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A molecular analysis of the LPS resistance of the mouse species *Mus spretus*.

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Thesis submitted in partial fulfillment of the requirements for the degree of

Doctor in Sciences: Biotechnology

Promotor: Prof. Dr. Claude Libert

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Cover illustration: LPS resistant (BxS)F_1 mice

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AA	amino acid
AAF	IFN-α activated factor
AKT	thymoma viral proto-oncogene 1
AP-1	activator protein-1
APC	antigen-presenting cell
APC	activated protein C
ARE	AU-rich element
ARM	armadillo repeat motif
AT	antithrombin
ATF	activating transcription factor
B	C57BL/6
BC	Before Christ
BIR	baculovirus inhibitor of apoptosis repeat
BLP	bacterial lipoprotein
(BxS)F_1	(C57BL/6xSPRET/Ei)F_1
BMDM	bone marrow-derived macrophages
BMP	bone morphogenetic protein
bp	basepairs
BPI	bactericidal permeability increasing protein
C1	complement 1
CAP	cathelicidin antimicrobial peptide
CARD	caspase-recruitment domain
CBP	CREB-binding protein
CCL5	chemokine (C-C motif) ligand 5
CCR	CC-chemokine receptor
cDC	conventional DC
cDNA	complementary DNA
C/EBP	CCAAT/enhancer binding protein
C.elegans	*Caenorhabditis elegans*
CHO	Chinese hamster ovary
CI	confidence interval
CIM	composite interval mapping
CITED	CBP/p300-interacting transactivators with E (glutamic acid)/D (aspartic acid)-rich carboxyl-terminal domain
CLP	cecal ligation and puncture
cM	centiMorgan
CR	complement receptor
CRD	carbohydrate recognition domain
CRE	cAMP response element
CREB	cAMP responsive element binding protein
CRF2	class II cytokine receptor family
CRID	coding region instability domain
CSS	chromosome substitution strain
C-terminal	carboxy-terminal
CTL	cytotoxic T lymphocyte
CXCL10	CXC-chemokine ligand 10
CXCR	chemokine (C-X-C motif) receptor

DAP	diaminopimelic acid
DBD	DNA binding domain
DC	dendritic cell
DD	death domain
d-GalN	d-galactosamine
DNA	deoxyribonucleic acid
DRAF	dsRNA activated factor
dsRNA	double-stranded RNA
EAE	experimental allergic encephalomyelitis
EBV	Epstein–Barr Virus
EC	endothelial cell
ECM	extracellular matrix
E.coli	*Escherichia coli*
ECSIT	evolutionarily conserved signalling intermediate in toll pathways
EIF4E	eukaryotic translation initiation factor 4E
ERK	extracellular signal-regulated kinase
ENU	ethyl-N-nitrosourea
ES	embryonic stem
EST	expressed sequence tag
FADD	Fas-associated death domain
FCS	foetal calf serum
G^-	Gram-negative
G^+	Gram-positive
Gal	galactose
GARG16	glucocorticoid-attentuated response gene 16
GAS	IFN-γ-activated site
GBP	guanylate binding protein
G-CSF	granulocyte colony-stimulating factor
GDF5	growth differentiation factor 5
GlcN	glucosamine
Glu	glucose
GluNac	N-acetylglucosamine
GlyCAM	glycosylation-dependent cell adhesion molecule
GM-CSF	granulocyte macrophage colony stimulating factor
GPI	glycerosylphosphatidylinositol
h	human (prefix)
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDL	high density lipoprotein
HEK	human embryonic kidney
Hep	heptose
HIV	human immunodeficiency virus
HMGB	high mobility group box protein
HMGI	high mobility group protein
HOS	homologue of slimb
HSP	heat shock protein
HSV	herpes simplex virus
IAD	IRF association domain
ICAM	intercellular adhesion molecule
IDDM	insulin-dependent diabetes mellitus

IFIT	interferon-induced protein with tetratricopeptide repeats
IFN	interferon
IFNAR	interferon (alpha and beta) receptor
Ig	immunoglobulin
I-κB	inhibitor kappaB
IKK	I-κB kinase
IL	interleukin
IL-1R	interleukin-1 receptor
IL-1Ra	IL-1R antagonist
IL-1RAcP	IL-1R accessory protein
iNOS	inducible nitric oxide synthetase
ip	intraperitoneal
IPC	IFN-α/β producing cells
IRAK	IL-1R-associated kinase
IRF	IFN regulating factor
IRG1	immunoresponsive gene 1
ISG15	interferon-stimulated gene 15
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response element
IU	international units
JAK	janus kinase
JNK	c-Jun N-terminal kinase
kb	kilobase
KD	kinase domain
kDa	kiloDalton
Kdo	ketodeoxyoctonate
LAM	lipoarabinomannan
LBP	LPS binding protein
LD100	100 % lethal dose
LDL	low-density lipoprotein
LOD	logarithm of the odds
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LRS	likelihood ratio statistic
LTA	lipoteichoic acid
m	mouse (prefix)
MadCAM	mucosal addressin cell adhesion molecule
MAL	MyD88-adaptor-like
MALP	mycoplasmal macrophage-activating lipopeptide
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MAPKAPK	MAPK-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MARCO	macrophage receptor with collagenous structure
Mb	megabases
MCMV	mouse cytomegalovirus
MD	myeloid differentiation
MDA	melanoma differentiation-associated gene
MDP	muramyl dipeptide

MEF	mouse embryonic fibroblast
MEK	MAPK/ERK kinase
MEKK	MEK kinase
µg	microgram
MHC	major histocompatibility complex
Min	minutes
MKK	MAP kinase kinase
M.m.	*Mus musculus*
MMP	matrix metalloproteinase
MMR	macrophage mannose receptor
MMTV	mouse mammary tumor virus
MNK1	MAPK-interacting protein kinase
MODS	multiple organ dysfunction syndrome
MOF	multiple organ failure
MPL	monophosphoryl lipid A
mRNA	messenger RNA
MRSA	methicillin-resistant *staphylococcus aureus*
MSK	mitogen- and stress-activated kinase
MSR	macrophage scavenger receptor
MyD88	myeloid differentiation factor 88
MyD88s	MyD88 short
Mx1	myxovirus (influenza virus) resistance 1
NAK	NF-κB activating kinase
NAP	NAK-associated protein
NBS	nucleotide-binding site
NBS-LRR	nucleotide-binding site and leucine-rich repeat
ND	not determined
NEMO	NF-κB essential modulator
NES	nuclear export signal
NF-IL6	nuclear factor IL-6
NF-κB	nuclear factor kappa B
NIK	NF-κB inducing kinase
NK	natural killer
NLS	nuclear localization sequence
NO	nitric oxide
Nod	nucleotide-binding oligomerization domain
NRD	negative regulatory domain
NRE	negative regulatory element
NRF	NF-κB regulating factor
N-terminal	amino-terminal
OAS	2'-5' oligoadenylate synthetase
OspA	outer-surface lipoprotein A
P	phosphate
PAF	platelet activating factor
PAMP	pathogen-associated molecular pattern
PCAF	p300/CBP-associated factor
PCR	polymerase chain reaction
pDC	plasmacytoid DC
PGN	peptidoglycan
PI3K	phosphatidylinositol 3-kinase

PIAS	protein inhibitor of activated STAT
PI-PLC	phosphatidylinositolphospholipase
PKR	dsRNA-dependent protein kinase
PMN	polymorphonuclear neutrophils
Poly(I:C)	polyinosinic-polycytidylic acid
PPARγ	peroxisome proliferator-activated receptor-gamma
PRD	positive regulatory domain
PRD-LE	PRD-like element
PRR	pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
PTP	protein tyrosine phosphatase
PYD	pyrin domain
Q-PCR	quantitative RT-PCR
QT	quantitative trait
QTL	quantitative trait locus
RA	rheumatoid arthritis
RFLP	restriction fragment length polymorphism
RIG-I	retinoic acid-inducible gene I
RIP	receptor interacting protein
RNA	ribonucleic acid
RNAi	RNA interference
RP-HPLC	reverse phase – high pressure liquid chromatography
RSV	respiratory syncytial virus
RT	room temperature
RT	reverse transcription
RT-PCR	reverse transcriptase PCR
S	SPRET/Ei
S.a.e.	*Salmonella abortus equii*
SAM	sterile α motif
SAP	serum amyloid P
SAPK	stress-activated protein kinase
SARM	sterile α and HEAT/armadillo motif containing protein
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	serine
SH2	SRC homology 2
SHIP	SH2-containing inositol 5-phosphatase
SHP1	SH2-domain-containing PTP1
SIGIRR	single Ig domain IL-1R related protein
SIM	simple interval mapping
SIRS	systemic inflammatory response syndrome
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
SOCS	suppressor of cytokine signalling
SR	scavenger receptor
SSCP	single strand conformation polymorphism
SSLP	simple sequence length polymorphism
STAT	signal transducer and activator of transcription
T2K	TRAF2-associated kinase
TAB	TAK-binding protein
TAK	TGF-β-activated kinase

TANK	TRAF family member associated NF-κB activator
TBK	TANK-binding kinase
TCPTP	T-cell PTP
TF	transcription factor
TFPI	tissue factor pathway inhibitor
TGF-β	transforming growth factor-β
T_H	T helper cell
TICAM	toll-like receptor adaptor molecule
TIR	toll/IL-1 receptor domain
TIR1	TIR domain-containing protein 1
TIRAP	TIR domain-containing adaptor protein
TIRP	TIR-containing protein
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFR	TNF receptor
TOLLIP	Toll-interacting protein
TPL2	tumour-progression locus 2
TRAF	TNFR-associated factor
TRAM	TRIF-related adaptor molecule
TRAILR	TNF-related apoptosis-inducing ligand receptor
TRIF	TIR domain-containing adaptor inducing IFN
TSA	trichostatin A
TYK2	tyrosine kinase 2
U	uridine
USA	United States of America
UTR	untranslated region
VRE	virus responsive element
VSV	vesicular stomatitis virus
WBC	white blood cells
WNV	West Nile virus
XIAP	X-chromosome linked inhibitor of apoptosis
YY1	yin yang 1

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I. GENERAL INTRODUCTION

1. INNATE IMMUNITY: REGULATING HOST RESPONSE TO PATHOGENS

1.1. History, chemical constitution and bioactivity of endotoxin

More than a century ago, endotoxin was discovered by Richard Pfeiffer as a heat-resistant, non-proteinaceous, toxin that is associated with the insoluble part of the bacterial cell. Pfeiffer stated that endotoxins were components of both Gram-negative and Gram-positive bacteria (Pfeiffer, 1892). Mainly through the efforts of Mary Jane Osborn and Hiroshi Nikaido, it is recognized nowadays that endotoxin is the principal glycolipid component of the outer membrane of Gram-negative bacteria (Figure I.1.1.) (Rietschel and Westphal, 1999). It took till the middle of the 20th century to purify endotoxin, which was then called lipopolysaccharide (LPS) because of the presence of lipid and polysaccharide components (Beutler and Rietschel, 2003), and this purification ultimately led to the characterization of the chemical structure of LPS (Rietschel et al., 1996).

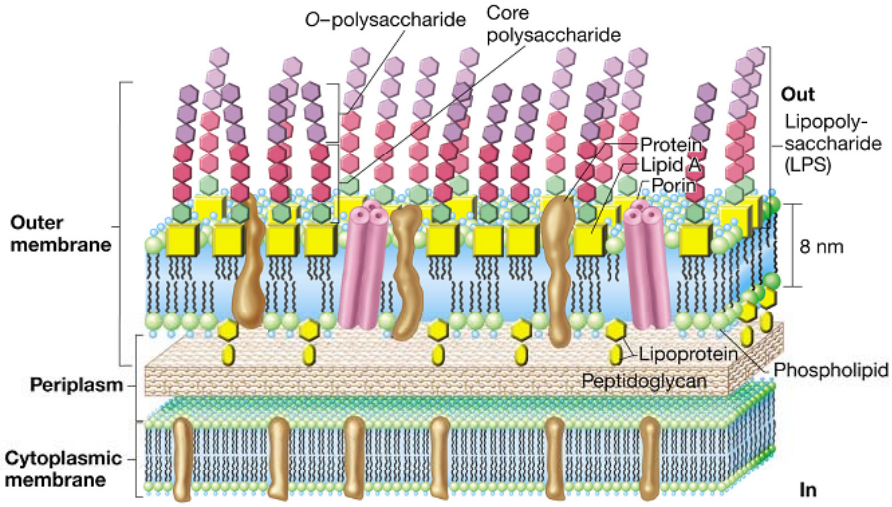

Figure I.1.1. – Overall structure of the Gram-negative cell envelope (Reprinted from textbook: Biology of Microorganisms, eighth edition, 1997).

LPS is composed of 2 major structural regions: a hydrophilic polysaccharide and a hydrophobic complex glycophospholipid known as lipid A, which anchors the molecule to the outer leaflet of the outer membrane. The polysaccharide portion is divided into a highly variable core oligosaccharide containing 10-12 sugars, which is further divided into outer and inner segments, and a more conserved O-specific chain, which contains dozens of identical oligosaccharide repeating units (Figure I.1.2.) (Caroff et al., 2002; Ulevitch and Tobias, 1995).

The classical lipid A moiety is represented by *Escherichia coli*. It has a backbone consisting of a β-1',6-linked D-glucosamine (GlcN) disaccharide carrying ester- and amide-linked fatty acids, and phosphate groups at positions C-1 and C-4' (Figure I.1.2). The hydroxyl groups in position 6' serve as the attachment site for the polysaccharide component (Ulevitch and Tobias, 1995). The lipid A moiety from other Gram-negative bacteria follows the same architectural principle, but might differ in structural details (Caroff et al., 2002). Thus, next to high variability in O-polysaccharides also lipid A heterogeneity is observed, even though lipid A is the most conserved region of LPS. Both can be achieved through variations at different levels. For example, O-polysaccharides can be modified with sugar moieties such as glucosyl and fucosyl residues, or they can vary in the number of repeating oligosaccharide units (Lerouge and Vanderleyden, 2002). Lipid A structures can be modified by, for example, the degree of acylation or the nature of fatty acids (Karibian et al., 1993; Zarrouk et al., 1997). In 1985, *E.coli* lipid A was chemically synthesized for the first time and it was found to be a molecule of about 1,2 kDa. Synthesized *E.coli* lipid A exhibited the same degree of biological activity as the wild-type lipid A (Galanos et al., 1985). In addition, it was demonstrated that any modification of the chemical structure of LPS does not lead to higher toxicity, but rather yields products of lower endotoxicity (Kotani et al., 1985). Thus, minor to moderate changes in lipid A structures can have a tremendous impact on their biological activity.

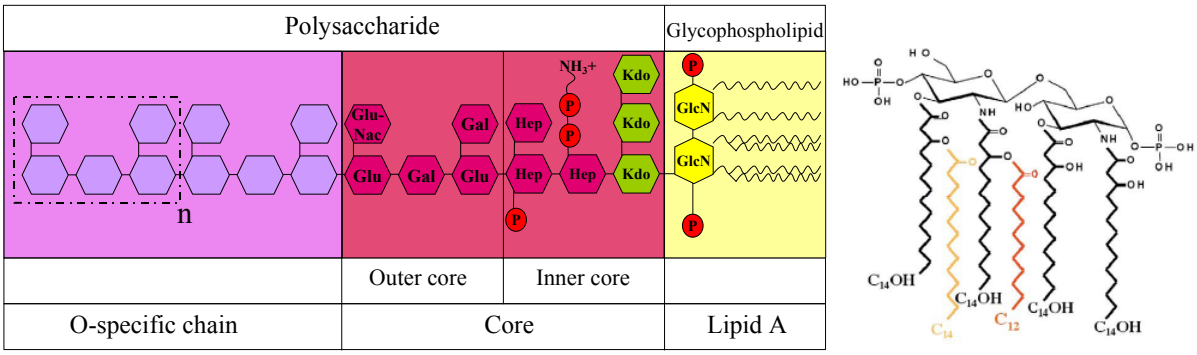

Figure I.1.2. – General overview of LPS and chemical structure of lipid A. The left panel shows a schematic representation of the basic chemical structure of LPS of Gram-negative bacteria. Glu, glucose; Gal, galactose; GluNac, N-acetylglucosamine; GlcN, glucosamine; Hep, Heptose; P, phosphate; Kdo, ketodeoxyoctonate; zig-zag lines, fatty acids. The right panel shows the chemical structure of lipid A from *Enterobacteria* such as *E.coli* and *Salmonella* (Reprinted from Caroff *et al*, Microbes and infection, 4, 915-926, 2002).

LPS molecules vary considerably in structural motifs from one genus, species or strain to another (Caroff et al., 2002). Moreover, they can also vary within one bacterial strain (Hackstadt et al., 1985). Furthermore, bacteria can produce, either naturally or because of genetic defects, incomplete LPS

lacking the inner core sugars or the O-specific chain. This incomplete LPS is called 'rough LPS' because of the morphology of the colonies the organisms form. LPS composed of the O-specific chain, inner core oligosaccharides and lipid A is called 'smooth LPS', because the organisms grow as smooth colonies (Dixon and Darveau, 2005).

Lipid A is known as the bioactive component of LPS (Ulevitch and Tobias, 1995). Bioactivity of lipid A is not only dependent on the composition of lipid A, but also on the molecular conformation determined by steric factors, negative charges and hydrophobic domains. These features determine the distinct shape of the molecule, which in turn leads to formation of larger multimeric aggregates. Endotoxin aggregates in a cubic conformation, with individual molecules within the aggregates having a conical shape, are biologically highly active, whereas lamellar aggregates, with individual molecules within the aggregates having a cylindrical shape, are not active (Schromm et al., 1998). LPS is one of the most potent bioactive molecules. Extremely small amounts (ng to pg range) of LPS are sufficient to initiate the innate host defense system (Rietschel and Westphal, 1999) by activating myeloid (neutrophils, monocytes, macrophages, platelets) and/or non-myeloid cells (fibroblasts) (Ulevitch and Tobias, 1995) to produce primary (e.g. TNF, IL-6, IL-12 and IFN-γ) and secondary (e.g. NO, thromboxanes and PAF) mediators. Then these primary and secondary mediators cause the activation of the coagulation cascade, the complement cascade and the production of prostaglandins and leukotrienes (Hsueh et al., 1990; Levi and Ten Cate, 1999). Immense differences in toxicity are observed between species and especially between vertebrate classes. For example, reptiles, amphibians, birds and fish are unresponsive to LPS, while mammals are very sensitive (Berczi et al., 1966). LPS is able to reproduce many of the features of a Gram-negative infection, including fever, shock and other features of sepsis.

1.2. Sepsis, severe sepsis and septic shock: an enormous impact on society

Sepsis occurs when the body's response to an overwhelming infection becomes uncontrolled. The immune response, necessary to clear the pathogen and its toxins, actually causes damage to the host's own tissues when it becomes hyper-reactive. The response involves a complex network of circulating mediators such as pro-inflammatory cytokines and chemokines, the activation of plasma protein cascade systems, such as the complement system, the pathways of coagulation, and the fibrinolytic system (Tslotou et al., 2005). Hence, the immune response must be carefully controlled to prevent damage to the host.

The term SIRS (systemic inflammatory response syndrome) was developed to define a systemic clinical response to an inflammation or injury that can be infectious (bacterial, viral, fungal) or non-infectious (major surgery, burns, trauma, ischaemia/reperfusion, pancreatitis). SIRS is defined by having two of the following symptoms: hypothermia (<36°C) or hyperthermia (>38°C), tachycardia (>90 beats/min), tachypnea (>20 breaths/min or P_{CO2}<32mm Hg) and the alteration of white blood cell count (<4 x 10^9 cells/liter or >12 x 10^9 cells/liter) or the presence of >10% immature neutrophils. Sepsis is defined as the presence of SIRS associated with infection. Sepsis, severe sepsis and septic shock are commonly used terms. Sepsis associated with organ dysfunction, tissue/organ hypoperfusion, or hypotension is called severe sepsis. Septic shock is characterized by a persistent arterial hypotension, despite adequate fluid resuscitation. Later, multiple organ failure/dysfunction syndrome (MOF/MODS), characterized by the presence of altered function of two or more organs, is developed and often results in death (Karima et al., 1999; Tslotou et al., 2005).

People at greatest risk of developing sepsis are those with a suppressed immune system due to cancer or immune deficiency diseases, but also neonates and elderly. Sepsis remains an important and life-threatening problem. Sepsis affects approximately 750.000 people in the United States of America (USA) each year and an additional 1.2 million in Europe and Japan. The rate of severe sepsis is expected to rise to 1 million cases a year in the USA alone by 2020 as the population ages. Each year, approximately 215.000 people in the USA die from severe sepsis, more than breast, colon/rectal, pancreatic, and prostate cancer combined (Angus et al., 2001). Each year sepsis costs 7.6 billion dollar in Europe and 17.4 billion dollar in the USA (Angus et al., 2001; Bone et al., 1992). The overall mortality is approximately 30%, rising to 40% in the elderly and 50% or more in septic shock patients (Angus et al., 2001).

There are three major types of sepsis characterized by the type of infecting organism. Gram-negative sepsis, the majority of these infections caused by *Escherichia coli*, Klebsiella species and *Pseudomonas aeruginosa*, and Gram-positive sepsis, caused mainly by staphylococci and streptococci, are the most common types of sepsis. The third major group includes fungi, most commonly *Candida*. Fungal infections cause a relatively small percentage of severe sepsis cases, but with a high mortality rate. Also mixed infections involving both Gram-negative and Gram-positive bacteria are common (Tslotou et al., 2005).

Therapies for sepsis at this moment are antimicrobial therapy, fluid resuscitation, glucose control, use of corticosteroids and use of recombinant human activated protein C. Early initiation of antibiotics for sepsis improves survival, but both timing and adequacy of antibiotics are critical contributors to ultimate survival with sepsis (Houck et al., 2004). Antibiotic resistance among pathogens is a growing problem in hospitals. A common cause of hospital infections is the methicillin-resistant *staphylococcus aureus*

(MRSA). Fluid resuscitation is used for patients with severe sepsis or septic shock. In the presence of acute organ dysfunction in case of severe sepsis, therapy with recombinant human activated protein C is considered. However, despite all these therapies, sepsis and SIRS remain the main causes of death in intensive care units (Bone et al., 1992). Various clinical trials in sepsis have failed. For example, administration of anti-endotoxin antibodies, PAF (platelet activating factor) inhibitor and anti-TNF antibodies failed in sepsis therapy (Riedemann et al., 2003). So, extensive studies to find novel strategies for the treatment of sepsis are still indispensable. A summary of clinical trials in sepsis is given in Table I.1.1.

Table I.1.1. - Therapeutic interventions for sepsis in clinical trials (Adapted from Riedemann *et al*., 2003).

INTERVENTION	TARGET	SUCCESS
Corticosteroids	Inflammatory system	+
Anti-endotoxin antibodies	Endotoxin	-
BPI (bactericidal permeability increasing protein)		-
LPS analogues		-
Anti-TNF antibodies	TNF-α	-
Soluble TNF receptor		-
IL-1 receptor agonist	IL-1	-
PAF inhibitor/antagonist	PAF	-
Prostaglandin E1	Arachidonic acid metabolites	-
Thromboxane (synthetase) inhibitors		-
Cyclooxygenase inhibitor		-
Antioxidants (N-acetylcysteine, Selenium)	Oxygen radicals	-
Inhibitors of NO synthase (NOS, iNOS)	NO	-
Inhibition of coagulation factors	Coagulation/Inflammation	
- tissue factor pathway inhibitor (TFPI)		-
- antithrombin (AT)-III		-
- activated protein C (APC)		+
IFN-γ	Neutrophil activation	-
G-CSF, GM-CSF		-
Phosphodiesterase inhibitor	Phosphodiesterase	-
Bradykinin antagonist	Bradykinin	-
C1 (complement 1) inhibitor	Complement system	-

1.3. Pattern-recognition receptors (PRRs)

1.3.1. Pattern-recognition receptors important in innate and adaptive immunity

Vertebrates possess two main intricate systems of host defense, termed the innate and adaptive immunity system (for an overview see Table I.1.2.). The adaptive immune system is unique to vertebrates, but the innate immune system is found in vertebrates, invertebrate animals and plants. This evolutionary ancient innate immunity system provides a first line of defense against infection. It uses sets of germ-line encoded receptors (pattern-recognition receptors or PRRs) to recognize highly conserved molecular structures shared by large groups of pathogens (pathogen-associated molecular patterns or PAMPs). PRRs can be secreted, located in intracellular compartments or expressed on the cell surface. The best known PRRs are the Toll-like receptors (TLRs) (see next section), but a lot of non-TLR PRRs such as proteins with leucine-rich repeat (LRR) domains, calcium-dependent lectin domains, or scavenger-receptor (SR) domains, are known at this moment (see further). PRRs are involved in performing specific tasks including opsonization, phagocytosis, apoptosis, activation of the complement cascade and the coagulation system and release of cytokines and chemokines (Medzhitov, 2001; Medzhitov and Janeway, 2000).

Table I.1.2. - Comparison of the properties of innate and adaptive immune systems (Adapted from Janeway and Medzhitov, 2002).

Property	Innate immune system	Adaptive immune system
Cells	Macrophages, DCs, NK cells	T-cells, B-cells
Receptors	Germline-encoded Rearrangement not necessary Non-clonal distribution	Encoded in gene segments Somatic rearrangement necessary Clonal distribution
Type of Response	Antigen-independent	Antigen-dependent
Response	Cytokines Chemokines Co-stimulatory molecules	Clonal expansion or anergy IL-2 Effector cytokines
Recognition	Conserved molecular patterns Selected over evolutionary time	Details of molecular structure Selected over lifetime of individual
Immunologic Memory	None	Exposure results in immunologic memory
Specificity	Broad spectrum	Antigen-specific
Action time	Immediate (hours)	Lag between exposure and response (days)
Evolution	Vertebrates and invertebrates	Only vertebrates

In vertebrates, a second line of defense, called the adaptive immune system, has evolved. The adaptive immune system is characterized by clonal selection of lymphocytes, specificity and memory. The system uses a diverse repertoire of antigen receptors on B- and T-cell lymphocytes. Each lymphocyte expresses a unique antigen receptor generated by random somatic gene rearrangements. Activation of a naïve lymphocyte is followed by clonal expansion to generate sufficient lymphocytes with relevant specificities, before differentiation into effector cells. This process, which is the basis of immunological memory, takes 3-5 days and is the reason why adaptive immune responses occur only after several days. Because there is a delay of 3-5 days, our body relies on the more general innate immune system to control infection during this period (Medzhitov and Janeway, 2000; Pasare and Medzhitov, 2004).

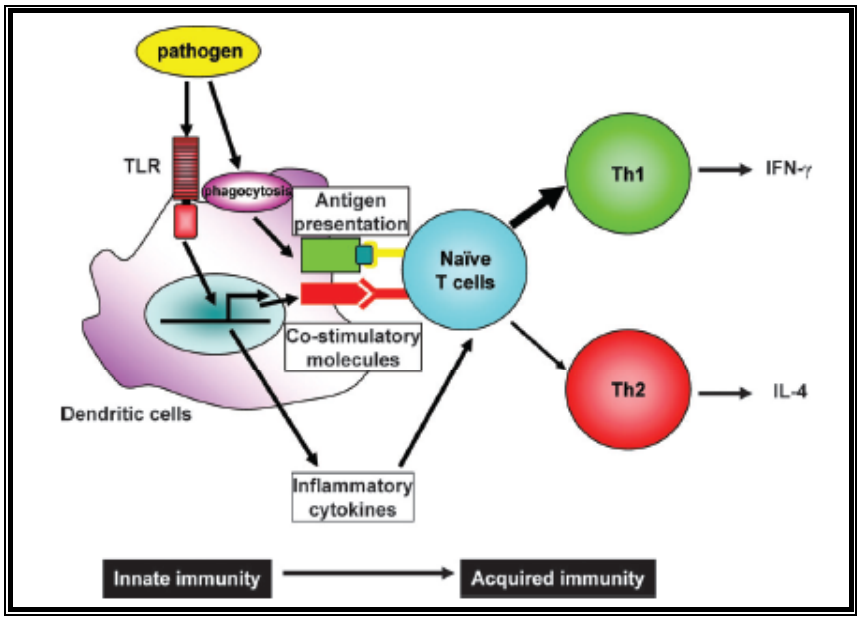

Figure I.1.3. – Contribution of the innate immune system to activation of adaptive immune responses. Phagocytosis of pathogens by antigen-presenting cells (APCs) such as DCs and macrophages triggers both degradation and subsequent presentation of pathogen-derived peptides. In addition, TLRs recognize pathogen-derived components, inducing the expression of genes such as co-stimulatory molecules and inflammatory cytokines. Antigen-presentation together with TLR-mediated gene expression of co-stimulatory molecules and inflammatory cytokines drives naïve T-cells to differentiate, especially into T_H1 cells (Reprinted from Takeda and Akira, 2005).

The innate immune system significantly contributes to activation of adaptive immune responses. Detection of microbial presence by TLRs of the innate immune system leads to downstream signalling events that initiate the process of dendritic cell (DC) maturation. The maturation process includes up-regulation of co-stimulatory molecules (CD40, CD80 and CD86) and major histocompatibility complex (MHC) molecules bearing pathogen-derived peptides, production of pro-inflammatory cytokines (e.g. IL-12 and TNF-α) and altered expression of chemokine receptors (CCR2, CCR5 and CCR7). Mature

antigen-presenting cells (APCs) such as DCs signal T-cells and induce clonal expansion of antigen-specific T-cells. Antigen-presentation in the absence of co-stimulatory molecules results in a state of non-responsiveness to the antigen, termed anergy. In addition to its contribution to co-stimulatory molecule induction, the innate immune system is also responsible for induction of chemokines and cytokines, such as IL-12 and IL-18, by APCs. Production of IL-12 and IL-18 drives naïve T-cells to differentiate in T_H1 cells (Akira et al., 2001) (Figure I.1.3.). Also, B-cells express receptors of the innate immune system and respond to their ligands by proliferation and expression of co-stimulatory molecules (Pasare and Medzhitov, 2004).

1.3.2. Toll/IL-1R family in mammals

The Toll/IL-1R superfamily is a family of type I transmembrane receptors characterized by a common cytoplasmic region of about 200 amino acids, which is known as the Toll/IL-1 receptor (TIR) domain. Within this domain are three conserved boxes that are crucial for signalling (Slack et al., 2000). In contrast, the extracellular regions are quite distinct: whereas the extracellular region of the TLR family contains leucine-rich repeats (LRR) that of the IL-1 receptor family (IL-1R) possesses three immunoglobulin (Ig)-like domains (Fig. I.1.4). The extracellular region is involved in ligand recognition and contains 19-25 tandem copies of the LRR motif. The LRR motif comprises 24-29 AAs containing the leucine-rich sequence XLXXLXLXX, where X denotes any AA. The LRR region is separated from the transmembrane region by a C-terminal domain characterized by a consensus motif CXC(X23)C(X17)C (Medzhitov, 2001). In spite of this conservation among LRR domains, TLRs can recognize the most diverse ligands, lacking any structural similarity (Bell et al., 2003).

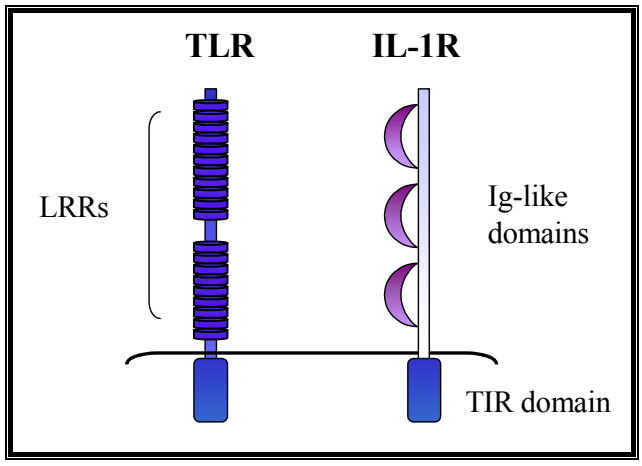

Figure I.1.4. - Toll/IL-1R superfamily. The Toll/IL-1R superfamily comprises the TLR family and the IL-1R family. The TLR family is characterized by leucine-rich repeats (LRRs) in their extracellular domain, while the IL-1R family has three Immunoglobulin (Ig)-like domains in their extracellular region.

The TIR domain is also found in a number of mammalian cytoplasmic proteins such as MyD88 (see further). In addition, cytoplasmic plant-disease-resistance proteins involved in plant immunity contain both TIR and LRR domains (Medzhitov, 2001). Also in the nematode *Caenorhabditis elegans* two genes encoding TIR domain proteins, which have a function in host defense, are found (Liberati et al., 2004; Pujol et al., 2001). The first TIR domain-containing protein identified was 'Toll' in *Drosophila*.
Toll was first identified in the early 1980s by Anderson, Jurgens and Nusslein-Volhard as an important factor in establishing the dorso-ventral axis in the early embryo of *Drosophila* (Anderson et al., 1985). The *Toll* gene was isolated and sequenced in 1988 (Hashimoto et al., 1988). In the 1990s, Toll was proved to be a type I transmembrane receptor containing blocks of LRRs in the ectodomain (Buchanan and Gay, 1996), flanked by characteristic cysteine-rich motifs and a cytoplasmic domain with similarities to that of the IL-1R, therefore referred to as the Toll/IL-1R (TIR) domain (Schneider et al., 1991). A study using Toll-mutant flies demonstrated that Toll is important in detecting fungal infection and demonstrated its important role in innate immune responses of *Drosophila* (Lemaitre et al., 1996).

1.3.2.1. Toll-like receptors, belonging to the Toll/IL-1R superfamily

One year after the discovery of *Drosophila* Toll, a mammalian homologue of the Toll receptor, now termed TLR4, was found to induce the expression of genes involved in inflammatory responses (Medzhitov et al., 1997). After the characterization of the first mammalian TLR, several structurally related proteins, named Toll-like receptors, were identified (Rock et al., 1998). TLRs belong to the Toll/IL-1 receptor superfamily. To date, thirteen Toll-like receptors have been reported, ten in humans and twelve in mice (Tabeta et al., 2004) (Table I.1.3.). TLR1-9 are conserved between human and mouse. In contrast to human TLR10, mouse TLR10 is non-functional. Human TLR11 is not produced, because of a stopcodon in the *Tlr11* gene. Mouse TLR11 is functional (Zhang et al., 2004). Mice express TLR12 and TLR13, but both TLRs are absent in humans (Tabeta et al., 2004). The TLR family is specialized for functioning in immune responses and comprises a family of cell-surface (TLR1, TLR2, TLR4, TLR5, TLR6, TLR11) and endosomally (TLR3, TLR7, TLR8, TLR9) expressed receptors.

