer which is closely associated with human papillomavirus (HPV) and liver cancer which is caused mainly by chronic hepatitis B or hepatitis C infection. Knowledge of host-virus interactions and antiviral immunity is important to provide improved strategies for antiviral vaccines, and more effective treatments for viral infectious disease as well as virus-associated cancer.

The immune system is a collection of mechanisms within an organism that protects against foreign pathogens as well as endogenous malignant cells. The human body possesses both innate and adaptive immune defences which comprise distinct mechanisms. The adaptive immune system is acquired and has the feature of specific long-lasting memory, but it takes time before it reaches its maximum efficiency. Meanwhile an immediate response to invading pathogens is required to prevent replication and spread of the infectious agent. This shows the importance of the germ-line encoded mechanisms referred to as the innate immune system. All these processes rely mainly on specialized cells and molecules inside the body.

1.1 Pattern recognition of the innate immune system

The main function of the innate immune system is to recruit immune cells to sites of infection through the induction of cytokines and interferons (IFNs) prior to activation of an adaptive immune response. Subsequently foreign substances are identified and removed by specialized phagocytic cells, such as neutrophiles, macrophages and dendritic cells (DCs). To mount an appropriate defence against invading pathogens, the phagocytic cells are dependent on germ-line encoded receptors termed pattern recognition receptors (PRRs). PRRs recognize pathogen associated molecular patterns (PAMPs) that distinguish foreign organisms from host cells.

Such molecular patterns include viral nucleic acids, parts of the bacterial cell wall, bacterial flagellar proteins and more. However, this detection system is not foolproof and may be inadversely activated by host molecules secreted during stress or by dying cells (1). Such endogenous stress signals are termed damage-associated molecular patterns (DAMPs). Inadvertent activation of PRRs by DAMPs may have an important role in autoimmune and inflammatory diseases (2). Upon pattern recognition and activation of PRRs, specific adaptor molecules are recruited to the receptor leading to the initiation of distinct signaling cascades that regulate the transcription of pro- and anti-inflammatory cytokines necessary for the early immune response.

The most studied PRRs are the Toll-like receptors (TLRs). At present 11 different TLRs (numbered 1-11) are described in human, all recognizing different molecular patterns and initiating specific signaling cascades. The TLRs are found either at the plasma membrane where they recognize invading pathogens on the surface of the cell, or in the endosomes/lysosomes where they signal viruses entering the cell by endocytosis. However, both bacteria and viruses may at some point enter the cytoplasm in which the TLRs are poorly represented. Recently several cytoplasmic pattern recognition receptors have been discovered. They have been divided into two families, the NLR family (nucleotide-binding oligomerization domain (NOD)-like receptor family) and the RLR family (RIG-I like receptor family). The NLR family consists of at least 23 members, either NOD receptors generally activating NF-κB, or NALPs (NACHT-, LRR-and pyrin-domain containing proteins) known to activate caspase-1 to control the processing of the pro-inflammatory cytokines IL-1β and IL-18 (3). The RLR family is formed by at least two members named RIG-I and Mda5, both important for the recognition of viral RNA. In 2007, the double-stranded DNA-binding protein DAI (DNA-dependent activator of IFN-regulatory factors) was identified as a candidate cytoplasmic DNA sensor with roles in infections *in vivo*, but its functions remain to be shown (4). Very recently, the PYHIN family member absent in melanoma 2 (AIM2) was identified as a receptor for cytosolic DNA which regulates caspase-1 (5). Knock-down of Aim2 abrogates caspase-1 activation in response to cytoplasmic dsRNA and dsRNA vaccinia virus.

1.1.1 Toll-like receptors

TLRs are evolutionary conserved from the worm *Caenorhabditis elegans* to mammals (6;7). The founding TLR member Toll was originally identified as a protein important for the development of embryonic dorsoventral polarity in *Drosophila* (8). Later it was found to be critical for the response against fungal infection in flies (9). Next, a mammalian homolog of the Toll receptor was shown to induce expression of genes involved in inflammatory responses (10). This homolog was identified as the key receptor for LPS (11) and is today known as TLR4.

TLRs are capable of sensing organisms ranging from bacteria to fungi, protozoa, and viruses. They are transmembrane signaling receptors that are expressed on cellular membranes; either on the plasma membrane or on intracellular vacuolar membranes. Their structure consists of extracellular leucin-rich repeats (LLRs), which differ between the TLRs, and a cytoplasmic Toll-interleukin-1 receptor (TIR) domain (12). Upon ligand binding the TLRs utilize their common TIR domain to transmit intracellular responses through the homologous recruitment of TIR-contaning adaptor proteins (6;13). The pattern of the response depends on the combination of PAMPs and TLRs. In general, TLR4 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria, TLR3 senses viral double-stranded RNA (dsRNA), TLR7 and TLR8 senses viral singel-stranded RNA (ssRNA), whereas TLR9 specifically responds to bacterial nonmethylated CpG DNA motifs (TLR9) (11;14-18). The TLRs utilize TIR-domain containing adaptor proteins to propagate the signal. All TLRs except TLR3 activate downstream signaling components through MyD88. TLR3 on the other hand utilizes the adaptor protein TRIF (19-22), which is mainly responsible to type-I IFN. TLR4 signals through both MyD88 and TRIF. In addition TLR4 use the TRIF-related adaptor molecule (TRAM), which functions to recruit and activate TRIF (23). In contrast to TLR4 signaling, TLR3 recruits TRIF directly. The intracellular signaling cascades initiated by the TLRs often involve protein kinases and ligases. The pathways diverge at the level of the adaptor proteins. However, there is complex cross-talk between the distinct pathways. Signals generated from MyD88 mainly regulate NF-κB dependent transcription of pro-inflammatory cytokines, whereas TRIF-induced pathways generally regulate interferon regulatory factor (IRF) 3 and IRF7 dependent transcription of type-I IFNs. However, TLR-stimulated signaling through TRIF is also capable of activating NF-κB.

The Toll-like receptors are localized in different organelles. TLR1, TLR2, TLR4, TLR5 and TLR6 recognize the invading pathogen on the surface of the cell and show localization to the plasma membrane. TLR3, TLR7, TLR8, and TLR9, on the other hand, localize to the endoplasmic reticulum (ER) in resting cells, but a fraction enters the endosomal pathway to capture the entering virus and transmit an antiviral signal. The mechanism by which the receptors are trafficked to the endosomes/lysosomes is not understood. However, it has been reported that a missense mutation in the membrane protein UNC93B1 abrogates translocation of TLR7 and TLR9 from the ER to endolysosomes (24). Very recently it was shown that proteolytic cleavage of TLR9 is a prerequisite for its signaling (25), demonstrating the importance of lysosomal localization. The TLRs localizing to endosomes/lysosomes share the ability to recognize viral and bacterial nucleic acids, nevertheless they have the potency to respond to endogenous nucleic acids (15;26). Importantly, their localization to intracellular vesicles could prevent them from responding to self components (26).

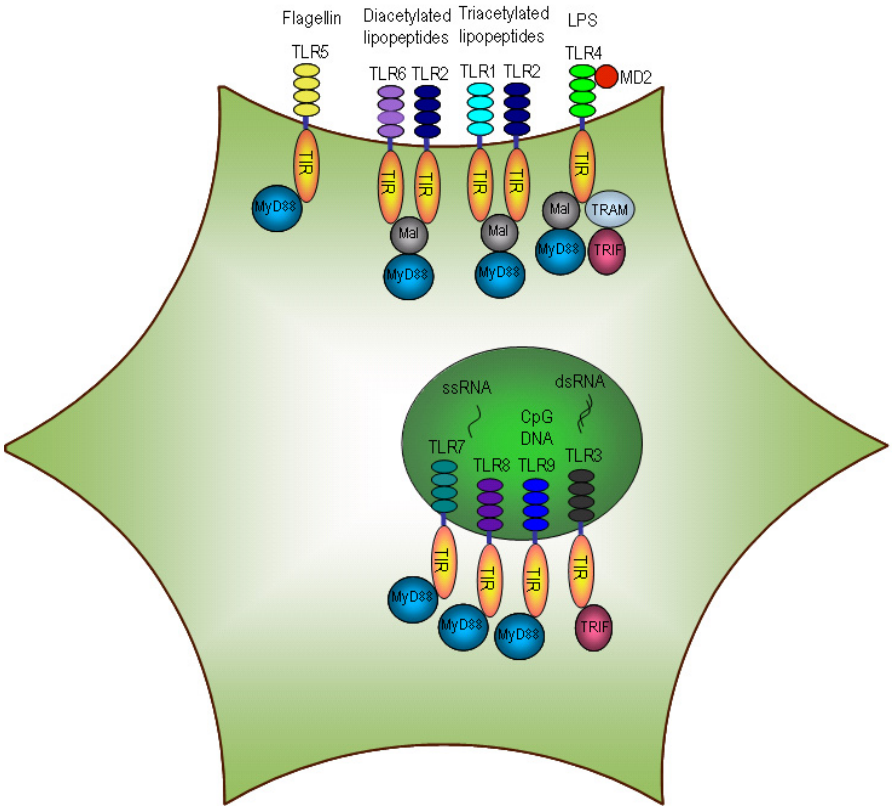

Figure 1: Overview of TLR 1-9, their ligands and the TIR adaptors they utilize for downstream signaling.

Bacterial sensing by TLRs

Bacteria may be classified as Gram-negative and Gram-positive bacteria based on the characteristics of their cell wall. Some of the unique cell-wall components are potent PAMPs for PRRs in immune cells. LPS from Gram-negative bacteria is the most potent immunostimulant among these cell-wall components, in particular due to a lipid portion termed lipid A. LPS is the ligand of TLR4, which is highly expressed on the cell surface of monocytes (27-29). LPS interacts with LPS binding protein (LBP) and binds CD14, a protein expressed on the surface of phagocyting cells. LPS is then transferred to MD2 which

associates with the extracellular domain of TLR4. TLR4 undergoes oligomerization and initiates LPS signaling (11;30).

Lipoteichoic acid (LTA), a cell-wall component of Gram-positive bacteria, seems to function in a similar manner as LPS as an immune activator. TLR2 is expressed on the cell surface of monocytes and myeloid DCs (27-29;31), and is the major detectant of Gram-positive bacteria such as *Staphylococcus aureus* and *Mycobacteria*. TLR2 is reported to recognize a variety of microbial components, including LTA, lipoprotein, peptidoglycan, zymosan and porins. TLR2 interacts physically and functionally with TLR1 and TLR6, which may explain its wide repertoire of ligands (32-34). TLR2/TLR1 heterodimerization occurs in response to triacetylated lipopeptides, whereas TLR2/TLR6 heterodimerization occurs upon stimulation with diacetylated lipopeptides and LTA (33-35).

Flagellin, the motility apparatus of many microbial pathogens, is another potent immune stimulator. TLR5, also expressed on the cell surface of monocytes and myeloid DCs (27;28), recognizes flagelling and is reported to specifically detect the D1 domain of the flagellin, which consists of well conserved central α-helical chains (36;37). Another bacterial immunostimulant is unmethylated CpG-DNA. The CpG motif is abundant in bacterial genomes, but not in mammalian DNA. Moreover, mammalian, but not bacterial CpG-DNA, is highly methylated (38). TLR9, specifically expressed intracellularly in plasmacytoid DCs (pDCs) (27;29), recognizes and interacts directly with CpG-DNA that has been delivered to intracellular vesicles (39;40). As previously mentioned, it was demonstrated that the TLR9 ectodomain is cleaved in the endolysosome, and that the truncated receptor rather than the full-lenght form is functional and recruits MyD88 (41). This may represent a strategy to restrict receptor activity to the endosomes/lysosomes, thus preventing TLRs from responding to self molecules.

Viral sensing by TLRs

Viruses contain genetic material, either DNA or RNA, encoding viral structural components, such as replication enzymes and proteases. Viral PAMPs recognized by PRRs are mainly DNA, ssRNA, dsRNA and surface proteins. Among the TLR family, TLR3, TLR7, TLR8 and TLR9 are able to respond to viral infections. DNA viruses that contain genomes rich in

unmethylated CpG motifs, such as herpes simplex virus 1 (HSV-1), HSV-2 and mouse cytomegalovirus (MCMV) are recognized by TLR9. This activates induction of type I IFNs. However, TLR9-mediated IFN-α production in response to HSV-1 and HSV-2 is cell-type specific and occurs only in pDCs. Macrophages also induce type I IFNs upon HSV infection, suggesting that cells other than pDCs have TLR9 independent systems for antiviral responses.