The TLRs are evolutionary conserved to recognize PAMPs ranging from bacteria (Hayashi et al., 2001; Poltorak et al., 1998a; Takeuchi et al., 1999; Zhang et al., 2004) to viruses (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2003; Lund et al., 2004; Tabeta et al., 2004) to fungi (Meier et al., 2003) to parasites (Campos and Gazzinelli, 2004). The innate immune system uses not only TLRs for detecting infection, but also for detecting sterile inflammation, driven by ligands derived from damaged cells which

are usually not present in the extracellular environment. Examples are heat-shock proteins, β-defensins and oxidized lipids. Some TLRs are able to form heterodimeric complexes to activate innate immunity (Ulevitch, 2004).

Toll-like receptors and their (synthetic) ligands

TLR2. TLR2 recognizes a broad range of microbial components (see Table I.1.3.), but is primarily activated by PGN and lipoproteins. Its expression is regulated and seems to be restricted to APC and endothelial cells (Muzio et al., 2000). TLR2-deficient mice respond to LPS as wild-type mice, but are hypo-responsive to Gram-positive bacterial cell wall PGN and highly susceptible to *Staphylococcus aureus* infection (Takeuchi et al., 2000; Takeuchi et al., 1999). This is confirmed by a report showing that over-expression of human TLR2 in Chinese hamster ovary (CHO)/CD14 cells activated response to PGN from *Staphylococcus aureus* (Yoshimura et al., 1999). TLR2 can also recognize atypical LPS from *Leptospira interrogans* and *Porphyromonas gingivalis*, which differ from the typical LPS by the number of acyl chains. However, it was recently observed that LPS from *P. gingivalis* is only a poor activator of TLR2 (Hashimoto et al., 2004).

The broad range of microbial components recognized by TLR2 can be explained by 2 mechanisms. The first explanation is that TLR2 cooperates with two other structurally related TLR family members: TLR6 and TLR1 (Ozinsky et al., 2000; Takeuchi et al., 2001). Combinations of TLRs can recognize agonists not effectively recognized with individual TLRs. Both TLR1 and TLR6 are expressed constitutively on many cell types (Muzio et al., 2000). The second explanation of this broad range of ligands recognized by TLR2 is the functional cooperation of TLR2 with distinct types of receptors such as Dectin-1, a PRR recognizing the fungal cell wall component β-glucan (Gantner et al., 2003).

TLR1/2. TLR1 and TLR2 are co-expressed in normal human monocytes and functionally cooperate with each other. Co-transfection of TLR1 and TLR2 into HeLa cells leads to enhanced responsiveness to soluble factors released from *Neisseria meningitides*, while expression of only TLR1 or TLR2 is not sufficient to confer responsiveness (Wyllie et al., 2000). Using TLR1-deficient mice, it became clear that TLR1 is involved in the recognition of triacylated lipopeptides as well as lipoproteins from mycobacteria. TLR1-deficient mice were most impaired in response to lipopeptides with an N-palmytoyl-S-dilauryl cysteine residue, demonstrating that TLR1 discriminates a subtle difference in the lipid moieties of lipopeptides (Takeuchi et al., 2002). In addition, it was shown that TLR1 is important in the recognition of the outer-surface lipoprotein (OspA) of the pathogen *B. burgdorferi* (Table I.1.3.) (Alexopoulou et al., 2002).

Table I.1.3. - Toll-like receptors and their (synthetic) ligands (Adapted from Takeda *et al*., 2003).

TLRs	Ligands	References
TLR1	Triacyl lipopeptides (bacteria, mycobacteria)	(Takeuchi et al., 2002)
	Soluble factors (*Neisseria meningitides*)	(Wyllie et al., 2000)
	Triacyl lipopeptides (synthetic)	(Ulevitch, 2004)
TLR2	Pepidoglycan (G+)	(Schwandner et al., 1999; Takeuchi et al., 1999)
	LTA (G+)	(Schwandner et al., 1999)
	Zymosan (yeast)	(Underhill et al., 1999)
	Lipoproteins/lipopeptides (a variety of pathogens)	(Aliprantis et al., 1999; Brightbill et al., 1999)
	LAM (*Mycobacteria*)	(Means et al., 1999)
	A phenol-soluble modulin (*Staphylococcus epidermis*)	(Hajjar et al., 2001)
	Porins (*Neisseria*)	(Massari et al., 2002)
	LPS (*Leptospira interrogans, Porphyromonas gingivalis*)	(Hirschfeld et al., 2001; Werts et al., 2001)
	GPI (*Trypanosoma cruzi*)	(Coelho et al., 2002)
	Glycolipids (*Treponema maltophilum*)	(Opitz et al., 2001)
	Di- and triacyl lipopeptides (synthetic)	(Ulevitch, 2004)
TLR3	DsRNA (virus)	(Alexopoulou et al., 2001)
	Poly (I:C) (synthetic)	(Alexopoulou et al., 2001)
TLR4	LPS (G-)	(Hoshino et al., 1999; Poltorak et al., 1998a)
	Taxol (plant)*	(Kawasaki et al., 2000)
	Fusion protein (RSV)	(Kurt-Jones et al., 2000)
	Envelope proteins (MMTV)	(Rassa et al., 2002)
	Heat-sensitive cell-associated factor (*M. tuberculosis*)	(Means et al., 1999)
	HSP60 (*Chlamydia pneumoniae*)*	(Bulut et al., 2002)
	MPL, a mimetic of LPS/Lipid A (synthetic)	(Ulevitch, 2004)
	E5564 (Synthetic lipid A)	(Ulevitch, 2004)
TLR5	Flagellin (bacteria)	(Hayashi et al., 2001)
TLR6	Diacyl lipopeptides (mycoplasma)	(Takeuchi et al., 2001)
	LTA (G+)	(Ozinsky et al., 2000; Schwandner et al., 1999)
	Zymosan (yeast)	(Ozinsky et al., 2000)
	Diacyl lipopeptides (synthetic)	(Ulevitch, 2004)
TLR7	(G+U)-rich ssRNA (virus)	(Diebold et al., 2004; Heil et al., 2004)
	Imidazoquinoline (synthetic)	(Hemmi et al., 2002)
	Loxoribine (synthetic)	(Heil et al., 2003)
	Bropirimine (synthetic)	(Heil et al., 2003)
TLR8	**(**G+U)-rich ssRNA (virus)	(Heil et al., 2004)
	Imidazoquinoline (synthetic)	(Jurk et al., 2002)
TLR9	Unmethylated bacterial CpG DNA (bacteria and virus)	(Hemmi et al., 2000)
	CpG oligodeoxynucleotides (synthetic)	(Ulevitch, 2004)
TLR10(h)	ND	(Hasan et al., 2005)
TLR11(m)	Uropathogenic bacteria	(Zhang et al., 2004)
	Profilin-like protein (Toxoplasma gondii)	(Lauw et al., 2005)
TLR12(m)	ND	(Tabeta et al., 2004)
TLR13(m)	ND	(Tabeta et al., 2004)

*It is possible that these ligand prepariations were contaminated with LPS and/or other potent microbial components.

TLR6/2. TLR2 and TLR6 physically interact in the cell, as demonstrated by co-immunoprecipitation of TLR2 and TLR6. Both receptors cooperate in the response to PGN of Gram-positive bacteria and zymosan of the yeast cell-wall (Ozinsky et al., 2000). Recognition of a phenol-soluble modulin from *Staphylococcus epidermidis* by TLR2 is enhanced by TLR6 (Hajjar et al., 2001). Using TLR6-deficient cells, it was proven that these cells are unresponsive to diacylated mycoplasmal macrophage-activating lipopeptide (MALP)-2, but retain their normal responses to triacylated lipopeptides of other bacterial origins. Reconstitution experiments in TLR2/TLR6 double-knockout embryonic fibroblasts demonstrated that both TLR2 and TLR6 are required for MALP-2 responsiveness. In conclusion, association of TLR6 with TLR2 is necessary to discriminate between diacyl and triacyl lipopeptides (Table I.1.3.) (Takeuchi et al., 2001).

TLR10. Human TLR10 is most closely related to TLR1 and TLR6 and is located in the *Tlr6-Tlr1-Tlr10* gene cluster. TLR10, primarily expressed on B-cells and pDCs, also heterodimerizes with TLR1 and TLR2 (Hasan et al., 2005). The specific ligands and functions of TLR10 are currently unknown.

TLR3. TLR3 is located in the endosomal membranes (Matsumoto et al., 2003) and has unique features among the mammalian TLRs. First, TLR3 is different from other TLRs in its genomic organization and it also lacks the conserved proline residue at codon 712 common to other TLRs. A mutation in this proline residue in the *Tlr4* gene of C3H/HeJ mice results in hypo-responsiveness to LPS (see further). Also, equivalent substitutions in some other TLRs abrogates their inflammatory responses (Ozinsky et al., 2000; Underhill et al., 1999), suggesting that TLR3 signalling might differ from the signalling mechanisms of other TLRs. TLR3 indeed differs from the other TLRs in not requiring MyD88 for signalling, but recruiting TRIF as the critical adaptor protein (Yamamoto et al., 2002). Secondly, TLR3 is expressed in specific celtypes. It is expressed in specific myeloid cells, vascular endothelial cells and airway epithelial cells. Also, there are differences between mice and humans. Mouse TLR3 is highly expressed in macrophages, whereas human TLR3 is highly expressed in immature dendritic cells (DCs) (Muzio et al., 2000; Rehli, 2002). In mice, TLR3 expression is induced by LPS and IFNs, particularly IFN-β, while in human its expression is only induced by IFNs, and also particularly IFN-β (Heinz et al., 2003).

TLR3 is a major mediator of cellular response to certain viral infections, because it responds to dsRNA, produced as an essential intermediate in RNA synthesis or as a by-product of replication of some viruses. TLR3 activation leads to induction of type I IFN (IFN-α/β), which exerts anti-viral and

immunostimulatory activities, transcription of IFN-inducible genes and maturation of DCs. Alexopoulou *et al*. showed that some synthetic dsRNA molecules, such as poly (I:C), have similar activity as dsRNA. TLR3-/- mice showed reduced responses to poly (I:C), resistance to the lethal effects of poly (I:C) when sensitized with d-galactosamine (d-GalN), and reduced production of inflammatory cytokines (Alexopoulou et al., 2001). Enforced expression of human TLR3 in the dsRNA-non-responsive cell line HEK293, enables the activation of NF-κB and the IFN-β promotor in response to dsRNA and poly (I:C) (Alexopoulou et al., 2001; Matsumoto et al., 2002).

TLR3 plays a role in clearing a mouse cytomegalovirus (MCMV) infection (Tabeta et al., 2004) and is important in the response to influenza virus infection (Guillot et al., 2005). On the other hand, Edelmann *et al*. claimed that TLR3 is not required for the generation of antiviral responses after MCMV, reovirus, vesicular stomatitis virus (VSV) and lymphocytic choriomeningitis virus (LCMV) infection (Edelmann et al., 2004). TLR3 can also recognize West Nile virus (WNV), a mosquito-borne ssRNA flavivirus. TLR3-deficient mice were more resistant to lethality following WNV infection. Furthermore, TLR3-deficiency leads to impairment of inflammatory cytokine production and enhanced viral load in the periphery, whereas in the brain, viral load, inflammatory responses and neuropathology were reduced compared to wild-type mice (Wang et al., 2004).

TLR4. In 1965, it was observed that mice of the C3H/HeJ strain were highly resistant to the lethal effect of LPS (Heppner and Weiss, 1965). Later it became clear that responses to LPS were impaired by a single mutation affecting a locus that was called *Lps* (Watson and Riblet, 1974). By the adoptive transfer of bone marrow cells from LPS-sensitive mice (C3H/HeN) to LPS-resistant mice (C3H/HeJ), it was demonstrated that C3H/HeJ mice can be rendered sensitive to LPS. So, this suggested that lymphocytes and/or macrophages play a primary role in responsiveness to LPS (Michalek et al., 1980). Also, C3H/HeJ mice showed enhanced susceptibility to infection by Gram-negative bacteria, suggesting that recognition of LPS is essential for clearing of the infection (O'Brien et al., 1980). In 1998, *Tlr*4 on mouse chromosome 4 has been identified as the gene encoded by *Lps* by positional cloning in the LPS-non-responsive C3H/HeJ mouse (Poltorak et al., 1998b). A point mutation in the third exon of *Tlr4* modifies the protein within the TIR domain and creates a co-dominant inhibitory effect on LPS signal transduction. The mutation results in amino acid change from proline to histidine at position 712 (Poltorak et al., 1998a). The role of TLR4 in LPS-induced responses was further supported by the demonstration that TLR4-null mice had a phenotype similar to that of C3H/HeJ mice (Hoshino et al., 1999). In addition, a second spontaneous mutation that caused LPS susceptibility was identified. C57BL/10ScCr mice were shown to have a deletion of *Tlr4*, yielding a recessive loss of the LPS response. Also, polymorphisms in both the ectodomains and the cytoplasmic domains of human TLR4

have been identified and these are correlated with an increased susceptibility to Gram-negative infections (Agnese et al., 2002). In addition to LPS, TLR4 recognizes several other molecules (Table I.1.3.). TLR4 is expressed in a variety of cell types, most predominantly in myeloid cells, including macrophages and DCs (Medzhitov et al., 1997).

TLR5. TLR5 is involved in recognition of flagellin, a conserved monomeric protein of bacterial flagella, which is a very complex structure that extends out from the outer membrane of certain Gram-negative bacteria. Recognition of an evolutionary conserved domain of flagellin is mediated through close physical interaction between TLR5 and flagellin (Smith et al., 2003). TLR5 can recognize bacterial flagellin from both Gram-positive and Gram-negative bacteria. Enforced expression of human TLR5 in CHO cells enables the response to bacterial flaggelin (Hayashi et al., 2001). Interestingly, TLR5 is expressed on the basolateral, but not the apical side of intestinal epithelial cells (Gewirtz et al., 2001), where it is able to recognize flagellin from pathogenic bacteria. Pathogenic, but not commensal, microorganism can cross the epithelial barrier, which enables the host to discriminate between commensals and pathogens. The intestinal endothelial cells of the subepithelial compartment also express TLR5 (Maaser et al., 2004). A common stop codon polymorphism in the ligand-binding domain of TLR5 unables flagellin signalling and is associated with susceptibility to pneumonia caused by *Legionella pneumophila*, a flagellated bacterium (Hawn et al., 2003). TLR5 forms homomeric complexes as well as heteromeric complexes with TLR4. Flagellin induces distinct patterns of inflammatory mediators depending on the nature of the TLR5 signalling complex. For example, the induction of NO by flagellin involves signalling via TLR5/TLR4 complexes (Mizel et al., 2003).

TLR7, TLR8 and TLR9. TLR7, TLR8 and TLR9 compose a subfamily of TLRs based on phylogenetic analysis. TLR7 and TLR8 are the most closely related TLR family members (Du et al., 2000). TLR7/8/9 are strongly expressed by pDCs (Liu, 2005) and primarily in the endosomal compartment (Ahmad-Nejad et al., 2002; Diebold et al., 2004). The endosomal location of TLR9 in a mouse macrophage cell line was revealed using a monoclonal antibody against TLR9 (Ahmad-Nejad et al., 2002). Signalling by TLR7/8/9 is inhibited by chloroquine, indicating the requirement for endosomal acidification in this process (Heil et al., 2003). Not only in pDCs, but also in other celtypes such as in immature B-cells and in macrophages expression is found of TLR7/9 (Applequist et al., 2002).

By using TLR-deficient mice and genetic complementation, the natural ligand of murine TLR7 and human TLR8 was observed to be guanosine (G)- and uridine (U)-rich ssRNA oligonucleotides derived from viruses such as human immunodeficiency virus (HIV), VSV and influenza virus (Diebold et al.,

2004; Heil et al., 2004; Lund et al., 2004). Mammalian DNA has a low frequency of CpG dinucleotides. Mammalian CpG dinucleotides are mostly methylated on their cytosine residues and therefore mammalian DNA does not have immuno-stimulatory activity. In contrast, bacteria lack cytosine methylation and thus, unmethylated CpG DNA might signal the presence of microbial infection. DNA containing unmethylated CpG motifs is common to both bacterial and viral genomes. Using TLR9-deficient mice it was demonstrated that TLR9 is a receptor for unmethylated CpG DNA (Hemmi et al., 2000). Signalling by CpG DNA requires its internalization into late endosomal or lysosomal compartments (Hacker et al., 1998). Another interesting aspect of CpG DNA recognition is that human and mouse cells are optimally activated by slightly different sequence motifs flanking CpG dinucleotides (Krieg and Wagner, 2000). This can be explained by species differences among TLR9s. Enforced expression of human or mouse TLR9 in the CpG DNA-unresponsive cell line HEK293, enabled the cells to respond respectively to the optimal human or mouse CpG sequences. Therefore, TLR9 can presumably recognize CpG DNA directly (Bauer et al., 2001). Also, it was shown that herpes simplex virus (HSV)-2 stimulates IFN-α production by pDCs through TLR9 (Lund et al., 2003).

TLR7 and TLR8 can also detect synthetic compounds. Using TLR7 knockout mice it was demonstrated that TLR7 is able to respond to synthetic Imidazoquinolines (e.g Imiquimod and Resiquimod), which have structures similar to nucleic acids (Hemmi et al., 2002). Two other compounds, Loxoribine and Bropirimine, are also recognized by TLR7 (Heil et al., 2003). Stimulation with those synthetic compounds induces cytokine production and upregulation of co-stimulatory molecules and MHCI/II (Lee et al., 2003). Some of these compounds are in clinical use. For example, Bropirimin is being used against renal cell carcinoma (Sarosdy, 1993) and Imidazquinolines are used for treatment of genital warts associated with viral infection (Hemmi et al., 2002). This indicates that a screen for TLR-ligands can be useful for clinical applications. Human TLR8, but not murine TLR8, is also activated by an Imidazoquinoline (Jurk et al., 2002).

TLR11. The first ligand for mouse TLR11 identified is a profilin-like protein from *Toxoplasma gondii* (Lauw et al., 2005). TLR11-null mice are highly susceptible to infection with uropathogenic *Escherichia coli*. TLR11 is expressed in bladder epithelial cells, liver, kidney and macrophages. There is no functional TLR11 protein in humans (Zhang et al., 2004).

Toll-like receptors and endogenous ligands

An increasing number of studies implicate TLRs as being involved in the immune response to self-molecules that have in some way been altered from their native state or accumulate in non-physiologic sites or amounts, although questions have been raised about possible contaminants in certain of these studies.

It was suggested that TLR2 can interact with endogenous ligands such as high mobility group box 1 protein (HMGB1) and HSPs, such as HSP60, HSP70 and GP96 (Asea et al., 2002; Park et al., 2005; Vabulas et al., 2001; Vabulas et al., 2002b). However, activation of TLR2 by HSPs has recently been determined to be due to contamination with pathogen-associated molecular patterns (Table I.1.4.). TLR3 is also involved in the recognition of endogenous RNA released from or associated with necrotic cells or generated by *in vitro* transcription and induces immune activation (Table I.1.4.) (Kariko et al., 2004).

Table I.1.4. - Toll-like receptors and endogenous ligands.

TLRs	Endogenous ligands	References
TLR2	HSP60*	(Vabulas et al., 2001)
	HSP70*	(Asea et al., 2002)
	GP96*	(Vabulas et al., 2002b)
	HMGB1	(Park et al., 2005)
TLR3	mRNA	(Kariko et al., 2004)
TLR4	HSP60*	(Vabulas et al., 2001)
	HSP70*	(Asea et al., 2002)
	GP96*	(Vabulas et al., 2002b)
	Oligosaccharides or hyaluronic acid	(Termeer et al., 2002)
	Fibrinogen	(Smiley et al., 2001)
	Type III repeat extra domain A of fibronectin	(Okamura et al., 2001)
	Polysaccharide fragments of heparan sulphate	(Johnson et al., 2002)
	Surfactant protein-A	(Guillot et al., 2002)
	β-Defensin 2	(Biragyn et al., 2002)
	HMGB1	(Park et al., 2005)

*These putative endogenous ligands of TLR2 and TLR4 have recently been discovered to be due to contamination with pathogen-associated molecular patterns. Thus, more data are needed to conclude that TLRs recognize these endogenous ligands.

Numerous studies have identified a role for TLR4 in endogenous ligand recognition (Table I.1.4.). For example, it has been suggested that TLR4 is involved in the recognition of HSP60 and HSP70 (Asea et al., 2002; Vabulas et al., 2001; Vabulas et al., 2002a). Also, a role for TLR4 in recognition of

extracellular matrix (ECM) components, including the type III repeat extra domain A of fibronectin, oligosaccharides of hyaluronic acid, and polysaccharide fragments of heparan sulfate has been demonstrated (Johnson et al., 2002; Okamura et al., 2001; Termeer et al., 2002). Fibrinogen is another endogenous ligand of TLR4 (Smiley et al., 2001). All of these components can be released upon inflammation, suggesting that TLR4 is also involved in inflammatory processes even in the absence of infection. However, to activate TLR4 very high concentrations of all of these ligands are necessary. Therefore the possibility remains that these preparations might be contaminated with LPS. Multiple studies have demonstrated that contamination of HSP preparations with LPS was the reason for TLR4 activation (Bausinger et al., 2002; Gao and Tsan, 2003). In addition, TLR4-deficient C57BL/10ScCr mice still bind HSP60 on their macrophages, but do not demonstrate HSP60-induced production of inflammatory cytokines (Habich et al., 2002). Therefore, more studies have to be performed to confirm the ability of TRL4 to recognize endogenous ligands.

1.3.2.2. The IL-1R family, belonging to the Toll/IL-1R superfamily

The IL-1R family comprises true receptors such as IL-1R1 and IL-18Rα, decoy receptors such as IL-1R2 and accessory proteins such as IL-1R accessory protein (IL-1RAcP). Single Ig domain IL-1R related protein (SIGIRR or TIR8) and ST2 (IL-1RL1), both involved in negative regulation of IL-1 and LPS signalling, belong also to this family (Sims, 2002). The IL-1R1 is a receptor for IL-1, refering to both IL-1α and IL-1β molecules, and IL-1 receptor antagonist (IL-1Ra), while the IL-1R2 can only bind IL-1. IL-1α and IL-1β are agonists and IL-1Ra is a specific receptor antagonist. IL-1α, IL-1β and IL-1Ra belong to the large IL-1 gene family. Binding of IL-1Ra to the receptor complex prevents or disrupts the complex between IL-1 and the receptor (Dinarello, 1996). The IL-1R1 is an integral membrane protein containing a single membrane-spanning segment of 21 amino acid residues. The extracellular ligand-binding domain of the receptor consists out of three Ig-like domains (319 amino acid residues). The intracellular domain contains 217 amino acid residues. The human and murine IL-1 receptors are very similar (Sims et al., 1989). Similar to the IL1-R1, the IL-1R2 has three extracellular Ig-like domains and a short transmembrane region. In contrast, the IL-1R2 has a truncated cytoplasmic domain of only 29 intracellular amino acid residues. IL-1 mediated signalling occurs exclusively through IL-1R1 and the IL-1R2 is not able to transduce signals (Sims et al., 1993). Instead, IL-1R2 acts as a decoy receptor, inhibiting IL-1 signalling by capturing and blocking IL-1 (Re et al., 1996). It is likely that heterodimerization of the intracellular domains of IL-1R1 and IL-1RAcP triggers IL-1 signal transduction. IL-1R1 is mainly expressed on endothelial cells, epithelial cells, fibroblasts, epidermal dendritic cells, and T lymphocytes, while IL-1R2 is predominantly present on B-cells, monocytes, bone marrow cells

and neutrophils (Dinarello, 1996). IL-1 is a proinflammatory cytokine that elicits its pleiotropic effects through activation of the transcription factors NF-κB and activator protein-1 (AP-1). IL-1 is involved in several inflammatory and autoimmune pathologies, such as in septic shock, rheumatoid arthritis, inflammatory bowel diseases, psoriasis, autoimmune diabetes, osteoporosis, leukemias and solid tumors and many other pathological conditions (Dinarello, 1996).
Similar to IL-1, IL-18, another member of the IL-1 family, participates in both innate and acquired immunity. IL-18 activates the IL-18 receptor (IL-18R) complex. This IL-18R complex consist out of IL-18Rα, a member of the IL-1R family, and a signalling chain, also a member of the IL-1R family. Binding of IL-1/IL-18 to their receptors results in recruitment of the adaptor protein MyD88 and activates a signalling pathway similar to that of TLRs (Dinarello, 1999).

1.3.3. Non-TLR pattern-recognition receptors recognizing PAMPs of different microorganisms

Several non-TLR PRRs can recognize LPS. Among them are some members of the macrophage scavenger receptor (MSR) family. Scavenger receptors are integral membrane proteins expressed by mammalian monocytes/macrophages and certain endothelial cells. They play an important role in lipid transport, but also in host defence and in the regulation of adaptive immunity. They bind to bacterial cell walls and their products including Gram-negative bacteria (LPS), Gram-positive bacteria (lipoteichoic acid or LTA), intracellular bacteria and CpG DNA. They are essential in the clearing of bacteria from the circulation (Gordon, 2002; Peiser et al., 2002). The scavenger receptor family comprises at least eight different subclasses (A-H). The different subclasses bear little sequence homology to each other but recognize common ligands (Murphy et al., 2005). Macrophage scavenger receptor class A types I and II (MSR-A) knockout mice have smaller atherosclerotic lesions and are also more susceptible to infection with *Listeria monocytogenes* (Kobayashi et al., 2000; Suzuki et al., 1997). MARCO, a MSR-A family member, is a macrophage receptor with a collagenous structure encoded by a distinct gene. MARCO binds to a range of Gram-negative and Gram-positive bacteria to clear them of circulation (Gordon, 2002).

The macrophage mannose receptor (MMR), a member of the calcium-dependent lectin family, is an endocytic PRR expressed by macrophages, DCs and some endothelial cells. It functions as a secreted PRR and specifically recognizes microbial carbohydrates through its carbohydrate recognition domains (CRD) and mediates their phagocytosis by macrophages (Gordon, 2002). In addition, it recognizes LPS of *Klebsiella pneumoniae* (Zamze et al., 2002). Type 3 complement receptor (CR3), also known as Mac-

1 or CD18/CD11b, is a β2 integrin expressed on cells of myeloid origin. It is mainly a phagocytic receptor for complement opsonised particles and contributes to the clearance of apoptotic cells (Gordon, 2002). It can also interact with LPS (Wright and Jong, 1986). Malhotra and colleagues showed that L-selectin on neutrophils (Malhotra et al., 1996) and P-selectin on platelets (Malhotra et al., 1998) are able to bind LPS. A receptor on the plasma membrane of rat hepatocytes can also bind with heptose residues in the inner core of LPS (Parent, 1990).

LPS binding protein (LBP) is important in the LPS response by accelerating the binding of LPS to CD14, a glycerosylphosphatidylinositol (GPI)-anchored protein (see further) (Yu and Wright, 1996). Other LPS-binding proteins such as bactericidal/permeability-increasing protein (BPI), cathelicidin antimicrobial peptide (CAP)18, CAP37, serum amyloid P (SAP), lactoferrin and lysozyme have been characterized (Van Amersfoort et al., 2003).

The nucleotide-binding site and leucine-rich repeat (NBS-LRR) family of proteins, involved in intracellular recognition of microbes and their PAMPs, is characterized by a tripartite structure with a C-terminal LRR domain, a central nucleotide binding site (NBS) domain and an N-terminal effector domain, such as a pyrin (PYD), a caspase-recruitment domain (CARD) or a baculovirus inhibitor of apoptosis repeat (BIR) domain. The LRR domain is necessary for sensing microbial motifs. The NBS domain is essential for the oligomerization of the molecule and its subsequent transactivation capacity. The N-terminal CARD and PYD signal through homophylic interactions with other CARD/PYD containing molecules. Nucleotide-binding oligomerisation domain (Nod) molecules belong to this NBS-LRR family of PRRs. Nod-1 and Nod-2, are cytoplasmic surveillance proteins. Structurally the Nod proteins are similar to the R proteins of plants, involved in disease-resistance against pathogens (Philpott and Girardin, 2004). Nod-1 and Nod-2 both recognize bacterial peptidoglycan (PGN) although distinct motifs of PGN. The core structure recognized by Nod-1 is d-Glu-*meso*-DAP, a naturally occurring PGN degradation product of Gram-negative bacteria (Chamaillard et al., 2003). In contrast, Nod-2 recognizes muramyl dipeptide (MDP), the minimal bioactive PGN motif common to both Gram-positive and Gram-negative bacteria. Before, it was thought that Nod-1 and Nod-2 could recognize LPS, but recent reports have demonstrated that this was due to contamination of LPS preparations with PGN motifs (Girardin et al., 2003).

TLR3-deficient mice are only partially impaired in dsRNA recognition (Alexopoulou et al., 2001). Various molecules for the TLR3-independent recognition of dsRNA and viruses are known. PKR or dsRNA-

dependent protein kinase is an interferon-induced serine-threonine kinase implicated in dsRNA recognition. PKR stops protein synthesis by phosphorylating the alpha subunit of the translation initiation factor eIF2 (Taylor et al., 2005). Retinoic acid-inducible gene I (RIG-I) is a DexD/H box RNA helicase containing a caspase recruitment domain (CARD). It was demonstrated using RNAi knockdown experiments and ectopic expression of RIG-I, that RIG-I is important in dsRNA- and virus-induced type I IFN expression (Yoneyama et al., 2004). In fibroblasts and conventional dendritic cells (cDCs), RIG-I induces type I IFNs by activating IRF-3 via IκB kinase-related kinases. In contrast, plasmacytoid DCs (pDCs), which produce large amounts of IFN-α, use TLR3 rather than RIG-I for viral detection (Kato et al., 2005). Melanoma differentiation-associated gene (MDA) 5 is another CARD and helicase domain containing protein which senses viral RNA (Yoneyama et al., 2005).

Table I.1.5. gives an overview of PRRs recognizing PAMPs of different microorganisms and some other of their endogenous and exogenous ligands.

Table I.1.5. – Pattern-recognition receptors and their ligands.

Receptor (Family)	Example(s)	Ligand(s)
Scavenger receptors and related	MSR-A I/II	LPS, LTA, oxidized LDL, apoptotic cells
	MARCO	G^-/G^+ bacteria, LPS
C-type lectins and related	MMR	*Klebsiella* LPS, lipoarabinomannan, mannosyl, fucosyl
β2 Integrins	CR3 (CD18/11b)	LPS, C3bi, C3b, ICAM-1/2, zymosan
L-selectin		LPS, LTA, GlyCAM-1, CD34, MAdCAM-1
P-selectin		LPS, PSGL-1 (sialyl Lewis-X moiety)
Heptose receptor		LPS
GPI-anchored proteins	CD14	LBS, PGN, LBP, apoptotic cells, HDL, HSP, fibrinogen
LPS binding molecules	LBP, BPI, CAP18, CAP37, SAP, lactoferrin, lysozyme	LPS
NBS-LRR	Nod-1	LPS(?), PGN of G^-
	Nod-2	LPS(?), PGN of G^-/G^+
PKR		dsRNA
RIG-I		dsRNA
MDA5		dsRNA

1.4. Recognition of LPS

1.4.1. Requirement of LBP, MD-2 and CD14

TLR4 forms a complex with several proteins on the cell surface to recognize and respond to LPS. Overexpression of TLR4 in HEK293 cells is not sufficient for LPS-signalling, indicating the necessity for additional factors (Kirschning et al., 1998).

LBP. Until the purification of LBP from rabbit serum in 1986, it was believed that LPS activates immune cells through spontaneous intercalation of lipid A into the mammalian lipid bilayer (Tobias et al., 1986). LBP was demonstrated to bind with the lipid A moiety of rough and smooth LPS (Tobias et al., 1989). It is constitutively synthesized in hepatocytes as a single polypeptide, glycosylated, and secreted into the bloodstream as a 60-kD glycoprotein (Grube et al., 1994; Ramadori et al., 1990). In all species, including human, LBP behaves as an acute phase protein (Schumann et al., 1996). Its synthesis is under control of cytokines (e.g IL-1β and IL-6) and steroid hormones (e.g. glucocorticoids) (Grube et al., 1994; Schumann et al., 1996). Upon infection, the levels of LBP in serum greatly increases (Gallay et al., 1993). Besides the liver, LBP is also produced in the lung, kidneys and heart (Su et al., 1994).
LBP is closely related to bactericidal/permeability-increasing protein (BPI), a protein found in the granules of polymorphonuclear neutrophils (PMN) and able to bind LPS with high affinity (Elsbach and Weiss, 1993). In contrast to LBP, BPI neutralizes the LPS activity (Gallay et al., 1993). LBP has opsonic activity, but its importance as a protein involved in LPS response lies with its abilities to accelerate the binding of endotoxin to either membrane or soluble CD14 (Yu and Wright, 1996). LPS bound to LBP is 100-to 1000-fold more potent, as measured by the response of $CD14^+$ cells (Martin et al., 1992). The C-terminal part of LBP is necessary for interaction with CD14. A truncated form of human LBP comprising amino acid residues 1-197, binds LPS but does not transfer LPS to CD14 (Han et al., 1994). Residues 91-108 of the N-terminal part of the molecule are necessary for LPS binding (Taylor et al., 1995), while the LPS binding site of LBP is localized in the N-terminal part.
The importance of LBP in LPS signalling is obvious from studies with LBP knockout animals, which are hypersusceptible to invasion by otherwise harmless numbers of Gram-negative bacteria (Fierer et al., 2002; Jack et al., 1997). This sentinel function of LBP is shared by CD14 (Moore et al., 2000). LBP has been shown to contribute to LPS toxicity in experimental endotoxemia. Blockade of LBP activity with polyclonal Abs was found to protect mice from lethal endotoxemia (Gallay et al., 1993; Gallay et al., 1994). LBP contributes to the toxicity of high doses of LPS, and the transfer of LPS to HDL is not

sufficient to prevent the activation of cells via the LPS/LBP/CD14 pathway. LBP can transfer LPS also to high density lipoproteins and LPS is nearly biologically inactive when complexed to HDL (Vesy et al., 2000). Since the affinity of CD14 for LPS-LBP is greater than that of HDL, it is not clear whether the trafficking of LPS to HDL is a major pathway *in vivo*. High ratios of LBP to LPS may also inhibit the binding of LPS to lipid membranes and decrease the stimulatory effects of LPS on mononuclear cells (Gutsmann et al., 2001). CD14 greatly enhances the formation of LPS-TLR4-MD-2 complexes.

MD-2. Myeloid differentiation-2 (MD-2) is a 25-kDa secreted glycoprotein that exists on the cell surface in complex with transmembrane proteins. Following synthesis, MD-2 is either secreted directly into the medium as a soluble, active protein, or binds directly to TLR4 in the endoplasmic reticulum before migrating to the cell surface. MD-2 was first identified as a molecule similar to MD-1, a protein that binds with the B-cell specific receptor RP105. RP105 contains LRRs similar to *Drosophila* Toll and is involved in B-cell activation. It was thought that an analogous protein might associate with TLR4. Shimazu *et al.* identified that MD-2 physically associates with the extracellular leucine-rich repeats of TLR4 and augments TLR4-dependent LPS responses *in vitro* (Shimazu et al., 1999). MD-2-knockout mice do not respond to LPS, but are susceptible to *Salmonella typhimurium* infection. These phenotypes are identical to TLR4-knockout mice, demonstrating an absolute requirement for MD-2 in TLR4-dependent LPS responses *in vivo* (Nagai et al., 2002a). B-cells deficient for RP105 or MD-1 have also an impaired LPS response, revealing that B-cells require both TLR4/MD-2 and RP105/MD-1 clusters for intact LPS signalling (Nagai et al., 2002b).

LPS binds directly to cell surface TLR4-MD2 (Akashi et al., 2003; da Silva Correia et al., 2001). Furthermore, other groups have indicated that MD-2 binds to LPS and that the soluble MD-2/LPS complex behaves as an active ligand for TLR4 (Kennedy et al., 2004; Viriyakosol et al., 2001; Visintin et al., 2003). The amino-terminal region of TLR4 seems to be crucial for its association with MD-2, which is essential for the cell surface expression of the receptor and hence the recognition of LPS (Fujimoto et al., 2004).

CD14. CD14 belongs to the family of leucine-rich proteins. It is a 55-kDa glycerosylphosphatidylinositol (GPI)-anchored glycoprotein expressed on the plasma membrane of most cell types of the myeloid lineage (Tobias et al., 1995). Other LPS-responsive cells such as endothelial cells, smooth muscle cells, and some epithelial cell lines do not express this membrane-bound CD14 (mCD14), but contain the soluble form of CD14 (sCD14), which circulates in plasma without a GPI-tail (Pugin et al., 1993). Treatment with phosphatidylinositolphospholipase C (PI-PLC) cleaves CD14 between phosphatidylinositol and the diacylglycerol moiety and releases the protein from the cell surface. The

transfer of LPS from LBP-LPS complexes to mCD14 or sCD14 is catalyzed by LBP. Transgenic mice over-expressing CD14 are hypersensitive to LPS-induced endotoxin shock (Ferrero et al., 1993). In addition, CD14-deficient mice were found to be highly resistant to shock induced by either live Gram-negative bacteria or LPS. Surprisingly, despite the inability to respond to LPS and Gram-negative bacteria, CD14-null mice have an accelerated clearance of Gram-negative bacteria from the blood and tissues (Haziot et al., 1996). This is due to an enhanced infiltration of neutrophils that is normally delayed in CD14-expressing mice (Haziot et al., 2001). However, at very high concentrations of LPS or bacteria, responses through non-CD14 receptors could be detected (Haziot et al., 1996). In CD14-null macrophages, CD11b/CD18 receptors can compensate for deficiency in CD14 in response to whole bacteria (Moore et al., 2000). Blocking of CD14 only partially inhibits LPS-binding (Lynn et al., 1993). Neutralization of CD14 prevents lethal shock by LPS. However, after blocking LBP and CD14, mice challenged with virulent *Klebsiella pneumoniae* or *Salmonella typhimurium* did not survive. Thus, host responses to Gram-negative bacteria are not identical to that of LPS (Heumann et al., 2003). Also, CD14 is important in the response on TLR2-mediated peptidoglycan signalling (Dziarski and Gupta, 2005). CD14 lacks transmembrane and intracellular domains, which disables this molecule to transduce a signal inside the cell. So another molecule, namely TLR4, is necessary for signal transduction.

1.4.2. A receptor cluster as a model for TLR4 activation

Different studies have suggested that LPS must interact with a complex of transmembrane receptors for signal transduction. An activation cluster comprising of TLR4, CD14, CD11b/CD18, CD55, Hsp70, Hsp90, growth differentiation factor 5 (GDF5) and chemokine receptor 4 (CXCR4) was formed in response to LPS. The composition of these supramolecular activation clusters may change with the activation state of the cell. Stimulation with penta-acyl lipid A, which is a known LPS antagonist, triggered the recruitment of hsp70, hsp90, CD55 and, to a lesser extent, TLR4. CD11b/CD18, CD81, GDF5 and CXCR4 were not recruited within the cluster of receptors, thus suggesting that different shapes of LPS promote different protein–protein receptor associations (Pfeiffer et al., 2001; Triantafilou and Triantafilou, 2002). Another model described formation of an activator cluster composed of TLR4, CD14, CD11b/CD18, CD55, CD16a, Fcγ RIIIa, CD36 and CD81, Fcγ-receptors CD32 and CD16a after LPS or LTA stimulation (Pfeiffer et al., 2001).

1.5. References

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2. TLR/IL-1R SIGNALLING

2.1. TIR domain-containing adaptors

The first TIR domain-containing adaptor characterized was MyD88. At this moment, four additional TIR domain-containing adaptors have been identified. These include TIRAP/Mal, TRIF, TRAM and SARM (Figure I.2.1.) and will be discussed next.

MyD88. Myeloid differentiation factor 88 (MyD88) was originally identified as a myeloid differentiation primary response gene induced rapidly during IL-6-stimulated differentiation of M1 myeloleukaemic cells into macrophages (Lord et al., 1990). The protein contains a death domain (DD) in the N-terminal region and a TIR domain in the C-terminal region. Subsequently, MyD88 was cloned and proven to be an adaptor molecule involved in recruitment of IRAK to the IL-1R complex after IL-1 stimulation. MyD88 associates with the TIR domain of TLRs/IL-1Rs and links them with downstream signalling molecules containing DDs, an event leading to NF-κB activation (Wesche et al., 1997). MyD88 forms homodimers through its DD domain (DD-DD interaction) or through its TIR domain (TIR-TIR interaction), and is recruited as a homodimer to the receptor complex (Dunne et al., 2003). It is a common adaptor for all TLRs, except TLR3 (Medzhitov et al., 1998). In response to several TLR ligands, MyD88-deficient mice show an impaired pro-inflammatory cytokine (TNF-α, IL-12 and IL-6) production (Akira et al., 2001). In addition, a role for MyD88 in apoptosis has been proposed. Bacterial lipoproteins induce apoptosis of monocytic cells through a TLR2- and MyD88-dependent pathway and it is demonstrated by co-immunoprecipitation studies that Fas-associated death domain protein (FADD) and MyD88 interact through their DDs. Thus, an apoptotic signal is mediated via a MyD88 - FADD - caspase 8 pathway (Aliprantis et al., 2000). Also, a cellular splice variant of MyD88 (referred to as MyD88s), lacking only the short intermediate domain separating the DD and TIR domains, has been identified. MyD88s has been demonstrated to be involved in the regulation of IL-1R- and TLR4-mediated signalling. Over-expression of MyD88s inhibits IL-1- and LPS- induced NF-κB activation (Janssens and Beyaert, 2002; Janssens et al., 2002).

TIRAP/Mal. MyD88-deficient mice fail to produce inflammatory cytokines in response to LPS. On the other hand, activation of NF-κB and mitogen-activated protein kinase (MAPK) was still able, although with delayed kinetics (Kawai et al., 1999). This study suggested a role for an additional adaptor in LPS-induced signalling and led to the search for homologues of MyD88. Fitzgerald *et al.* discovered *Mal* (MyD88-adapter-like) during high-throughput sequencing of a human DC cDNA library as a gene with

sequence similarity to MyD88. Mal has a C-terminal TIR domain but lacks, in contrast to MyD88, a DD in its N-terminal region (Figure I.2.1.). Through their C-terminal TIR domains, Mal and MyD88 can form homo- and/or heterodimers. A dominant-negative form of Mal inhibits NF-κB activation by LPS, but it does not inhibit activation of NF-κB by IL-1RI or IL-18R. In addition, TLR4 has been shown to recruit Mal. Also, it was demonstrated that IRAK-2, but not IRAK-1, is necessary for NF-κB activation by Mal. Thus, Mal is a specific adaptor for TLR4 signalling (Fitzgerald et al., 2001). At the same time, another group also discovered Mal, but named it TIR domain-containing adaptor protein (TIRAP). They confirmed the important role for Mal/TIRAP in NF-κB activation by TLR4. In addition, they showed that Mal/TIRAP interacts with PKR, which is activated upon LPS-stimulation in a MyD88-independent manner (Horng et al., 2001).

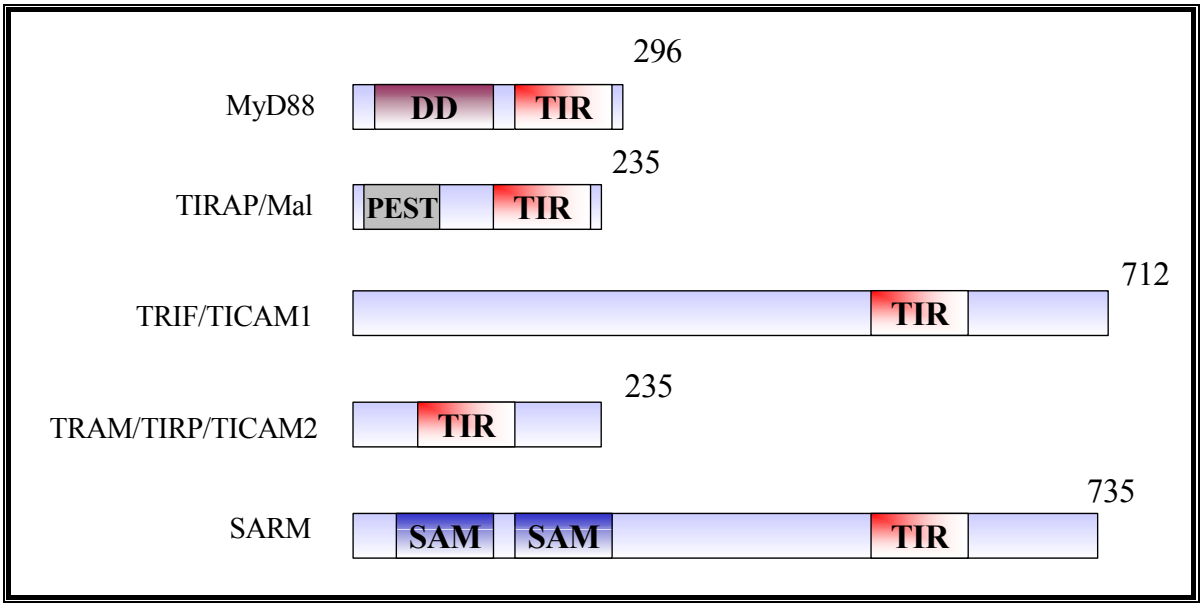

Figure I.2.1. - TIR domain-containing adaptor molecules. A schematic representation of the TIR domain-containing adaptor molecules MyD88, Mal/TIRAP, TRIF/TICAM1, TRAM/TICAM2/TIRP, and SARM. MyD88 is an essential adaptor used by all TLRs except TLR3. Mal/TIRAP is restricted to TLR2- and TLR4-dependent signalling to NF-κB. TRIF is an adaptor for TLR3 and TLR4 signalling, which regulates both NF-κB and IRF-3. TRAM is specific for TLR4 signalling. A function for SARM has yet to be described. The AA lengths are indicated on the right side.

Initially it was thought that TIRAP mediates the MyD88-independent signalling from TLR4. However, subsequent studies using TIRAP-deficient macrophages showed that expression of IFN-β and IFN-inducible genes is intact (Yamamoto et al., 2002a). Also, TIRAP-deficient mice respond normal to ligands of TLR3, TLR5, TLR7 and TLR9, as well as to IL-1 and IL-18. In response to ligands of TLR2 there's a defect in NF-κB activation, MAPK activation and subsequent cytokine production, but not in expression of IFN-inducible genes (Horng et al., 2002). Even in mice lacking both MyD88 and

Mal/TIRAP, expression of IFN-inducible genes by LPS is not impaired. Thus, Mal/TIRAP is essential for the TLR2- and TLR4-mediated MyD88-dependent signalling pathway, but not for the MyD88-independent signalling.

TRIF. A third TIR domain-containing adaptor, (TIR domain-containing adaptor inducing IFN) TRIF, was identified by a database search (Yamamoto et al., 2002b). Another group identified the same adaptor as a molecule associated with TLR3 by a yeast two-hybrid screen and termed it TIR-containing adaptor molecule-1 (TICAM-1) (Oshiumi et al., 2003a). Over-expression of TRIF, but not MyD88 or TIRAP, in HEK293 cells led to activation of the IFN-β promoter. Therefore, this molecule was named TIR domain-containing adaptor inducing IFN-β or TRIF. Furthermore, over-expression of TRIF, as well as MyD88 and TIRAP, resulted in the activation of the NF-κB-dependent promoter. Also, a dominant-negative TRIF inhibited TLR3-dependent activation of the IFN-β promoter. These *in vitro* studies showed that TRIF is important in the MyD88-independent pathway to induce IFN-β.
In TRIF-deficient mice, TLR3- en TLR4-mediated expression of IFN-β and IFN-inducible genes and activation of IRF-3 was severely impaired (Yamamoto et al., 2003a). Analysis of mutant *Lps2* mice, generated by random germline mutagenesis using the alkylating agent N-ethyl-N-nitrosourea (ENU), also led to the conclusion that *Trif* is responsible for TLR3- and TLR4-mediated responses (Hoebe et al., 2003). In addition, TRIF-deficient mice have a defective TLR4-mediated inflammatory cytokine production, although LPS-induced IRAK-1 phosphorylation and early phase NF-κB activation is normal. Therefore, the TLR4 signalling pathway requires activation of both the MyD88-dependent and MyD88-independent components to induce inflammatory cytokines.

TRAM. A fourth TIR domain-containing adaptor has been identified through sequence homology by different groups (Bin et al., 2003; Fitzgerald et al., 2003b; Oshiumi et al., 2003b; Yamamoto et al., 2003b). This molecule was called TRIF-related adaptor molecule (TRAM), TIR-containing protein (TIRP), and TICAM-2. *In vitro* studies using RNAi-mediated knockdown of TRAM expression showed that TRAM is involved in the MyD88-independent pathway from TLR4, but not TLR3 (Fitzgerald et al., 2003b; Oshiumi et al., 2003b). In addition, similar to TRIF-deficient mice, TRAM-deficient mice showed impaired activation of IRF-3 and diminished expression of IFN-β and IFN-inducible genes in response to LPS. However, TRAM-deficient mice showed normal responses to a TLR3 ligand. Also, TRAM-deficient mice are deficient in production of pro-inflammatory cytokines in response to LPS, although they show normal activation of IRAK-1 and early phase NF-κB activation (Yamamoto et al., 2003b). These data

prove that TRAM is specific for the TLR4-mediated MyD88-independent pathway and that TRAM is involved in the TLR4-mediated production of inflammatory cytokines (Yamamoto et al., 2003b).

SARM. The fifth known TIR adaptor protein is also known as SARM (sterile α and HEAT/armadillo motif containing protein) (O'Neill et al., 2003). Human SARM consists of about 700 amino acids, with the TIR domain located in the C-terminal region. At present, its function is entirely unknown. SARM is the most distant of the TIR adaptors, displaced from the other members of the family by a great evolutionary distance. The TIR motif of SARM is most similar to a TIR motif observed in the *Caenorhabditis elegans* TIR domain-containing protein (TIR1). TIR1 mediates the expression of genes that encode antimicrobial peptides, but this response is independent of the *C.elegans* TLR (Couillault et al., 2004). Next to its TIR domain, SARM also contains two sterile α motif (SAM) domains and an armadillo repeat motif (ARM).

2.2. TLR signalling

Stimulation of TLRs/IL-1Rs induces activation of signalling pathways. After ligand binding, TLRs/IL-1Rs form dimers and undergo a conformational change necessary to recruit downstream adaptor molecules. TLR2 can form heterodimers with TLR1 and TLR6 (Akira and Takeda, 2004). TLR10 can form heterodimers with TLR1 and TLR2 (Hasan et al., 2005). In other cases, TLRs form homodimers. MyD88 was the first adaptor molecule proven to be important in the signalling pathways originating from the TIR domain of the TLRs/IL-1Rs. It was demonstrated that MyD88-deficient mice fail to produce inflammatory cytokines such as TNF, IL-1 and IL-6, but are able to activate NF-κB and JNK, although with delayed kinetics, in response to LPS (Kawai et al., 1999). In addition, MyD88-null mice still induce IFN-dependent genes such as GARG16 (glucocorticoid-attentuated response gene 16), IRG1 (immunoresponsive gene 1) and CXCL10 (CXC-chemokine ligand 10), co-stimulatory molecules and maturation of DCs in response to LPS (Kaisho et al., 2001; Kawai et al., 2001). This indicated that although MyD88 is important for LPS-induced production of inflammatory cytokines, there must be a pathway independent of MyD88. Four different TIR domain-containing adaptor molecules, including MyD88, TIRAP/Mal, TRAM and TRIF, determine the signalling specificity of the response. (Akira and Takeda, 2004; Fitzgerald et al., 2001; Kawai et al., 1999; Oshiumi et al., 2003a; Yamamoto et al., 2003a; Yamamoto et al., 2002a; Yamamoto et al., 2003b; Yamamoto et al., 2002b). The MyD88-dependent pathway is used by all TLRs, except TLR3 (Moynagh, 2005). However, there are some differences in the use of signalling molecules in the MyD88-dependent pathway from TLR7 and TLR9 in pDCs. To conclude, two main pathways can become activated upon TLR stimulation: a MyD88-dependent pathway that promotes fast activation of NF-κB and induction of pro-inflammatory molecules

and a MyD88-independent pathway that mediates slow activation of NF-κB and activates IFN-regulatory factor (IRF-3), which leads to induction of IFN-β, IFN-inducible genes and co-stimulatory molecules. This MyD88-independent pathway is specific for the TLR3- and TLR4-signalling pathways (Akira et al., 2001).

2.2.1. The MyD88-dependent pathway

2.2.1.1. Important molecules belonging to the MyD88-dependent pathway

IRAK family. In mammals, four members of the IL-1R-associated kinase (IRAK) family have been identified: IRAK-1, IRAK-2, IRAK-3 or IRAK-M, and IRAK-4 (Li et al., 2002). IRAKs contain an N-terminal DD and a central serine/threonine kinase domain. IRAK-1 and IRAK-4 are catalytically active since they harbour a critical aspartate residue in their kinase domain. IRAK-2 and IRAK-M lack this critical residue, which causes them to be catalytically inactive (Janssens and Beyaert, 2003). In contrast to IRAK-4-deficient mice which are completely defective in their response to ligands that stimulate TLR2, TLR3, TLR4 and TLR9 (Suzuki et al., 2002), IRAK-1-deficient mice show diminished but not abolished cytokine production in response to IL-1 and LPS (Swantek et al., 2000; Thomas et al., 1999). Introduction in 293RI cells of a dominant-negative IRAK-4 molecule results in an impaired IRAK-1 degradation after IL-1 stimulation. Thus, IRAK-1 is a direct substrate of IRAK-4 but not vice versa (Li et al., 2002). In addition, the kinase activity seems not essential for signalling, as seen by strong NF-κB activation after over-expression of a kinase-defective IRAK-1 mutant (Li et al., 1999a). Also, children with an inherited IRAK-4 deficiency fail to respond to IL-1, IL-18, or stimulation of TLR2, TLR3, TLR4 and TLR5, and as a consequence develop infections caused by pyogenic bacteria (Picard et al., 2003).
IRAK-M is primarily expressed by monocytes and macrophages. IRAK-M-deficient mice show an increased production of pro-inflammatory cytokines in response to TLR ligands and an exaggerated inflammatory response to bacterial infection, demonstrating its role as a negative regulator in TLR signalling (Kobayashi et al., 2002a).

TRAF6. TNFR-associated factor (TRAF) proteins belong to a family of evolutionary conserved proteins that mediate cytokine signalling pathways. This family contains seven members and is characterized by a N-terminal coiled-coil domain, called TRAF-N, and a conserved C-terminal domain, called TRAF-C. The TRAF-C domain is necessary for interaction with upstream molecules and for interaction with TRAF proteins. The TRAF-N domain contains a RING (really interesting new gene)-finger/zinc-finger region, essential for downstream signalling events (Bradley and Pober, 2001). TRAF6 was first identified as a protein binding to the N-terminal region of CD40, a member of the TNFR superfamily (Ishida et al.,

1996). Next to its involvement in CD40 signalling, TRAF6 is important in TLR/IL-1R signalling through its interaction with IRAKs. The consensus sequence for the TRAF-6-binding domain is Pro-X-Glu-X-X-aromatic/acidic residue, and is found three times in IRAK-1, two times in IRAK-2 and once in IRAK-M (Ye et al., 2002). In TRAF6 deficient mice, LPS-induced inflammatory cytokine production is reduced and NF-κB and MAPK activation is observed with delayed kinetics. In response to ligands for TLR2, TLR5, TLR7 and TLR9, NF-κB and MAPK activation or inflammatory cytokine production is impaired (Gohda et al., 2004).

TAK1 and TABs. TGF-β-activated kinase 1 (TAK1), also called TRAF2-associated kinase (T2K), is a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family, originally identified in the TGF-β signalling pathway (Yamaguchi et al., 1995). TAK1 binding proteins, TAB1 and TAB2 are two proteins involved in the activation of transcription factors NF-κB and activator protein 1 (AP-1) by TRAF6 (Deng et al., 2000; Shibuya et al., 1996). TAB2 functions as an adaptor molecule linking TRAF6 and TAK1 and hereby facilitating activation of TAK1. However, TAB2 deficient embryonic fibroblasts show no impaired IL-1/LPS- or TNF-induced NF-κB activation. TAK1 becomes activated by the E2 ligases Ubc13 and Uev1A, which interact with the E3 ubiquitin ligase TRAF6. The Ubc13 and Uev1A complex catalyzes the assembly of a lysine63-linked polyubiquitin chain of TRAF6, thereby inducing TRAF6-mediated TAK1 activation (Deng et al., 2000; Wang et al., 2001) (Figure I.2.2). Over-expression studies show that LPS and IL-1 can activate TAK1, which in turn activates the IKK complex (Deng et al., 2000; Lee et al., 2000b).

NF-κB. The NF-κB family is composed of five members of the Rel transcription factor family: p65 (RelA), RelB, cytoplasmic Rel (c-Rel), p50 (and its precursor p105) and p52 (and its precursor p100). Active NF-κB transcription factors are composed of homodimeric or heterodimeric combinations of these members. Heterodimers of p50 and p65 are the most abundant (Akira and Takeda, 2004). B-cells from mice deficient in p50, RelA, c-Rel or RelB show an impaired growth response to LPS (Ghosh et al., 1998). Mice deficient in p50, RelB or p52 were found to be highly susceptible to microbial infections (Caamano et al., 1999; Franzoso et al., 1998; Sha et al., 1995). Members of the inhibitory IκB family sequester NF-κB dimers in the cytosol in an inactive form, until they become phosphorylated on serine residues by the IκB kinase (IKK) complex. The IKK complex contains two catalytic subunits, IKK-α and IKK-β, as well as a regulatory subunit, IKK-γ or NF-κB essential modulator (NEMO). Phosphorylation of IκB leads to its polyubiquitinylation, followed by its degradation by the 26S proteasome. Degradation of IκB releases NF-κB and this is followed by translocation of NF-κB to the nucleus where it induces pro-

inflammatory and co-stimulatory molecules (Akira and Takeda, 2004) (Figure I.2.2). Stimulation of a human monocytic cell line with LPS enhances IKK activity (O'Connell et al., 1998). In contrast to NF-κB activation in IKK-α-null mice, NF-κB activation in IKK-β- or IKK-γ-null mice is impaired in response to LPS or IL-1 (Li et al., 1999b; Rudolph et al., 2000).

MAPKKK/MAPKK/MAPK. Extracellular signal-regulated kinase (ERK1/2)/MAPK, c-Jun N-terminal kinase (JNK)/stress-activated protein kinase 1 (SAPK1) and p38/SAPK2 belong to the MAPK family. Activation of a MAPK involves a three-part intracellular signal transduction cascade. A MAP/ERK kinase kinase (MEKK) or Raf phosphorylates a MAP/ERK kinase (MEK) or MAP kinase kinase (MKK), which in turn phosphorylate a specific tyrosine and threonine residue on a MAPK (Figure I.2.2). MEKK3-deficient MEFs have an impaired IL-6 production and defective NF-κB, JNK and p38 MAPK activation in response to LPS. Stimulation with LPS also induces the association of MEKK3 with TRAF6 (Huang et al., 2004). Tumour-progression locus 2 (TPL2) is also involved in TLR4-induced ERK activation (Dumitru et al., 2000). MKK4 and MKK7 are involved in phosphorylation of JNK/SAPK1 (Kishimoto et al., 2003). MKK6 has been implicated in TAK1-induced JNK and p38 activation (Wang et al., 2001). MKK3 is involved in p38 activation in LPS-stimulated neutrophils (Nick et al., 1999).
The AP-1 family of transcription factors contains c-Jun and c-Fos proteins which form homo- and heterodimers (Karin et al., 1997). Jun proteins can also form heterodimers with the cAMP responsive element binding protein/activating transcription factor (CREB/ATF) family of transcription factors, such as ATF-2, which enables them to bind to the cAMP response element (CRE). Phosphorylation of AP-1 transcription factors by the MAPK JNK enhances its activity (Karin, 1995). LPS or PGN stimulation enhance the transcriptional activity of AP-1 and the CREB/ATF family (Mackman et al., 1991). Viral infection and dsRNA activate the transcriptional activity of AP-1 through induction of JNK (Chu et al., 1999) and p38 (Moynagh, 2005). Recently it has been shown that p38 is needed for phagocytosis (Doyle et al., 2004).

2.2.1.2. TLR-induced MyD88-dependent signalling (Figure I.2.2.)

The IL-1R complex and all TLRs, except TLR3, activate the MyD88-dependent pathway upon stimulation. It has been demonstrated that TLR7 and TLR9 use a slightly different MyD88-dependent pathway in pDCs. After ligand binding, MyD88 associates with the cytoplasmic TIR domain of TLRs/IL-1Rs. TIRAP/Mal, a second TIR domain-containing adaptor, is involved in the MyD88-dependent signalling pathway through TLR2 or TLR4.

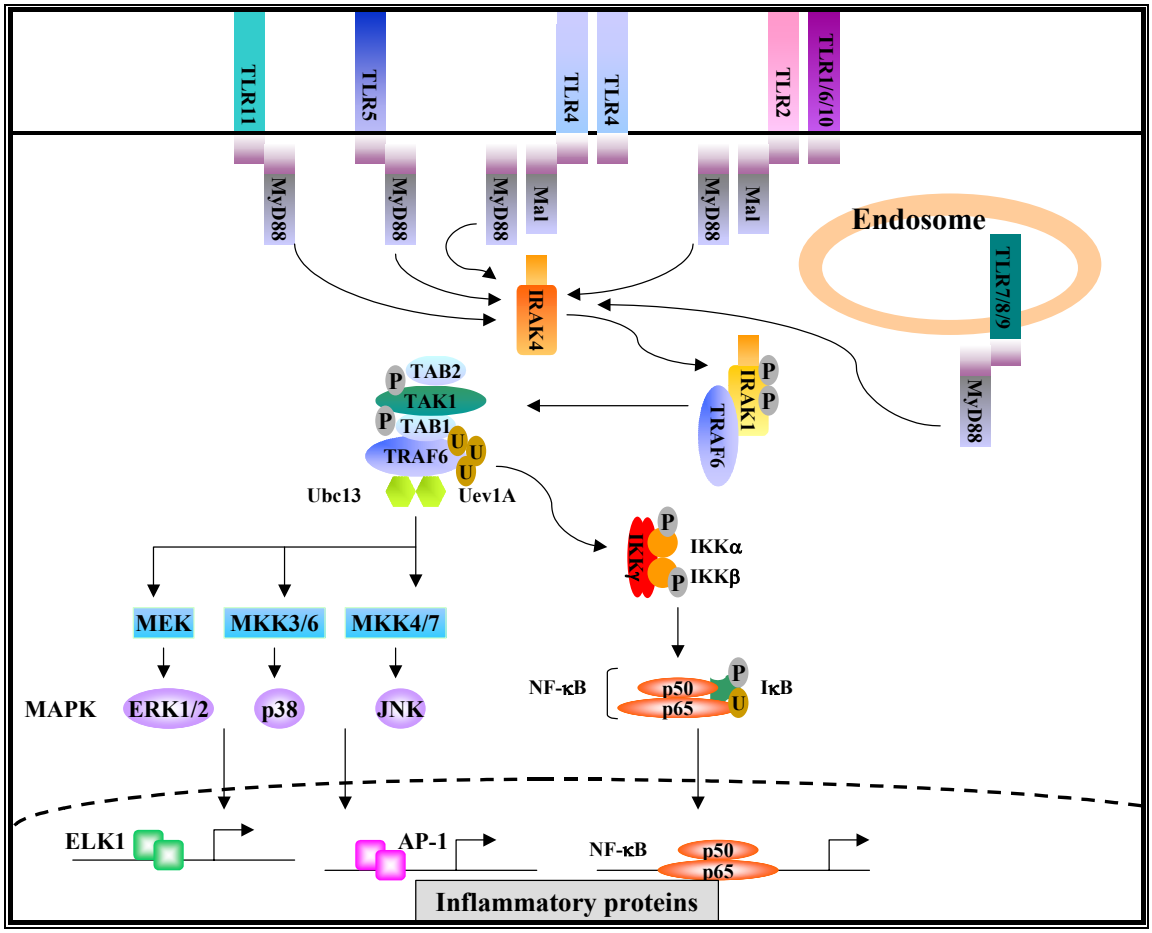

Figure I.2.2. - TLR-mediated MyD88-dependent signalling pathway. This pathway is used by all TLRs except TLR3. It is demonstrated that TLR7 and TLR9 use a slightly different MyD88-dependent pathway in pDCs. Upon stimulation, TLRs homodimerize and undergo conformational changes to recruit adaptor molecules. TLR2 can also form heterodimers with TLR1, TLR6 and probably also with TLR10. MyD88 binds to the cytoplasmic portion of TLRs through interaction between individual TIR domains. TIRAP/Mal, a second TIR domain-containing adaptor, is involved in the MyD88-dependent signalling pathway through TLR2 and TLR4. The MyD88-dependent pathway leads to NF-κB and MAPK activation to induce expression of inflammatory cytokines. Both AP-1 and CREB/ATF transcription factors as well as NF-κB are required for cytokine production.

Next, MyD88 facilitates the binding of IRAK-4 with the receptor complex through interaction of the DDs of both molecules. Binding of IRAK-4 to the receptor complex leads to IRAK-4-mediated phosphorylation of IRAK-1. This triggers IRAK-1 autophosphorylation and enables IRAK-1/TRAF6 interactions. TRAF6 is recruited to the receptor complex upon IRAK-1 phosphorylation. Then, the IRAK-1/TRAF-6 complex dissociates from the receptor and associates with TAK1 and TAK1-binding proteins, TAB1 and TAB2, at the membrane portion, inducing phosphorylation of TAB2 and TAK1. IRAK-1 mediates translocation of this complex (consisting of TRAF6, TAK1, TAB1 and TAB2) to the cytosol, while IRAK-1 itself stays at the plasma membrane and is degraded after ubiquitinylation. In the cytosol,

the TRAF6/TAK1/TAB1/TAB2 complex recruits other proteins such as the E2 ligases ubiquitin-conjugating enzyme 13 (Ubc13) and ubiquitin-conjugating enzyme E2 variant 1 (Uev1A). These ligases catalyze the synthesis of a Lys63-linked polyubiquitin chain of TRAF6, leading to the activation of TAK1. TAK1, in turn, phosphorylates the IKK complex, which consists of IKK-α, IKK-β and IKK-γ, and also MAP/ERK kinases. The IKK complex induces phosphorylation of IκB, which sequesters the transcription factor NF-κB in the cytoplasm. Phosphorylation of IκB leads to its ubiquitinylation and subsequent degradation, triggering the nuclear translocation of NF-κB to induce expression of inflammatory cytokines (Takeda and Akira, 2004a; Takeda and Akira, 2004b). MAP/ERK kinases in turn phosphorylate specific tyrosine and threonine residues on MAPK. Concomitantly, members of the AP-1 transcription factor family, such as c-Jun and c-Fos, and members of the CREB/ATF family of transcription factors, such as ATF-1 and ATF-2, are activated by MAPK JNK and p38. Both AP-1 and CREB/ATF transcription factors as well as NF-κB are required for cytokine production (Figure I.2.2.) (Guha and Mackman, 2001). TRAF6 also interacts with two other proteins, p62 and evolutionarily conserved signalling intermediate in toll pathways (ECSIT), which link TRAF6 respectively to protein kinase ζ and MEKK1 (Janssens and Beyaert, 2002). Also, TLR2 induces apoptosis through MyD88 via FADD and caspase 8 (Aliprantis et al., 2000).

2.2.1.3. TLR7- and TLR9-induced MyD88-dependent signalling in pDCs (Figure I.2.3.)

TLR7 and TLR9 are strongly expressed by pDCs (Liu, 2005) and locate in the endosomal compartment (Ahmad-Nejad et al., 2002; Diebold et al., 2004). Also in other cell types such as in immature B-cells and in macrophages expression is found of TLR7 and TLR9 (Applequist et al., 2002). pDCs produce large amounts of type I IFN, especially IFN-α, in response to virus infection. Lund *et al.* demonstrated by using TLR7-deficient mice that ssRNA viruses, such as VSV or influenza virus, stimulate type I IFN responses through TLR7 (Heil et al., 2004; Lund et al., 2003). Furthermore, recognition of these ssRNA viruses requires endosomal acidification (Lund et al., 2004). In response to TLR9 ligands such as the DNA viruses MCMV and HSV-1/-2, and unmethylated CpG DNA motifs, pDCs also produce large amounts of IFN-α (Lund et al., 2003). In contrast to pDCs, macrophages and cDCs respond to TLR7/8 and TLR9 stimulation by activating NF-κB to produce pro-inflammatory cytokines, but they produce less type I IFN (Asselin-Paturel et al., 2005; Heil et al., 2004). Within the endosomal compartment, TLR7 and TLR9 signalling is entirely dependent on MyD88 (Lund et al., 2004). MyD88 forms a complex with IRF-7, which is constitutively expressed in pDCs, to trigger its activation and induce type I IFN (Honda et al., 2004; Kawai et al., 2004). IRF-7 seems to be phosphorylated by IRAK-1 (Honda et al., 2005). IRAK-4

and TRAF6 are also part of the complex and the ubiquitin ligase activity from the latter is necessary for IRF activation (Honda et al., 2004; Kawai et al., 2004) (Figure I.2.3.).