Viral ssRNA is recognized by TLR7 and TLR8. TLR7 signals in response to ssRNA in both human and mouse, whereas TLR8 may function only in human (16;42). The genes encoding TLR7 and TLR8 are highly homologous to each other and are both located on the X chromosome. However, these two TLRs show differences in cell-type specific expression in human. TLR7 is expressed on intracellular vesicles in pDCs, whereas TLR8 is expressed intracellularly in monocytes and mDCs (27;29). Both TLR7 and TLR8 mediate response to ssRNA derived from human immunodeficency virus (HIV), and to synthetic antiviral imidazoquinoline components (R848, Imiquimod). TLR7 is also reported to respond to ssRNA from influenza virus, synthetic poly U and small interfering RNAs (15;16;18;43;44).

DsRNA and a synthetic dsRNA analogue, polyinosinic acid:cytidylic acid (poly IC), are recognized by TLR3 (14). DsRNA is synthesized during replication of many viruses, either as a replication intermediate of ssRNA viruses (*e.g.* EMCV and West Nile viruses) or as a by-product of symmetrical transcription in DNA viruses (*e.g.* MCMV). In addition, TLR3 is able to recognize dsRNA derived from dsRNA viruses such as reovirus (45-47). Besides expression in epithelial cells, TLR3 is specifically expressed in natural killer (NK) cells and conventional DCs, but not in pDCs (48-52). The 3-dimensional structure of the TLR3 ectodomain (ECD) has recently been solved. It is a horseshoe-shaped solenoid consisting of 23 LRRs. TLR3 is masked by carbohydrates, but one side of the ECD is glycosylation-free (53;54). Mutational analysis has located the dsRNA binding site within the glycan-free, lateral surface towards the C-terminal region of TLR3 (55). TLR3 ectodomains dimerize on oligonucleotides of 40-50 base pairs, which is suggested to be the minimum length of the dsRNA sequence required for signal transduction (56). In contrast to TLR4, TLR3 binds its ligand directly (54).

Finally, viral envelope glycoproteins are recognized by TLR4 and TLR2. This, however, leads to the production of pro-inflammatory cytokines rather than type I IFNs. Thus, detection of viral glycoproteins results in inflammation, but not specific antiviral responses (57).

1.1.2 RIG-I-like helicases

RIG-I and Mda5 are members of the DEAD-box RNA helicases. They are both composed of two N-terminal caspase recruitment domains (CARDs) and a C-terminal RNA helicase domain (58-60). Despite the structural similarities the RIG-I like helicases (RLHs) display high degree of specificity on virus detection. Cytokine secretion is markedly reduced in RIG-I -/- cells infected with Sendai virus (SV), Newcatle disease virus (NDV), Vesicular stomatitis virus (VSV), Influenza A virus and Japanese encephalitis virus (JEV). On the other hand, cytokine production in response to EMCV, Thyler`s virus and Mengo virus, all Picornaviruses, is almost absent in Mda5 -/- cells (61). Additionally, it is believed that RIG-I discriminates between self- and non-self-RNA by specifically binding RNA, both ds and ss, containing free 3-phosphate on its 5`-end (62;63). RIG-I and Mda5 utilize the adaptor protein MAVS (also called Cardif, VISA and IPS) in a CARD-CARD dependent manner to trigger antiviral response (64-67). MAVS is anchored to the mitochondrial outer membrane through a short part of its C-terminal, and recruits signaling molecules crucial for the activation of NF-κB and IRF7 and/or IRF3, and subsequent induction of type-I IFNs. It has been suggested, as for the TLRs, that RIG-I and Mda5 signaling pathways diverge on the level of the adaptor protein.

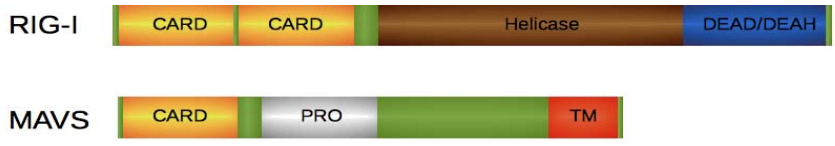

Figure 2: Domain structure of RIG-I and MAVS. RIG-I contains two N-terminal CARD domains and a C-terminal DEAD-box/helicase domain. MAVS contains one N-terminal CARD-domain in addition to a proline-rich region and a C-terminal transmembrane region.

1.1.3 NOD-like receptors

The NOD like receptors (NLRs) comprise two subclasses, the NODs (numbered 1-5) and the 14 members of the NALPs, giving a total number of 22 NLRs identified so far (68). NLRs contain three domains: an N-terminal CARD or pyrin effector domain, a nucleotide-binding and oligomerization domain called NACTH domain, and a number of C-terminal LRRs. The best studied NLRs are NOD1 and NOD2 that recognize bacterial peptidoglycan and activate MAPK and NF-κB through a CARD-dependent recruitment of RIP2 (69;70). The NALP proteins, at least some of them, have important roles in activation of pro-inflammatory caspases through formation of complexes called inflammasomes. These complexes are identified as the NALP1 and the NALP2/NALP3 inflammasomes. The NALP1 inflammasome consists of NALP1, the adaptor protein ASC, caspase 1 and caspase 5 (71), whereas the NALP2/NALP3 inflammasome is composed of NALP2 or NALP3 in addition to CARDINAL, ASC and caspase 1 (72). Both inflammasomes control the processing of the pro-inflammatory cytokines IL-1β and IL-18 (3). Interestingly it seems that not only PAMPs but also the presence of additional danger signals or DAMPs, *e.g.* silica crystals, salt aluminium crystals, asbestos and uric acid, can be sensed by inflammasomes (73-75).

1.2 Antiviral signaling pathways

Cell signaling comprises a complex system of communication within cells and between cells. This communication governs basic cellular activities and coordinates cell actions. The ability to respond to extracellular as well as endogenous signals is the basis of important processes such as development, immunity, tissue repair, cell growth, metabolic regulation and cellular homeostasis. A signal is generally received by a receptor which in turn initiates protein-protein interactions. One major mechansim in signaling is tyrosine phosphorylation. The human genome encodes 90 different tyrosine kinases (76), which may be divided into receptor-type and non-receptor-type tyrosine kinases. These proteins are crucial to important cell signaling pathways, *e.g.* the Epidermal growth factor receptor (EGFR), which regulates cellular growth. Increased activation of EGFR is strongly related to development of cancer (77), illustrating the importance of tyrosine kinases in cell signaling.

Viral recognition by PRRs initiate the activation of signaling pathways that lead to production of type I IFNs and cytokines. Two types of PRRs that recognize invading viruses have been identified; TLRs and RLHs. Virus sensing TLRs including TLR3, TLR7, TLR8 and TLR9 are localized on intracellular vesicles like endosomes/lysosomes suggesting that these TLRs recognize viral particles upon internalization and lysing of viruses. RLHs on the other hand detect viral components in the cytoplasma. In order to initiate downstream signaling pathways, TLRs and RLHs interact with adaptor proteins through TIR domains and CARD domains, respectively. The adaptor proteins utilized by TLRs are TRIF, MyD88, MAL, TRAM and SARM. However, the TLR signaling pathways are commonly divided into TRIF-dependent and MyD88-dependent signaling. The RLHs recruit the adaptor protein MAVS to mount antiviral signaling.

1.2.1 TRIF-dependent antiviral signaling

TLR4 is able to signal through TRIF. Still, TLR3 is the only TLR that exclusively utilizes TRIF to initiate signaling (38). In response to stimulation by dsRNA or poly IC, TLR3 recruits TRIF directly to its cytoplasmic TIR domain initiating a signaling cascade that activates several transcription factors including IRF3, NF-κB, AP-1 (19;22), and probably additional unidentified transcription factors. IRF3 controls transcription of type I IFN genes, whereas NF-κB and AP1 regulate expression of genes encoding inflammatory cytokines. TRIF forms a complex (either directly or indirectly) with the cytoplasmic adaptor molecule TNF-receptor associated factor 3 (TRAF3) and recruits non-canoncial IKKs, TBK1 and IKKε (also called IKKi) (78-81). This in turn results in phosphorylation of IRF3 on several sites. Phosphorylated IRF3 forms a dimer that translocates into the nucleus to induce expression of immune effector genes, such as IFN-β (82-85) and the chemokine IP10 (86;87).

The NF-κB family consists of five members: Rel A (p65), Rel B, C-Rel, p105 (precursor of p50) and p100 (precursor of p52). NF-κB proteins form homo- or heterodimers to regulate the expression of effector molecules (88). The most frequently activated form of NF-κB in TLR responses is a heterodimer composed of Rel A and p50 (89). Under unstimulated conditions, NF-κB is sequestered in the cytoplasma through interaction with inhibitory IκB proteins. Alternative to IRF3 activation, TRIF may induce the NF-κB pathway through the K63 linked

ubiquitin ligase TRAF6. TRAF6 activates TAK1 in an ubiquitin dependent manner (90;91). Subsequently TAK1, which is a member of the MAPKKK familiy, activates canoncial IKKs (IKKα and IKKβ). This leads to the phosphorylation and degradation of IκBs, nessecary for NF-κB to enter the nucleus. At the same time TAK1 is able to activate the MAPK pathway including JNK, p38 and ERK leading to phosphorylation and activation of AP1 transcription factors. Moreover, the C-terminal region of TRIF exhibits RHIM motifs which bind RIP1. RIP1 is involved in TNFR-mediated NF-κB activation and forms complex with TRAF6 and TAK1 (92;93). Thus the interaction between TRIF and both TRAF6 and RIP1 is likely to be important for maximal NF-κB and MAPK activation.

1.2.2 MyD88-dependent antiviral signaling

TLR7, 8 and 9 are able to detect viral patterns and initiate antiviral signaling. In contrast to TLR3, these PRRs interact with the adaptor protein MyD88 in response to ligand recognition. Upon interaction with TLR7 and TLR9, MyD88 immediately recruits members of the IL-1R associated kinase (IRAK) familiy (IRAK1 and IRAK4). The signaling pathways downstream TLR7/TLR8/TLR9/MyD88 may be divided into NF-κB dependent -, IRF7 dependent - and IRF5 dependent signaling. For activation of NF-κB, the IRAK family recruits TRAF6 which subsequently activates TAK1 in the same manner as decsribed above for TRIF-dependent NF-κB activation.

IRF7, which is the IRF family member most homologous to IRF3, potentially activates transcription of IFN-α and IFN-β, particularly in pDCs. MyD88 has been shown to be essential for the activation of IRF7 by TLR7, TLR8 and TLR9 (94-96). However, unlike IRF3 the expression of IRF7 is not ubiquitously, but it is induced in response to viral infections (84;97). IRF7 has been found in complex with MyD88, IRAK1, IRAK4, and TRAF6. Moreover, IRAK1 but not IRAK4 was shown to phosphorylate IRF7 (95;98). IKKα is able to phosphorylate IRF7 *in vitro*, but the functional relationship between IKKα and IRAK1 is not known (99). Additionally, TRAF3 is required for IRF7 activation and type I IFN production in response to TLR7 and TLR9 ligands (80). TBK1 and IKKε are reported to phosphorylate IRF7, thus activating the IFN-α promoter (79). However, TLR9-mediated IFN-α production is unaffected in TBK1 and IKKε deficient mice (95).

MyD88 signaling also leads to activation of IRF5, which is essential for the induction of a range of pro-inflammatory genes such as IL-6, IL-12 and TNF-α. Recent studies show that IRF5 is subjected to K63-linked ubiquitination by TRAF6, and that interaction with IRAK1 is required for the ubiquitination and activation of IRF5 (100). Ultimately, IRF5 translocates to the nucleus where it binds to ISRE motifs in promotor regions of cytokine target genes (101). IRF4 negatively regulates IRF5 by competing for MyD88 (102).