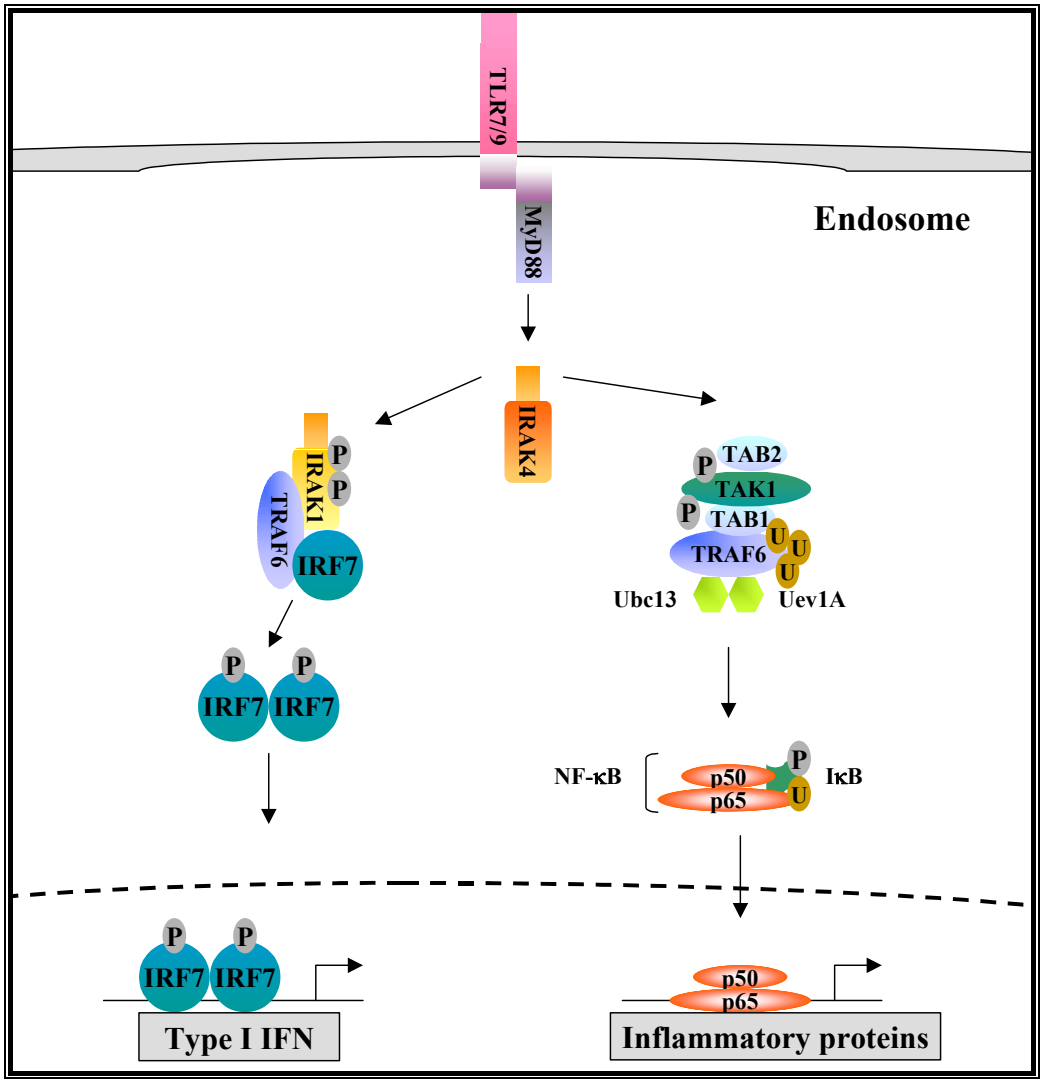

Figure I.2.3. - TLR7 and TLR9 signalling pathways. TLR7 and TLR9 are primarily expressed in the intracellular endosomal compartement of pDCs. Both TLR7 and TLR9 use a MyD88-dependent, but IRAK-1-independent pathway to induce NF-κB. This results in the phosphorylation, ubiquitination and degradation of the IκB proteins, allowing NF-κB to translocate to the nucleus and induce the expression of inflammatory proteins. TLR7 and TLR9 use also a MyD88- and IRAK-1-dependent pathway to phosphorylate IRF-7. Phosphorylation of IRF-7 leads to its translocation to the nucleus to induce type I IFN genes.

In IRAK-1-deficient mice, TLR7- and TLR9-mediated IFN-α production is abolished, while inflammatory cytokine production is not impaired (Uematsu et al., 2005). In IRAK-1-deficient pDCs, IRF-7 is not activated by a TLR9 ligand, whereas NF-κB and MAPK activation is normal. This shows that the kinase activity of IRAK-1 is necessary for transcriptional activation of IRF-7 and that IRAK-1 is a specific regulator for TLR7- and TLR9-mediated IFN-α induction (Uematsu et al., 2005). Thus, IRAK-1 is not

involved in NF-κB-mediated induction of inflammatory genes. Also, it is still unclear whether other kinases, such as TBK1 and IKK-ι, might play a role in IRF-7 activation.

2.2.1.4. Other molecules involved in MyD88 signalling

Pellino. Pellino was originally identified in *Drosophila* as a protein that interacts with Pelle, a homologue of mammalian IRAK. At this moment, three Pellino proteins have been identified. Pellino-1 and Pellino-2 are observed to interact with IRAK-1 after IL-1 signalling, and in the case of Pellino-2 also after LPS signalling (Jiang et al., 2003; Yu et al., 2002). Pellino-3 physically interacts with IRAK-1, TRAF6, TAK1, and NF-κB-inducing kinase (NIK) in an IL-1-dependent manner, displaying its role as a scaffolding protein. Two alternatively spliced Pellino-3 mRNAs, Pellino-3a and Pellino-3b, are widely expressed (Jensen and Whitehead, 2003). Recently, it was found that Pellino-3 acts as a novel upstream regulator of the p38 MAPK pathway (Butler et al., 2005).

ECSIT. Evolutionary conserved signalling intermediate in Toll pathways (ECSIT) was identified in a yeast two-hybrid screening of TRAF6-interacting molecules. A homologue of mammalian ECSIT is identified in *Drosophila*. ECSIT can interact with the MAP kinase kinase MEKK1, which can phosphorylate and activate the IKK complex (Kopp et al., 1999). ECSIT-deficient mice die on about embryonic day 7.5 (Xiao et al., 2003). This adaptor protein plays not only a role in Toll/IL-1 signalling, but also in bone morphogenetic protein (BMP) signalling. Expression of a dominant-negative mutant and knock-down of ECSIT with short hairpin RNA inhibits both BMP and Toll signalling (Xiao et al., 2003).

Bruton's tyrosine kinase. Bruton's tyrosine kinase (BTK) belongs to the SRC-related TEC-family of protein tyrosine kinases. BTK has an essential role in B-cell receptor mediated signalling and in B-cell development. It has been observed to interact with the TIR domain of TLRs, such as TLR4, TLR6, TLR8 and TLR9 (Jefferies et al., 2003) and of signalling molecules such as TIRAP and MyD88. Also, BTK is tyrosine phosphorylated upon LPS stimulation. Expression of a dominant-negative form of BTK inhibits LPS-induced NF-κB activation (Meng and Lowell, 1997).

2.2.2. TLR3- and TLR4-mediated MyD88-independent pathway

2.2.2.1. Some important molecules belonging to the MyD88-independent pathway

TRIF and TRAM. See section 2.1

TBK1 and IKK-ε/IKK-i. Hiscott and colleagues tried to identify molecules that interact with IRF-3 by two-hybrid screening, and found that IRF-3 was associated with two non-canonical IKKs, TANK-binding kinase 1 (TBK1) and IKK-ε/-i (Sharma et al., 2003). TBK1, also called NF-κB-activating kinase (NAK), and IKK-ε/IKK-i have distinct kinase activities compared with the canonical IKKs, IKKα and IKK-β (Takeda and Akira, 2004b). McWhirter and colleagues showed that purified recombinant IKK-ε and TBK1 directly phosphorylate the critical serine residues in IRF-3. In response to LPS, mouse embryonic fibroblasts (MEFs) derived from TBK1-deficient mice show impaired production of type I IFN and IFN-inducible genes, but not of pro-inflammatory cytokines (McWhirter et al., 2004). However, IKK-ε knockout mice show normal production of these genes. MEFs from IKK-ε/TBK1-doubly deficient mice were completely defective in the induction of IFN-β as well as IFN-inducible genes in response to poly (I:C) stimulation. Activation of IRF-3 in response to LPS and poly (I:C) was abolished in IKK-ε/TBK1 double deficient cells (Hemmi et al., 2004). These observations demonstrate that IKK-ε/TBK1 signalling is essential for LPS and dsRNA-induced IFN responses (Fitzgerald et al., 2003a; Sharma et al., 2003).

IRF-3. IRF-3 was identified through a search of an expressed sequence tag (EST) database for IRF-1 and IRF-2 homologues (see further). IRF-3 is constitutively expressed in all tissues and is not induced by viral infection or IFN treatment (Au et al., 1995). It is present in the cytoplasm due to a continuous nuclear export mediated by a nuclear export signal (NES). IRF-3 also contains a nuclear localization sequence (NLS), an IRF association domain (IAD) and a DNA binding domain (DBD) (Yoneyama et al., 1998). The C-terminal regulatory domain is activated upon phosphorylation of specific serine residues by TBK1 and IKK-ε. The serine residues, which become phosphorylated, differ between the TLR3 and TLR4 pathways. The serine residue 396 (Ser396) in the C-terminal portion of IRF-3 is only phosphorylated after stimulation with dsRNA, while Ser386 is phosphorylated both upon LPS and dsRNA stimulation (Mori et al., 2004; Servant et al., 2003). However, the critical serine residue in IRF-3 seems to be Ser386 (Mori et al., 2004). Phosporylation of IRF-3 causes a change in conformation, revealing the IAD and DBD domains. This allows IRF-3 to form dimers, to translocate rapidly to the nucleus and to bind to IRF-3 elements in relevant promoters (Yoneyama et al., 1998). The prominent phosphorylation sites have been mapped to Ser385, Ser386 and the distal region; the mutations of

these residues abolish virus-induced activation (Lin et al., 1998; Yoneyama et al., 1998). Stimulation with LPS activates IRF-3 in a MyD88-independent manner, as observed using MyD88-deficient cells (Hoshino et al., 2002). Expression of a dominant-negative form of IRF-3 in a macrophage cell line interferes with the LPS-mediated induction of the IFN-β gene. Also, DCs of IRF-3 deficient mice show a defect in LPS-mediated IFN-β gene induction. Moreover, IRF-3-deficient mice show resistance to LPS-induced endotoxin shock (Sakaguchi et al., 2003).

2.2.2.2. MyD88-independent signalling (Figure I.2.4. and I.2.5.)

TRIF and TRAM, two TIR domain-containing adaptors, are used in the MyD88-independent pathway. TRAM is specific for TLR4-signalling, while TRIF can be recruited by both TLR3 and TLR4. Recruitment of TRAM and TRIF to the TLR4 receptor leads to activation of the kinases TBK1 and IKK-ε, followed by phosphorylation of the transcription factor IRF-3 and thereby inducing IFN-β (Moynagh, 2005). The N-terminal portion of TRIF is needed for its association with TBK1 and IKK-ε (Sato et al., 2003). A recent study suggested that NAK-associated protein 1 (NAP1) might facilitate the interaction of both kinases with TRIF (Sasai et al., 2005). It is also shown that TRIF recruits TRAF6-TAK1-TAB2 through its TRAF6-binding site lying in the N-terminal portion of TRIF (Sato et al., 2003). Recruitment of TRAF6 is required for late phase NF-κB activation, but not for IRF-3 activation (Jiang et al., 2004). The IRF-3 transcription factor is ubiquitously expressed and resides in the cytoplasm in an inactive state (Au et al., 1995). Upon activation through phosphorylation of critical serine residues in the C-terminal regulatory domain of IRF-3, IRF-3 molecules form a dimer, which is transported to the nucleus where it activates transcription of type I IFN genes by recruiting co-activators p300 and CREB-binding protein (CBP). This complex of IRF-3, CBP and p300 is called 'dsRNA-activated factor 1' or DRAF1. The serine residues phosphorylated in IRF-3 differ between the TLR3 and TLR4 pathways. The IRF-3 activation following TLR3 stimulation is more rapid and potent then following TLR4 stimulation. This correlates with the higher IFN-β production after TLR3 stimulation (Doyle et al., 2003).

The gene induction is primarily due to transcriptional activation of the IFN-α/β promoter sequences, termed virus-inducible enhancers (referred to as PRDI and III for IFN-β) (Fan and Maniatis, 1989), to which IRF-3 (and IRF-7) binds. Transcription of IFN-β is dependent on transcriptional activator proteins such as IRF-3, but also on NF-κB (p50/p65) and ATF-2/c-jun and they induce IFN-β promoter activation in a synergistic manner. They bind co-operatively and form the enhanceosome on the IFN-β promoter (Thanos and Maniatis, 1995). The secreted type I IFN activates the expression of IFN-inducible genes such as CXCL10 and IRG-1 through a JAK-STAT signalling pathway (see further).

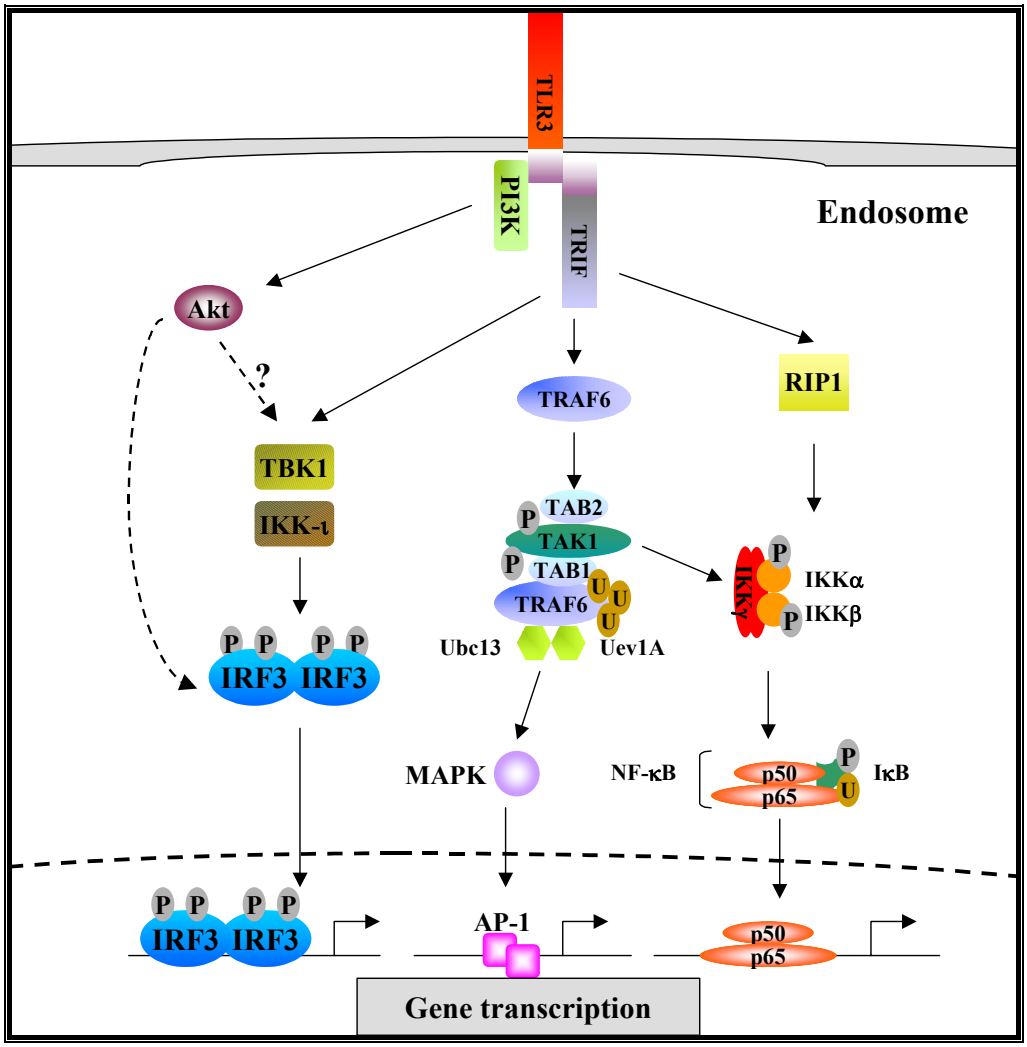

Figure I.2.4. - TLR3 signalling pathway. TLR3 is located in the intracellular endosomal membranes and is the only TLR not able to recruit the adaptor molecule MyD88. Upon TLR3 stimulation the adaptor molecule TRIF is recruited to the membrane. Non-typical IKKs, TBK1 and IKK-i, mediate phosphorylation of the transcription factor IRF-3 downstream of TRIF. Activated TLR3 can also recruit PI3K through its phosphotyrosines. Recruitment of PI3K activates the PI3 kinase-Akt pathway, leading to further phosphorylation and maximal activation of IRF-3 by an unknown mechanism. Phosphorylation of IRF-3 leads to its dimerization and translocation to the nucleus where it induces IFN-β, IFN-dependent genes and co-stimulatory molecules. TRIF can activate through a TRAF6-dependent mechanism three classes of kinases (JNK, p38 and IKK), which in turn activate their corresponding transcription factor to initiate gene induction. In addition, TRIF can activate NF-κB through a TRAF6-independent mechanism, involving the recruitment of RIP1.

In addition, the proteins of the CITED family show nuclear or nucleocytoplasmic localization and bind to CBP/p300 transcriptional integrators through their conserved C-terminal acidic domain (Shioda et al., 1997; Yahata et al., 2000). CITED proteins do not appear to bind to DNA directly but function as transcriptional co-activators. CITED stands for CBP/p300-interacting transactivators with E (glutamic acid)/D (aspartic acid)-rich carboxyl-terminal domain (Shioda et al., 1997; Yahata et al., 2000). CITED1, or MSG1, and CITED2 (splice isoforms known as MRG1 or p35srj) belong to this family (Sun et al.,

1998). Because their expression is regulated by cytokines and stress, they are predicted to have a possible role in modifying CBP/p300-dependent transcription in a variety of biological events. For example, it has been demonstrated that CITED1 directly binds to CBP and p300, which in turn bind to the Smads and enhances Smad-mediated transcription (Yahata et al., 2000).

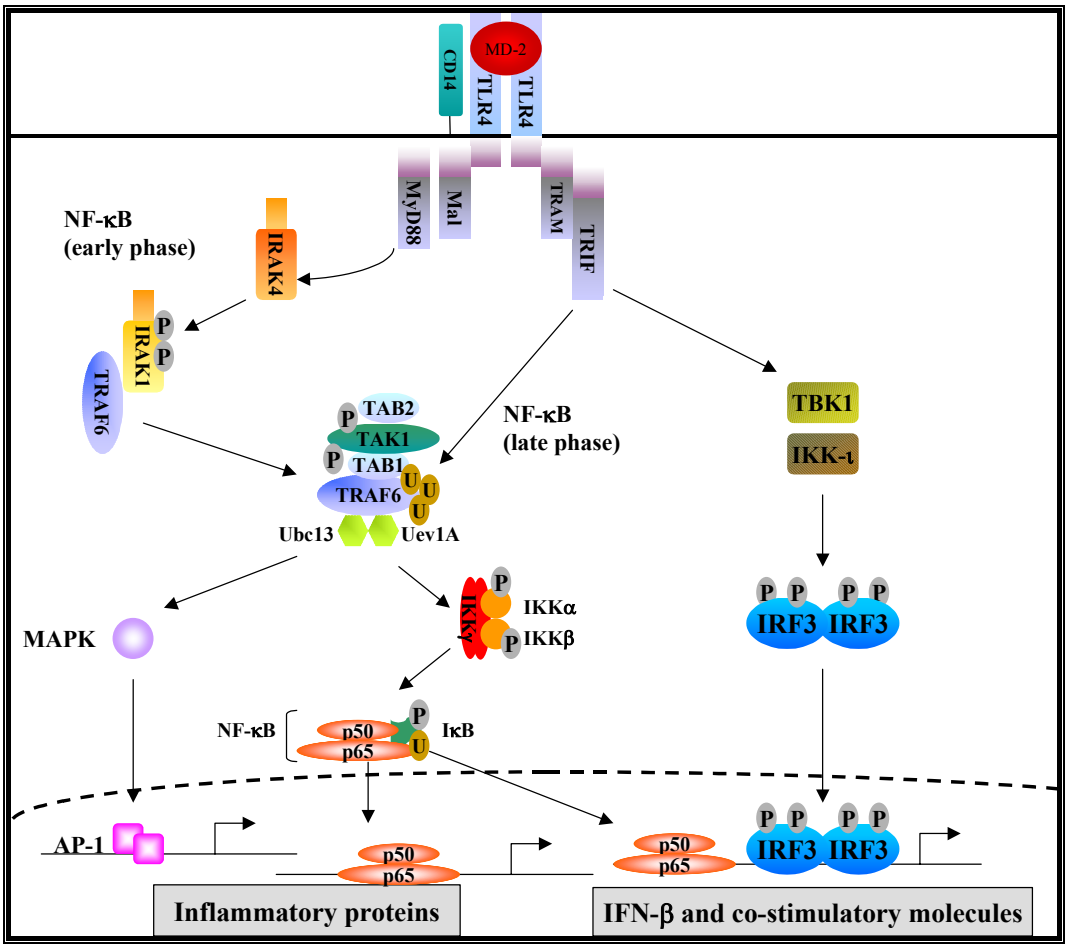

Figure I.2.5. - TLR4 signalling pathway. Stimulation of TLR4 facilitates the activation of two main pathways: the MyD88-dependent and MyD88-independent pathways. The MyD88-dependent pathway involves the early activation of inflammatory proteins through early phase NF-κB activation. The MyD88-independent pathway involves the recruitment of two adaptor molecules, TRAM and TRIF. TRAM is specific for the TLR4-mediated MyD88-independent (TRIF-dependent) pathway. TRIF is essential for both TLR4 and TLR3-mediated signalling pathways (see Figure I.2.4. for TLR3-mediated signalling).The MyD88-independent pathway activates IRF-3 and is involved in NF-κB late phase activation. Both transcription factors are needed for production of IFN-β, co-stimulatory molecules and the expression of IFN-inducible genes. For details of the MyD88-dependent pathway see Figure I.2.2. and for details of the TRIF-dependent pathway see Figure I.2.4.

2.2.2.3. Other signalling pathways: role of PI3K, RIP1 and RIP2 (Figure I.2.4.)

The phosphatidylinositol 3-kinase (PI3K) p85 regulatory subunit can directly interact with TLRs through its SRC homology 2 (SH2) domain. The subsequent association of the p110 catalytic subunit of PI3K is important for its complete activation and results in the activation of its downstream target Akt (Koyasu, 2003). By using LY294002 and wortmannin, two PI3K inhibitors, it was demonstrated that *ISG56* induction by dsRNA was abolished. Dominant-negative mutants of PI3K or its downstream target kinase Akt, inhibited *ISG56* induction. Thus, PI3K is required for TLR3 signalling. However, PI3K activity is not sufficient for TLR3 signalling, as seen by a non-induction of *ISG56* after over-expression of a constitutively active mutant of PI3K (Sen and Sarkar, 2005). Upon stimulation by dsRNA, TLR3 is phosphorylated by an unknown kinase on two specific tyrosine residues at the cytoplasmic domain. Phosphorylation of TLR3 is essential for initiating two distinct signalling pathways. One involves activation of TBK1 via TRIF and the other recruits and activates PI3K and the downstream kinase, Akt. Both are involved and necessary for the complete phosphorylation of IRF-3. Incompletely phosphorylated IRF-3 fails to bind the promoter of the target genes in dsRNA-stimulated cells (Sarkar et al., 2004).

Also, a role for PI3K in LPS signalling has been suggested. In macrophages, lauric acid-induced TLR4 signalling is inhibited by blocking PI3K/Akt (Lee et al., 2003). In the absence of PI3K, TLR4-induced NF-κB release and nuclear translocation is observed, but no gene induction (Ojaniemi et al., 2003). In human neutrophils, JNK activation and induction of some genes in response to LPS is dependent on the PI3K pathway (Arndt et al., 2004). TLR2 signalling is also dependent on PI3K. PI3K is needed for both TLR2-induced NF-κB activation and receptor tyrosine phosphorylation (Arbibe et al., 2000). TLR2-induced ERK1/2 activation, but not JNK1/2 or p38, requires PI3K (Martin et al., 2003). In neutrophils, PI3K is important for TLR2-induced Ser536 phosphorylation of the p65 subunit of NF-κB (Strassheim et al., 2004). Also, PI3K plays an important role in shuttling CpG DNA to TLR9 (Ishii et al., 2002).

RIP1-knockout MEFs have an impaired NF-κB activation in response to a TLR3 ligand. In its C-terminal domain, TRIF has a RIP homotypic-interaction motif, thus RIP1 binds with the C-terminal domain of TRIF (Meylan et al., 2004). RIP1 mediates NF-κB activation through TRIF after TLR3 stimulation. RIP2 was originally identified as a kinase that associates with TRAFs and with TNFR family members to induce NF-κB activation and apoptosis (McCarthy et al., 1998). RIP2-deficient mice are partially impaired in their response to LPS, PGN and dsRNA (Chin et al., 2002; Kobayashi et al., 2002b).

2.2.3. IRF-5 in TLR signalling (Figure I.2.6.)

IRF-5 is structurally most related to IRF-6 and is induced after stimulation with IFN-α/β. Cells from IRF-5 knockout mice show a diminished pro-inflammatory cytokine production (e.g. TNF-α, IL-6 and IL-12) after stimulation with TLR3, TLR4, TLR5, TLR7 and TLR9 ligands. However, IFN-α is still induced upon TLR9 stimulation in IRF-5 -/- pDCs. In addition, IRF-5 -/- mice are more resistant to lethal shock induced by LPS or unmethylated DNA than wild-type mice. IRF-5 interacts with MyD88 and TRAF6, becomes phosphorylated and translocates to the nucleus to cooperate with NF-κB to induce pro-inflammatory cytokines (Takaoka et al., 2005).

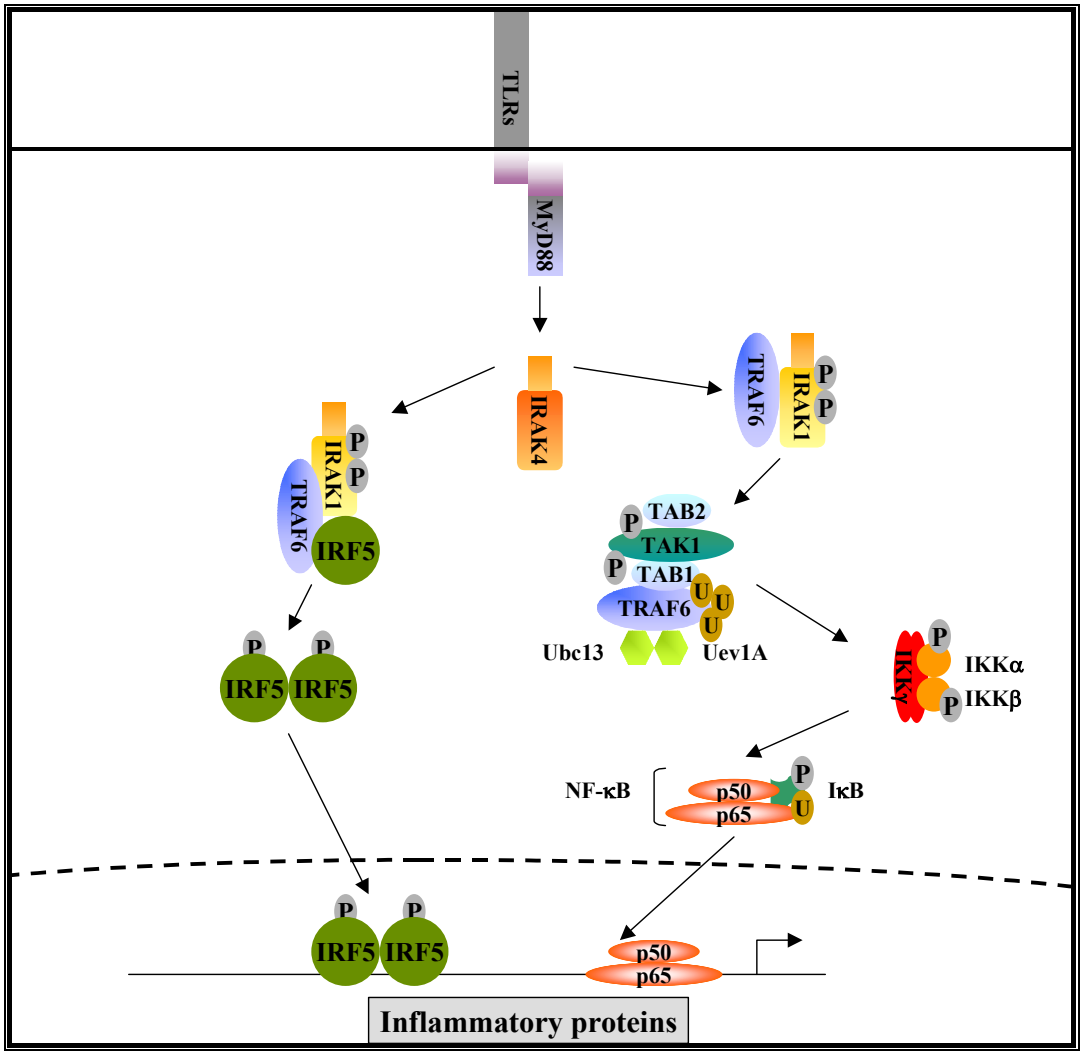

Figure I.2.6. - Role of IRF-5 in TLR signalling. All TLRs, except TLR3, use a MyD88-dependent pathway to activate NF-κB. This involves phosphorylation of the inhibitory IκB proteins, which leads to translocation of NF-κB to the nucleus and induction of inflammatory proteins. Recently, it has been demonstrated that TLRs can induce IRF-5 phosphorylation through a MyD88-dependent pathway involving IRAK-1 and TRAF6. Activation of IRF-5 leads to its translocation to the nucleus and co-operation with NF-κB in the induction of inflammatory genes.The mechanism underlying the possible TLR3-mediated activation of IRF-5 still awaits further clarification.

Putative IRF-5-binding sites are found in the promoters of several pro-inflammatory cytokines. The kinase responsible for phosphorylation of IRF-5 is not yet clear at this moment. TBK1 and IKK-ε can phosphorylate IRF-5, but this phosphorylation does not lead to nuclear localization or activation of IRF-5 (Lin et al., 2005). IRF-5 becomes activated primarily after TLR7, TLR8 and TLR9 stimulation. Recently, Schoenemeyer and colleagues showed a role for IRAK-1 and TRAF6 in TLR7-induced IRF-5 activation (Schoenemeyer et al., 2005). They claim that IRF-5 is not activated upon TLR3 stimulation, which is in contrast to data from other groups. All TLRs, except TLR3, use a MyD88 dependent pathway to activate IRF-5 and NF-κB to induce pro-inflammatory cytokine production, such as TNF, IL-6 and IL-12 (Napolitani et al., 2005) (Figure I.2.6).

Napolitani and co-workers show a synergistic effect between TLR agonists to optimize transcriptional regulation of particular target genes important in immune response to pathogens. By giving suboptimal doses of TLR agonist, unable to induce IL-12p70 when given alone, it was demonstrated that 20- to 50-fold more IL-12p70 is induced when TLR agonists are given together than when a single agonist is given (Napolitani et al., 2005). However, another study showed that the co-operation between different TLR pathways is explained through synergistic action of TLRs and type I IFN receptors (Gautier et al., 2005). Using cells from IFNAR- and STAT1-knockout mice it was shown that an autocrine and/or paracrine loop of type I IFN, induced after stimulation of TLR3 or TLR4, is necessary for production of IRF-7. Next, IRF-7 can be activated by engagement of TLR7/8 or TLR9, leading to secretion of bioactive IL-12p70 and optimal type I IFN (Gautier et al., 2005).

2.3. Negative regulation of TLR signalling

TLRs serve to recognize PAMPs of different microorganisms to clear the invasion of these organisms in the body. The TLR-mediated response needed to clear the infection also leads to production of inflammatory cytokines. When those inflammatory cytokines are released in excess, this can induce serious systemic disorders such as endotoxin shock associated with a high mortality rate. Thus it is not surprising that TLR signalling is under tight negative regulation. There are different layers of negative regulation of TLR signalling, going from soluble decoy receptors to intracellular inhibitors, membrane-bound suppressors, degradation of TLRs, and TLR-induced apoptosis (Table I.2.1.).

Soluble decoy receptors. Naturally produced soluble decoy TLRs provide powerful negative regulatory mechanisms, reminiscent of soluble chemokines and cytokine receptors. They might provide a first-line defense by preventing a direct contact between TLRs and their PAMPs. So far, only soluble decoy

TLR4 and TLR2 have been identified. The mechanism by which they attenuate TLR2 and TLR4 function remains obscure (Liew et al., 2005).

Intracellular negative regulators. The most studied group of regulators are the intracellular negative regulators. Some of them are constitutively expressed to regulate TLR activation at a physiological level, whereas others are upregulated and attenuate TLR signalling in a negative-feedback loop. At this moment this group includes MyD88s, IRAK-M, IRAK2c, IRAK2d, SOCS-1, Nod-2, PI3K, TOLLIP, A20, RIP3, IRF-4, β-arrestin, IκBNS and FLN29. They function at various stages of the TLR signalling cascade but concentrate principally on the MyD88-dependent pathway.

MyD88s. MyD88 short (MyD88s), an alternatively spliced form of MyD88 that lacks only the short intermediate domain separating the DD and TIR domains, is induced upon LPS stimulation and is primarily expressed in the spleen (Janssens et al., 2002). Expression of MyD88s leads to shutdown of IL-1/LPS-induced NF-κB activation. MyD88s is not able to interact with IRAK-4, which disables IRAK-1 phosphorylation and subsequent NF-κB activation (Burns et al., 2003).

IRAK-M, IRAK2c and IRAK2d. Whereas other IRAKs are expressed ubiquitously, IRAK-M expression is restricted to monocytes/macrophages and is induced upon TLR stimulation. IRAK-M inhibits the dissociation of the IRAK1-IRAK4 complex from MyD88, thereby preventing the formation of an IRAK1-TRAF6 complex. IRAK-M-knockout mice show increased cytokine production to bacterial infection and lack of endotoxin tolerance (Kobayashi et al., 2002a). The mouse *Irak2* gene has four splice variants: *Irak2a*, *Irak2b*, *Irak2c*, *Irak2d*. Over-expression of IRAK2c and IRAK2d, lacking the DD of fullength IRAK2, inhibits LPS-induced NF-κB activation in fibroblast cells. LPS-stimulated mouse macrophages show an increased level of IRAK2c, but not IRAK2a (Liew et al., 2005).

SOCS-1. Suppressor of cytokine signalling 1 (SOCS-1) belongs to the SOCS family involved in the negative regulation of signal transduction by a variety of cytokines (Yasukawa et al., 2000). SOCS-1 is found to be rapidly induced by LPS or CpG-DNA stimulation in macrophages (Crespo et al., 2000; Dalpke et al., 2001). Furthermore, SOCS-1 knockout mice are more sensitive to LPS-induced shock. In addition, over-expression of SOCS-1 suppressed LPS-induced NF-κB activation, showing that SOCS-1 plays an important role in the down-regulation of LPS signal transduction (Kinjyo et al., 2002; Nakagawa et al., 2002). Previously it was thought that the mechanism of SOCS-1 inhibition of TLR4- and TLR9-signalling occurred by targeting IRAK-1. It was also demonstrated that SOCS-1, induced by TLR stimulation, limits the extent of TLR signalling by inhibiting type I IFN signalling, but not the main NF-κB pathway (Baetz et al., 2004). In addition, recently it was reported that SOCS-1 mediates Mal degradation. Mal is specifically involved in TLR2 and TLR4 signalling. Mal becomes phosphorylated by

Btk. Phosphorylation of Mal leads to its interaction with SOCS-1, which results in Mal polyubiquitination and degradation (Mansell et al., 2006).

NOD-2. The role of Nod-2 as a negative regulator of TLR2 signalling is probably not universal. Watanabe and co-workers showed a Nod-2-induced suppression of TLR2-ligand-induced T_H1-cell responses in wild-type mice, but not in Nod2-deficient mice (Watanabe et al., 2004). However, a recent report failed to confirm Nod-2 as a negative regulator in TLR2 signalling (Kobayashi et al., 2005).

PI3K. PI3K functions at the early phase of TLR signalling and modulates the magnitude of the primary activation (Fukao and Koyasu, 2003). Also, PI3K inhibits IL-12 synthesis and prevents the over-expression of a T_H1 response. The exact mechanism by which PI3K inhibits TLR signalling remains unknown, but might involve the suppression of p38, JNK, ERK1/2 and NF-κB (Fukao et al., 2002).

TOLLIP. Toll-interacting protein (TOLLIP) was first described for its role in IL-1 signalling (Burns et al., 2000). After IL-1 stimulation, a membrane-proximal signalling complex is formed consisting out of two different IL-1Rs, IL-1RI and IL-1RAcP, MyD88, IRAK and TOLLIP (Burns et al., 2000). Later, it was discovered that TOLLIP not only interacts with the IL-1Rs, but also with TLR2 and TLR4 through their cytoplasmic TIR domain. TOLLIP act as a negative regulator in IL-1 and TLR-signalling by inhibiting the phosphorylation and kinase activity of IRAK-1. In quiescent cells, TOLLIP pre-associates with IRAK, thus preventing it from being phosphorylated and activated on the TLR receptor complex. Once activated on the receptor, TOLLIP-IRAK1 complexes are recruited to the receptor and IRAK1 phosphorylates TOLLIP. Phosphorylation of TOLLIP might then lead to the dissociation of TOLLIP from IRAK and the receptor complex, allowing IRAK to bind to downstream TRAF6. Thus, TOLLIP is thought to maintain immune cells in a quiescent state (Zhang and Ghosh, 2002).

A20. Mice deficient for A20, a cytoplasmic zinc finger protein, develop severe inflammation and are hypersensitive to both LPS and TNF, and die prematurely (Lee et al., 2000a). A20 is a negative inhibitor of TLR4 signalling as showed by the fact that full length A20 inhibits the ability of TLR4 to activate NF-κB and AP-1 (O'Reilly and Moynagh, 2003). A20 interacts with several proteins including ABIN-1, ABIN-2, TRAF6 and IKK-γ, and its expression is controlled by NF-κB itself (Heyninck et al., 1999; Van Huffel et al., 2001). The effect of A20 is mediated by its deubiquitinating activity. A20 is a deubiquitinating enzyme that cleaves ubiquitin chains from TRAF6, thereby terminating TLR-induced NF-κB signalling (Boone et al., 2004).

RIP3. RIP3 acts by down-regulating TLR3-induced TRIF-mediated NF-κB activation. RIP3 and TRIF compete which each other for binding with RIP1. RIP3 acts as a negative inhibitor of TRIF-dependent NF-κB activation (Meylan et al., 2004).

IRF-4. Another recently identified negative regulator of TLR signalling is IRF-4. IRF-4 mRNA is induced upon TLR stimulation and competes with IRF-5 for interaction with MyD88. In IRF-4-knockout macrophages, TLR-dependent induction of pro-inflammatory cytokines is enhanced. Moreover, IRF-4-deficient mice show hypersensitivity to DNA-induced shock, with elevated serum pro-inflammatory cytokine levels. Thus, IRF-4 is an important factor in the negative-feedback regulation of TLR-induced pro-inflammatory cytokine production (Negishi et al., 2005).

β-arrestins. β-arrestins, a family of multifunction proteins, directly interact with TRAF6 in response to LPS or IL-1β stimulation *in vivo* and *in vitro*. Formation of a β-arrestin-TRAF6 complex prevents TRAF6 autoubiquitination and oligomerization to negatively regulate the TLR-IL-1R signalling pathways. As a consequence, β-arrestins negatively regulate activation of NF-κB and AP-1. β-arrestin 2-knockout mice produce higher levels of pro-inflammatory cytokines and are more susceptible to endotoxic shock than are wild-type mice (Wang et al., 2006).

IκBNS. IκBNS, a TLR-inducible nuclear IκB protein, is involved in termination of NF-κB activity and thereby it inhibits a subset of TLR-dependent genes that are induced late such as IL-6, IL-12p40 and IL-18. Termination of NF-κB activity is caused by the degradation of p65 by IκBNS. IκBNS-deficient mice are highly susceptible to LPS-induced endotoxic shock and to intestinal inflammation (Kuwata et al., 2006).

FLN29. Recently another TLR signalling inhibitor, FLN29, was discovered. FLN29 is an IFN- and LPS-inducible gene, which contains a TRAF6-related zinc finger motif and TANK-related sequences. By interacting with TRAF6, FLN29 attenuates NF-κB activation and TLR signalling (Mashima et al., 2005).

Transmembrane regulators. Transmembrane protein regulators include ST2L, SIGIRR, TRAILR and RP105. These proteins inhibit TLR functions either by sequestration of adaptor proteins (ST2L) or transcription factors (TRAILR), or by interfering with the binding of TLR agonists to their respective TLRs (RP105).

ST2L. It was demonstrated that the membrane bound form of ST2 (ST2L) negatively regulates IL-1RI and TLR4, but not TLR3, signalling by sequestrating the adaptors MyD88 and Mal. In contrast to wild-type mice, ST2-deficient mice fail to develop endotoxin tolerance and show increased inflammatory cytokine production.

SIGIRR. SIGIRR negatively modulates TLR and IL-1R signalling. The mechanism of inhibition is not yet clear but it is shown that SIGIRR can bind with TLR4, IRAK and TRAF6. SIGIRR does not affect the MyD88-independent pathway (Liew et al., 2005). Cells from SIGIRR-null mice show enhanced activation in response to either IL-1 or certain Toll ligands (Wald et al., 2003). SIGIRR-deficient mice are hyper-responsive to IL-1 injection or LPS challenge.

Table I.2.1. - Negative regulators of Toll-like receptor signalling (Adapted from Liew *et al*.).

Negative Regulator	Possible mechanism	Affected TLR
sTLR2	Antagonist of TLR2	TLR2
sTLR4	Blocks interaction of TLR4 and MD2	TLR4
MyD88s	Inhibits IRAK-4 recruitment	TLR4
IRAK-M	Prevents the formation of IRAK1-TRAF6 complex	TLR4, 9
IRAK2c	Suppression of IRAK function	TLR4
IRAK2d	Suppression of IRAK function	TLR4
SOCS-1	Supresses IRAK	TLR4, 9
	Indirect: inhibition of type I IFN signalling	TLR2, 3, 4, 9
	Degradation of Mal	TLR2, 4
NOD-2	Supresses NF-κB	TLR2
PI3K	Inhibition of p38, JNK and NF-κB	TLR2, 4, 9
TOLLIP	Autophosphorylates IRAK	TLR2, 4
A20	Deubiquitinylation of TRAF6	TLR2, 3, 4, 9
RIP3	Inhibition of RIP1/TRIF interaction	TLR3
IRF-4	Compete with IRF-5 for MyD88	TLR4, 7, 9
β-ARRESTINS	Prevents TRAF6 oligomerization	TLR4
IκBNS	Terminates NF-κB activity	TLR2, 4, 9
FLN29	Sequesters TRAF6	TLR4
ST2L	Sequesters MyD88 and Mal	TLR2, 4, 9
SIGIRR	Interacts with TLR4, TRAF6 and IRAK	TLR4, 9
TRAILR	Stabilizes IκBα	TLR2, 3, 4
RP105	Inhibiting binding of LPS to TLR4	TLR4
TRIAD3A	Ubiquitylates TLRs	TLR4, 9

TRAILR. TNF-related apoptosis-inducing ligand receptor (TRAILR) belongs to the TNF superfamily and does not have a TIR domain. Stimulation of macrophages with ligands for TLR2, TLR3 and TLR4, but not TLR9, results in TRAIL up-regulation and enhanced cytokine production in TRAILR-deficient cells. The TRAILR seems to inhibit TLR signalling by stabilizing IκBα and decreases in this way the nuclear translocation of NF-κB (Diehl et al., 2004).

RP105. RP105 is a TLR4 homologue and is not only expressed in B cells as originally proposed, but is widely expressed on APCs as this is the case for TLR4. RP105 is a specific inhibitor of TLR4 signalling in HEK 293 cells and DCs. Moreover, RP105 and its helper molecule, MD-1, interact directly with the TLR4 signalling complex, disabling its binding to LPS (Divanovic et al., 2005).

Reduction in TLR expression. A reduction in TLR expression can be realized by ubiquitination (TRIAD3A) promoting the proteolytic degradation of TLRs, or through inhibition of the transcription or stability of TLR-encoding mRNAs by anti-inflammatory cytokines such as IL-10 and TGF-β. TRIAD3A is a RING finger protein, which act as an E3 ubiquitin ligase. It enhances ubquitination and proteolytic degradation of TLR4 and TLR9, but not TLR3 or TLR2 (Chuang and Ulevitch, 2004).

Apoptosis. The last level of negative regulation is self-destruction. TLR-induced apoptosis might be important in the control of excessive TLR activation. Association of MyD88 and FADD through their DD domains might trigger the recruitment of caspase-8 and subsequent caspase-dependent apoptosis. The MyD88-independent signalling pathway can trigger caspase-independent apoptosis. The MyD88-dependent pathway will also enhance cell survival through NF-κB activation. Thus, TLR signalling can be regulated by the balance of apoptosis and anti-apoptosis signals induced by TLR (Liew et al., 2005).

2.4. Importance of endotoxin tolerance

LPS activates monocytes and macrophages to produce pro-inflammatory cytokines and other mediators. This inflammatory response is critical to control the growth of pathogenic microorganisms. However, excessive and uncontrolled production of these inflammatory cytokines and mediators in response to LPS is regarded as the cause of sepsis and septic shock. Gram-negative sepsis is a major cause of death throughout the world (see section 1.2.).

Beeson (1946) first defined 'endotoxin tolerance' as a reduced endotoxin-induced fever following repeated injections of typhoid vaccine in humans (Beeson, 1947a). He described the same phenomenon in experimental animals (Beeson, 1947b). 'Endotoxin tolerance', also called 'LPS desensitisation' or 'deactivation' or 'reprogramming', is now defined as a transient state of cellular hypo-responsiveness towards a second stimulation with a high dose LPS after a preceding stimulation with a sub-lethal dose of LPS. As a consequence of endotoxin tolerance, the host is protected from developing a shock syndrome caused by hyper-activation of macrophages and other immune cells, and has a significant survival advantage (Zeisberger and Roth, 1998). On the other hand, it has been demonstrated that suppressed IL-12 expression by monocytes and DCs, associated with endotoxin tolerance, may result in an inability to respond appropriately to secondary infections in survivors of sepsis (Karp et al., 1998). Thus, endotoxin tolerance can be a huge problem in the treatment of patients with Gram-negative sepsis. Patients recovering from Gram-negative septic shock are often hypo-

responsive to LPS, but have a reduced response to opportunistic pathogens leading to high mortality (Docke et al., 1997).

Using animal models, it became clear that macrophages/monocytes are the principal mediators in tolerance (Cavaillon, 1995). In addition, both macrophage cell lines (RAW 264.7, Mono-Mac-6 cells) and *ex vivo* isolates of macrophages can be tolerized for LPS (Haas et al., 1990; Li et al., 1994; Virca et al., 1989). One of the main characteristics of endotoxin tolerance is a change (up- or down-regulation) in production of pro-inflammatory (e.g. TNF-α, IL-1, IL-12 and IL-6), anti-inflammatory (e.g. IL-10) and other mediators (e.g. peroxisome proliferator-activated receptor-gamma (PPARγ), NO, TNFRII and IL-1Ra) in cells of the myeloid lineage. Also, alterations to the LPS-induced activation of signalling cascades have been noticed. So, endotoxin tolerance does not totally inhibit cellular functions, but rather represents a reprogramming of cells, possibly, as a means of adaptation to bacterial infection (Schade and E. Dominguez-Fernandez, 1999; Shnyra et al., 1998).
In animal models, an early and a late stage of endotoxin tolerance has been demonstrated. The later stage of endotoxin tolerance depends on specific O-antigen antibodies, whereas the initial stage of endotoxin tolerance has not been elucidated yet (Flohe et al., 1999; West and Heagy, 2002). The early stage of endotoxin tolerance is associated with low levels of inflammatory mediators. Endotoxin-tolerant animals fail to induce IL-6 and TNF-α after LPS treatment. In contrast, IFN and IL-1 synthesis were only partially inhibited (Erroi et al., 1993; Flohe et al., 1999). In LPS-tolerized rats there was a diminished iNOS production due to the elevation of endogenous glucocorticoid levels (Szabo et al., 1994). *In vitro* studies demonstrated that sometimes IL-1 and IL-6 levels are augmented and sometimes they are decreased. These contrasting results may be due to differences in potential LPS treatment and differences in the cellular phenotype and genotype. TNF-α expression is inhibited during endotoxin-tolerance (Peck et al., 2004; West and Heagy, 2002).
The cytokine IL-12 was also suppressed in endotoxin-tolerant monocytes (Karp et al., 1998). The CC chemokines MIP-1α, MIP-1β and RANTES are permanently down-regulated in endotoxin-tolerant monocytes. In contrast, the chemokine IL-8 is unaffected (Kaufmann et al., 2000). Other possible mechanisms of endotoxin tolerance are a decreased Gi protein content and activity (Makhlouf et al., 1998), and a diminished activation of protein kinase C leading to a diminished TNF-α secretion (West et al., 1997).

Endotoxin tolerance has been widely investigated, but to date, the mechanism underlying this phenomenon remains poorly understood. One of the proposed molecular mechanisms of LPS tolerance

is the down-regulation of the LPS receptor complex. It has been demonstrated that TLR4 is down-regulated on the surface of LPS-tolerant macrophages (Nomura et al., 2000). During tolerance, the expression of LBP, CD14 and MD-2 is unchanged or up-regulated (Labeta et al., 1993). However, a down-regulation of the surface expression of the TLR4/MD-2 complex has been demonstrated in LPS-tolerant macrophages (Fujihara et al., 2003). In contrast, in CD14-expressing CHO cells that over-express human TLR4/MD-2, LPS pre-treatment caused a reduction of NF-κB activation while there was no reduction of TLR4/MD-2 cell surface expression (Medvedev et al., 2001). Also in HEK293T cells that over-express CD14, TLR4 and MD-2, reduction of NF-κB activation was demonstrated (Medvedev and Vogel, 2003). Thus, down-regulation of the surface expression of the TLR4/MD-2 complex alone is not sufficient for endotoxin tolerance, and other mechanisms must be involved.

Another possible molecular mechanism behind the endotoxin tolerant phenomenon is an alteration to the LPS intracellular signalling pathway. This can be an impaired activation of the MyD88-dependent and/or MyD88-independent pathway. A diminished quantity of the IRAK-1 signalling protein is seen in LPS-tolerant THP-1 cells, and as a consequence interaction of IRAK-1 with MyD88 is abolished (Li et al., 2000). Furthermore, a reduction in LPS-induced MAPK, SAPK1, and p38 kinase activation is noticed (Kraatz et al., 1999). Medvedev and colleagues also showed that MAPK phosphorylation, NF-κB and AP-1 activation was inhibited in endotoxin-tolerized murine macrophages stimulated with LPS. In addition, a suppressed activation of IKK and decreased degradation of IκB isoforms is demonstrated. The LPS-inducible IκB kinase activity is not detectable in endotoxin-tolerant cells. IκB remains in the cytoplasm where it sequesters NF-κB. Thus, the IκB kinase may play an important role in endotoxin tolerance (Kohler and Joly, 1997). At the nuclear level, accumulation of p50/p50 NF-κB homodimers, which lack trans-activation activity, instead of the transcriptionally active p50-p65 heterodimer leads to less TNF and a decrease in the levels of NO release (Goldring et al., 1998; Ziegler-Heitbrock et al., 1994).

Another mechanism to regulate the endotoxin tolerant phenomenon is the induction of specific anti-inflammatory proteins and signalling pathways. The anti-inflammatory proteins TGF-β and IL-10 are mediators of endotoxin tolerance. IL-10 is up-regulated and is involved in the down-regulation of pro-inflammatory cytokines (Cavaillon et al., 2003). Although, some studies have shown a down-regulation of the IL-10 protein (Randow et al., 1995). Prostaglandins and glucorticoids are also mediators of endotoxin tolerance (Cavaillon et al., 2003). Also, increased activation of peroxisome proliferator-activated receptor-gamma (PPARγ) has been linked to the tolerance phenotype (Fan and Cook, 2004).

It has also been demonstrated that IRAK-M (Escoll et al., 2003), SOCS-1 (Crespo et al., 2000), and PI3K signalling (Bowling et al., 1995) are induced in the endotoxin tolerant phenomenon. IRAK-M becomes rapidly up-regulated in tolerant monocytes and its expression is regulated by PI3K (Escoll et al., 2003). Experiments with SH2-containing inositol 5-phosphatase (SHIP) -/- mice show a correlation between the duration of endotoxin tolerance and elevated SHIP levels. In SHIP -/- mice, inhibition of both NF-κB activation and STAT1 activation, two of the principal end points of LPS signalling, is not observed in contrast to wild-type SHIP +/+ mice. Thus, SHIP is also an important molecule in endotoxin tolerance (Beutler, 2004).

Cross-tolerance is also an interesting phenomenon. In RAW264.7 macrophages, LTA, LPS and CpG-DNA induce tolerance towards a second stimulation with the same stimulus used for priming, as well as cross-tolerance towards a second stimulation with other stimuli. In an *in vivo* model of dGalN-induced liver damage, CpG-DNA does not induce cross-tolerance towards LTA or LPS. In contrast, LTA/LPS can induce cross-tolerance in the same model (Dalpke et al., 2005). Also, tolerance induced by bacterial lipoprotein (BLP) protects mice against BLP-induced lethality, but also LPS-, live bacteria- sepsis-induced lethality (Wang et al., 2003).

2.5. References

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3. TYPE I IFNs

3.1. Overview of the IFN family

About fifty years ago interferon (IFN) was discovered as a soluble factor that served to inhibit viral replication (Isaacs and Lindenmann, 1957). For a long time, it was believed that this was the only function of IFN. Recently a lot of progress has been made in understanding the mechanisms and signalling pathways of IFN. The IFNs were initially classified as classical or type I IFN and immune or type II IFN (Ho and Armstrong, 1975). The type I IFNs, predominantly α and β (IFN-α/β) belong to the class II family of α-helical cytokines. This family consists out of 6 cytokines of the IL-10 family (IL-10, IL-19, IL-20, IL-22, IL-24 and IL-26), multiple type I IFNs, 1 type II IFN, 3 IFN-λs and IL-22BP, which is the soluble receptor which can neutralize the action of one of the cytokines of the IL-10 family (Kotenko and Langer, 2004).

The mouse type I IFN multi-gene family comprises 14 IFN-α genes, single IFN-β, IFN-κ, IFN-ζ (limitin) and IFN-ε genes (Hardy et al., 2004; Kelley and Pitha, 1985; Takahashi et al., 2001; van Pesch et al., 2004; Vassileva et al., 2003). Based on sequence similarity and their anti-viral activity IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B) are called IFN-like molecules. However, because IL-29, IL-28A and IL-28B use a different receptor, have another chromosomal location and gene structure they form a new cytokine family (Kotenko et al., 2003; Sheppard et al., 2003). In addition to these IFNs, IFN-δ in pigs, IFN-τ in ruminants and IFN-ω in mammals with placenta (eutherian mammals) have been identified (Table I.3.1.) (Pestka et al., 2004).

3.2. The IFN-β promoter

3.2.1. Sequence and regulatory domains

The functional IFN-β promoter is contained within a 170-bp DNA fragment located 5' of the coding sequence. The murine IFN-β promoter has a virus-responsive element (VRE) containing the four positive regulatory domains (PRDs I to IV) of the IFN-β promoter. The VRE region corresponds to the minimal region necessary for virus-induced activation of the promoter. Also, two negative regulatory domains, NRDI and NRDII, are found in the promoter. Within NRDI a negative regulatory element, called the NRE, is identified (Nourbakhsh et al., 1993). PRDI and III are related sequence elements that bind members of the IRF family. PRDII and PRDIV are composite regulatory units binding NF-κB/Rel

family members (PRDII) and ATF-2 homodimers or c-Jun/ATF-2 heterodimers (PRDIV). All PRD elements act in a synergistic manner to induce IFN-β promoter activation (Du et al., 1993). The high mobility group protein HMGI(Y) enhances binding of c-Jun/ATF-2 and NF-κB to the IFN-β promoter through binding in the minor grooves of the DNA helix in the proximity of PRDII and PRDIV (Figure I.3.1.) (Du and Maniatis, 1994; Thanos and Maniatis, 1992).

Table I.3.1. - The IFNs and IFN-like molecules (Adapted from Pestka *et al*., 2004).

	Gene number	Receptor	Species (mammals)
Type I IFN		IFN-αR1, IFN-αR2	
IFN-α	Multiple		All
IFN-β	One (except cows)		All
IFN-δ	Multiple		Pigs
IFN-ε	One		Eutherian mammals
IFN-κ	One		All (?)
IFN-τ	Multiple		Ovine
IFN-ω	One (primates) Multiple (cat, pig, cow)		Eutherian mammals
IFN-ζ (limitin)	Multiple		Mouse
Type II IFN		IFN-γR1,IFN-γR2	
IFN-γ	One		All
IFN-like molecules		IL-28RI, IL-10R2	
IL-28A (IFN-λ2)	One		All (?)
IL-28B (IFN-λ3)	One		All (?)
IL-29 (IFN-λ1)	One		All (?)

First, it was demonstrated that IRF-1 binds to PRDI and PRDIII (Reis et al., 1992). Later, it was suggested that both IRF-3 and IRF-7 are involved in the transcriptional induction of IFN-β (Lin et al., 1998; Sato et al., 1998b; Wathelet et al., 1998). Another IRF family member, IRF-2, can also bind with the IFN-β promoter (see further) (Harada et al., 1989). The NRDI domain physically overlaps with PRDII and acts as a constitutive and position-independent silencer of PRDII (Nourbakhsh et al., 1993). Using co-immunoprecipitation assays, two transcriptional co-activators CBP and p300 were found to associate with IRF-3. CBP and p300 molecules do not have the ability to bind DNA directly but can recruit the histone acetyltransferase (HAT) p300/CBP-associated factor (PCAF) (Bannister and Kouzarides, 1996)

that is important in histone acetylation, chromatin conformation, and acetylation of specific transcription factors.

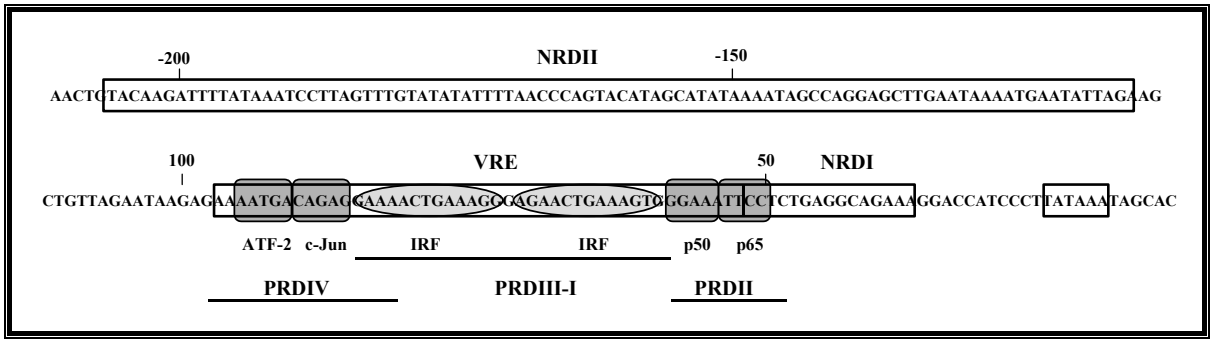

Figure I.3.1. - DNA sequence and regulatory regions of the murine IFN-beta promoter. The DNA sequence of the murine IFN-β promoter is spanning from the TATA box to position –210. The DNA binding sites of transcription factors NF-κB (p50 and p65), IRF proteins, and ATF-2/c-Jun are indicated. Positions of the VRE and NRDs are also indicated (Reprinted from Bonnefoy *et al*., Mol Cell Biol, 19, 2803-2816, 1999).

3.2.2. Activation and termination of IFN-β gene expression

Virus infection activates multiple transcriptional activator proteins such as NF-κB (p50/p65), ATF-2/c-jun and IRF proteins. In the presence of the architectural protein HMGI(Y), they bind co-operatively and form the enhanceosome on the IFN-β enhancer (Thanos and Maniatis, 1995). HMGI(Y) is the essential architectural component for the assembly and stability of the IFN-β gene enhanceosome (Du et al., 1993; Thanos and Maniatis, 1992; Thanos and Maniatis, 1995). Two molecules of HMGI(Y) bind to four sites within the enhancer in a highly co-operative fashion by employing both intra- and intermolecular co-operativity (Yie et al., 1997).

In the inactive state, the enhancer is nucleosome-free but is flanked by two nucleosomes. One of these nucleosomes (nucleosome II) masks the TATA box and the initiation site necessary for transcriptional activation, whereas the other lies upstream of the enhancer (Agalioti et al., 2000). Following the enhanceosome assembly, the activation domains of all the activators form a novel surface that constitutes a high-affinity binding site and thus function co-operatively to recruit the GCN5/PCAF complex, which acetylates the nucleosomes. PCAF has an intrinsic HAT activity and acetylate histones of nearby nucleosomes. Hyper-acetylation of histone tails in nucleosomes upon virus infection is an important mechanism by which transcription of IFN-β is induced (Parekh and Maniatis, 1999). Recruitment of this GCN5/PCAF complex is followed by recruitment of CBP/p300, which has also intrinsic HAT activity, and CBP-associated proteins. Recruitment of CBP by the enhanceosome tethers

the PolII holoenzyme via its interaction with the carboxyl terminus of CBP. In turn, the nucleosome acetylation facilitates interaction of CBP with the Swi/SNF complex resulting in nucleosome modification. By binding of another molecule, TBP, DNA bending is induced and nucleosome II slides to a new position, unmasking the TATA box and enabling transcription from the IFN-β promoter (Agalioti et al., 2000; Bode et al., 1986; Lomvardas and Thanos, 2001). At the same moment of recruitment of the PolII holoenzyme complex, other components of the basal transcription machinery, such as transcription factor (TF) IID, TFIIA, TFIIB and USA of the TFIID/A/B complex are recruited (Figure I.3.2.) (Agalioti et al., 2000; Merika et al., 1998). The treatment of cells with trichostatin A (TSA), an inhibitor of histone deacetylases, induced strong, constitutive derepression of the murine IFN-β gene promoter stably integrated into a chromatin context (Shestakova et al., 2001).

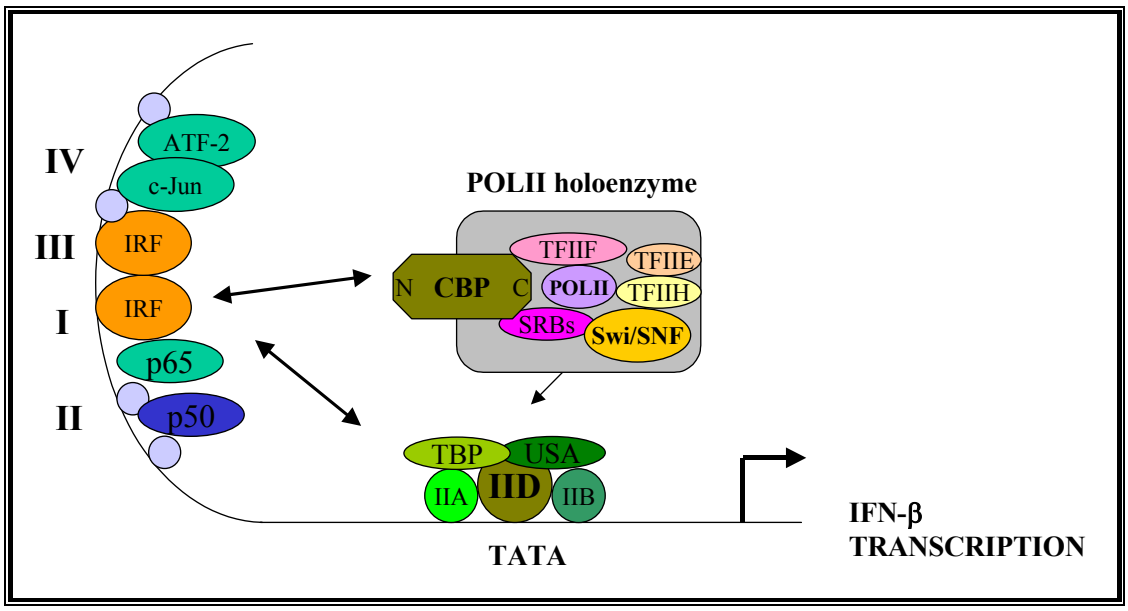

Figure I.3.2. – A model for the synergistic activation of transcription by the IFN-β enhanceosome. The IFN-β gene requires the assembly of a higher order nucleoprotein complex, the enhanceosome, which consists of the transcriptional activators NF-κB (p50/p65), ATF-2/c-jun, IRF proteins, architectural protein HMGI(Y), and the coactivator CBP. HMGI(Y) helps them to bind co-operatively to the enhancer to form an enhanceosome HMGI(Y) organizes the enhanceosome into a structure that optimally interacts with chromatin-modifying activities and general transcription factors (Adapted from (Kim and Maniatis, 1997).

Termination of IFN-β gene expression depends on the recruitment of CBP that acetylates HMGI(Y) at distinct lysine residues. Both PCAF and CBP can acetylate HMGI(Y), but the lysine residues acetylated by CBP and PCAF differ. Acetylation of HMGI(Y) by CBP, but not by PCAF, disrupts the HMGI/NF-κB complex and thus the enhanceosome stability (Munshi et al., 2001; Munshi et al., 1998).

3.2.3. Regulatory mechanisms of the IFN-β promoter

The production of IFN-β is considered to be subject to stringent control, and essentially IFN-β is maintained in a constitutive transcriptional silent state in the absence of external stimuli. This silent state is controlled through the inhibitory effects of an NF-κB regulating factor (NRF). NRF, an active transcription repressor that binds to NRE, abolishes the transcriptional activity of the bordering NF-κB-binding sites by a direct protein-protein interaction between NRF and proximal bound NF-κB preventing the transcriptional activation (Nourbakhsh and Hauser, 1997). The promoters of human IL-8 and human inducible nitric oxide synthetase (hiNOS) contain also such a NRE site overlapping partially with the NF-κB response element. NRF is involved in transcriptional silencing of the human IL-8 and hiNOS promoters, but it plays an additional role acting as an activator of IL-1-induced IL-8 gene expression (Feng et al., 2002; Nourbakhsh et al., 2001).

YY1 is a transcription factor that binds the IFN-β promoter at positions -90 and -122 through the recognition of a specific consensus sequence. YY1 has been shown to bind to transcriptional co-repressors such as histone deacetylases (HDACs) or co-activators such as HATs, and a wide variety in transcription factors, such as c-Myc, SP1 (Seto et al., 1993) and E1A (Lewis et al., 1995). *In vivo* YY1 binds to HDAC2 and *in vitro* to HDAC1, HDAC2, and HDAC3 (Davie and Chadee, 1998). YY1 also interacts with HATs p300 and CBP (Yao et al., 2001). YY1 has a dual activator/repressor role in the transcriptional capacity of the IFN-β promoter depending on its posttranslational modifications through HATs and HDACs, its binding site and on the moment after virus infection (Weill et al., 2003).

IRF-1 and IRF-2 are functionally distinct factors, but they show a high degree of structural similarity in their N-terminal DNA-binding domains. Both factors bind to the same DNA element, called IRF-E (consensus sequence: G(A)AAA$^{G}/_{C}$ $^{T}/_{C}$GAAA $^{G}/_{C}$ $^{T}/_{C}$). The IRF-E element resembles the interferon-stimulated response element (ISRE; consensus sequence: $^{A}/_{G}$ NGAAANNGAAACT) in IFN-dependent genes (Tanaka et al., 1993). Both factors are constitutively expressed at low levels in a variety of cell types. Upon virus infection or IFN stimulation both IRF-1 mRNA and IRF-2 mRNA are induced, and particularly IRF-1 mRNA. The IRF-1 promoter has binding sites for STAT and NF-κB transcription factors (Harada et al., 1994; Pine et al., 1994). In STAT1-deficient cells, no induction of IRF-1 mRNA was seen after IFN stimulation (Meraz et al., 1996). IRF-2 is also inducible by IFN-α/β and this is mediated by an NF-κB and ISRE element in its promoter region (Harada et al., 1994).

IRF-1 can activate IFN-α/β promoters as shown by IRF-1 cDNA expression experiments. High level expression of the cloned mouse *Irf-1* gene in monkey COS cells and mouse ECs resulted in the induction of endogenous IFN-α and IFN-β without viral stimulation (Fujita et al., 1989; Harada et al., 1989; Harada et al., 1990). In contrast, IRF-2 represses IRF-1 induced transcription of IFN-α/β promoters (Harada et al., 1990). Thus, IRF-1 and IRF-2 act as transcriptional activator and repressor for the IFN-α/β genes.