1.2.3 RIG-I-dependent antiviral signaling

The recent characterization of RIG-I has defined a novel antiviral signaling pathway. RIG-I activates NF-κB and IRF3 through its N-terminal CARD domains. Upon viral recognition RIG-I recruits the adaptor protein MAVS through CARD-CARD interaction (64-67). MAVS is anchored to the mitochondrial outer membrane through a hydrophobic C-terminal region (66). Its mitochondrial localization is apparently crucial for downstream signaling. The NS3/4A protease of hepatitis C virus (HCV) cleaves the C-terminal region of MAVS causing disruption in RIG-I-mediated IRF3 activation, probably due to mislocalization of cleaved MAVS from the mitochondria (65;103). MAVS is found to associate with TRAF3 (80;81;104). TRAF3 recruits the downstream kinases TBK1 and IKKε which regulates the phosphorylation of IRF3 and IRF7 (78;79). Phosphorylation of IRF3 and IRF7 induces the formation of homodimers or heterodimers that translocate into the nucleus for transcription of type I IFN genes (96). MAVS also interacts with the death domain-containing adaptor FADD which is involved in death receptor signaling (64). FADD forms a complex with caspase-8 and caspase-10 and is responsible for the activation of NF-κB downstream of MAVS (105). Very recently, MAVS and RIG-I were found to associate with the autophagy components Atg5-Atg12 (106), suggesting a link between innate immunity and autophagy.

1.2.4 TLR3 and RIG-I signaling: converging pathways

TLR3 and RIG-I are crucial to the innate immune response against viruses. Even though TLR3 signals from intracellular vesicles and RIG-I signals from the cytoplasma, both initiate the production of type I IFNs mainly through the activation of IRF3. The phosphorylation of IRF3 is mediated by TBK1 and IKKε. However, it remains unclear how these two kinases are

activated. Other members of the IKK family such as IKKα and IKKβ are phosphorylated by members of the MAPKKK family, which in turn are activated by TRAF6. Despite of the requirement for TRAF6 in TLR-mediated IKKα/β activation, activation of IRF3 downstream of TRIF is independent of TRAF6 (107). TRAF3 was originally identified many years ago as a protein that binds CD40, a TNFR family member important for adaptive immune responses (108;109). Later studies showed that TRAF3 was required for TLR3-mediated IRF3 activation, but not NF-κB activation. Thus, TRAF3 was important for TRIF-dependent responses (80). Also, MAVS, the RIG-I adaptor protein, has been shown to bind TRAF molecules through TRAF interacting motifs (TIMs) (67;104). Thus, multiple viral receptors may bind TRAF3 through direct or indirect mechanisms making TRAF3 a converging point for antiviral signaling pathways leading to IRF3 activation and type I IFN production. TRAF3 was also found to be required for IRF7 activation in response to TLR7 and TLR9 signaling (80), extending the role of TRAF3 in antiviral responses. The nature of the interaction between TRAF3 and other components of antiviral signaling pathways is to a large extent unknown. However, the deubiquitinase DUBA was recently shown to deubiquitinase TRAF3 and by this suppress RIG-I-mediated signaling (110). It has been speculated that TRAF3, like TRAF6, acts as an E3 ubiquitin ligase in antiviral signaling (110;111). Very recently, it was shown that TRAF3 is an adaptor that links an ubiquitin ligase complex to NIK, thus acting as an adaptor and not an E3 ligase in this process (112). Another protein, TRAF associated NF-κB activator (TANK), has been shown to interact with TBK1 and IKKε (113;114). This suggests that a multimeric complex comprised of at least TRAF3, TANK, TBK1 and IKKε might be important for both TLR3 and RIG-I-mediated phosphorylation of IRF3.

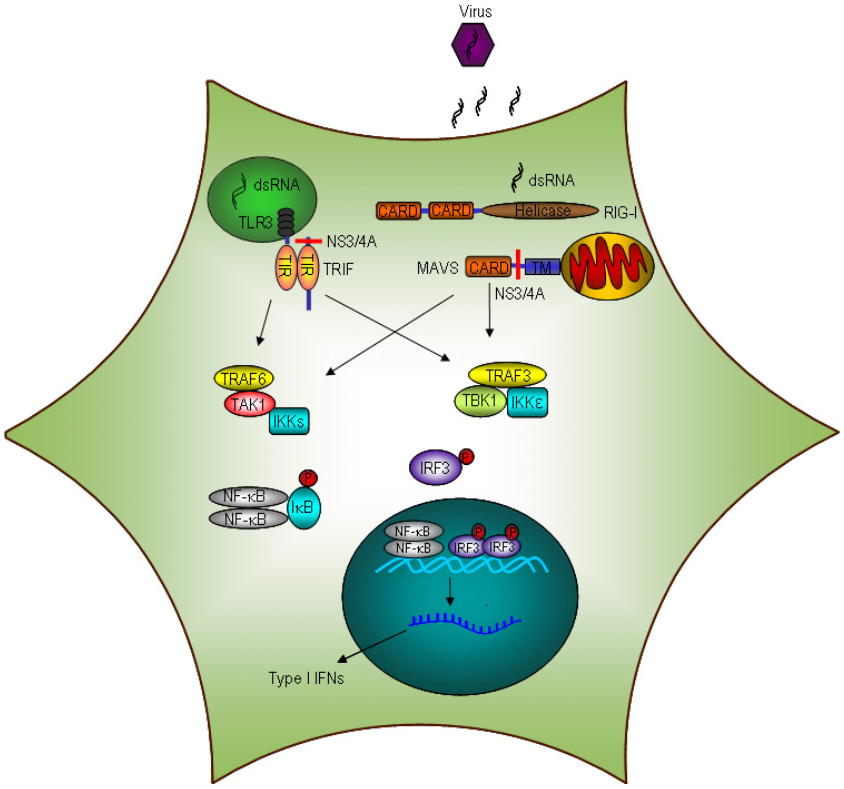

Figure 3: Sensing of viral dsRNA by TLR3 and RLHs. TLR3 binds dsRNA in endosomal compartments and recruits the adaptor protein TRIF. Signaling from TLR3 activates NF-κB through TRAF6 and the IKK complex, or IRF3 through TBK1/IKKε. RLHs recognize viral RNA in the cytoplasma and recruits the mitochondrial adaptor protein MAVS. RLH-dependent signaling activates NF-κB and IRF3 via the TRAF6/IKK complex or TBK1/IKKε, respectively. Both pathways induce production of type I IFNs. During an HCV infection, both TRIF and MAVS are cleaved and inactivated by the HCV NS3-4A protease (adapted from Meylan *et al.* 2006 (115)).

1.2.5 Accessory molecules in antiviral signaling

Proteins that accompany and assist other proteins, *e.g.* in activation and interaction, are commonly termed accessory molecules. Accessory molecules are required for recognition of

PAMPs, subsequent signaling and regulation of immune responses. Several accessory molecules have been described for TLR signaling, regulating events on the cell surface, *e.g.* MD2, or in the ER, *e.g.* UNC93B, and by acting directly on TLR ligands, *e.g.* CD14 (30;116-118). Recently, a number of accessory molecules have been identified and shown to act in discrete subcellular compartments at different levels of the signaling pathways leading to IRF3 activation and IFN-β production. A protein termed suppressor of IKKε (SIKE) was identified as a suppressor of both TLR3 and RIG-I-dependent IRF3 pathways. SIKE interacts with TBK1 and IKKε and inhibits both RLH- and TLR-dependent IRF3 activation by preventing interactions between IKKε or TBK1 with TRIF, RIG-I and IRF3. In contrast, SIKE has no effect on NF-κB activity (119). Also DEAD box protein 3 (DDX3) was shown to alter TBK1-and IKKε-mediated IRF3 activity. The vaccinia virus protein K7 inhibits TLR- and RIG-I-triggered IFN-β induction by preventing TBK1- and IKKε-mediated IRF3 activation, and DDX3 was identified as a target for K7. Impairment of DDX3 attenuates antiviral responses to dsRNA and SV, and DDX3 associates with IKKε upon virus infection (120). In addition, SINTBAD and NAP1 have been proposed to link TBK1 and IKKε to virus-activated signaling cascades (121;122). Hence, SIKE, DDX3, SINTBAD and NAP1 represent novel regulators of antiviral kinases; SIKE functions to suppress TBK1 and IKKε activity, whereas DDX3, SINTBAD and NAP1 positively alter their function.

The RIG-I adaptor protein, MAVS, is anchored to the mitochondrial outer membrane. A novel protein that also localizes to the mitochondrial outer membrane was recently identified and termed mediator of IRF3 activation (MITA). MITA was shown to induce IRF3 activation and be important for antiviral immune responses. Morover, MITA interacts with MAVS and IRF3, and recruits TBK1 to the complex. TBK1 phosphorylates MITA *in vitro*, an event that is required for MITA-induced IRF3 activation (123). Another study identified the same protein as an ER resident protein and termed it STING (124). In this study, STING was found to activate NF-κB and IRF3, and to induce type-I IFNs. Ablation of STING abrogates RIG-I dependent antiviral responses, but does not affect the TLR3 pathway. Interestingly, STING interacts with translocon-associated proteins, and the translocon appears to be important for antiviral response. A member of the NOD-like receptors, NLRX1 (also called NOD5), contains a putative mitochondrial targeting sequence within the first 20 amino acids instead of the typical *N*-terminal CARD domain. A recent study proposed that the NACHT domain of

NLRX1 interacts with the CARD domain of MAVS, suggesting that NLRX1 competes with RLHs. NLRX1 was observed to reside on the mitochondrial outer membrane. Overexpression of NLRX1 interferes with SV-induced RLH-MAVS interaction, as well as RLH- and MAVS-induced IFN-β production. Depletion of NLRX1 induces type-I IFN production and decreases viral replication. The TLR3-dependent pathway is not affected by NLRX1 (125). Still, the exact function of NLRX1 is controversial. It has also been reported that NLRX1 amplifies NF-κB and JNK pathways induced by TNF, apparently through increased generation of reactive oxygen species (ROS) in the mitochondria. This study did not report any function of NLRX1 on the IFN-β or NF-κB pathways (126). Nevertheless, MITA/STING and NLRX1 are novel regulators of RLH-dependent antiviral signaling. Both appear to modulate the function of signaling components on membranes, however, they display opposite effects on antiviral responses.

1.3 PRR-stimulated responses

Signaling from PRRs leads to the induction of cytokine genes and establishment of innate immunity. TLR and RLR responses stimulate both production of pro-inflammatory cytokines including IFNs, IL-1, TNFα and IL-12, and anti-inflammatory cytokines such as IL-10. Antiviral signaling typically activates IRF3 and IRF7 which regulate the expression of type I IFNs (IFN-α and IFN-β). DCs produce high amounts of type I IFNs leading to an antiviral state in the cells. The antiviral state is characterized by inhibition of both viral replication and cell proliferation, and also enhancement of the ability of NK cells to lyse virally infected cells.

1.3.1 Type I interferons and the antiviral state

In the late 50s a soluble factor produced by cells in response to inactive, non-replicating virus was described to inhibit subsequent infection with live virus (127-129). Today this interfering factor is known as Type I IFN (IFN). The type I IFN familiy consists of a growing group of IFN proteins wherein the best described types are IFN-α and IFN-β (130). Several IFN-α subtypes are found, whereas IFN-β is encoded by only a single gene (131). The transcriptional regulation of type I IFNs is best understood for IFN-β. In response to virus or viral components a complex termed the enhanceosome is assembled on the IFN-β promoter (132;133). The enhanceosome comprises at least three classes of transcription factors; ATF-2,

NF-κB and IRF3. Once secreted, IFN-β binds the IFN-α/-β receptor (IFNAR) in an autocrine and paracrine manner, initiating a positive feedback loop that results in further production of type I IFNs. The IFNAR, which consists of IFNAR-1 and IFNAR-2, activates the janus kinase (JAK) family members JAK1 and Tyk2. This in turn leads to phosphorylation of signal transducer and activator of transcription1 (STAT1) and STAT2. STAT1 and STAT2 form a complex with IRF9, termed interferon stimulated gene factor 3 (ISGF3), which initiates the transcription of more than 300 IFN stimulated genes (ISGs) on interferon sensitive response elements (ISRE), including genes encoding 2′-5′-oligoadenylate-synthetase (OAS), PKR (double-stranded RNA–dependent protein kinase), MxA (myxovirus resistance A), ISG15, IRF-7 and IP-10 and more. These proteins elicit an antiviral state by inhibiting different stages of virus replication and spread (134).

Among the numerous antiviral mechanisms mediated by ISG encoded proteins, four main effector pathways of IFN-mediated antiviral response have been distinguished; the Mx GTPase pathway, the 2´-5´-oligodenylate-synthetase (OAS)-directed ribonuclease L pathway, the PKR pathway and the ISG15 ubiquitin-like pathway. These pathways individually modify protein function, block viral transcription, degrade viral RNA and inhibit translation. ISG15, which is one of the most highly induced ISGs, was initially identified as an ubiquitin homolog (135). ISG15 mediates an IFN-induced ubiquitin-like protein response referred to as ISGylation. Through the action of specific enzymes, ISG15 is activated and conjugated to substrate proteins. All components of the ISGylation pathway are induced by type I IFNs. Through conjugation to target proteins, ISG15 modifies important mechanisms of the host immune system and the viruses, *e.g.* virus-mediated degradation of IRF3 which is prevented by ISGylation (136). The Mx GTPases belong to a class of proteins termed guanine hydrolysing proteins that is involved in scission to mediate vesicle budding, oranogenesis and cytokinesis. In humans, only MxA have been shown to elicit antiviral activity. Upon IFN-mediated induction, MxA proteins accumulate as oligomers in the cytoplasma where they partly associate with a subcompartment of the ER (137). During viral infection, MxA monomers are released and bind viral components such as nucleocapsids. Consequently the virus is trapped and prevented from replicating at early time points (138). MxA may for instance associate with subunits of the influenza virus polymerase, thus inhibiting viral gene transcription (139). The OAS proteins are expressed at low levels in unstimulated cells, but

are induced by virus infection. They activate RNase L which in turn cleaves mRNA (140;141). Hence, OAS in combination with RNase L constitutes an antiviral RNA decay pathway. RNA degraded by RNase L can activate other PRRs, including RIG-I and Mda5, thus inducing type I IFN gene expression. PKR is constitutively expressed, and is also induced by type I IFNs (142). It accumulates in the cytoplasma as an inactive monomer, and is activated directly by viral RNA and other ligands (143-145). Activated PKR forms a dimer and functions in viral defence by phosphorylating eukaryotic translation initiation factor 2α (EIF2α), and by that inhibiting translation.

NK cells are an important component of the antiviral immune defence with the ability to eliminate virus-infected cells. NK cells are traditionally considered as effector cells with its rapid activation and cytotoxic potential. Early activation of NK cells is mediated by cytokines, *e.g.* type I IFNs produced by DCs in the antiviral state. Activated NK cells kill infected cells by exocytosis of small proteins such as perforin and granzymes. In addition, activated NK cells secrete interferon-γ (IFN-γ) and augment pathogen clearance by macrophages (146;147).

1.3.2 PRR-stimulated responses: linking innate and adaptive immunity

Type I IFNs are important for antiviral innate immune responses. In addition, this class of cytokines has several effector functions in the development of adaptive immunity. Adaptive immune responses are initiated by antigen-presenting cells, such as DCs and macrophages. DCs and macrophages arise from monocytes, which circulate in the body and, depending on the correct signal, differentiate into DCs or macrophages. It has been reported that IFNα/β may act very early to stimulate DC differentiation from monocytes (148;149). DCs in peripheral tissues capture exogenous antigens and PRR ligands, and migrate to the lymph nodes to present the processed antigen to naive T lymphocytes via MHC molecules. This migration is regulated by PRR-induced chemokines (150-152). Upregulation of the chemokine receptor CCR7, which renders DCs sensitive to the chemokines CCL19 and CCL21, has been observed in human mDCs upon treatment with Type I IFNs (153). At the same time the DCs undergo a maturation process turning them into immunostimulatory antigen-presenting cells able to prime effector T cells. Type I IFNs produced after internalization of virus or viral nucleic acid, *e.g.* CpG and dsRNA, has been shown to induce phenotypic maturation of DCs, assessed by upregulation of co-stimulatory molecules such as

CD40, CD80 and CD86 (154-156). The upregulation of CD40 is markedly diminished in DCs from STAT knock-out mice after stimulation with TLR4 and TLR9 agonists. This suggests that DC activation upon TLR signals may largely depend on the establishment of an autocrine or paracrine positive type I IFN feedback-loop (154;157). Overall, type I IFNs exhibit multiple effects on DCs, including differentiation, migration and maturation.

Besides their influence on DC function, type I IFNs have been implicated in cross-presentation of viral antigens leading to cell-mediated cytotoxicity and killing of viral-infected cells. During infection with lymphocytic choriomeningitis virus (LCMV), cross-presentation occurs by a mechanism that depends on type I IFNs (158). Also, another study showed that cross-priming induced by TLR3 and TLR4 ligands is completely dependent on Type I IFNs (159). Shulz *et al.* demonstrated that immunization with virus-infected cells or cells containing synthetic dsRNA lead to increase in CD8+ T cell cross-presentation against cell-associated antigens, which is largely dependent on TLR3 expression by APCs (46).

1.4 Regulation of interferon transcription

The IFN-β gene induction is a highly ordered process and includes tight regulation by several different transcription factors. The IFN-β promoter consists of at least four positive regulatory domains (PRDs), termed I, II, III and IV. In comparison, the IFN-α promoter contains only PRD I- and III- like elements. IRF family members bind to PRD I and III, NF-κB to PRD II and AP1 (also called ATF2/c-Jun) to PRD IV. These transcription factors assemble a complex with the high mobility group protein (HMG)-I(Y). This complex is commonly termed the enhanceosome, and is responsible for the regulation of interferon transcription. The enhanceosome recruits histone acetyl transferases (HATs), including the general-control-of-amino-acid synthesis 5 (GCN5) and CREB binding protein (CBP), to the nucleosome. Histone acetylation leads to the recruitment of a nucleosome modification complex termed the Brahma-related gene (BRG)-Brahma (BRM) associated factor (BAF) complex, which forces displacement of the nucleosome from the transcription start site. At the same time the transcription complex TFIID binds to the promoter. Subsequently, transcription of the IFN-β gene can take place (160).

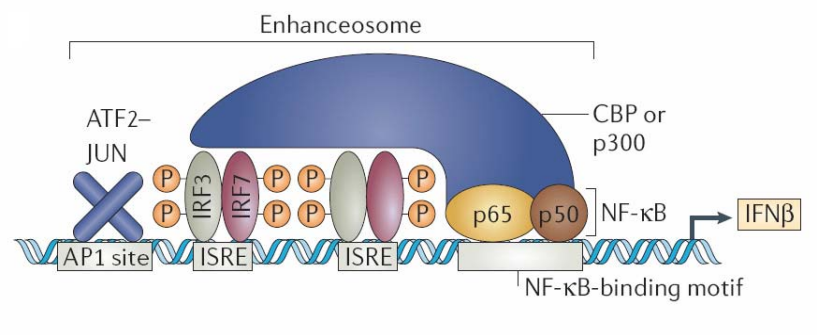

Figure 4: The enhanceosome. One NF-κB complex (p50 and p65) and two IRF dimers (IRF3 homodimers, IRF7 homodimers or IRF3-IRF7 heterodimers) bind independently to their respective binding elements. These transcription factor complexes cooperatively interact with each other and the co-activators CBP or p300 to form the enhanceosome. Assembly of the enhanceosome induces transcription of the IFN-β gene (160).

1.4.1 The IRF family

To date nine members of the IRF family, IRF1-9, have been identified (161;162). They contain a well-conserved *N*-terminal DNA binding domain (DBD) (162;163) which forms a helix-turn-helix and recognizes similar DNA sequences (164). The *C*-terminal region of the IRFs, except for IRF1 and IRF2, comprises an IRF association domain (IAD) which enables them to form homodimeric and heterodimeric interactions with familiy members or other transcription factors. IADs show structural similarities with Mad-homology 2 (MH2) domains of the Smad family of transcription factors (165;166), and Smad 3 was recently found to interact with IRF7 for induction of type I IFN production (167). The specificities of IRFs appear to be mediated by their cell type specific expression, distinct interaction with other IRFs, other transcription factors or cofactors, and discrete regulatory mechanisms. Four of the IRFs, IRF1, IRF3, IRF5 and IRF7, are categorized as positive regulators of type I IFN gene transcription. IRF3 and IRF7 are the main inducers of type I IFNs and will be discussed in

more detail later. In addition, both IRF1 and IRF5 have been implicated in type I IFN gene induction by viruses.

IRF5 was identified to be crucial for induction of inflammatory cytokines and type I IFNs by all TLRs. Still, studies in knock-out mice revealed that IRF5 is involved in induction of cytokines such as IL-6, IL-12 and TNF-α rather than in type I IFN induction (101). In contrast to IRF3 and IRF7, the activation mechanism for IRF5 is poorly understood. It has been shown that IRF5 acts in a trimeric complex with TRAF6 and MyD88, and that IRF5 is subjected to ubiquitination by TRAF6 (100). Also it has been suggested that IRF5 is activated through IRAK kinases, in particular IRAK1 which was required for IRF5 ubiquitination and activation (100). In contrast, IRF4 negatively regulates IRF5 by competing for MyD88 (102). Nevertheless, the precise role of IRF5 in type I IFN gene induction remains to be assessed.

1.4.2 The interferonic IRFs: IRF3 and IRF7

IRF3 and its close homolog, IRF7, are regarded as the main transcription factors for type I IFN gene induction by viruses. IRF3 is constitutively expressed in the cytosol. Upon viral challenge, it undergoes phosphorylation, dimerization and translocation to the nucleus (82;84;168). Potential phosphorylation sites have been identified in the *C*-terminal region of IRF3; serine 385, 386, 396, 398, 402 and 405, and threonine 404. Based on a crystal structure, two models for the activation and dimerization of IRF3 have been presented. One is the «phosphorylation-induced dimerization model», where the S385 and S386 of one monomer of IRF3 interact with a hydrophobic pocket of another monomer of IRF3 or IRF7 (166). The other model is called «the autoinhibitory model» and suggests that interaction between *N*- and *C*-terminal α-helical structures flanking a β-sandwich IAD core in IRF3 form a hydrophobic structure in the inactive state. This structure opens upon multiple phosphorylations of the suggested threonine and serines which generate massive negative charges (165). In whatever way, upon an antiviral response the dimeric form of IRF3, either a homodimer or a heterodimer with IRF7, translocates to the nucleus where it forms a complex with the coactivators CBP and/or p300 and binds to its target DNA sequences in type I IFN genes in addition to some cytokine and chemokine genes. The different dimers act differentially on the type I IFN gene family members. IRF7 is expressed at low levels in most cell types, but is strongly induced by type I IFN responses that includes the IFNAR (84;97).

The IFNAR recruits the ISGF3 complex which in turn activates gene expression of IRF7. Like IRF3, IRF7 is expressed in the cytosol where it undergoes phosphorylation and dimerization upon virus infection. The IRF7 hetero- or homodimer translocates to the nucleus to activate gene expression. IRF3 activates the IFN-β gene rather than the IFN-α genes, whereas IRF7 is a strong activator of both IFN-β and IFN-α genes (96). Recently it was demonstrated that IRF3 and IRF7 are involved in the induction of a new class of IFN family members, type III IFNs, including IFN-λ1, IFN-λ2 and IFN-λ3. This new class of IFNs shows similar biological antiviral function to type I IFNs (169).

1.4.3 The ATF/CREB family

The ATF/CREB family consists of a large group of basic-region leucine zipper (bZIP) transcription factors with a diverse set of biological functions. Despite their diverse activities they share a common ability to respond to stress signals. Apart from its role in the enhancosome, ATF2 is mainly involved in transcriptional control of stress response genes, as is ATF3 (170). ATF6 plays a role in the regulation of gene induction upon endoplasmic reticulum stress and serum-responses (170-172). CREB and ATF1 regulate transcription in response to intracellular cAMP, whereas ATF4 is a negative regulator of cAMP levels (173). All ATFs bind to the ATF/CRE consensus sequence TGACGTCA (172). Transcription of ATF3 is induced by proinflammatory cytokines and stress signals. Interestingly, in contrast to other ATF family members ATF3 has been shown to repress rather than activate transcription from promoters with ATF/CRE binding sites (172). In accordance with this, ATF3 was recently implicated in regulation of immune responses and was identified as a negative regulator of TLR4-elicited signaling (174).

1.5 C-Src tyrosine kinase

In 1911, Peyton Rous observed a microscopic particle, later identified as a virus that could induce sarcoma in chicken (175). The virus was named Rous sarcoma virus (RSV). In 1976, the RSV genome was found to encode an oncogene nearly identical to a human proto-oncogene (176). The viral gene v-Src and the human gene c-Src (cellular Src) were shown to display differences in their C-terminal sequences. Further studies showed that the proteins

encoded by these genes were tyrosine protein kinases (177), and ultimately that v-Src exhibits constitutive tyrosine kinase activity (178).