IRF-1-/- and IRF-2-/- mice are resistant to LPS-induced lethality (Cuesta et al., 2003; Senaldi et al., 1999). Using IRF-1-/- macrophages, it was demonstrated that IRF-1 is necessary for iNOS production (Kamijo et al., 1994). However, IRF-1 is not essential for type I IFN production *in vivo*, because virus- or dsRNA-induced type I IFN was unimpaired in IRF-1-/- mice (Reis et al., 1994). IRF-2-/- mice develop a $CD8^+$ T-cell mediated-inflammatory skin disease and show elevated expression of IFN-dependent genes (e.g. *Irf-7* and *Oas*) (Hida et al., 2000). IFN induction after viral infection is more efficient in IRF-2-deficient fibroblasts than in wild-type fibroblasts (Matsuyama et al., 1993). IRF-2 and IFN-stimulated gene factor 3 (ISGF3) compete with each other for the ISRE sites on IFN-inducible genes and loss of IRF-2 leads to a continuing expression of IFN-dependent genes (Hida et al., 2000). IRF-2 can also act directly on the IFN-β promoter to inhibit its induction (Senger et al., 2000). To conclude, in contrast to IRF-1, which is a positive factor, IRF-2 does not function as an activator but suppresses the function of IRF-1 under certain circumstances (Harada et al., 1989).

Another regulator of the IFN-β promoter is IRF-7, which is most closely related to IRF-3 in terms of the primary structure (for IRF-3, see section 2.2.2.1.) (Nguyen et al., 1997). IRF-7 was first described to bind and repress the Qp promoter region of the Epstein–Barr Virus (EBV)-encoded gene EBNA-1, which contains an ISRE-like element. IRF-7 is ubiquitously expressed, but unlike IRF-3 its expression is dependent of IFN-α/β signalling (Zhang and Pagano, 1997). Upon IFN-α/β stimulation, IRF-7 becomes phosphorylated in its C-terminal domain, which is highly homologous to the IRF-3 C-terminal end, and undergoes nuclear translocation. Sato and colleagues expressed a deletion mutant of IRF-7, in which the region containing the potential sites of inducible phosphorylation between amino acids 411 and 453 was truncated. The mutant IRF-7 is incapable of translocation upon virus infection of the cells, demonstrating an essential role of this region in nuclear translocation. In addition, *de novo* synthesis of IRF-7 is important for positive-feedback regulation of IFN-α/β gene induction (Sato et al., 1998a). For a more extensive explanation of IRF-7 and its importance in amplification of the type I IFN response see section 3.3.4.

3.2.4. Posttranscriptional regulation of IFN-β expression

IFN-β mRNA is transiently expressed after virus infection or poly(I:C) stimulation (Hayes et al., 1979). The down-regulation of IFN-β mRNA synthesis is due to both transcriptional repression and rapid mRNA degradation. Degradation of transiently expressed mRNAs involves two steps. First, the mRNAs are deadenylated and this is followed by a rapid degradation of the deadenylated mRNA, which is facilitated by the presence of an AU-rich element (ARE) in the 3' untranslated region (UTR) (Raj and Pitha, 1993). ARE motifs are found in many transiently expressed mRNAs such as in proto-oncogenes (e.g. c-fos, c-myc and c-jun), and in cytokine/lymphokine mRNAs (e.g. TNF-α). IFN-β has two destabilizing sequences: an AU-rich element in the 3' UTR, and the coding region instability domain (CRID) in the 3' end of the coding region. The AU-rich element in the 3'UTR of the IFN-β mRNA is highly homologous to the ARE region of other transiently expressed mRNAs. It targets the mRNA for rapid degradation and affects its translatability. AREs are classified into distinct categories based on the number and distribution of AUUUA pentamers. IFN-β ARE belongs to class II AREs, which are characterized by the accumulation of poly(A)$^-$ intermediates. The CRID region is also found in other unstable mRNAs such as c-fos and c-myc. The degradation of the IFN-β mRNA is under the control of these two cis-acting elements, which act independently (Paste et al., 2003; Raj and Pitha, 1993).
The posttranscriptional regulation of the IFN-β mRNA is also mediated by a 65 kDa cytoplasmic protein that binds on three AU-rich domains of 19, 20 and 29 nucleotides long. One of these binding sites is the CRID domain, and the two other sites are in the 3'UTR. The deadenylation and degradation of IFN-β mRNA is not coupled to its translation or to viral infection but requires a nuclear event (Raj and Pitha, 1993).

3.3. The IFN-α promoter

In humans and mice, multiple functional IFN-α gene subtypes exist. All IFN-α subtypes interact with the same receptor complex, termed the IFNAR complex. The PRDII and PRDIV elements, which are found in the IFN-β promoter and are activated by NF-κB and ATF-2/c-jun, are not identified in the IFN-α promoters (Nakaya et al., 2001). On the other hand, PRDI- and III-like elements (PRD-LEs), which bind members of the IRF family, have been identified in IFN-α promoters (Braganca et al., 1997). The murine IFN-α4 gene contains a VRE element located in the promoter proximal (-120 to -43) region comprising four PRD-LE elements (A-D). Cooperation among these binding sites is required for maximal IFN-α4

gene trans-activation. The AB elements bind preferentially IRF-7, while the C element is preferentially recognized by IRF-3. On the other hand, in the IFN-α11 gene there are point mutations in the C and D elements. This causes weaker activation of the IFN-α11 subtype and explains the molecular basis for differential expression of IFN-α genes following virus infection (Morin et al., 2002). At this moment it is still not clear whether or not other transcription factors are involved in the induction of these genes.

Next to IRF-3 and IRF-7, IFN-α promoters can also bind IRF-1, IRF-2 and IRF-5 proteins. IRF-5 can synergize with the virus-mediated induction of IFN-α genes. It has also been demonstrated that human IRF-5 can activate IFN-α genes after virus infection in the absence of IRF-7 and that this is virus-specific. IRF-5 can form heterodimers with IRF-3 and IRF-3/IRF-5 heterodimers can induce IFN-α genes in virus-infected cells (Barnes et al., 2001). Barnes and colleagues showed that also IRF-5/IRF-7 heterodimers can be formed that modulate the expression profile of IFN-α subtypes (Barnes et al., 2003). In addition, the functions of IRF-5 and IRF-7 are not redundant, since they each induce distinct IFN-α subtypes (Barnes et al., 2001).

It seems that *in vitro* (monocytes) no IFN-α is induced after LPS stimulation (Bogdan et al., 2004; Hayes et al., 1991). Also *in vivo* it was demonstrated that LPS-induced IFN-α4 expression is very low (Karaghiosoff et al., 2003). In contrast, IFN-α is induced after TLR3 stimulation. The mechanisms of TRIF-mediated IFN gene induction via TLR4 and via TLR3 are not the same. While TLR3 activation results in the induction of IFN-α4 as well as IFN-β, TLR4 induces only IFN-β (Doyle et al., 2002).
IFN-α is also strongly induced upon activation with TLR7 and TLR9 ligands in pDCs (Asselin-Paturel et al., 2005; Krug et al., 2001). To efficiently induce IFN-α production a signalling complex, consisting of IRF-7, is formed.

3.4. Type I IFN signalling

3.4.1. The classical JAK-STAT pathway in type I IFN signalling and importance of IFN-β in sepsis

Manuscript submitted by Tina Mahieu and Claude Libert

'Must we inhibit type I IFNs in endotoxemia and sepsis?'

Must we inhibit type I IFNs in endotoxemia and sepsis?

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Type I interferons (IFNs) are well-known potent antiviral cytokines, but our knowledge about their roles in the host response to bacterial lipopolysaccharides (LPS) has increased rapidly only over the last few years. Bacterial infections, if they succeed in deregulating host responses, can lead to sepsis, severe sepsis or septic shock, and possibly high mortality rates. Here, we revisit the available data that suggest that type I IFNs are absolutely essential in LPS induced endotoxemia. This review will discuss the importance of the molecules involved in the induction of type I IFNs, the IFNs themselves, the IFN-responsive JAK-STAT molecules, and the downstream genes in endotoxemia. We also discuss the potential advantages and disadvantages of treating septic patients with antibodies that block type I IFNs or their receptors.

Introduction

Infectious agents (viruses, bacteria, lower eukaryotes, worms etc.) are omnipresent, posing a continuous threat to vertebrates, including humans. Many examples of disastrous outbreaks of plague or viral epidemics colour our history books, for example the plague of the 1340s, which killed one third of the European population, and the Spanish flu of 1918. Many infectious agents have come under strict control, but many others that remain unchecked cause many casualties, e.g. HIV, SARS, Influenza and malaria.

Sepsis is defined as a systemic inflammatory response syndrome (SIRS) resulting from infection. Every year, more than 750,000 people in the U.S. and the E.U. develop sepsis, severe sepsis (sepsis associated with acute organ failure) or septic shock (severe sepsis with a dependency on vasopressors). All three syndromes are characterized by an overwhelming systemic response to infection that can rapidly lead to organ dysfunction and eventually death. Severe sepsis and septic shock strike hard and kill quickly. Each year, approximately 215,000 people in the U.S. die from severe sepsis/septic shock, which exceeds those dying from breast, colon/rectal, pancreatic, and prostate cancer combined (1). Over the recent decades, mortality rates due to severe sepsis have remained constant or decreased slightly, but incidence rates have grown steadily. Despite advances in our knowledge of infectious diseases and critical care, and despite numerous attempts to develop new treatments, the rate of mortality from severe sepsis and septic shock remains unacceptably high (2). Gram-negative sepsis, the leading cause of sepsis, has a case fatality rate of about 35%. Gram-positive pathogens are the second major cause of sepsis.

Vertebrates, as well as invertebrates and plants, have developed mechanisms to detect and respond to intruders (3, 4). Clearly, inflammation and innate and adaptive immune responses are aimed at destroying the intruders. Gram-negative bacteria contain lipopolysaccharides (LPS) in their outer membranes (5). LPS, which has been studied extensively, is considered as the prototypic activator of innate immunity. Picomolar concentrations of LPS are sufficient to stimulate cells of the immune, inflammatory and vascular systems (6). LPS belongs to the group of molecules produced by pathogens and containing so-called “pathogen-associated molecular patterns” (PAMPs). PAMPs are recognized by one or more members of a family of transmembrane signalling receptors known as the Toll-like receptor family (TLR), as well as by intracellular PAMP-detecting molecules, such as nucleotide-binding oligomerization domain (Nod)-1, Nod-2, and retinoic acid inducible gene I (RIG-I). (7-10). To date, 13 different mammalian TLRs have been recognized and cloned. (11). Activation of TLRs induces intracellular signalling pathways that lead to the production of specific sets of pro-inflammatory cytokines and chemokines, as well as type I IFNs and IFN-inducible gene products (12).

Interferon (IFN) was discovered about 50 years ago as a soluble factor that inhibited viral replication upon induction of specific anti-viral genes, such as *Oas* and *Mx*, in infected cells. IFNs are classified in two distinct types. Type I IFNs consist of multiple IFN-α proteins, and single IFN-β, ε, κ, ζ (also called limitin), and ω subtypes, as well as δ and τ subtypes found in pig and ovine, respectively (13). IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B) function somewhat like type I IFN, but belong to a new cytokine family (14).

The last few years of research have not only led to a much better characterization of the classical antiviral activities of IFN, but have also revealed a number of surprises concerning other biologically important immune regulatory functions of type I IFNs. Together, these results led to the conclusion that type I IFNs are essential links between the early innate responses and the subsequent, more specific adaptive immune responses (15, 16). Type I IFNs induce MHC class I expression and have important effects on maturation and function of dendritic cells (DCs). They also lead to amplification of their own induction, as well as that of IL-15 and a high-affinity form of the IL-12 receptor, and activate natural killer (NK) cell cytotoxicity (17-21). Indeed, recent studies have shown that a clear connection exists between type I IFN and antigen-presenting DCs at two levels. First, a specific DC precursor, the plasmacytoid pre-DC (p-preDC), was identified as a cell type that, following stimulation with infectious agents, can secrete very large amounts of type I IFNs. Second, type I IFNs have been shown to act as differentiation and maturation factors for DCs. The signal for up-regulation of co-stimulatory surface molecules, including CD40, CD80 (B7-1) and CD86 (B7-2), is initiated by LPS, but it is mediated by IFN-β and the type I interferon receptor (IFNAR) signalling axis (21). Furthermore, type I IFNs are crucial in inducing cytotoxic activity and proliferation of NK cells (17), and may also play key roles in induction of effective B-cell responses (22). Taken together, the available data suggest that type I IFNs serve as a link between the innate immune response to infection and the adaptive immune response.

In this review, we discuss the importance of type I IFNs in LPS-induced lethal endotoxemia and sepsis, and the rationale for treating endotoxemia and sepsis by blocking type I IFN production or activity.

TLR4-mediated type I IFN production

Bacterial lipopolysaccharide (LPS) is an important structural component of the outer membrane of Gram-negative bacteria. It is considered as the principal active agent in the pathogenesis resulting from infection with Gram-negative bacteria. Indeed, injection of LPS leads to endotoxemia and endotoxic shock, which closely resemble sepsis and septic shock (23). We will briefly review the major factors involved in induction of type I IFNs upon LPS stimulation, and describe the major factors involved in responding to these IFNs.

Beutler and co-workers demonstrated that the genetic defects in two LPS hyporesponsive strains of mice are linked to TLR4 (24, 25). C3H/HeJ mice have a point mutation in the coding region of the *Tlr4* gene, resulting in a substitution of a highly conserved proline at codon 712 by histidine, whereas in C57BL/10ScCr mice the *Tlr4* gene is deleted. These mutations render these strains resistant to endotoxin (24). It has been demonstrated that activation of macrophages by LPS results in the release of a variety of inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, IL-8, IL-12 and interferon (IFN)-β, in addition to smaller mediators such as prostaglandins and nitric oxide (NO) (26).

After binding to LPS, TLR4 dimerizes and undergoes a conformational change required for the recruitment of downstream TIR domain-containing adaptor molecules (see Figure 1). These include myeloid differentiation primary-response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP), TRIF-related adaptor molecule (TRAM) and TIR-domain-containing adaptor protein inducing IFN-β (TRIF), which together determine the signalling specificity of the response (12). All TLRs, except TLR3, recruit MyD88 in order to activate both NF-κB and the mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) (12). MyD88 recruits IL-1 receptor-associated kinases (IRAKs) through interaction of the death domains. IRAKs are activated by phosphorylation, after which they associate with TRAF6, leading to activation of the IκB kinase (IKK) complex, degradation of IκB, nuclear translocation of NF-κB, and expression of inflammatory cytokines (10, 27).

Stimulation with LPS also leads to recruitment of TRIF to the TLR4 receptor complex, and consequently to activation of IFN-regulatory factor (IRF)-3. This transcription factor induces expression of the IFN-β encoding gene (12). Hiscott and colleagues identified the kinases responsible for activation of

IRF-3. Using two-hybrid screening, they found that IRF-3 was associated with two IKKs (28, 29), namely TANK-binding kinase 1 (TBK1) and IKK-ε/IKK-i, whose activities are distinct from those of the canonical IKK-α and IKK-β (10). In response to LPS, mouse embryonic fibroblasts (MEFs) derived from TBK1-deficient (TBK1-/-) mice are impaired in the production of type I IFNs and IFN-inducible gene products, but not of pro-inflammatory cytokines (Table 1) (30). IKK-ε-/- mice show normal expression of these genes, but MEFs from IKK-ε/TBK1-double deficient mice, upon specific TLR3 stimulation with poly(I:C), were unable to produce IFN-β and IFN-inducible proteins (note that the TLR3 receptor does not use the MyD88 pathway, but only the TRIF pathway). Moreover, in IKK-ε/TBK1 double deficient cells, LPS failed to activate IRF-3 (31). It was also shown that after TLR4 stimulation TRIF can recruit TRAF6-TAK1-TAB2 through its TRAF6-binding site, which is different from its IRF-3 activating site (32). Upon LPS stimulation, phosphorylated IRF-3 dimerizes and translocates to the nucleus. IRF-3 dimers become transcriptionally active after association with p300/CBP co-activators. Activated IRF-3, along with NF-κB, induce the expression of the IFN-β gene (33).

The JAK-STAT pathway

Once produced, type I IFNs bind to the interferon-alpha receptor (IFNAR) 1 and IFNAR2 and initiate a signalling cascade mediated by the tyrosine kinases janus kinase (JAK) 1 and tyrosine kinase (TYK) 2, which activate the signal transducer and activator of transcription (STAT) 1 and STAT2 to form a STAT1/STAT2 heterodimer (34). Other pathways, most notably the p38 MAPK and phosphatidylinositol 3-kinase (PI3K) pathways, are also induced (35). STAT1/STAT2 complexes associate with a p48 protein, identified as IRF-9, to form the interferon-stimulated gene factor-3 (ISGF-3). This factor recognizes interferon stimulated response elements (ISREs) in promoter regions of interferon responsive genes (ISGs) encoding proteins such as PKR (double-stranded RNA-dependent protein kinase), OAS (2',5'-oligoadenylate synthetase), Mx1 (myxovirus (influenza) resistance 1) and IRF-7 (34). In addition to being part of ISGF-3, STAT1 also forms homodimers that bind to a distinct promoter element, the IFN-γ-activated site (GAS). STAT1 homodimers, called the AAF (IFN-α activated factor) complex, induce the IRF-1 gene, another transcriptional activator (36, 37). When IRF-7 is induced by ISGF3, it becomes phosphorylated and is translocated to the nucleus (38), where it activates the IFN-α/β promoters (20, 39). Induction of serum IFN-α/β by viruses is severely impaired in IRF-7 knockout mice, which shows that IRF-7 is essential for the induction of type I IFN after virus infection (40). IFN-α/β gene induction is more severely impaired by blocking IRF-7 expression than by introducing an IRF-3 null mutation (41). Thus, IRF-7 plays a crucial role in the massive IFN-α/β production through a positive feedback loop (see Figure 1) (20, 38, 42).

The JAK-STAT pathway is negatively regulated by distinct regulatory proteins, including the suppressors of cytokine signalling (**SOCS)-1 and SOCS-3**, which inhibit the kinase activity of JAK1. STAT1 tyrosine phosphorylation following TLR triggering is severely impaired by SOCS-1, and to a lesser extent SOCS-3. Thus, SOCS proteins, which can be induced by cytokines as well as by TLR ligands such as LPS and CpG, limit the extent of TLR signalling by inhibiting the type I IFN signalling pathway but not the main NF-κB pathway (43).

JAK1 phosphorylation is also negatively regulated by protein tyrosine phosphatases (**PTPs**), such as SRC homology 2 (SH2)-domain-containing PTP1 (**SHP1), SHP2, CD45 and T-cell PTP (TCPTP)** (44).

STAT1 is not only regulated by the PTPs SHP2 and TCPTP, but also by protein inhibitor of activated STAT (PIAS). STAT1-mediated gene activation is regulated by PIAS1 and PIASY. **PIAS1** blocks the DNA-binding activity of STAT dimers and inhibits STAT1-mediated gene activation in response to IFN-γ (45). **PIASY** acts as a transcriptional co-repressor of STAT by recruiting co-repressor proteins such as histone deacetylase (HDAC) (46).

SH2-containing inositol 5-phosphatase (**SHIP**) is a negative regulator of the PI3K-pathway, which stimulates a number of other pathways, including the MyD88-dependent (47) and the TRIF-dependent pathways (48). Phosphorylation of STAT1 has recently been shown to be PI3K dependent (49).

IFN-β as a critical mediator in lethal endotoxemia

Type I IFNs have long been known as potent antiviral molecules. In the last few years, however, a critical role for these interferons in LPS-induced endotoxemia has been elucidated. It has become clear that non-viral PAMPs, such as LPS, induce expression of the type I IFN genes. The essential molecules involved in inducing type I IFNs and in responding to them have also become known. The role of these molecules in endotoxemia, as well as that of the IFNs themselves, is now being investigated in knockout mice and in cells. Table 1 lists

the phenotypes relevant to this review. Figure 1 depicts molecules whose knockout affects LPS responsiveness.

A mutant mouse, called *Lps2* and generated by random mutagenesis with ENU in the laboratory of Dr. B. Beutler, appears to contain a distal frameshift error in a Toll/interleukin-1 receptor/resistance (TIR) adaptor protein, now known as **TRIF** or TICAM-1. $Trif^{Lps2}$ homozygote mice are markedly resistant to endotoxemia, and fail to produce type I IFNs in response to LPS. LPS-induced STAT1 phosphorylation and IRF-3 dimerization are also impaired in these mice (50). TRIF-deficient mice, generated in the laboratory of Dr. S. Akira, are also defective in TLR4- and TLR3-induced expression of IFN-β and activation of IRF-3. Furthermore, TRIF-deficient macrophages are impaired in the production of inflammatory cytokines in response to the TLR4 ligand, but not in response to ligands of TLR2, TLR7 and TLR9. Poly (I:C)-induced NF-κB activation was severely impaired. In contrast, induction of NF-κB and MAPK JNK by LPS was almost normal. This might be due to an intact MyD88-dependent early NF-κB activation (51).

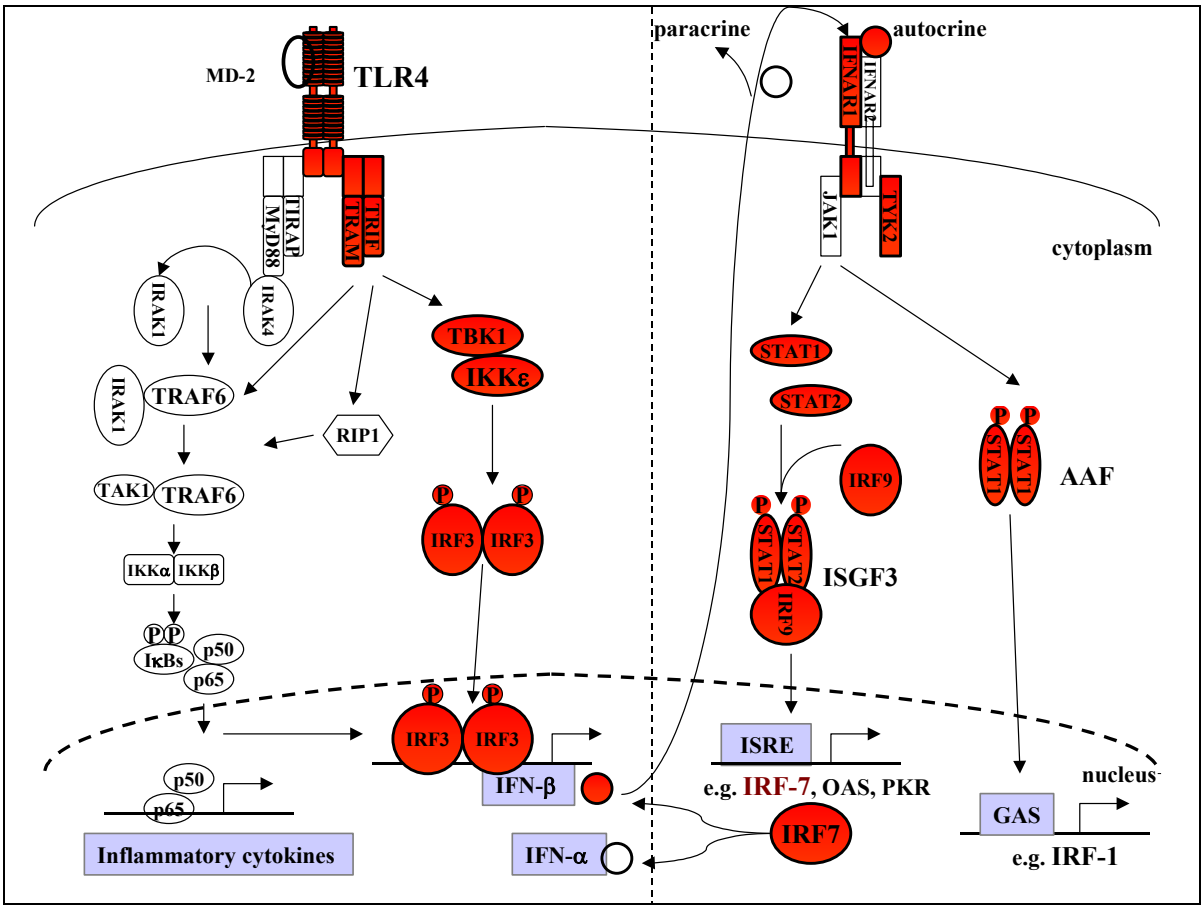

Figure 1. Representation of TLR4-signalling through the MyD88-dependent and MyD88-independent pathways. The MyD88 independent/TRIF-dependent pathway leads to induction of IFN-β. IFN-β then binds to the IFNAR complex in an autocrine or paracrine way, leading to activation of ISGF3 and AAF. The former binds to ISRE elements of IFN-inducible genes, such as those encoding IRF-7, OAS and PKR. IRF-7 can bind to promoter elements of the genes encoding IFN-β and IFN-α. The molecules in color are those whose deletion in mice leads to resistance to endotoxemia.

TRAM-deficient mice, also generated by Dr. Akira and his colleagues, are defective in LPS-induced cytokine production (TNF-α and IL-6). In TRAM-deficient macrophages (52) LPS failed to induce IFN-β and IFN-stimulated genes (*Ifit2*, *Cxcl10*, *Ccl5*), and to activate STAT1.

TBK1 deficiency resulted in TNF-mediated liver degeneration and consequent embryonic mortality (53). In TBK1$^{-/-}$ macrophages, the LPS induced activation of IRF-3 and STAT1 was absent or greatly diminished. Also, in response to LPS, TBK1$^{-/-}$ macrophages failed to up-regulate transcription of IFN-β and IFN-mediated transcription of genes encoding CXCL10, CCL5, IFN-α5, IRF-7, IL-15 and Mx1. However, they activate NF-κB normally in response to LPS (54).

It was also shown recently, using **IRF-3** knockout mice, that IRF-3 is indeed essential for LPS-mediated IFN-β gene Embryonic fibroblasts from **IKK-ε**$^{-/-}$ mice responded normally to LPS with respect to IRF-3 activation and induction of type I IFN. However, expression of IRG-1 and CXCL10 mRNA in IKK-ε$^{-/-}$ TBK1$^{-/-}$ cells was severely impaired, and induction of IFN-β, IFN-α, and ISG54 mRNA after stimulation with poly (I:C) was abolished (31). Loss of IRF-3 also affects the expression profile of other genes, such as some IFN-α subtypes, CXCL10 and IL-15. As would be expected, IRF-3-deficient mice are resistant to LPS-induced endotoxic shock (55).

As described above, the JAK-STAT pathway is involved in transduction of the signal induced by the IFNAR1 and leading to expression of IFN-responsive genes as well as massive up-regulation of IFN-α/β genes. Absence of several molecules of the JAK-STAT pathway causes resistance to LPS-induced endotoxemia. In addition, over-expression of negative regulators of the JAK-STAT pathway (see above) can shut down IFN signalling and hence are expected to protect against endotoxemia.

Table 1: Summary of the biological effects, relevant to endotoxemia, observed in mice or cells deficient in genes that are centrally involved in induction of type I IFNs, downstream signaling molecules or the IFNs themselves.

	LPS stimulation					
-/-	**IFN-β**	**IFN-dependent genes (e.g. IFNα)**	**Pro-inflammatory Cytokines (e.g. TNF)**	**Lethality**	**Gram – Sepsis**	**References**
TLR4	ND	ND		R	Sensitive	**23, 52, 53**
TRIF(m) **TRIF (c)**	↓↓ ↓↓	↓↓ ↓↓	↓↓ ↓↓	R	ND	**54, 55**
TRAM (c)	↓↓	↓↓	↓↓	ND	ND	**56**
TBK1 (c)	↓↓	↓↓	√ (NF-κB)	ND	ND	**58**
IKK-ε/ι (c)	↓↓	↓↓	ND	ND	ND	**30**
IRF-3	↓↓	↓↓	√	R	ND	**59**
IFNAR1	ND	↓↓	ND	R	ND	**60**
TYK2	↓↓	↓↓	√ (IκB degrad.)	R	ND	**62**
STAT1	↓↓	ND	ND	R (±)	ND	**62**
STAT2	ND	↓↓	ND	ND	ND	**63**
IRF-7	ND	ND?	ND	ND	ND	**42**
IFN-β	↓↓	ND	√	R	ND	**62**
SOCS-1	ND	ND	√ (IκB)	Sensitive	ND	**64**

Legend: ↓↓ mRNA strongly decreased; √ : Inflammatory cytokine production OK; R: resistant to LPS ; m: mice; c: cells; ND: not done.

IFNAR1 -/- mice are highly susceptible to viral infection. In cells from IFNAR1 -/- mice, no signalling in response to type I IFN was detectable as measured by induction of OAS. Also, bone marrow macrophages from IFNAR1 -/- mice respond abnormally to LPS (56). We indeed found that IFNAR1-deficient mice completely resist LPS-induced lethal endotoxemia **(Mahieu et al., 2006)**. In addition, using macrophages from IFNAR1$^{-/-}$ mice, Hertzog and colleagues found that type I IFNs mediate the induction of cyclin D2 by LPS (58).

Tyk2 knockout mice are resistant to shock induced by high doses of LPS. Induction of IL-1β, IL-6, IL-12, TNF and NO in serum were comparable in wild-type and Tyk2 null mice. LPS-induced MyD88-dependent signalling *in vitro* was intact, as shown by normal TNF-secretion, IκB degradation and phosphorylation of p38, ERK1/2 and JNK. In Tyk2 null macrophages, LPS-induced expression of IFN-β and IFN-α4 mRNA was diminished, and induction of IFN-γ mRNA was low. Moreover, phosphorylation of IRF-3 was normal, but induction of IRF-1 and IRF-7 mRNA was reduced (59).

Mice defective in **STAT1** are resistant to LPS, but not as much as Tyk2 knockout mice. As would be expected, LPS-induced expression of IFN-β mRNA was reduced in the absence of STAT1. But, in contrast to Tyk2 null macrophages, STAT1 null macrophages were not impaired in IFN-γ expression (59).

STAT2 null mice also show a loss of the type I IFN autocrine/paracrine loop, which afffects several aspects of the immune response (60). Furthermore, it was shown in **IRF-7** knockout mice that the transcription factor IRF-7 is essential for the induction of IFNα/β genes after virus infection (40). The most solid evidence that type I IFNs are central mediators in endotoxemia was provided by Karaghiosoff and co-workers, who showed that **IFN-β** knockout mice are resistant to lethal endotoxemia induced by high doses of LPS, and that they have less serum TNF, NO and IFN-γ after LPS challenge compared to WT animals (59).

Deficiency in **SOCS-**1 leads to early-onset fatal disease. Experiments on cells from SOCS-1 knockout mice demonstrated that SOCS-1 is necessary for inhibition of IFN-α/β receptor signalling through effects on Tyk2. As expected, SOCS-1-/- mice did not resist LPS, nor did SOCS1-/- IFN-γ-/- mice (61). On the contrary, SOCS-1 is strongly induced by LPS and is an essential protective molecule since SOCS-1-/- mice were found to be supersensitive to LPS (62, 63). In fact, SOCS-1--/- mice develop severe inflammatory disease, which appears to be solely the result of overactivity of the type I IFN signaling cascade and not due to enhanced type II IFN activity (64). Overexpression of SOCS-1 or SOCS-3 down-regulated the IFN-induced phosphorylation of STAT1 and STAT3. Overexpression of SOCS-1 in cells abolished mRNA expression of both OAS and Mx1 and overexpression of SOCS-3 inhibited mainly OAS mRNA expression. Thus, SOCS-1 and SOCS-3 have important negative regulatory effects on the type I IFN-induced activation of the JAK-STAT pathway (65). In macrophages for example, overexpressing SOCS-1 or SOCS-3, induction of the IFN-β-dependent gene CXCL10 was defective, and LPS-induced STAT1 phosphorylation was abolished (43). Interestingly, recent data suggest that protein therapy using cell permeable SOCS-3 has a curative effect in mouse models of endotoxemia (66).

Not much is known about the potential role of the other JAK-STAT inhibitory molecules SHP1, SHP2, CD45, TCPTP, the PIAS proteins and SHIP in endotoxemia. It was shown that **SHIP-/-** macrophages are hyper-responsive to LPS, that they are not endotoxin tolerant, and that their STAT1-phosphorylation is not diminished after a second exposure to LPS (67).

IFN-α/β as a therapeutic tool and target

Almost 50 years of intense research have made it clear that type I IFNs are absolutely essential in the defence of vertebrates against many viruses. First, the IFN genes are strongly conserved, and their orthologues have been found in different species, e.g. fish and birds. Second, deficiency of IFN receptor genes leads to dramatically increased sensitivity for many viruses (56, 68). Third, exogenously added IFNs have antiviral effects, such as the curative effects on hepatitis C infected patients (69). However, type I IFNs are also important in controlling other diseases and pathologies. In leishmaniasis, low doses of IFN-β protect mice from progressive cutaneous and fatal visceral disease after infection with *Leishmania major* parasites (70). Also, IFN-β is the most commonly used therapy for relapsing Multiple Sclerosis (71) and it can also inhibit collagen-induced arthritis in mice (72). Finally, type I IFNs inhibit proliferation of several human cancers, a therapy that has been evaluated in clinical trials, e.g. for renal cancer (73-75). From the above, one can conclude that type I IFNs are molecules that should certainly not be absent from the body and that should be given to patients under certain pathological conditions.

It is now also clear that resistance to endotoxemia can be induced by deletion of genes encoding IFN-β, IFNAR1, Tyk2 or other genes involved in the induction of, or in the response to, type I IFNs. The data support the idea that type I IFNs have to be considered as mediators in endotoxemia. The mechanism by which they mediate LPS, is still an open question. However, several studies have clearly shown that type I IFNs are able to induce the expression of genes encoding other inflammatory molecules, such as NF-IL6 and a large set of chemokines (76, 77). Hence, one would also like to block type I IFNs or their up-stream or down-stream mediators. A recent study clearly shows that this idea can have therapeutic application in the treatment of endotoxemia (66). Two central questions, however, need to be addressed.