1.5.1 Domain structure and kinase activation

The Src family of non-receptor protein tyrosine kinases have been implicated in numerous cellular processes, including gene transcription (179;180) adhesion (181), migration (182;183), cell cycle control (184-186) apoptosis (187;188), differentiation (189;190) and more. The family consists of nine members; Src, Fyn, Yes, Fgr, Lyn, Hck, Lck, Blk and Yrc. Src, Yes, Fyn and Yrc are ubiquitously expressed, whereas the others are expressed in more restricted patterns (191). In vertebrates all members of the Src family display similar structure (178). They show several conserved domains in addition to the tyrosine kinase domain (SH1, Src homology 1). The kinase domain is responsible for tyrosine kinase activity and plays a crucial role in substrate specificity. The amino acid sequence preferably phosphorylated by c-Src is EEEIY$^{G}/_{E}$EFD (192). The SH2 domain binds phosphotyrosine-rich sequences on other proteins, whereas the SH3 domain binds proline-rich regions. Some proteins interacting with c-Src contains an optimum binding sequence, pYEEI. However, not all proteins binding to the c-Src SH2 domain harbours such phosphorylated sequence. All known SH3 ligands carry the proline-rich consensus sequence PXXP, and the amino acid adjacent to proline determine the specificity of the SH3 domain. In addition the Src proteins consist of an *N*-terminal variable region that is modified by myristotylation and palmitoylation (also called the SH4 domain), as well as a flexible tyrosine containing tail (178;193). The *N*-terminal region is responsible for membrane binding. The SH2 and SH3 domains are important for regulation of the catalytic activity of the c-Src. In order to stabilize an inactive conformation, the SH3 domain interacts with the catalytic domain and the linker region between the catalytic domain and the SH2 domain. In this inactive state the SH2 domain binds phosphorylated tyrosine 527 (Y527) in the C-terminal region. Dephosphorylation of Y527 leads to stimulation of c-Src catalytic activity. Hence, the substitution of the tyrosine on position 527 by another amino acid residue constitutively activates c-Src (194). However, the regulation of c-Src activity occurs at two sites. Phosphorylation of tyrosine 416 (Y416), which is probably mediated by autophosphorylation, stimulates maximal activation of c-Src and genreates a binding site for SH2 domains of other proteins. Thus the two regulating phosphorylation events of c-Src have opposite results; phosphorylation of Y416 in the activating loop of the kinase domain

activates the enzyme, whereas phosphorylation og Y527 in the C-terminal region causes its inactivation.

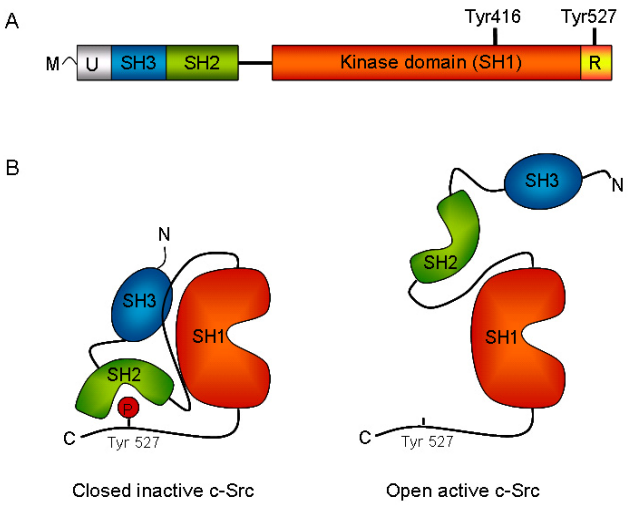

Figure 5: Domain structure and activity model of c-Src. A) The domain structure of c-Src includes a kinase domain, a SH2 domain, a SH3 domain and an N-terminal unique region. B) Schematic diagram illustrating inactive and active c-Src. In the inactive assembled form, the SH3 domain interacts with the kinase domain and the linker region between the kinase domain and the SH2 domain. The SH2 domain binds to phosphorylated Tyr527. Dephosphorylation of Tyr527 and autophosphorylation of Tyr416 results in the activation of c-Src. In the active form the SH2 and SH3 domains are displaced and free to interact with external ligands (Adapted from Young *et al.* 2001 (195)).

1.5.2 Roles of c-Src in innate immunity

C-Src was the first proto-oncogene to be identified and also the first protein shown to possess intrinsic kinase activity. Its role in tumorigenesis have been extensively studied, and chemical inhibitors targeting c-Src PTKs have been developed as potential drugs for cancer treatment. Several studies demonstrate that c-Src PTKs may modulate functions of the immune system as well. Tyrosine phosphorylation is an important post-translational mechanism that regulates diverse intracellular pathways in the innate immune system. The functions of TLRs are

modulated by tyrosine phosphorylation of *e.g.* TLR2, TLR3, TLR4, TLR8, TLR9 and Mal (196-203), but the exact molecular mechanisms involved have not been disclosed. Nevertheless, it has been shown that inhibition of c-Src PTKs can attenuate sepsis, which is caused by excessive production of inflammatory cytokines (204). In particular, c-Src tyrosine kinases have been implicated in immune responses induced by LPS. Mice deficient of c-Src family kinases Hck and Fgr are resistant to endotoxic shock (205), whereas mice expressing constitutively active Hck display enhanced immune responses to LPS (206). Moreover, the c-Src family member Lyn is activated by LPS, and is coupled to the LPS receptor CD14 in human monocytes (207). Another study shows that Hck and Lyn kinase activity is increased by LPS, and that inhibition of PTK activity reduces LPS-induced responses including TNF-α production in monocytes and macrophages (208-210). Still other studies show that NF-κB activation is unaffected in macrophages from wild type and hck-/-fgr-/-lyn-/- triple knock out mice (211). This indicates that c-Src PTKs may be important, but not critical for LPS signaling in monocytes and macrophages.

During inflammation, tissue cells are important mediators of the host response. Epithelial cells can produce host inflammatory effectors such as monocyte chemoattractant protein 1, IL-6 and IL-8 in response to inflammatory stimuli (212). These responses are partly regulated by c-Src PTKs. In human airway epithelial cells TNF-α stimulation increases activation of c-Src (213). In human lung epithelial cells, TNF-α activates c-Src through protein kinase C (PKC) on one hand, and NF-κB-inducing kinase (NIK) via TRAF2 on the other hand. These two pathways converge at IKKβ, suggesting that NIK phophorylate two serine residues on IKKβ, whereas c-Src PTKs phosphorylate two tyrosine residues. IKKβ in turn activates NF-κB to induce inflammatory genes (213;214). In conclusion, c-Src PTKs may be important mediators of innate immune responses at several levels. However, the specific role of each c-Src family member is largely unknown.

2 AIMS OF STUDY

In light of the considerable global health threat that viral infections represent in regard to inflammatory diseases and cancer, it was of interest to study the signaling mechanisms induced upon the immediate interaction between the host and the virus. It was required to delineate signaling components downstream pattern recognition receptors, and study the mechanisms regulating the early response to viral infections induced by these receptors. The study of molecular mechanisms behind host-virus interactions is important in the development of new strategies for antiviral therapeutics and vaccines, as well as cancer treatment. This is exemplified through the clinical development of a number of substrate-based peptidomimetic agents targeting virally encoded proteins, *e.g.* the HCV NS3/4A protease.

In particular, we aimed to:

1) Examine if the tyrosine kinase c-Src is involved in TLR3-mediated antiviral signaling, and characterize its possible role in dsRNA-induced antiviral responses (***Paper I***)
2) Characterize the intracellular localization of dsRNA, TLR3 and c-Src, and study the uptake mechanisms of dsRNA in monocyte-derived dendritic cells (***Paper I***).
3) Elucidate if c-Src is important in antiviral responses mediated by the cytoplasmic receptor RIG-I, and delineate its possible function in antiviral signaling pathways (***Paper II***).
4) Compare the role of c-Src in activation of IRF3 and IRF5 in TLR-mediated transcription (***Paper III***).

3 SUMMARY OF PAPERS

PAPER I: Toll-like receptor 3 associates with c-Src tyrosine kinase on endosomes to initiate antiviral signaling

Viral dsRNA initiates antiviral signaling through TLR3. The signaling pathways downstream of TLR3 comprise several components, including kinases. In this paper we examine the role of the tyrosine kinase c-Src in TLR3-mediated antiviral signaling. We show that c-Src tyrosine kinase is activated by viral dsRNA in human monocyte-derived dendritic cells, and is recruited to TLR3 in response to dsRNA. Treatment with c-Src chemical inhibitors reduces IRF3 activation. Further, nuclear translocation of IRF3 is abolished in c-Src deficient mouse embryonic fibroblasts, as is activation of STAT1, suggesting a role of c-Src in antiviral immunity. Expression of a c-Src kinase inactive mutant shows that c-Src elicits its function downstream of TRIF. We also demonstrate that c-Src tyrosine kinase conveys and regulates dsRNA-mediated Akt activation, but not dsRNA-induced MAPK activation. This shows that c-Src differentially regulates TLR3 responses. Studies using confocal scanning microscopy reveal that TLR3 is expressed in endoplasmic reticulum in resting cells and that a fraction of TLR3 translocates to endosomes/lysosomes upon dsRNA treatment. DsRNA is internalized into intracellular vesicles by clathrin-mediated endocytosis and is recognized by TLR3 in the endosomal pathway. Moreover, c-Src tyrosine kinase localizes to endosomes/lysosomes where it interacts with TLR3/dsRNA in order to transmit antiviral signaling.

PAPER II: The tyrosine kinase c-Src enhances retinoic acid inucible gene-I (RIG-I)-elicited antiviral signaling

TLR3 transmits antiviral signaling from intracellular vesicles such as endosomes/lysosomes. However, cytoplasmic sensing of virus or viral products is conveyed by RIG-I like helicases. In this paper we study the role of c-Src tyrosine kinase in RIG-I-mediated antiviral response, and delineate molecular mechanisms behind the function of c-Src in antiviral signaling pathways. We show that c-Src is activated upon infection with Sendai virus (SV). Impairment of c-Src through chemical inhibition or transient expression of a c-Src kinase inactive mutant attenuates IRF3 activation as well as IP-10 and IFN-β production after SV-infection or

expression of RIG-I and its adaptor protein MAVS. Also, treatment with c-Src siRNA impairs SV-induced IFN-β synthesis and IRF3 nuclear translocation. Likewise, the induction of IP-10 and IFN-β mRNA is abolished in c-Src deficient cells, as is nuclear translocation of IRF3. Immunoprecipitation studies reveal that c-Src interacts with components of the RIG-I signaling pathway, including RIG-I, MAVS, TRAF3 and TBK1, indicating that c-Src functions in a signaling complex downstream of RIG-I. Finally, using TRAF3 deletion mutants, we show that the interaction between c-Src and TRAF3 occurs within the RING domain of TRAF3.

PAPER III: Activating transcription factor 3 is regulated by c-Src tyrosine kinase and confers differential gene expression downstream Toll-like receptors

Transcription of cytokines which modulate immune responses is regulated by a complex network of transcription factors, including several members of the IRF family. In this study we compare the effect of c-Src inhibition on TLR-mediated IRF-3 and IRF-5 activation. Interestingly, we find that chemical inhibition of c-Src enhances unmethylated CpG-induced IRF5 activation, whereas it has the opposite effect on CpG-induced IRF3 activation. Moreover, chemical inhibition of c-Src attenuates transcription of the IRF5-regulated gene IL-6 in response to CpG, whereas transcription of the IRF3-regulated gene IP-10 is elevated. On the other hand, we show that inhibition of PI3-kinase reduces both CpG-mediated IRF3 and IRF5 activation. Also PI3-kinase inhibition attenuates mRNA transcripts of IL-6 and IP-10, suggesting that c-Src and PI3-kinase exert distinct roles in the regulation of IRF5 activation. Next, we find that c-Src inhibition results in enhanced IL-6 mRNA induction for all TLR ligands investigated, whereas TLR-elicited IP-10 induction is abrogated. This indicates that c-Src exerts a similar role downstream of both MyD88-dependent and -independent pathways. To extend these findings, we show that the TLR ligands LPS, R484, poly IC and CpG enhance IL-6 mRNA induction in c-Src deficient mouse embryonic fibroblasts (MEFs). In contrast, levels of IP-10 in response to TLR ligands are reduced in the same cell-line. Ultimately, we present that TLR stimulated induction of activating transcription factor 3 (ATF3) is positively regulated by c-Src. Depletion of ATF3 by siRNA markedly enhances IL-6 production and attenuates IP10 production upon CpG stimulation, indicating that c-Src

regulates intracellular ATF3 levels and through ATF3 differentially affects gene expression upon TLR-stimulation.