First, to what extent will an inhibitory molecule, such as a neutralizing antibody to IFN-β or an IFNAR1 antagonist, compromise the anti-viral status of the organism? Studies using IFNAR1-knockout mice indeed indicate that these mice may be resistant to endotoxemia (Mahieu and Libert, unpublished data) and supersensitive to viral infections (68). Further studies with experimental animals or clinical trials will have to provide an answer this first question, but possibilities can be contemplated. Firstly, e.g. in the case of the IFNAR1, an inhibition of 90% could suffice to protect against endotoxemia, but that the remaining 10% of receptor activity could be enough to mount an adequate anti-viral response. Whether a sufficiently strong inhibition of the IFNAR1 will be possible has to be evaluated first. Inhibition of ligand could be problematic too, as many type I IFNs may play a role in endotoxemia. Luckily, the resistance of IFN-β deficient mice to endotoxemia resembles that of IFNAR1-knockouts, indicating that IFN-β is the type I IFN playing the predominant role in endotoxemia. Moreover, IFN-β is the major type I IFN induced by LPS (56), though also induction of IFN-α4 by LPS was observed in macrophages (59). Furthermore, septic patients may be treated with IFN-blocking agents over a short time interval in strictly contained conditions to prevent viral infection.

Second, do type I IFNs also play a mediating role in real sepsis and not just in endotoxemia? To our knowledge, no studies endorsing this hypothesis in the case of Gram-negative sepsis have been published. However, it was clearly demonstrated that type I IFNs are an essential component in the lethal response of mice to a Gram-positive *Listeria monocytogenes* infection, probably because macrophages are sensitized to cell death by production of type I interferon induced by *Listeria monocytogenes* (78). Although our knowledge of the role of type I IFNs in endotoxemia indicates that they also play a mediating role in sepsis, experimental evidence is needed to confirm this hypothesis.

Finally, endotoxemia (and probably sepsis) are not the only disorders in which type I IFNs play a detrimental role, and in which the type I IFN system should be blocked. Given the diverse and potent effects of type I IFNs in the innate and adaptive immune systems, it is not surprising that they play a pivotal pathogenic role in several autoimmune diseases. Increased serum levels of IFN-α were found to correlate with exacerbation of systemic lupus erythematosus (SLE) (79, 80) and insulin-dependent diabetes mellitus (IDDM) (81, 82) in humans and rodents, and IFN-α over-expression in beta cells at the onset of diabetes has been reported in human patients (83).

Conclusion

Many recent data suggest that type I IFNs as well as several molecules involved in inducing these cytokines and in responding to them play essential mediating roles in endotoxemia induced by Gram-negative cell wall components, and in several autoimmune disorders. These data suggest that key molecules, such as IFN-β and the IFNAR1, may be considered as new therapeutic targets in endotoxemia and sepsis, provided that their essential role in antiviral defence and in activation of the immune system are not compromised.

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3.4.2. Other pathways involved in type I IFN signalling

Next to the classical JAK-STAT pathway, it has been demonstrated that other pathways such as the p38 and PI3K pathways are involved in responses to type I IFN (for review see Platanias, 2005). The p38 MAPK seems to play an important role in type I IFN signalling. After activation of p38 MAPK through a cascade of kinases, p38 may subsequently activate downstream molecules such as MAPK-activated protein kinase 2 (MAPKAPK2), MAPKAPK3, mitogen- and stress-activated kinase 1 (MSK1) and MAPK-interacting protein kinase 1 (MNK1). These molecules have a possible role in respectively *Isg15* gene induction, posttranscriptional regulation of expression of various genes, histone H3 phosphorylation and phosphorylation of the eukaryotic translation-initiation factor 4E (EIF4E) in IFN-mediated signalling. Other MAPK-signalling pathways, such as the MEK-ERK pathway, seem to have a role in type I IFN signalling. PI3K has a role in IFN-dependent transcriptional activation by STAT1, apoptosis, mRNA translation and regulation of IFN-β-dependent phosphorylation of the p65 subunit of NF-κB. Furthermore, instead of the classical STAT1-STAT2 and STAT1-STAT1 complexes, other STAT complexes are formed upon type I IFN stimulation. This includes the formation of STAT3-STAT3, STAT4-STAT4, STAT5-STAT5 and STAT6-STAT6 homodimers, as well as, STAT-1-STAT3, STAT1-STAT4, STAT1-STAT5, STAT2-STAT3 and STAT5-STAT6 heterodimers. All these complexes bind to the IFN-γ-activated site (GAS) element in some genes (Platanias, 2005).

3.4.3. Regulation of IFNAR expression

The type I IFNs bind to a receptor consisting of two sub-units, IFNAR1 and IFNAR2. There is a single form of the IFNAR1 subunit and there are three forms of the IFNAR2, namely one transmembrane (muIfnar-2c) and two soluble isoforms (muIfnar-2a/2a'). IFNAR2 has ligand-binding capacity, but is not able to transduce signals (Owczarek et al., 1997). Hardy and colleagues proposed a model by which stimulation of cells with IFN-γ leads to activation of ISGF3. Then, the increased level of ISGF3 can allow up-regulation of muIfnar-2c in response to type I IFN via ISRE and GAS elements in the promoter of the *mulfnar-2* gene. At first, muIfnar-2c is upregulated on the cell surface, leading to increased biological activity in response to type I IFN. Later, soluble muIfnar-2a mRNA is induced, acting as an inhibitor of type I IFN signalling (Hardy et al., 2002).

Down-regulation of activated receptors is important for retaining the duration of the signal. At this moment, there is still little information on the modulation of IFN receptor expression. Recently, some data on IFNAR1 down-regulation have been published. In the distal cytoplasmic tail of the IFNAR1 is a

motif that can be recognized by homologue of Slimb (HOS) F-box-containing protein, which acts as a receptor for SCF^{HOS} E3 ubiquitin ligase that has been demonstrated to be essential for ubiquitination, internalization, proteolysis and down-regulation of IFNAR1 in response to IFN-α (Kumar et al., 2003). It has also been reported that TYK2 slows down IFNAR1 degradation, a process that is at least partly due to inhibition of IFNAR1 endocytosis (Ragimbeau et al., 2003).

3.4.4. Amplification of the type I IFN response and importance of IRF-7

After virus infection, IFN-α/β and many IFN-inducible genes are expressed to elicit anti-viral responses. These genes contain similar cis-acting IRF-binding elements: interferon-stimulated response element (ISRE), PRD and PRD-like (PRD-LE) elements. ISREs reside usually in the promoters of IFN-inducible genes; PRDI and PRDIII elements are found in the IFN-β promoter and PRD-LE elements in the IFN-α promoters. A complex of IRF-3 and co-activators CBP/p300 can directly induce some IFN-inducible genes. This complex is called DRAF1. Also, the IFN-α/β-induced ISGF3 complex, composed of STAT1, STAT2 and IRF-9, can induce IFN-inducible genes and the *Irf-7* gene by binding the ISRE elements in their promoters. IRF-7 can induce IFN-α genes by binding the PRD-LE sites and IFN-β by binding PRD elements. Thus, ISRE, PRD and PRD-LE elements differentially utilize IRF family members. IFN-α/β and IFN-inducible genes are classified into different groups in terms of their activation. The first group is the 'ISGF3 only' group of genes totally dependent on IFN-α/β-induced ISGF3 formation. OAS, PKR and IRF-7 belong to this group. The second group is called the 'ISGF3/IRF-3' group and comprises ISG15, ISG54, guanylate binding protein (GBP) and IP10. The third group is called the 'IRF-3/IRF-7' group, to which IFN-β belongs. IFN-α belongs to the 'IRF-7 only' group (Nakaya et al., 2001).

Type I IFNs are expressed rapidly after infection and plays a key role in innate defence against pathogens. The regulation of IFN-α/β expression is through a two-step activation model. In uninfected cells, IRF-3 is constitutively expressed. IRF-3 becomes activated upon virus infection and translocates to the nucleus to induce IFN-β, but not IFN-α genes (except IFN-α4), resulting in the initial production of IFN-β (and IFN-α4). Type I IFN can act in an autocrine or paracrine manner through binding to the IFNAR1/IFNAR2 complex and induces *Irf-7* gene expression via activation of ISGF3. The expression of IRF-7 is ubiquitous, however, it is totally dependent on IFN-α/β. IRF-7 can bind on the PRD and PRD-LE elements in the IFN-β and IFN-α promoters. Thus, secreted IFN-α4 and IFN-β proteins can bind on their common receptor to induce activation of IRF-7, which will lead to the induction of a set of delayed IFN-α genes which includes IFN-α2, -α5, -α6 and -α8 genes (Marie et al., 1998; Taniguchi and

Takaoka, 2002). The *de novo* synthesis of IRF-7 is crucial for the up-regulation of IFN-α/β production by a positive feedback mechanism. The induced IRF-7 resides in the cytoplasm, but undergoes phosphorylation in its carboxy-terminal region on two distinct sets of Ser/Thr residues. The C-terminal region of IRF-7 is highly homologous to the corresponding region of IRF-3. Phosphorylation of IRF-7 leads to its dimerization, nuclear accumulation, DNA binding, and transcriptional activation (Taniguchi and Takaoka, 2002; Wathelet et al., 1998). Thus, IRF-7 is induced upon type I IFN stimulation and further enhances the production of IFN-α/β through a positive feedback loop (Sato et al., 1998a). The IRF-7 protein is short-lived, and must be continuously produced (Sato et al., 2000).

IRF-7-knockout mice are more susceptible to viral infection than IRF-3- or MyD88-knockout mice. In IRF-3-knockout cells IFN-α/β is still present, although in diminished levels, because of the weakly activated ISGF3 through spontaneously produced IFN-α/β. So, in uninfected cells spontaneously produced IFN-α/β maintains the level of IRF-7 expression at an appropriate level, so that the positive feedback loop can be initiated immediately upon viral infection (Sato et al., 1998a; Sato et al., 2000). The spontaneous production of IFN-α/β in absence of viral infection is observed both *in vitro* and *in vivo* (Gresser, 1990; Gresser et al., 1995). The mechanism of spontaneous IFN-α/β production is not known (Figure I.3.3.). Even in the absence of both IRF-3 and -7, there is a low constitutive level of IFN-α/β (Sato et al., 2000).

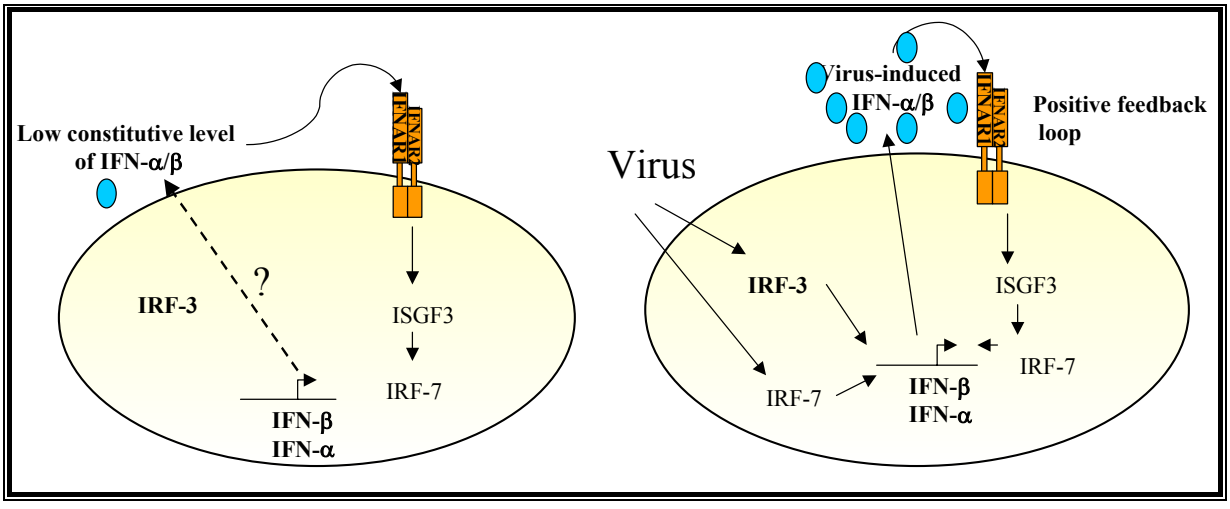

Figure I.3.3. – A weak constitutive level of IFN-α/β is necessary for effective amplification of the IFN-α/β signal after stimulation. The presence of a low constitutive level of IFN-α/β sustaines a weak activated ISGF3 and IRF-7 expression. This is important for a robust response of the cells to stimuli like viral infection. Upon viral infection the positive feedback loop can be rapidly initiated because of this sustained IRF-7 expression. The mechanism of the spontaneously produced IFN-α/β remains unknown.

Using cells lacking IRF-9, an essential component of ISGF3, it was shown that ectopic expression of IRF-7 but not IRF-3 can rescue IFN-α gene expression (Sato et al., 1998a). Using IRF-7-knockout mice, it was demonstrated that IRF-7 is important in type I IFN induction after virus-activated MyD88-independent signalling, but also in TLR9-induced MyD88-dependent signalling (Honda et al., 2005b). IRF-7 is also important in co-operation between TLRs and IFNAR for optimal induction of IL-12p70 (Gautier et al., 2005). There is also an important role for IRF-7 in the TLR9-mediated induction of the antigen-specific $CD8^+$ T-cell response in pDCs. Spatiotemporal regulation of MyD88-IRF-7 signalling is critical for type I IFN induction in response to TLR9 activation. The TLR9 ligand, CpG-A, is retained together with MyD88-IRF7 in the endosomal vesicles of pDCs for a long time, making it possible to activate this signalling pathway. In cDCs, CpG-A localizes to lysosomes and is not able to induce IFN (Honda et al., 2005a).

3.5. References

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4. MAPPING IN THE MOUSE

4.1. Mouse domestication

At the dawn of civilization, humans developed the ability to farm, to build store grain and foodstuffs. The mouse was attracted by this environment with unlimited food and was well adapted to steal food from granaries. Thus, humans needed to protect their food-storages from the 'bad mouse'. So it is not at all a surprise that the word mouse comes originally from the Sanskrit *mush* meaning to steal, which became *mus* in Latin and *mys* in Greek.

The ancestral *Mus musculus* followed human migration to all corners of the world (Figure I.4.1.). Several distinct subspecies of *Mus musculus* have evolved to dominate non-overlapping segments of the world. The wild house mouse, *Mus musculus* (*M.m.*) *musculus*, lives in Eastern Europe and Asia and *M.m. domesticus* is common in Western Europe, Africa and the near-East and is transported by man to the Americas and Australia. Asian *musculus* and European *domesticus* mice dominate the world but have evolved separately over about 1 million years (Ferris et al., 1983). These subspecies are not separated by a major geographical boundary and only live together in a small stroke of land in Eastern Europe. The subspecies *M.m. castaneus* lives from Sri Lanka to South East Asia.

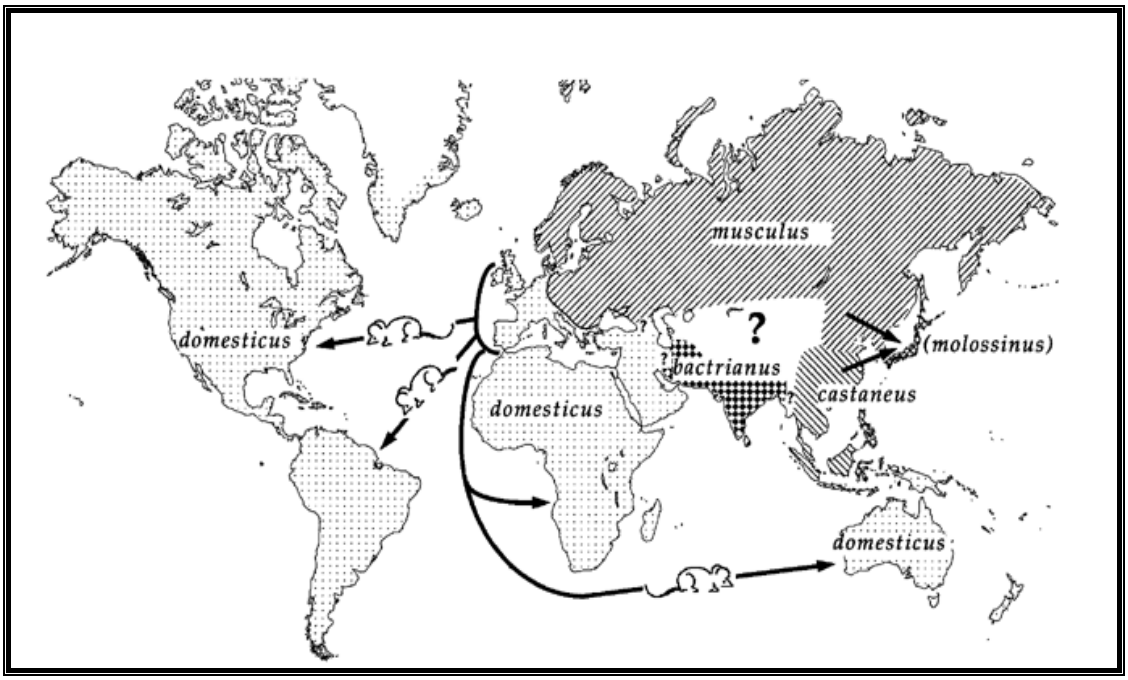

Figure I.4.1. - Geographical distribution of the different subspecies of *Mus musculus* (Reprinted from Silver, Mouse Genetics, concepts and applications, 1995).

In nature, those different subspecies are not completely isolated genetically. As a consequence, there is genetic exchange between subspecies in the regions where they meet. In Japan, the two subspecies *M.m. castaneus* and *M.m. musculus* have hybridized extensively, giving rise to a unique population referred to as *M.m. molossinus* (Guenet and Bonhomme, 2003; Silver, 1995).

4.2. Historical roots of inbred mice

In 1664, the mouse was used for the first time as a laboratory animal. Robert Hooke used the first laboratory mice in a study on the properties of air (Morse, 1978). By the 1700s, many varieties of mice were domesticated as pets in China and Japan. They were the first to raise unusual mice, in particular mice with coat-colour mutations (e.g. albino, yellow and dominant-spotting) and waltzing mutants. Already in 1100 B.C. the spotted mouse was mentioned in a Chinese lexicon. Europeans imported these 'fancy mice', so that by the 19th century 'fancy mouse breeding' became a popular hobby and it spread to the USA by the beginning of the 20th century. Abbie Lathrop, a retired schoolteacher, started to collect and breed these fancy mice at Granby, Massachusetts in 1900. Thousands of years of fancy mouse breeding resulted in highly homogeneous versions of these wild mice being traded and ending up in Lathrop's schoolhouse (Figure I.4.2.). A number of mouse colonies were generated, including some of which develop tumours.

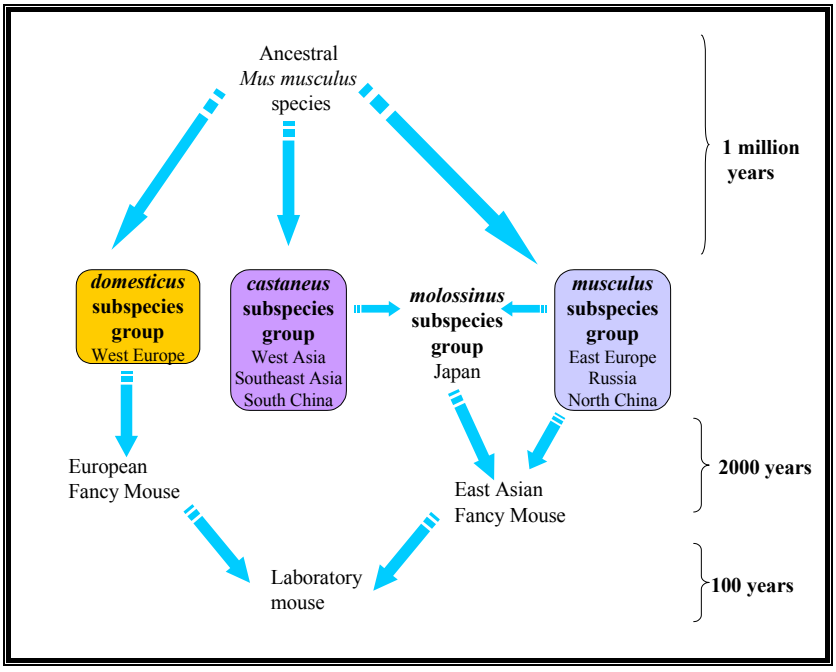

Figure I.4.2. – Origin of the inbred laboratory mouse strains. The genome of inbred strains is a mosaic from segments derived from both European (*domesticus*) and Asian (*musculus* and *castaneus*) subspecies because the mice were derived from a few mouse breeders in Asia or Europe who originally bred 'fancy' mice (Wade et al., 2002).

In 1900, with the rediscovery of Mendel's laws of inheritance, a rush to verify these genetic theories in other animals was created. It was shown that the variation of 'fancy' mice was analogous to that of Mendel's peas. From 1902 on, William Castle of the Harvard University, a customer of Abbie Lathrop, started studying inheritance in mice. Mating programs were established to create inbred strains that resulted in many of the modern, well-known strains used in medical research. Clarence C. Little established the first inbred strains by brother-sister mating. Castle, Little and others formed the most commonly used inbred strains from the Lathrop stock (Silver, 1995). The DBA mouse (which has the coat-colour alleles, dilute, *d*, brown, *b*, and non-agouti, *a*) was the first real inbred strain produced by Little in 1909. Other inbred strains were generated over the next decade, including C57BL, C3H, CBA and BALB/c (Staats, 1966). Abbie Lathrop also conducted her own experimental breeding program, giving rise to strains such as C57BL/6 and C57BL/10. This hybrid breeding between *M.m. domesticus*, *M.m. musculus* and other subspecies led to the creation of progenitors of modern laboratory mice.

The gene pools of most inbred laboratory mouse strains are small, as they were derived from a mixed, but limited founder population in a few laboratories (Beck et al., 2000). The genomes of these inbred strains are mosaics with the vast majority of segments derived from both European (*domesticus)* and Asian (*musculus and castaneus)* subspecies because the mice were derived from Asian or European 'fancy' stocks collected by a few mouse suppliers. So the genome represents a mosaic with unequal contributions of several *Mus musculus* subspecies (Figure I.4.2.). The pattern of genetic variation in the genome of classical inbred strains has a mosaic structure, with regions of low levels of polymorphism and regions of high levels of polymorphism (Wade et al., 2002). Most of the inbred strains carry a *musculus* Y chromosome (Bishop et al., 1985) and *domesticus* mitochondrial DNA (Ferris et al., 1982).
An inbred strain is defined as a strain that has been maintained for more than 20 generations of brother-sister mating and is essentially homozygous at all genetic loci, except for mutations arising spontaneously. The inbreeding coefficient achieved is at least 0.99. After breeding 20 generations about 98.6% of the loci in each mouse are homozygous. Many strains have been bred for more than 150 generations and thus are essentially homozygous at all loci. Many of these inbred mice are bred for a specific phenotype. For example, the C57BL/6 mice have a preference for alcoholics and narcotics (Peirce et al., 1998). Some mouse strains are useful in transgenic and ES cell technology: FVB/N mice because of their large pronuclei which facilitate microinjection of DNA (Taketo et al., 1991) and 129/Sv mice because of successful contribution of ES cells to the germline. BALB/c and C3H mice are very sensitive to mutagenesis and useful in ethyl nitrosourea (ENU) projects, for example the large-scale ENU-mutagenesis screen in the German Human Genome Project.

Over 450 inbred strains of mice have been described. Ninety years after the generation of the first inbred strain, a chart of the origin and relationship of all the known inbred strains was constructed (Beck et al., 2000). In 1929, the Jackson Laboratories were established to serve as a repository for inbred stocks and mutant lines for experimental purposes.

4.3. The mouse as a model organism

The mouse is the model organism most closely related to humans. The mouse and human genomes are approximately the same size (2.5 Gb compared with 2.9 Gb), contain the same number of genes (about 30.000) and show extensive synteny (conserved gene order). Most mouse genes have human counterparts (about 99%), and the functions of these genes are closely related. Mutations that cause diseases in humans often cause similar diseases in mice. Importantly, mice have genes that are not represented in other animal models (the fruit fly and nematode), including the genes of the immune system.

The similarities discussed above probably apply to most mammals, but the mouse has further properties that make it an ideal model organism. Mice do not pose a significant threat (non-aggressive) and there are fewer ethical issues associated with mice. With nonhuman primates, there are greater restrictions that must be followed. Mice are small, easy to maintain and breed in the laboratory and they have, compared to most mammals, a short breeding cycle of about 2 months. They can produce 10-15 offspring per litter and approximately one litter every month. Furthermore, the breeding of mice is relatively inexpensive.

All these properties make the mouse suitable for genetic analysis. Many mutants are available and new mutations can be introduced easily by irradiation, feeding with chemical mutagens or inserting DNA fragments into the genome to interrupt genes. In the early 1980s researchers began to produce transgenic animals by inserting human genes into fertilized mouse eggs. Now, the technique of homologous recombination is been used to target human genes into the mouse genome.

4.4. Use of wild-derived mouse strains

The mouse is the model organism most closely related to humans and so the common laboratory inbred strains of mice are extensively used in genetic and biomedical research. However, genetic diversity between the different inbred laboratory mouse strains is very low, as they were derived from a mixed, but limited ancestral pool as shown by single nucleotide polymorphisms (SNP) analysis (Beck et al., 2000; Wade et al., 2002). The common laboratory strains have limited levels of genetic variation when

compared with human populations, making it a serious disadvantage. Mouse inbred strains may be divided into two groups, classical and wild-derived. Wild-derived mouse inbred strains are inbred strains that have been derived from wild mice trapped at different times and locations, from different populations (Bonhomme and Guenet, 1996). Interspecific hybrids generated by crossing wild-derived *Mus spretus* and the classical laboratory strain *Mus musculus* are viable and have a high level of genetic diversity (Bonhomme et al., 1978). The usefulness of these wild-derived strains in genetic research stems from the high level of diversity, introducing new allelic variants at genes that might code for functional or non-functional variant enzymes. The levels of variation between wild-derived and common laboratory strains are significantly higher than among common laboratory strains (Wiltshire et al., 2003). For a good review about laboratory and wild mice, see Bonhomme (Bonhomme and Guenet, 1996).

Wild-derived strains are frequently used for genetic research in immunology (Bagot et al., 2002a; Bagot et al., 2002b; Campino et al., 2005; Jin et al., 1999; Kozak, 1985; Kozak et al., 1984; Sangster et al., 1998; Sebastiani et al., 2002; Sebastiani et al., 1998; Urosevic et al., 1999; Velupillai et al., 1999) and oncology (Ewart-Toland et al., 2003; Manenti et al., 1994; Manenti et al., 1996; Nagase et al., 1995; Nagase et al., 1999; Nagase et al., 2003; Nagase et al., 2001; Pataer et al., 1996; Santos et al., 2002). When looking at the size variability of microsatellites between *Mus spretus* and common laboratory strains, 70-90% of the sequences show size variations (Cornall et al., 1991; Hearne et al., 1991; Love et al., 1990; Montagutelli et al., 1991; Santos et al., 1995). Only about 50% of the sequences is polymorphic among different laboratory strains (Hearne et al., 1991; Love et al., 1990; Montagutelli et al., 1991). When compared to the classical inbred strains, inbred strains derived from *Mus spretus* have a SNP in every 80-100 basepairs (Guenet and Bonhomme, 2003). This high degree of genetic polymorphism made strains derived from *Mus spretus* (the most popular being SEG/Pas and SPRET/Ei) the species of choice to conduct a mapping experiment. In 1994, a large *Mus spretus*/C57BL/6 backcross of 982 progeny was set up. This was constructed to create a high-resolution genetic map of the mouse genome achieving a resolution of 0.1 centimorgan (cM) at the 95% confidence level (approximately 200 kb in the mouse genome) (Group, 1994; Rhodes et al., 1998).

However, some practical difficulties occur using *Mus spretus*-derived strains. SPRET/Ei mice are very poor breeders and litters are mostly very small. In addition, F1 hybrid males are sterile and only hybrid females are fertile (Guenet et al., 1990). This sterility of F1 hybrid males is concordant with Haldane's rule (Haldane, 1922). This rule states that when two different animal races are crossed and one of the sexes of the F_1 offspring is absent, rare or sterile, this sex is the XY or heterozygous sex. This F1 male sterility makes it impossible to do intercross-experiments. Also a considerably large amount of

backcross males are sterile due to the segregation of hybrid sterility factors (Bonhomme et al., 1982; El-Nahas et al., 2002; Fossella et al., 2000; Hemberger et al., 1999; Matsuda et al., 1991; Olds-Clarke, 1997; Redkar et al., 1998). In addition, sometimes there are difficulties in generating consomic mice or interspecific recombinant congenic strains starting from *Mus spretus*. Wild-derived strains belonging to the *Mus musculus* species (e.g. *M.m. castaneus* (CAST/Ei) or *M.m. musculus* (PWK/Ph)) also show a high level of diversity compared to the classical laboratory strains (Montagutelli et al., 1991), but suffer much less from these problems. Intersubspecific consomic and semi-consomic mice have been successfully generated from CAST/Ei (Iakoubova et al., 2001) and PWK/Ph.

Agouti SPRET/Ei (Figure I.4.3.) was made inbred in 1988 by Eva Eicher. The wild-derived inbred strain SPRET/Ei is an inbred strain derived from *Mus spretus*. Other wild-derived strains are CAST/Ei (*castaneus*) and CzechII/Ei (*musculus*). *Mus spretus* and *Mus musculus* are diverged from each other about 1,5 million years ago (Figure I.4.4.) and this has led to a great amount of genetic polymorphisms, which allow mapping to the subcM level of resolution (Bonhomme et Guénet, 1996). Other subspecies belonging to the *Mus spretus* group are SEG/Pas and STF/Pas (originating from the Institut Pasteur). The *Mus spretus*-derived inbred strains SPRET/Ei, SEG/Pas, STF/Pas are frequently used in crosses with common inbred strains (e.g. C57BL/6) to create highly polymorphic panels for genetic mapping.

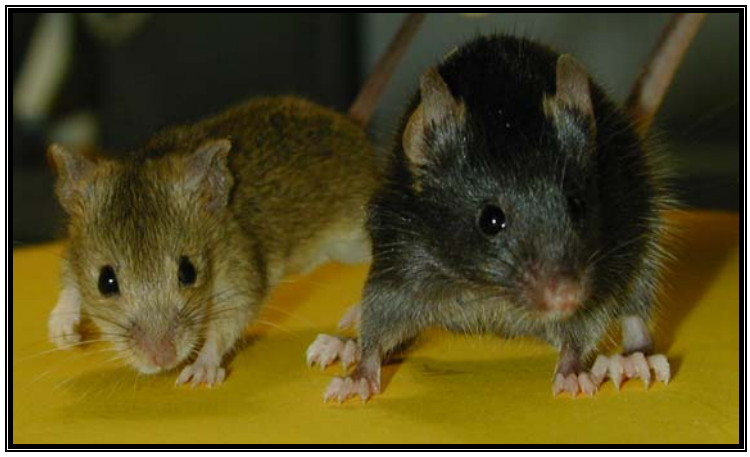

Figure I.4.3. - Picture of agouti SPRET/Ei (*Mus spretus*) and black C57BL/6 (*Mus musculus*) (Photograph by Jan Staelens).

Three independently derived strains of *Mus spretus*, SPE/Pas, SEG/Pas and SPR/Smh, were analysed by microsatellites for polymorphisms. SPE/Pas and SEG/Pas are very close (3% polymorphism), whereas the third one, SPR/Smh, is very different from the other two strains (33% polymorphism)

(Montagutelli et al., 1991). Staelens and colleagues from our group published that SPRET/Ei mice are resistant against high doses of TNF-α (Staelens et al., 2002). In addition, (C57BL/6 x SPRET/Ei)F_1 mice are resistant against TNF-induced arthritis and partially resistant against ovalbumin-induced asthma (Staelens et al., 2004).

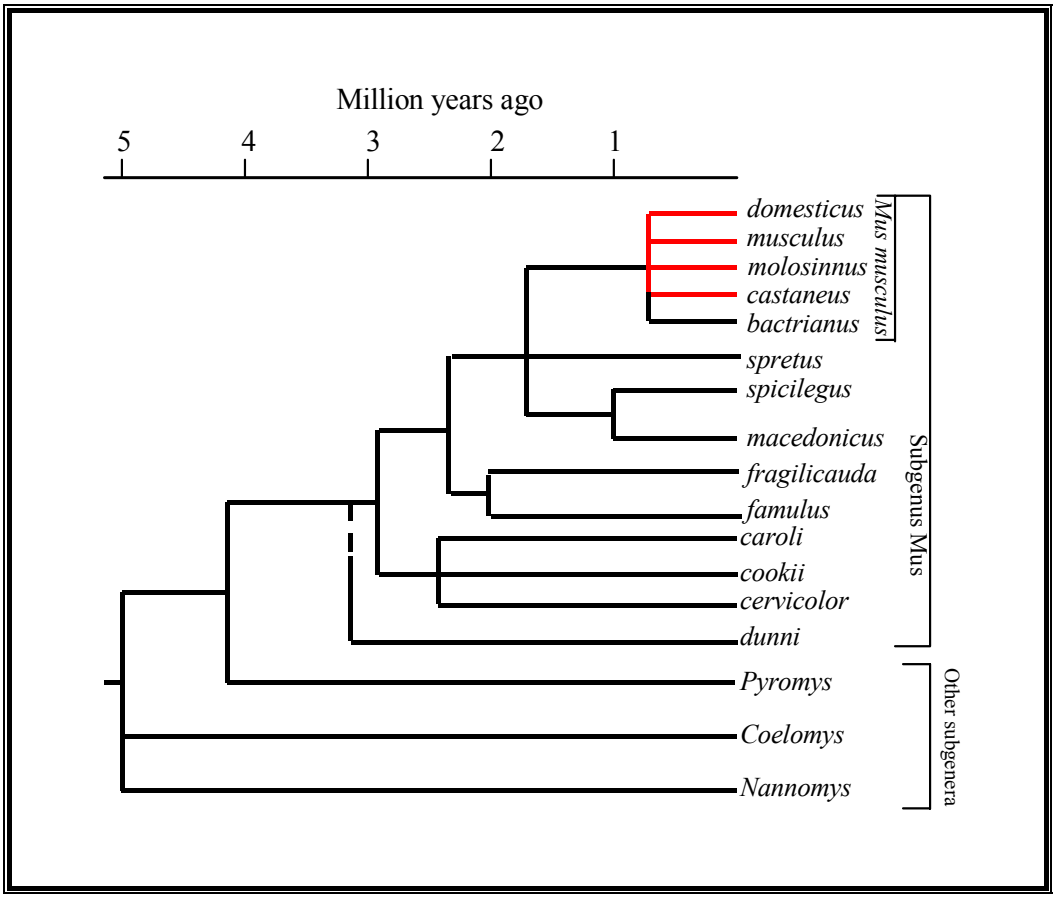

Figure I.4.4. – Phylogenetic relationships in the genus *Mus* (Reprinted from Guénet and Bonhomme, Trends in Genetics, 19, 24-31, 2003).