4 DISCUSSION

4.1 TLR3 translocates to dsRNA containing endosomal compartments

The innate immune system comprises a complex set of coordinated events necessary to mount an immediate defence against invading pathogens. These events include sensing of PAMPs, uptake of the pathogen, and signal transduction leading to the induction of immune response genes. Upon infection with certain viruses, *e.g.* EMCV and West Nile viruses, viral dsRNA is recognized by TLR3. In ***paper I*** we studied the dynamics of viral dsRNA internalization in human mDCs by confocal microscopy. We observed that dsRNA was internalized into vesicles that were transported from the cell periphery towards the centre of the cell and subsequently trafficked into tubular structures identified as lysosomes. The fluorescently labelled dsRNA sequence (dsRNA-Cy5) used in this study corresponds to a sequence from human rhinovirus. Leonard *et al.* reported that it requires a dsRNA length of at least 40 to 50 base pairs to bind a TLR3-ECD dimer and induce signaling (215). On the other hand, it has previously been shown that shorter chains of poly IC and even oligomeric IC can induce receptor signaling (216). The dsRNA-Cy5 sequence used in ***paper I*** is only 34 base pairs and could be unable to bind TLR3 and initiate antiviral response. Moreover, the fluorescent Cy5 dye is a bulky label. This may affect the binding between the ligand and the receptor. We proved that the sequence was biologically active, as it stimulated IFN-β production in TLR3 expressing cells and induced IFN-β and TNF in human mDCs. Finally, we found that dsRNA co-localized with markers for early and late endosomes/lysosomes, and showed extensively overlap with CpG-DNA, which is reported to be internalized by endocytosis (40). Collectively, these results show that the TLR3 ligand dsRNA is internalized into mDCs by endocytosis and is trafficked through the endosomal pathway.

The general believe has been that the nucleic acid recognizing TLRs, TLR3, TLR7, TLR8 and TLR9, localize to endosomal membranes and recognize their ligands in the endosomal lumen. However, only TLR9 is unambiguously shown to localize to endosomes (39;40). The same study stated that TLR9 is expressed in the endoplasmic reticulum (ER) in nonstimulated cells. Likewise, we demonstrated in ***paper I*** that TLR3 was expressed in the ER in resting cells, both in HeLa cells stably expressing TLR3 and in human mDCs. In cells stimulated with dsRNA, a fraction of TLR3 translocated to endosomes/lysosomes. The mechanism by which

TLR3 translocates from the ER to the endosomes/lysosomes still remains unexplained. Newly synthesized proteins are normally released from the ER and travel to different compartments, *e.g.* endosomes/lysosomes or the plasma membrane, through the Golgi apparatus via the secretory pathway. As presented in ***paper I***, Golgi-dependent mechanisms could not be detected for TLR3 translocation. Thus, TLR3 could be trafficked through direct delivery of ER membranes to endosomes, similar to the mechanism suggested for ER-mediated phagocytosis (217;218). However, another study failed to observe contact between the ER membrane and the plasma membrane. The same study showed that the plasma membrane is the main constituent of phagosomes, and that ER membranes are almost absent in phagosomes/endosomes (219). Hence, it is possible that other mechanisms may be responsible for the translocation of TLR3. If it is a signal-mediated event, TLR3 could either receive a signal elicited by dsRNA that triggers trafficking to the endsomes/lysosomes, or an additional molecule could trigger translocation of TLR3. However, TLR9 travels normally to the endosomes in CpG-DNA treated cells even in MyD88 deficient DCs (40). Therefore, if translocation of TLR9 is mediated by CpG-DNA, it occurs in a MyD88-independent way. Recently, autophagy was reported to be necessary for TLR7 dependent recognition of certain ssRNA viruses and for production of IFN-α in pDCs, by facilitating transport of cytosolic viral replication intermediates into lysosomes (220). It is possible that autophagy mediates signal-induced or constitutive uptake of TLR3 from the ER or the cytosol. In that way, TLR3 could be trafficked into the endosomal pathway through autophagy. Interestingly, the ER-resident membrane protein UNC93B was shown to interact with TLR3, 7 and 9, and to be required for appropriate signaling from these TLRs (118). Very recently, it was demonstrated that neither TLR7 nor TLR9 exits the ER in mice carrying a mutation in the UNC93B gene (24). Still, it is possible that TLR3 and TLR7/TLR9 travel by independent mechanisms, and that these processes are constitutive. Hence, it would be interesting to explore if the translocation of TLR3 from ER to endosomes/lysosomes is regulated, and determine receptors and signaling pathways that might be implicated.

4.2 C-Src tyrosine kinase associates with TLR3 on dsRNA containing endsomes/lysosomes

In ***paper I***, we found that c-Src tyrosine kinase was activated and recruited to TLR3 in response to dsRNA. Therefore, we prompted to study the subcellular localization of c-Src in

cells triggered by dsRNA. In particular, we addressed the cellular site of interaction between c-Src, TLR3 and dsRNA. In accordance with previous reports (221;222), we observed that c-Src was expressed on the plasma membrane and in intracellular vesicles. In ***paper I***, we found that treatment with dsRNA induced increased co-localization between c-Src and markers for early endosomes and late endosomes. Hence, endosomes/lysosomes might be the cellular site of action for c-Src in TLR3 dependent responses, given that c-Src interacts with TLR3 and/or TRIF upon dsRNA challenge to convey antiviral signaling. In this regard, we demonstrated that TLR3 encircled the surface of vesicles containing dsRNA in their lumen. These vesicles were identified as endosomes, as they were positive for the PI-3 phosphate-binding module FYVE, which is expressed on early endosomes. TLR3 was also found on vesicles positive for the late endosomal marker in dsRNA-stimulated mDCs. Interestingly, TLR3 co-localized with c-Src on these vesicular structures. Hence, we believe that a fraction of TLR3 translocates to the endosomal pathway where it recognizes dsRNA and associates with downstream signaling components, including c-Src, leading to an active signaling complex on the cytoplasmic tail of TLR3. Importantly, the TLR3-ECD binds dsRNA only at acidic pH (pH 6,5 and below) (215;216), indicating that TLR3 binds its ligand in endosomal compartments. There are several examples on signaling that occurs from endosomal membranes. Epidermal growth factor (EGF) receptors are known to preserve their dimerization and kinase activity on endosomes (223;224), and the kinases Raf, MEK and p42/44 MAP kinase are also associated with endosomes (225). Our findings regarding subcellular localization are based on confocal studies using markers for early and late endosomes. However, the marker for late endosomes also stains lysosomes, which makes it difficult to assess the exact site of TLR3 signaling within the endosomal pathway. The acidic environment in the endosomes/lysosomes is important as it allows for the degradation/uncoating of viruses and bacteria. Some viruses that penetrate through clathrin-mediated endocytosis fuse with the endosomal membranes in response to the pH drop, either in the early (pH 6,5 -6,0) or the late (pH 6,0 – 5,5) endosome (226). Subsequently the viral genetic material is released into the cytoplasm, thus avoiding degradation and detection by TLRs. Hence, the exact localization of TLRs within the endosomal pathway could be important. Based on the homology between TLR3 and TLR9, it is likely that TLR3-mediated signaling occurs from the same cellular compartment as TLR9-mediated signaling. A study by Honda *et al*. shows that retention of CpG in endosomal vesicles is required for activation of IRF7 and IFN-α production through TLR9 and MyD88

(227). Very recently, a study showed that conformational changes induced by CpG-DNA resulted in close apposition of the cytoplasmic signaling domains of TLR9 on endosomes (228), implying that TLR9 resides on this subcellular compartment when recruiting downstream signaling components. On the other hand, a study by Park *et al.* reported that proteolytic cleavage of TLR9 is a prerequisite for TLR9 signaling, implying that the signal is transmitted from the lysosomes (25). Nevertheless, it has been suggested that this pathway is a continuum in that the transitions from early endosomes to late endosomes and lysosomes involves transient interactions that occurs continuously (229). The exact site of signaling from TLR3 within the endosomal pathway remains to be explored, and may not have any implication for TLR3-mediated responses.

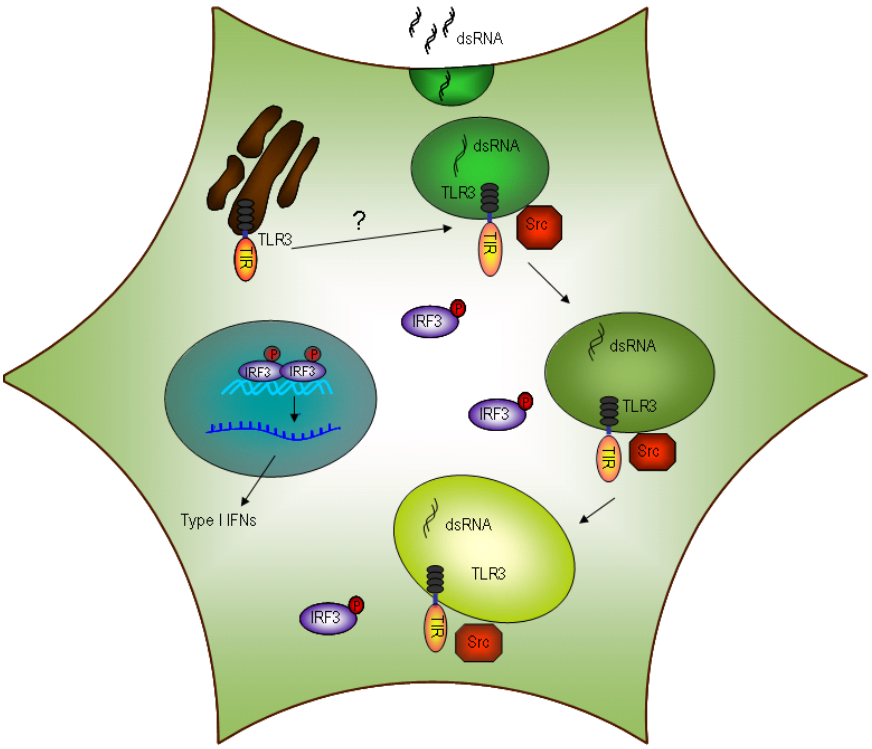

Figure 6: Schematic summary of paper I. C-Src tyrosine kinase is activated by dsRNA and associates with TLR3 upon dsRNA challenge. A fraction of TLR3 translocates from the ER to the endosomal pathway where it recognizes dsRNA and recruits downstream signaling components, including c-Src. C-Src is important for dsRNA-induced IRF3 phosphorylation and nuclear translocation.

4.3 C-Src tyrosine kinase is important for antiviral gene induction in response to diverse viruses

Upon ligand recognition, TLR3 recruits the adaptor protein TRIF to its cytoplasmic TIR domain to initiate antiviral signaling cascades including several kinases ultimately leading to activation of IRF3 and production of type I IFNs. Tyrosine phosphorylation regulates diverse intracellular pathways in the immune system, and is reported to modulate functions of TLRs and their signaling components, *e.g.* TLR2, TLR3, TLR4, TLR8, TLR9 and Mal (196-203). In particular it was shown that mutations of two specific tyrosine residues within the cytoplasmic domain of TLR3 affected dsRNA-mediated induction of the human 561 gene, which is regulated by IRF3 (201;202). In ***paper I***, we investigated the role of c-Src tyrosine kinase in dsRNA-induced responses. A major part of the insight into the biology of c-Src family kinases (SFKs) is provided from loss-of-function studies in mouse. Mice carrying mutations in all of the known SFKs have been generated (205). C-Src was the first SFK to be mutated. The main phenotype associated with this mutation was a bone remodelling disease termed osteopetrosis (190). This is the only severe phenotype observed due to a mutation in a single SFK, suggesting that different SFKs may compensate for each other. C-Src, Yes and Fyn are often co-expressed in the same cell type, and it is reported that at least in some cells these kinases are able to compensate for the loss of the other related kinases. Hence, it might be complicated to study specific functions of SFKs by genetic knockout (230). In our study, we used mouse embryonic fibroblasts (MEFs) devoid of c-Src, Yes and Fyn (SYF) and SYF cells in which c-Src has been introduced (c-Src) (185). Crossing of Src family mutants leads to more severe phenotypes compared to single mutants. Importantly, c-Src/Yes and c-Src/Fyn mutants die shortly after birth, whereas the Yes/Fyn mutant is viable but develop a renal disease (230). Hence, SYF and c-Src MEFs enabled us to study the function of c-Src. In ***paper I***, we demonstrated that c-Src was essential for IRF3 activation and nuclear translocation, as assessed using c-Src chemical inhibitor and cells devoid of c-Src. We showed that c-Src modulated IFN-β production downstream the TLR3 adaptor TRIF. Activation of IRF3 and production of type I IFNs are crucial to build an immediate antiviral response (160;231). Collectively, the results from ***paper I*** indicate that c-Src is an important component of TLR3-mediated innate antiviral defence mechanisms.

TLR3 was the first pattern recognition receptor characterized that was capable of signaling to IRF3 and NF-κB in response to dsRNA. However, *in vivo* antiviral responses to several viruses were found to be similar in TLR3 deficient mice compared to wild type mice (47). Hence, it soon became obvious that other dsRNA receptors may exist. The identification of the cytoplasmic PRRs, RIG-I and Mda5, revealed novel understanding of innate antiviral signaling pathways. In ***paper II*** we showed that c-Src tyrosine kinase has a functional role in RIG-I-mediated antiviral responses, thus modulating activation of IRF3 and production of type I IFNs through two distinct signaling pathways. Sendai virus (SV), which is a ssRNA virus of the paramyxoviridae family, induces antiviral gene expression through RIG-I (232;233). We found that SV activated c-Src tyrosine kinase, implying that c-Src is involved in RIG-I-mediated signaling. In contrast to poly IC, which activates c-Src within 1 hour, optimal activation of c-Src upon SV infection occurred after 8-12 hours. The difference in kinetics of activation is likely due to the necessity of viral endocytosis followed by its release into the cytosol for replication. We confirmed the role of c-Src in RIG-I dependent responses by using chemical inhibition, expression of kinase inactive c-Src and downregulation of c-Src mRNA by siRNA. Impairment of c-Src function abrogated IRF3 activation and production of the IRF3-regulated genes IFN-β and IP10. Also, IFN-β and IP10 transcription were significantly reduced in cells devoid of c-Src, as was nuclear translocation of IRF3. The effect of c-Src inhibition on IRF3 activation and IFN-β production could be due to effects of c-Src inhibition on SV entry or replication. However, we demonstrated that the mRNA levels of the SV-P gene were largely unaffected by c-Src chemical inhibition and in c-Src deficient cells. Taken together, the results presented in ***paper I*** and ***paper II*** imply that c-Src contributes to TLR3- and RIG-I-dependent signaling pathways. This suggests that c-Src may be involved in antiviral mechanisms induced by different PRRs and play a broader role in innate defence against diverse viruses.

4.4 C-Src tyrosine kinase participates in a multicomponent signaling complex to regulate antiviral gene expression

In ***paper II***, we found that molecular components of the RIG-I signaling pathway, RIG-I and MAVS, induced IRF3 dependent transcription that involved c-Src kinase activity. This was demonstrated by the ability of kinase inactive c-Src to suppress RIG-I- and MAVS-stimulated IRF3 activity and IFN-β and IP10 mRNA induction. Hence, c-Src seems to participate

downstream of RIG-I and its adaptor protein MAVS in antiviral responses. In ***paper I,*** found that c-Src acted downstream of or at the level of TRIF. This was demonstrated by the ability of kinase inactive c-Src to abbrogate TRIF-induced IFN-β production. TRAF3 has been reported as a critical molecule integrating TLR-dependent and -independent antiviral responses (80;81). As c-Src appears to be involved in both TLR3 signaling and RIG-I signaling, it is reasonable to speculate that c-Src and TRAF3 somehow collaborate in the regulation of IRF3 activity and IFN-β production. In light of that, we found that c-Src physically interacted with TRAF3 (***paper II***). TRAF3 is previously reported to interact with several nonrelated kinases, including TBK1 and IKKε which phosphorylate IRF3, IRAK1 which mediates MyD88 dependent pathways, NIK that activates the NF-κB pathway, and PKR that is known to bind dsRNA (80;234). NIK has been demonstrated to specifically bind to the TRAF domain of TRAF3. Still, the molecular mechanisms behind the interactions between TRAF3 and TBK1, IKKε, IRAK1 and PKR are not reported. We sought to explore the molecular determinants behind the interaction between TRAF3 and c-Src. We found that c-Src specifically bound to RING and zink-finger domains within TRAF3. In contrast, we observed that MAVS specifically bound the TRAF domain of TRAF3, consistent with a previous report showing that MAVS contains a TRAF interacting motif (TIM) (104). This illustrates that the different TRAF3 domains may harbour functional specificity in antiviral responses mediated by c-Src and MAVS. Moreover, different modular domains may link different kinases (NIK and c-Src) to TRAF3. Taken together, c-Src tyrosine kinase elicits its function in RIG-I dependent signaling downstream of MAVS, probably at the level of TRAF3 to which it specifically interacts with the RING domains. It is likely that TRAF3 is the converging point for c-Src-mediated regulation of TLR-dependent and -independent IRF3 activation and IFN-β production.

In addition to its interaction with TRAF3, we found that c-Src physically associated with other components of the RIG-I pathway, including RIG-I, MAVS, and TBK1. The results in ***paper II*** suggest that c-Src may be organized into IRF3-activating signaling complexes. The assembly of multicomponent signaling complexes downstream of ligand-receptor interactions is an important step in kinase activation and transcriptional regulation. Complexes containing TANK, TRAF3 and TRIF or MAVS have been suggested to trigger TBK1 which in turn phosphorylate IRF3 (114;121). C-Src has previously been shown to induce synergistic AP-1

activation in a signaling complex with TRAF6 in response to IL-1 (235). Moreover, Akt/PKB was reported to be activated by TRANCE through a complex involving TRAF6 and c-Src (236). In the same study TRAF3 interacted with c-Src. but the molecular mechanisms or functional importance of the latter interaction was not addressed. TRAF3 has been described as a regulator of IFN production (237) and is reported to interact with several nonrelated kinases, as discussed above. We show that c-Src is recruited to IRF3 activating complexes downstream the cytoplamic receptor RIG-I. In a similar manner, c-Src may convey TLR3-induced signals by interacting with TRAF3 and TRIF.

The exact function and phosphorylation target of c-Src in antiviral signaling pathways is, however, not assessed. C-Src could associate directly with TLR3 and RIG-I or through other signaling components, *eg.* TRIF, MAVS or TRAF3. The kinase domain of c-Src is responsible for its tyrosine kinase activity and plays an important role in substrate specificity. A possible interaction between c-Src and antiviral receptors and signaling components might be mediated by tyrosine phosphorylation. TLR3 contains several tyrosine-based sorting signals (YXXØ, Ø=hydrophobic) within its cytoplasmic domain, which normally target transmembrane proteins to the endosomal pathway (238). The amino acid sequence preferably phosphorylated by c-Src is EEEIY$^{G}/_{E}$EFD. However, this sequence is not found in human TLR3, TRIF, RIG-I, MAVS or TRAF3. It has recently been demostrated that Bruton`s tyrosine kinase (Btk) elicits tyrosine phosphorylation in TLR responses. The adaptor protein Mal is phosphorylated by Btk upon TLR2 and TLR4 signaling (198). Further, Btk is activated by and recruited to TLR8 and TLR9. Importantly, Btk-mediated tyrosine phosphorylation is required for NF-κB activation by TLR8 and TLR9 (203). Components of the TLR3 and RIG-I signaling pathways could undergo tyrosine phosphorylation by alternative/unidentified kinases, generating phosphorylated tyrosine residues that bind the SH2 domain of c-Src. Some proteins that bind to the SH2 domain of c-Src harbour the optimum binding structure YEEI. This binding structure is not present in human TLR3, TRIF, RIG-I, MAVS or TRAF3. However, not all proteins binding to the SH2 domain of c-Src possess such sequence. The platelet-derived growth factor (PDGF) and colony-stimulating factor (CSF)- I receptors that are phosphorylated at the sequences YIYV and YTFI, respectively, yet bind Src and Fyn through their SH2 domains (178;239;240). The SH3 domain of c-Src binds the common PXXP amino acid sequence of substrate proteins (241). TRIF and MAVS contain several such

proline-rich motifs that may mediate a possible interaction with c-Src. The cytoplasmic signaling protein TRAF6, which acts downstream of MyD88, interacts with c-Src and this interaction was reported to depend on a polyproline motif within TRAF6, as well as c-Src kinase activity (242). Taken together, c-Src may act as a positive effector of complex formation in TLR3- and RIG-I signaling at the level of TRIF/TRAF3 and MAVS/TRAF3, hence regulating downstream kinases, such as TBK1 and IKKε, and subsequent activation of IRF3.

Alternatively, c-Src may elicit its function at the level of TBK1 and IKKε. TBK1 is a serine/threonine kinase, but its activation mechanism is not known. It is possible that TBK1 undergoes autophosphorylation and activates itself. Moreover, TBK1 might be activated through sequential mechanisms, *e.g.* autophosphorylation subsequent to tyrosine phosphorylation, which could involve c-Src kinase activity. In light of that, TBK1 harbours a motif containing a serine close to a tyrosine (SLY) within its activation loop. It has also been suggested that alternative kinases are implicated in IRF3 activation through other residues than those targeted by TBK1 (243;244). c-Src could mediate activation of such kinases, as shown for Akt activation in ***paper I***. We demonstrated that treatment with dsRNA triggered activation of Akt, and that c-Src altered this activation. c-Src has previously been reported to directly phosphorylate Akt to control its activation (245). However, the role of c-Src in TLR-induced Akt activation has not been described. A study by Sarkar *et al.* demonstrated that the PI3-kinase/Akt pathway is necessary for maximal phosphorylation and activation of IRF3 downstream of TLR3, but does not involve TBK1 (201). This supports the hypothesis that activation of IRF3 may rely on additional kinases. Akt has also been implicated in antiviral signaling from other PRRs. Vesicular stomatitis virus (VSV) was recently reported to induce TLR4-dependent, MyD88-independent Akt activation (246). Moreover, Peters *et al*. showed that SV infection activates Akt. However, the latter study claimed that SV-induced IRF3 activity is unaffected by impairment of Akt function, and that Akt rather regulates caspase-8-mediated apoptosis upon SV infection. (247). Hence, regulation of IRF3 activity through the PI3-kinase/Akt pathway could be specific for TRIF responses and might not be implicated in RIG-I-mediated signaling.

Several publications illustrate the complexity of IRF3 regulatory mechanisms. TANK has been shown to undergo both phosphorylation and subsequent K63-linked ubiquitination (248). Also, additional components of innate antiviral signaling pathways are regulated by ubiquitination, *e.g.* RIG-I that is ubiquitinated by the E3 ligase TRIM25, a modification that is critical for type I IFN production. We and others (104;114) present a model of the assembly of antiviral signaling complexes in which TRAF3 is a central component. Still, it is not completely characterized how TRAF3 is activated and transmit signals to IRF3. TRAF3 was recently reported to become ubiquitinated in a K63-linked manner resulting in the recruitment of TBK1 (110). Similarly, the NF-κB pathway involves TRAF6-mediated K63-linked ubiquitination leading to activation of TAK1. Thus, signaling from TRAF interacting receptors is generally thought to involve K63-linked ubiquitination of target proteins in order to promote protein-protein interactions and activation of kinases in either the NF-κB pathway or the IRF3 pathway. In conclusion, TRAF3 might function as a platform recruiting kinases, *e.g.* c-Src, that elicits phosphorylation dependent post-translational modifications, such as ubiquitination.

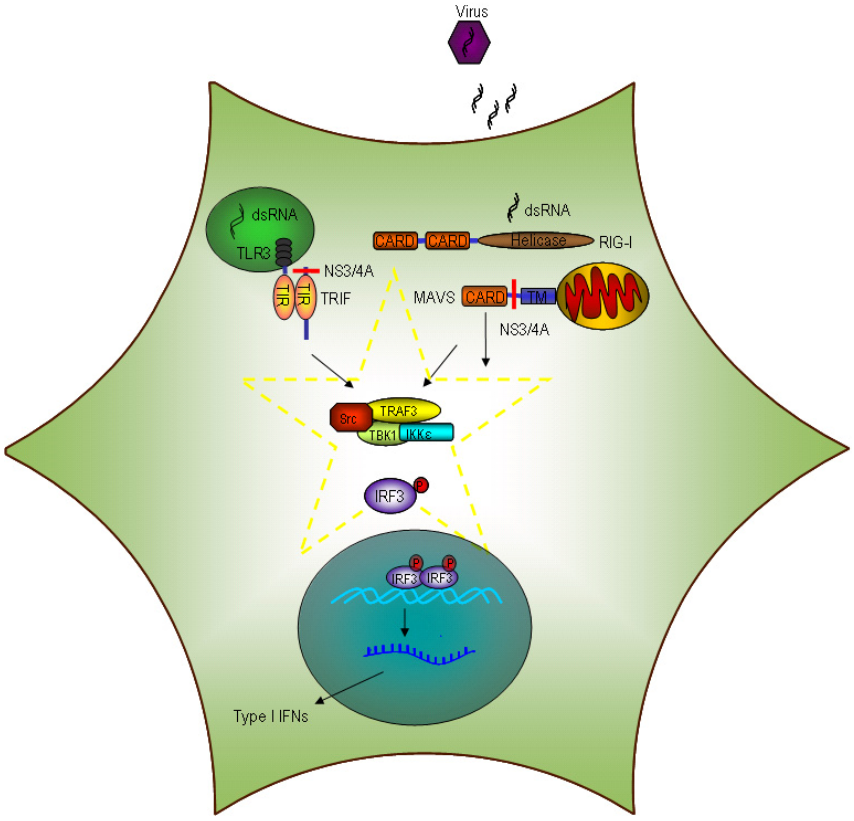

Figure 7: Schematic summary of paper II. C-Src tyrosine enhances RIG-I-mediated activation of IRF3 and induction of IRF3-regulated genes. C-Src associates with components of the RIG-I signaling pathway and specifically interacts with the RING domain of TRAF3. The present model proposes that c-Src functions in a multicomponent complex in both TLR3- and RIG-I-induced responses at the level of TRAF3.

4.5 C-Src tyrosine kinase differentially regulates TLR-dependent gene expression

Transcription of cytokines is regulated by a complex set of transcription factors and dynamic signaling networks. The IRF family consists of 9 members with distinct specificities depending on discrete regulatory mechanisms. So far we have discussed that c-Src positively regulates IRF3 activity downstream of TLR3 and RIG-I antiviral signaling pathways. Further, it was of interest to explore if c-Src did affect the regulation of other IRF family members. In

paper III, we demonstrated that c-Src tyrosine kinase differentially regulates TLR-mediated gene expression. Consistent with what observed for poly IC- and SV-induced IRF3 activation in ***paper I and II***, we found that CpG-induced activation of IRF3 was attenuated by chemical inhibition of c-Src. Interestingly chemical inhibition of c-Src had the opposite effect on IRF5 activation in response to CpG. The effect on IRF5, however, could not be assessed in the content of TLR3 as we and others found that poly IC failed to activate IRF5 (249). These findings suggest that c-Src may exert a negative regulatory role in IRF5 activation. The effect of c-Src inhibition on IRF5 required prolonged CpG treatment, indicating that c-Src regulates a delayed phase of CpG-induced gene transcription. Similarly, chemical inhibition of c-Src augmented CpG-mediated gene induction of IL-6 after prolonged stimulation, whereas CpG-triggered gene induction of IP10 was attenuated. IRF5 has been reported to regulate transcription of IL-6 in response to various ligands (101), and IRF3 has been suggested to be critical for TLR elicited synthesis of IP10 (87;250). We demonstrated that MEFs lacking expression of c-Src, Yes and Fyn (SYF) elicited enhanced IL6 induction from several TLR ligands, including LPS, R848, CpG and poly IC. On the other hand, IP10 transcription triggered by TLR ligands was abrogated in the same cell line. In general, TLRs are highly expressed in specialized immune cells, such as monocytes, macrophages and dendritic cells. Importantly, all TLRs are also expressed in the MEF cell line, and the MEFs are reported to respond to TLR ligand activation (251). Supporting the results from the MEF cell line, we found that chemical inhibition of c-Src enhanced the production of IL-6 and inhibited the production of IP10 in response to various TLR ligands. Collectively, we believe that c-Src enhances IRF3-dependent gene induction (IP10) and negatively regulates IRF5-dependent gene induction (IL6) in response to a range of PAMPs and downstream of both MyD88-dependent and -independent signaling.

4.6 C-Src tyrosine kinase regulates TLR-elicited induction of ATF3, a negative regulator of TLR signaling

A number of mechanisms have been described that downregulate cytokine signaling, thus preventing overaction of these immune effectors (252). Our results in ***paper III*** pointed towards a role for c-Src in inhibiting negative regulators of TLR signaling. Moreover, the effect of c-Src on IRF5 was observed after prolonged TLR stimulation. Signaling involved in the control of delayed TLR-responses may depend on induction of TLR-specific genes. IRF4

is known to negatively regulate IRF5 by competing for MyD88 (102). However, we could not observe any effect of TLR ligands or c-Src functional impairment on IRF4 mRNA levels. This indicates that the contribution of c-Src as a negative regulator of IRF5-dependent gene expression is not due to IRF4 induction. Still, we can not exclude that IRF4 may impact on IRF5 transcriptional control through protein-protein interactions. SOCS3 is reported to inhibit innate immune responses (252). In particular, SOCS3 overexpression inhibits NF-κB and IL-8 activities, as well as IFN-β expression during virus infection (253). Moreover, SOCS3 is reported to regulate secondary effects of TLR-mediated cytokine signaling, *e.g.* LPS-induced macrophage activation through regulation of IL-1, TGF-β, IL-10 and IL-6 (254). We found that TLR ligands were able to induce SOCS3 mRNA, and that the SOCS3 mRNA levels were extensively increased upon c-Src inhibition. Thus, enhanced transcription of IL-6 upon c-Src inhibition could not be explained by altered SOCS3 levels. The transcription factor ATF3 differs from the rest of the ATF family by its ability to repress rather than activate transcription from promoters with ATF/CRE binding sites (172). Interestingly, ATF3 was recently identified as a negative regulator of TLR4 signaling. In particular, ATF3 was shown to inhibit IL-12-β and IL-6 transcription (174;255). We found that ATF3 was rapidly induced in response to TLR ligands. Interestingly, CpG-induced ATF3 levels were elevated upon prolonged treatment. Given that ATF3 functions as a negative regulator in TLR responses, its high expression in response to prolonged stimulation may help the cell to tune down the response during late phases by inhibiting cytokine production. The elevated mRNA levels of ATF3 at delayed phases could also be explained by post-transcriptional regulation. Several studies have reported that the mRNA stability of cytokines and other protein transcripts is tightly regulated during inflammation (256;257). Nevertheless, impairment of c-Src by chemical inhibition and by using cells lacking c-Src expression abrogated induction of ATF3 mRNA. Also, c-Src activity regulated intracellular ATF3 levels, indicating that c-Src activity is necessary for induction of ATF3. The ATF3 promoter contains a consensus TATA box and a number of transcription factor binding sites including the AP-1, ATF/CRE, NF-κB, E2F, and Myc/Max binding sites. Actually, c-Src has previously been reported to modulate AP-1 activity triggered by IL-1 (235). Bioinformatics data showed that IL-6 and IL-12-β promoters harbour ATF or CRE sites. Thus, it is likely that ATF3 binds to these promoters, and possibly represses the production of IL-6 and IL-12-β upon TLR signaling. Moreover, binding of

ATF3 to specific promoters could be reduced by impaired c-Src function due to decreased ATF3 protein and consequently reduced ATF3 levels in the nucleus.

It was previously reported that ATF3 binds to the IL-6 and IL-12-β promoters upon LPS challenge (174). Still, the mechanisms behind ATF3-mediated repression of transcription are not fully understood. When it comes to LPS-triggered transcription, it was found that ATF3 was recruited to the promoters with slower kinetics than the NF-κB subunit Rel. This is in accordance with a possible role in delayed phases of TLR responses. Moreover, it was demonstrated that LPS triggered association between ATF3 and the chromatin modifying enzyme histone deacetylase in the nucleus (174). Hence, the molecular mechanism for ATF3-mediated repression of IL-6 transcription may be deacetylation of histones generating an inaccessible chromatin structure on the IL-6 promoter. In that regard, it was recently shown that chromatin modifications, such as histone methylations, acetylations and phosphorylations, differentially labelled TLR-elicited genes and might govern gene-specific rather than transcription factor dependent induction during distinct temporal phases (258-261) Also, it has been reported that NF-κB is recruited to promoters in either of two modes; fast recruitment to constitutive accessible promoters or slow recruitment to promoters that undergo stimuli-dependent modifications of chromatin structure (262). We believe that ATF3 binding to promoters represents a mechanism for control of chromatin accessibility. Thus, c-Src may play a role in chromatin-regulated transcription during late phases of TLR responses.

Currently, we have not studied the mechanisms by which c-Src positively regulates ATF3 transcription. It has been reported that stress-signals affect transcription of ATF3, probably via the JNK/SAPK-regulated pathways (263). We demonstrated that ATF3 induction in response to various TLR signals depends on c-Src. Also, several TLR ligands induce JNK/SAPK signaling. Therefore, ATF3 induction might be regulated through mechanisms common downstream of TLRs, involving c-Src tyrosine kinase. This also implies a possibly broad role of ATF3 in innate immune responses to pathogens.

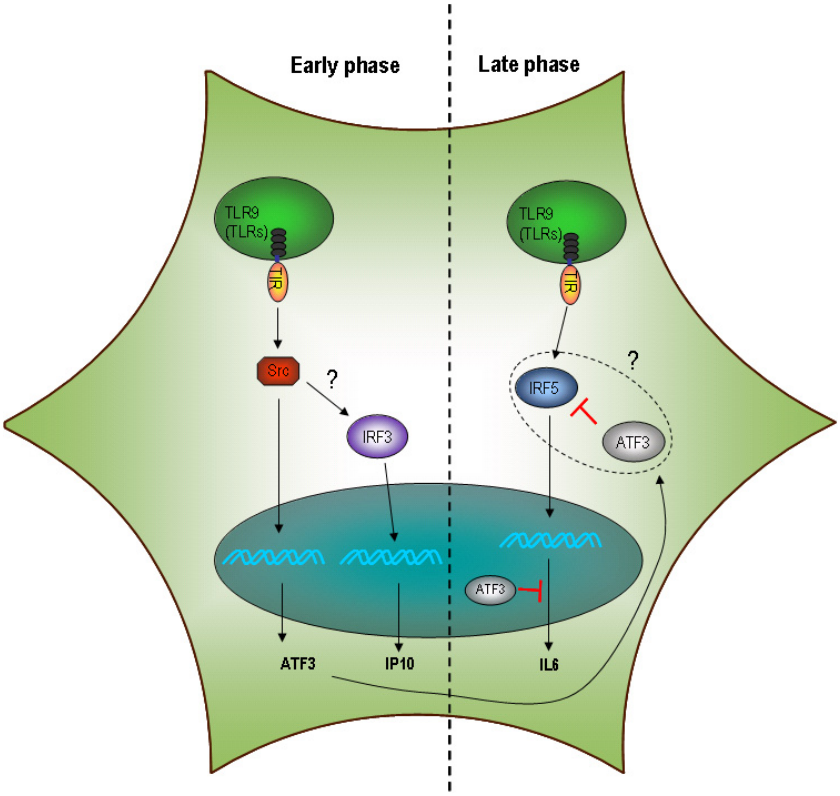

Figure 8: Schematic summary of paper III. During early phases of TLR responses, c-Src tyrosine kinase enhances induction of the IRF3-regulated gene IP10. Moreover, TLR-triggered induction of the transcription factor ATF3 is positively regulated by c-Src. ATF3 in turn, represses induction of the IRF5-regulated gene IL-6. Hence, we propose that c-Src negatively regulates the induction of the IRF5-regulated gene IL6 during late phases of TLR responses by controlling intracellular ATF3 levels.

5 CONCLUSIONS

In this study we demonstrate that c-Src tyrosine kinase is important for TLR3- and RIG-I-dependent activation of IRF3 and induction of type I interferons. We provide insight into the subcellular site of interaction between TLR3, dsRNA and c-Src. We show that c-Src associates with components of antiviral signaling pathways, and we reveal the molecular explanation behind its interaction with the cytoplasmic adaptor TRAF3. Further, we demonstrate that c-Src differentially regulates TLR-induced gene expression through different IRF family members, and we assess the biological explanation behind its negative control of the IRF5-regulated cytokine IL-6. Taken together, we provide insight into the role of c-Src in innate immune responses, in the regulation of antiviral signaling and the complex control of TLR-triggered cytokine production. Our results imply that c-Src modulates antiviral responses to diverse viruses and may play a broader role in innate immune defence mechanisms. We propose that this study adds to the general understanding of innate immune mechanisms, which could be of importance for the development of vaccines and new treatments against infectious diseases and cancer.

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