4.5. Mapping of a trait

4.5.1. Introduction: Linkage and recombination

Mendel's first law, also called 'the law of segregation', states that each individual has two copies of every gene and that only one copy is transmitted to the next generation. Cells or individuals with two copies of each gene, controlling a certain phenotype, are called 'diploid'. A germ cell can transform from the diploid state to the haploid state through a process called meiosis. At the end of meiosis the two copies of each gene are segregated from each other and the germ cell contains only one copy of each

gene. The probability to receive either allele is equal and one allele is dominant to the other which is said to be recessive.
Mendel's second law or 'the law of independent assortment' states that the segregation of alleles is independently from each other. For example, when we look at two genes (gene with alleles 'A' and 'a' and gene with alleles 'B' and 'b'), the possibility that a gamete will be AB, ab, Ab or aB is 25%. Today we know that Mendel's second law applies only to two genes that are located on different chromosomes.

Walter Sutton and Theodore Boveri demonstrated in the beginning of the 20th century that chromosomes segregate following Mendel's law. Then, Morgan and Brides showed that genes are located on chromosomes. In 1905 it became clear that the law of independent assortment applies only to chromosomes and not genes (Bateson et al., 1905).
When genes are located on the same chromosome they are 'linked'. Two allele combinations will be transmitted at a frequency different from 25%. In the case of extreme linkage, when the genes are very close to each other, only the 'parental combinations' of alleles will be transmitted, each at a frequency of 50%. At intermediate levels of linkage, termed 'incomplete linkage', the parental combinations of alleles will be transmitted at a frequency greater than 50%, but less than 100% because recombination events or 'crossovers' occur during meiosis. Crossovers occur at random sites across the chromosomes. The recombination frequency is related to the genetic distance between the two genes. Genetic distance is measured in cM and 1cM, which is about 2000kb in the mouse, is defined as the distance between two loci recombining with a frequency of 1%. In addition, when the distance between two loci increases, the probability increases to have double crossovers. Double or an even number of crossovers are not detectable from the original parental combination, while by an odd amount of crossovers a recombinant genotype can be detected. As a consequence, the observed recombination frequency will be less than the actual recombination frequency. The chance that a double crossover will happen between two loci that are separated by 20 cM is the product of the predicted frequencies for each locus alone, which is 0.20 (20%). So for this example, the probability to miss recombination events in gametes is 0.2 x 0.2 = 0.4 or 4%.
Also one recombination on a certain location on a chromosome can influence the initiation of other recombination events. This event is called interference and restricts the resolution of a linkage map. The resolution is calculated with the formula 100/N, where N is the number of meiotic events that are typed. For example, when 1000 backcross animals are analysed, the theoretical map will be 0.1 cM or 200kb. Linkage analysis can be used for the genetic mapping of a distinct phenotype.

4.5.2. Genetic markers

Mapping of a phenotype demands the cross of two parental strains that are phenotypically and also genetically distinct. Of course, tools are needed to detect what parts of the genome are from which parent. Any variation in sequence can be used to distinguish between strains. Two individuals of the same species have variations in about 1bp per 200-500bp. The frequency of variations between two individuals of different species is much higher.

The markers used to screen need to be abundantly present, to be easily genotyped and to have a high level of polymorphism. Some examples of molecular markers used in mapping are restriction fragment length polymorphisms (RFLPs), single stranded conformation polymorphisms (SSCPs), single nucleotide polymorphisms (SNPs) and simple sequence length polymorphisms (SSLPs) or microsatellites. Microsatellites have much of the requirements for a good screening system. Because of the high level of polymorphism associated with microsatellites, they are frequently used a genetic marker. Microsatellites are PCR-based DNA markers that can be rapidly typed in high amount of animals and are abundant throughout the genome. These are repeats of one till 4 nucleotides with the most common being CA-dinucleotide repeats. In the mouse genome, CA repeats are found once every 18kb on average. The length of these microsatellites can vary between 80 and 270bp. The flanking sequences of the microsatellites are quite conserved, making it possible to design PCR primers for amplification to determine the length of microsatellites in various mouse strains. The products can be visualized by gel electrophoresis or by RP-HPLC (Figure I.4.5.).

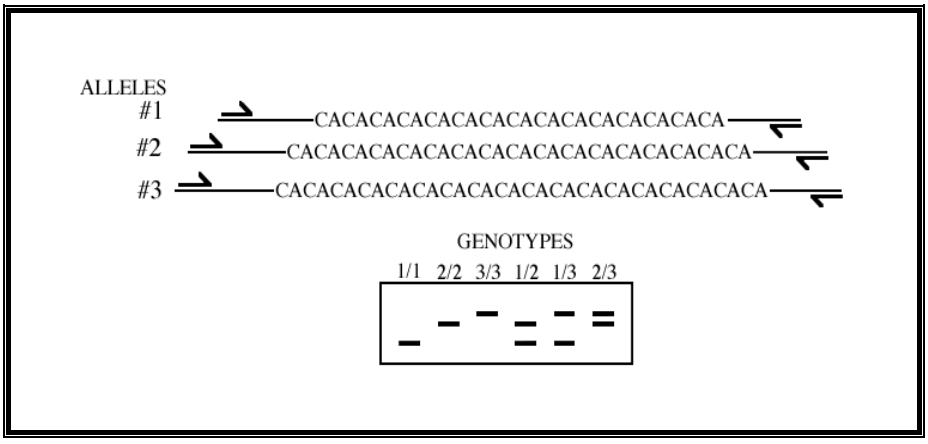

Figure I.4.5. – Genotyping of microsatellite alleles. The figure shows a microsatellite locus with 3 different alleles. PCR amplification of the locus results in 3 fragments of different lengths. The products can be visualized by gel electrophoresis as shown below (Reprinted from Silver, Mouse Genetics, concepts and applications, 1995).

About 14.000 microsatellites have been positioned on the genome, implying that every 0.1 cM a genetic marker can be found assuming that the mouse genome is about 1.500cM. About 50% of the microsatellites are polymorphic between the strains of the *Mus musculus* group, while the percentage can be as high as 90% between C57BL/6 and wild-derived inbred mouse strains such as *M.m. spretus* and *M.m. castaneus* (Love et al., 1990; Montagutelli et al., 1991).

Other popular genetic markers are SNPs, which are characterized by variation in a single nucleotide. SNPs are also abundantly scattered throughout the genome and are detected using PCR techniques, making it a technique that can be easily upscaled such as microsatellites (Syvänen, 2001). Approximately every 80-100 nucleotides a SNP is found between *M. spretus* and the common laboratory strains (Guenet and Bonhomme, 2003).

4.5.3. Mapping of a trait: initial crosses

The subject of my thesis is the hypo-responsiveness of SPRET/Ei mice to LPS. In contrast, C57BL/6 mice are susceptible to the toxic effect of LPS. The LPS-resistant phenotype is a dominant trait, because (BxS)F1 mice are also hypo-responsive to LPS. To map a phenotype you have essentially the choice between two crosses: a backcross or an intercross. However, C57BL/6 and SPRET/Ei mice can only produce fertile female hybrids making it impossible to intercross male F1 and female F1 mice.

In a backcross (Figure I.4.6.) the first step is to outcross the homozygous mutant mice (S/S) with the homozygous wild-type mice (B/B). Their (BxS)F1 progeny will be heterozygous for all loci (B/S) and, as mentioned before, are also resistant to LPS demonstrating the dominant character of the phenotype. Next, the (BxS)F1 progeny is crossed back to the wild-type strain (B). The N2 progeny**,** which are the result of one meiosis, are unique and can be homozygous (B/B, 50%) or heterozygous (B/S, 50%) for each locus. The mice that are still resistant to LPS shall be heterozygous for the locus responsible for the LPS-resistant phenotype. By determining the amount of LPS-sensitive and LPS-resistant mice a prediction can be made of the amount of loci linked with the LPS-resistant phenotype. The phenotype will be monogenic when 50 out of 100 mice are sensitive and the other 50 are resistant. When the number of resistant mice is lower than 50, the trait is complex (see further).

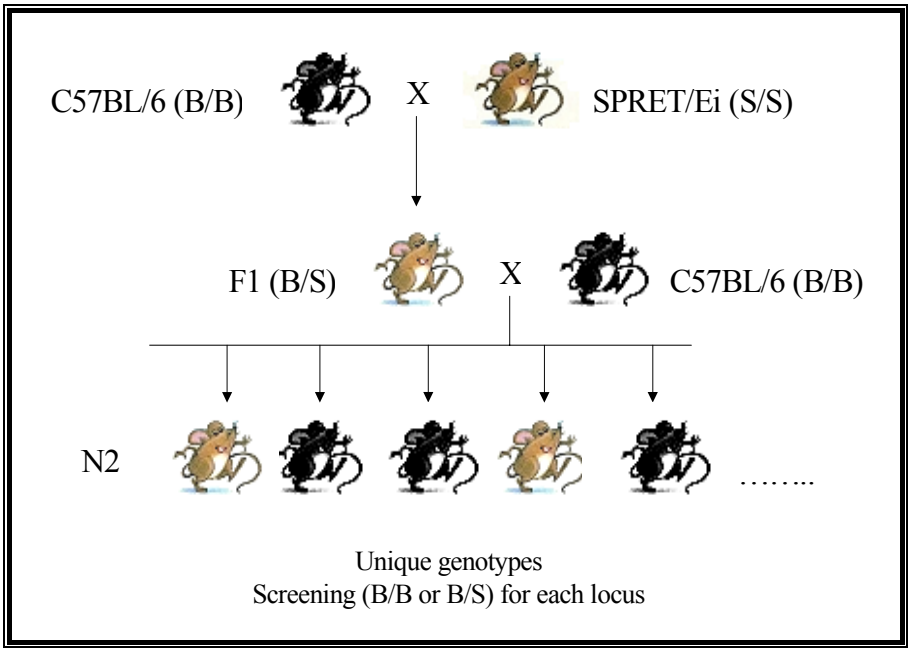

Figure I.4.6. – Illustration of a backcross experiment. The backcross mice (N2) have two possible genotypes for each locus (homozygous B/B or heterozygous B/S).

The swept radius can determine the number of markers that are necessary for an initial whole genome screen. The swept radius is the distance (in cM) that linkage can be observed between two loci and is related to the number of progeny tested. The smaller the number of progeny, the smaller the swept radius and the more markers are needed to ensure detection of linkage. Thus, the more mice are tested in a backcross, the less markers per chromosome are needed to span a whole chromosome. For example, when 72 backcross mice are tested the swept radius will be 20 cM. For a chromosome of 100 cM, 4 or 3 markers will be needed at a distance of 30 or 40 cM of each other to scan a whole chromosome (Figure I.4.7.). However, at a certain moment increasing the number of progeny does not lead to a reduction of markers needed for genotyping. An initial genome scan will be typically done on 50 to 100 animals with 75 markers spaced evenly over the whole genome. This will give an idea about the region, typical a 20 cM region, where the mutated gene is located.

For a monogenic trait, this region can be simply narrowed down by typing more genetic markers between the two genetic markers surrounding the mutated locus, and by analysing more (e.g. 1000) mice. This large amount of mice is necessary to find crossovers really close to the gene of interest. With a backcross of 1000 mice a resolution of 0.1 cM can be achieved (Silver, 1995). Next, the gene of

interest can be easily identified because the full sequence of the mouse genome is now available on the web.

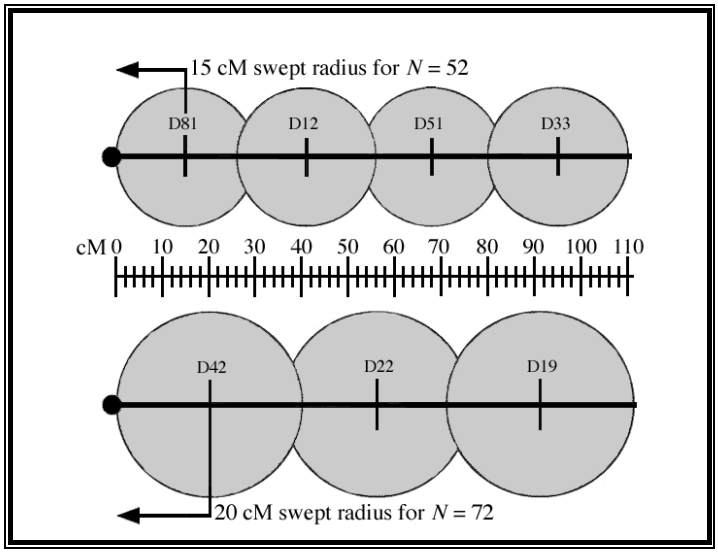

Figure I.4.7. – The swept radius determining the number of markers needed in a whole genome screen. Each circle represents the swept radius around a locus. With 72 backcross animals, three marker loci are necessary to cover mouse chromosome 2 (Reprinted from Silver, Mouse Genetics, concepts and applications, 1995).

However, most of the traits or diseases (e.g. asthma, diabetes, hypertension,…) are caused by multiple genes and this is called a complex trait. The term 'complex trait' is defined as any phenotype with no one-to-one relationship between genotype and phenotype. Often genes work together in an additive or epistatic way meaning that interactions between QTLs result in an effect on the trait that would not be predicted from the sum of the individual QTL effects. Traits can be qualitative or quantitative (e.g. temperature, IL-6 concentration, …), but qualitative traits can often be considered as quantitative traits. In this case, the quantitative trait (QT) has to reach a certain threshold to give rise to the qualitative trait (e.g. the development of a disease). The term 'quantitative trait locus' or QTL is used to define a locus that affects a complex trait in a strictly quantitative way or not (Nature review genetics, 4, 911-916, 2003).

A way to map QTLs in a complex trait is first to perform an initial backcross or intercross experiment to map at a subchromosomal level. Next, the QTLs will be isolated using consomic mice (or as Nadeau proposed 'chromosome substitution strains' or CSS strains). Nadeau and colleagues described this technique and it will be discussed in the next section. When the location of the QTL is confirmed using CSS strains, an additional cross can be done to allow fine mapping of the trait.

4.5.4. Isolation of QTLs from a complex trait in CSS strains (Nadeau et al., 2000)

CSS strains or consomic mice are homozygous inbred strains that are identical to strain 'B' except that 1 chromosome has been replaced by the corresponding chromosome of strain A (Figure I.4.8.). CSS strains are a very interesting tool in multigenic trait analysis. There are advantages and limitations of this approach over classical approaches. Advantages are that there is no need to perform a lot of crosses and that an unlimited amount of genetically identical mice are available. A limitation of CSS strains is that QTLs are assigned to an entire chromosome instead of a 20cM region as in a cross. However, the QTL can be narrowed by an additional backcross or intercross. In addition, CSS strains do not distinguish between multiple QTLs on the same chromosome.

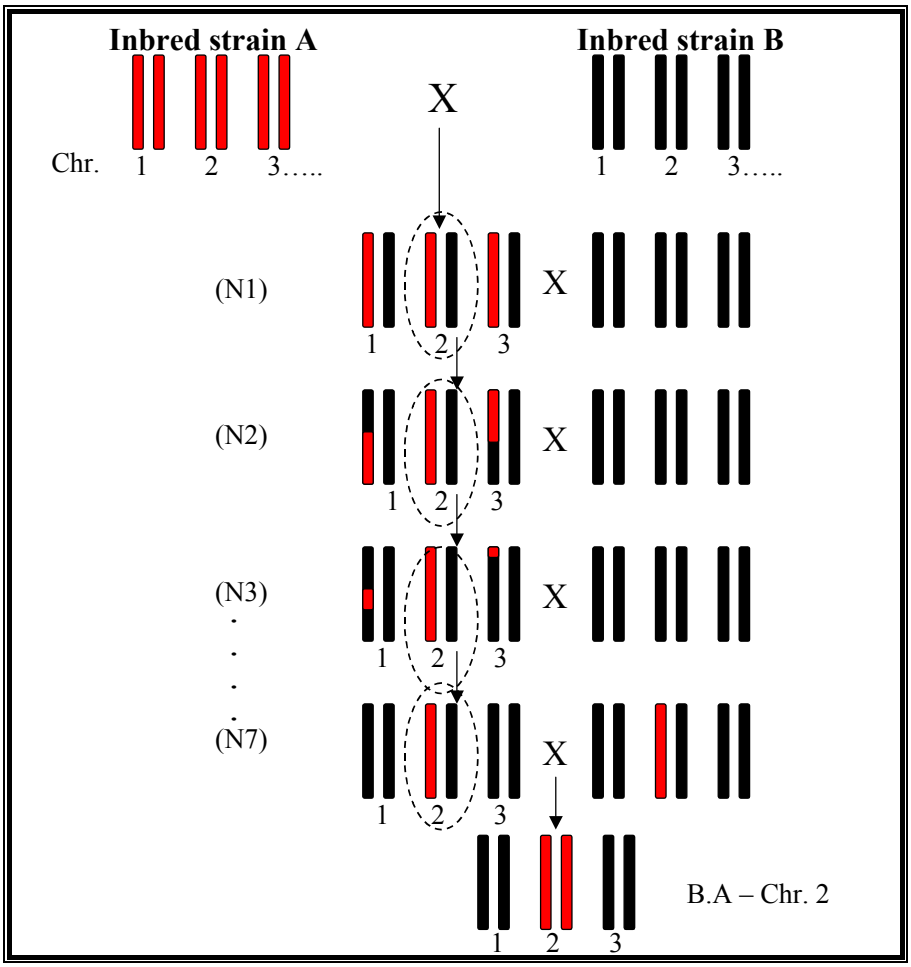

Figure I.4.8. – Strategy for the construction of CSS strains. The first step is the construction of F1 mice (AxB) which are then backcrossed to the recessive strain B. The progenies are screened for a specific chromosome, for example chromosome 2, using microsatellite markers. The mice that contain the entire chromosome of interest from the donor strain A are selected to cross back to strain B. For different generations, a selection is made for the mice containing the entire chromosome of interest. Each generation 50% of the donor genome is lost. After 7 generations mice are created that are for 99% donor strain, except for the chromosome for which a selection was performed. These mice are still heterozygous for the selected chromosome, so a brother-sister mating is necessary to receive a CSS strain homozygous for all loci and one chromosome from strain A. Expected is that for larger chromosomes such as chromosome 1, 2, 4 and 11, there will be less progenies that contain a whole chromosome because of the higher chance of a crossover.

Another advantage is that it is easy to investigate epistatic interactions between QTLs by crossing different CSS mice. It can give more information about the nature of the trait, but also about the combinations that are required. In the case of epistatic interactions between genes, the phenotype will not be fully present or even be completely absent in the consomic mice. In the absence of epistatic effects, it is also possible that the one QTL present has only a subphenotype of the original complex phenotype because of the absence of additive effects from the other QTL(s). By crossing different consomic mice it is possible to reconstitute the original QTL combination and phenotype or to determine which QTL combination is sufficient to obtain the parental phenotype. The creation of CSS strains goes as described in Figure I.4.8.

4.5.5. Some methods used in QTL mapping analysis

The following can be said about QTL mapping: 'Crossing two inbred lines creates associations between genetic marker loci and traits to allow localization of QTL'. Different methods can be used for QTL mapping. One method used in QTL mapping is the simple 'single-locus association' test (e.g using t-test, ANOVA and simple linear regression statistics). This test is used to evaluate the association between the genotype of each locus and a trait value. Each locus is analysed separately, so there is no requirement for mapping the loci relatively to each other. The marker locus being tested is called the target locus. Another level of QTL mapping is simple interval mapping (SIM). SIM does require the mapping of the loci relatively to each other. It evaluates the possibility of a hypothetical QTL at multiple analysis points across each inter-marker interval.
Results of these tests can be expressed by giving a LOD or LRS score. To give an example, a LOD-score of 4 means that there is 10^4 more chance that there is a QTL on the indicated location than that there is no QTL present. The likelihood ratio statistic (LRS) score is a value given to each association and follows an asymptotically χ^2 distribution. The logarithm of the odds (LOD) and LRS scores are effectively the same statistic. The correlation between the LOD and LRS scores is a factor 4.6 (LRS=LOD*4.6).

Although SIM is better for complex trait analysis than the single-locus association' test, because SIM can localize possible QTLs more accurately, both are still single-QTL methods. Because these tests cannot detect epistatic interactions between loci, other tests have been developed. The computer program (see below) we used in this work to analyze the data contains an algorithm, which detects epistatic interactions. Composite interval mapping (CIM) or multiple QTL mapping is like SIM but takes additional marker loci into account that have been shown to be associated with the trait. This test is

even better for complex trait analysis, but still cannot detect epistatic interactions between QTLs that by themselves do not show any linkage.

The terms for statistical significance levels such as 'point-wise significance' and 'genome-wide significance' were introduced by Lander and Kruglyak. A point-wise significance is the probability that one can find by chance a LOD or LRS at a certain locus. The genome-wide significance is the probability that one can encounter a specific LOD somewhere in a genome scan, and is obviously a more stringent significance level. A permutation test analysis is a method calculating the genome-wide significance of the LRS generated by one of the tests described above. It is a method where the phenotypic trait data are randomly distributed among the progeny, while the genetic map is kept intact. This breaks the relationship between the phenotypic trait data and the genotypes of the marker loci. This procedure can be repeated multiple times (e.g. 1.000 or 10.000 times). For each permutated dataset the maximal LRS is recorded at regular intervals throughout the genome. Values at appropriate percentile points of this empirical distribution are used as a threshold value above which the observed LRS is significant. The accepted levels for significance nowadays are: highly significant ($p<0.001$), significant ($p<0.05$) and suggestive ($p<0.63$). The suggestive level is the level at which on average one false positive is found per genome scan. At this level, the detected QTLs are not strong QTLs. The highly significant level is the level at which strong QTLs, or in large crosses the strongest moderate QTLs, will be detected.

To analyze the data obtained from large crosses a specific computer program containing these methods for QTL mapping, called Mapmanager QTX made by Dr. Manly, was used. This program, which is freely available, is used in this work to analyse the data (Manly et al., 2001). First, the genetic and phenotypic data obtained from a backcross are analysed using the simple 'single-locus association' test to predict the amount of loci linked with the LPS-resistant phenotype of SPRET/Ei. In a second step, the SIM test searches intervals defined by two adjacent markers at multiple analysis points and a permutation test is used to evaluate if a QTL is present at the location or not. For a monogenic trait, a trait caused by (a) mutation(s) in a single gene, this region can be simply narrowed down by setting up a large backcross and typing more genetic markers between the two genetic markers surrounding the mutated locus. The mapping of a complex trait (e.g. the LPS resistance of SPRET/Ei), generally caused by mutations in numerous genes, consists of several phases. After the initial genome scan, QTLs are isolated in consomic/congenic mice to confirm the location of the QTL. Congenic mice are mice where only a part of the chromosome is introgressed from the donor strain into the acceptor strain. Finally, when the location of the QTL is confirmed, a second cross involving the consomic/congenic mice will be set up to fine map the trait.

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II. AIM OF THE PROJECT

We have found that SPRET/Ei, an inbred strain derived from *Mus spretus*, can easily survive injection of 500 μg LPS, while C57BL/6 mice succumb to 50 μg LPS. SPRET/Ei mice exhibit an extreme, but also dominant resistance ((BxS)F_1 mice also survive a dose of 500 μg LPS) to LPS. This clearly demonstrates the presence of LPS resistance genes in SPRET/Ei. In addition, (BxS)F_1 mice are relatively resistant to a Gram-negative *Klebsiella pneumoniae* infection. Hence, SPRET/Ei is a very important new tool, because, in contrast to the LPS-resistant C3H/HeJ and C57BL10/ScCr strains, it not only resists the toxic effects of LPS, but can also clear infection of a Gram-negative pathogen. We reason that identifying the molecular basis of the LPS hypo-responsiveness might lead to new therapeutic possibilities for the treatment of sepsis, because SPRET/Ei mice are unresponsive to the toxicity of LPS and preserve the recognition and destruction of pathogens.

To identify the genes that are responsible for the LPS resistance of SPRET/Ei, we followed two different approaches.

A first approach was to map and clone the LPS resistance genes of SPRET/Ei. To map a trait, two parental strains are needed that are significantly different for a trait and that show significant genetic polymorphism. Thus, SPRET/Ei mice are ideal for mapping experiments due to the high degree of genetic polymorphism between SPRET/Ei and the common laboratory strains (e.g. C57BL/6). In addition, C57BL/6 and SPRET/Ei are significantly different in their response to LPS, since SPRET/Ei mice can resist a 10 times higher dose of LPS than C57BL/6 mice. First, we did an initial genome scan to detect the QTL(s) linked with the LPS resistance. To confirm the importance of the loci found in the initial backcross we started making consomic mice.

A second approach was to identify the molecular basis of the LPS resistance *in vitro*. Hypo-responsiveness of SPRET/Ei to LPS was also evident *in vitro* and the signalling pathways activated by LPS are well known. So, we were able to use an *in vitro* model to identify the molecular basis the LPS resistance of SPRET/Ei.

III. RESULTS

1. THE WILD-DERIVED INBRED MOUSE STRAIN SPRET/Ei IS RESISTANT TO LPS AND DEFECTIVE IN IFN-β PRODUCTION

1.1. PNAS article

Tina Mahieu, Jin Mo Park, Hilde Revets, Bastian Pasche, Andreas Lengeling, Jan Staelens, Andy Wullaert, Ineke Vanlaere, Tino Hochepied, Frans van Roy, Michael Karin and Claude Libert (2006) Proceedings of the National Academy of Sciences of the USA (*PNAS)* **203**: 2292-2297.

The wild-derived inbred mouse strain SPRET/Ei is resistant to LPS and defective in IFN-β production

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Although activation of Toll-like receptor 4 (TLR4)-positive cells is essential for eliminating Gram-negative bacteria, overactivation of these cells by the TLR4 ligand LPS initiates a systemic inflammatory reaction and shock. Here we demonstrate that SPRET/Ei mice, derived from *Mus spretus*, exhibit a dominant resistance against LPS-induced lethality. This resistance is mediated by bone marrow-derived cells. Macrophages from these mice exhibit normal signaling and gene expression responses that depend on the myeloid differentiation factor 88 adaptor protein, but they are impaired in IFN-β production. The defect appears to be specific for IFN-β, although the SPRET/Ei IFN-β promoter is normal. *In vivo* IFN-β induction by LPS or influenza virus is very low in SPRET/Ei mice, but IFN-β-treatment restores the sensitivity to LPS, and IFN type 1 receptor-deficient mice are also resistant to LPS. Because of the defective induction of IFN-β, these mice are completely resistant to *Listeria monocytogenes* and highly sensitive to *Leishmania major* infection. Stimulation of SPRET/Ei macrophages leads to rapid down-regulation of IFN type 1 receptor mRNA expression, which is reflected in poor induction of IFN-β-dependent genes. This finding indicates that the resistance of SPRET/Ei mice to LPS is due to disruption of a positive-feedback loop that amplifies IFN-β production. In contrast to TLR4-deficient mice, SPRET/Ei mice resist both LPS and sepsis induced with *Klebsiella pneumoniae*.

endotoxin | interferon | Toll | receptor | infection

Bacterial LPS, a Gram-negative cell wall component, is a potent activator of macrophages and dendritic cells (1–4). Genetic studies identified that Toll-like receptor 4 (TLR4) is the LPS receptor (5) and that two LPS-resistant mouse strains, C3H/HeJ and C57BL10/ScCr, are defective in TLR4. Activation of macrophages by LPS results in the release of a variety of inflammatory cytokines, IFNs, and chemokines (6). These molecules initiate inflammation and both innate and adaptive immune responses aimed at clearing the infection. However, cytokine production must be kept tightly controlled, because excessive production may lead to an exaggerated inflammatory response and consequent lethal shock (7).

Signal transduction through TLRs requires the presence of a Toll/IL-1 receptor (TIR) domain found in the cytoplasmic domain of TLRs and the IL-1 receptor. Several TIR domain-containing adaptor proteins can be recruited to the TIR domains of TLRs: myeloid differentiation factor 88 (MyD88), TIR-containing adaptor protein/MyD88 adaptor-like, TIR domain-containing adaptor inducing IFN (TRIF)/TLR adaptor molecule 1, and TRIF-related adaptor molecule/TLR adaptor molecule 2 (8–12). The association of MyD88 and TIR-containing adaptor protein with the TIR domain of TLR4 results in recruitment of IL-1 receptor-associated kinase, which associates with TNF receptor-associated factor 6 (13, 14). This association leads to activation of the canonical IKK-$\alpha\beta\gamma$ complex and ultimately to activation of Rel/NF-κB transcription factors and mitogen-activated protein kinase (MAPK) family members (11). TLR3 and TLR4 can signal independent of MyD88 to induce IFN-β and, to a lesser extent, NF-κB and MAPK activation (15–17). Genetic studies on mice demonstrated that TRIF is crucial in the MyD88-independent pathway that is shared by TLR3 and TLR4 and that TRIF is involved in the activation of IFN-regulatory factor 3 (IRF-3) (9, 18). IRF-3 is activated by phosphorylation by the noncanonical inhibitor κB (IκB) kinases (IKKs), TANK-binding kinase 1, and IKK-ε (also called IKK-ι) (19–21). This phosphorylation causes IRF-3 to homodimerize and interact with the coactivators CREB-binding protein and p300 (22, 23). The complex then translocates to the nucleus, where it activates promoters containing IRF-3 binding sites (24), such as the type I IFN promoters. Secreted IFN binds and stimulates the two receptor subunits IFNαR1 and IFNαR2 (25). This binding leads to activation of the tyrosine kinases (TYKs) Janus kinase 1 and TYK2, signal transducer and activator of transcription 1 (STAT1), and STAT2 to form a STAT1:STAT2 heterodimer (26). STAT1:STAT2 complexes associate with IRF-9 to form IFN-stimulated gene factor 3. IFN-stimulated gene factor 3 recognizes IFN-stimulated response elements in promoter regions of IFN-responsive genes, e.g., *Isg15* (encoding the protein IP17), *Cxcl10*, *Irf7*, and *Oas* (27). IRF-7, alone or in combination with IRF-3, can bind to the promoter region of type I IFNs, IFN-α/β. So a positive feedback loop is responsible for the *en masse* induction of IFN-α/β after exposure to LPS (28). Although first characterized by their potent antiviral functions, IFN-α and IFN-β also mediate a variety of immune regulatory effects. These immune modulating functions indicate that type I IFNs may form an important link between innate and adaptive immune responses (29). Recently, targeted mutations have generated mice deficient in their ability to produce type I IFNs or to respond to them, and several of these mutant mice have proved to be resistant to acute inflammation. This was the case for TRIF (18), IFN-β, and Tyk2 knockouts (30), all of which are LPS-resistant. These data illustrate that type I IFNs play an important role in inflammatory processes.

Several spontaneous and induced mutant mouse strains defective in LPS signaling have been described. Most of them are extremely resistant to LPS-induced lethal shock but hypersen-

Conflict of interest statement: No conflicts declared.

Abbreviations: TLR, Toll-like receptor; STAT, signal transducer and activator of transcription; IFNAR, IFN (α and β) receptor; BMDM, bone marrow-derived macrophage; DC, dendritic cell; TIR, Toll/IL-1 receptor; MyD88, myeloid differentiation factor 88; TRIF, TIR domain-containing adaptor inducing IFN; IκB, inhibitor κB; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; IRF, IFN-regulatory factor; TYK, tyrosine kinase; ISGF-3, IFN-stimulated gene factor-3; OAS, 2′-5′ oligoadenylate synthetase.

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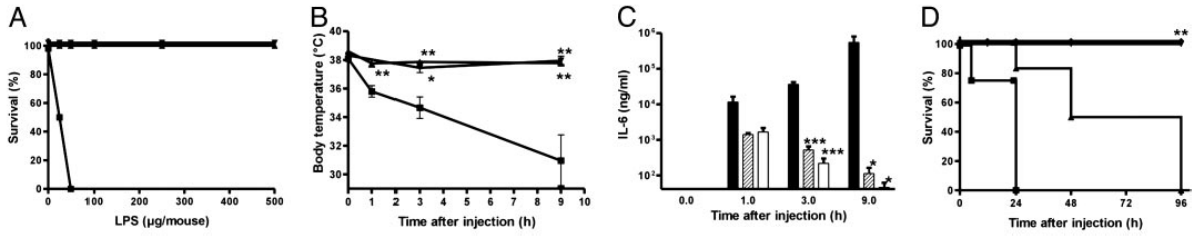

Fig. 1. Response of C57BL/6, (B × S)F_1, and SPRET/Ei mice to LPS. (*A*) Response of C57BL/6 (■), F_1 (▲), and SPRET/Ei (▼) mice (n = at least 5 for each dose) to increasing doses of LPS. Mortality was monitored up to 1 week after challenge (no further deaths occurred). (*B*) Induction of hypothermia after injection of 250 μg of LPS. ■, C57BL/6 (n = 5); ▲, F_1 (n = 5); ▼, SPRET/Ei (n = 5). (*C*) IL-6 concentrations in serum after injection of 250 μg of LPS. Black bars, C57BL/6 mice (n = 5); hatched bars, F_1 mice (n = 5); white bars, SPRET/Ei mice (n = 5). (*D*) Survival of C57BL/6 mice (▲; n = 4), F_1 mice (▼; n = 5), lethally irradiated F_1 mice reconstituted with C57BL/6 bone marrow (■; n = 4), or lethally irradiated F_1 mice reconstituted with F_1 bone marrow (♦; n = 5) after challenge with 250 μg of LPS. Significance was calculated for difference from C57BL/6.

sitive to bacterial infection, e.g., C3H/HeJ and C57BL10/ScCr, both of which carry mutations in *Tlr4* (5). Despite their endotoxin resistance, TLR4-deficient C3H/HeJ mice are extremely susceptible to infection by Gram-negative bacteria, most probably because the mutant TLR4 protein fails to sense bacteria, thereby failing to activate innate immune responses. We here describe our findings concerning the LPS resistance of the SPRET/Ei strain, an inbred strain derived from *Mus spretus*, to LPS-induced lethal shock.

Results

SPRET/Ei Mice Are Very Resistant to LPS. To determine the response of SPRET/Ei, C57BL/6, and F_1 mice to LPS, we injected mice i.p. with increasing amounts of LPS. Compared with C57BL/6, SPRET/Ei mice were highly resistant to the lethal effects of LPS (Fig. 1*A*). For C57BL/6 mice, LD_{50} is 25 μg and LD_{100} is 50 μg, but all SPRET/Ei and F_1 mice readily survived a dose of 500 μg of LPS, which also shows that LPS hyporesponsiveness is a dominant trait.

***In Vivo* Response of SPRET/Ei, F_1, and C57BL/6 Mice to LPS.** To study the resistance of SPRET/Ei mice in more detail, a dose of 250 μg of LPS was given i.p. After injection of LPS, the body temperature of C57BL/6 mice decreased dramatically. SPRET/Ei and F_1 mice were significantly protected against LPS-induced hypothermia (Fig. 1*B*). Injection of LPS induces high concentrations of IL-6 in serum (31). After injection of LPS, serum IL-6 levels were determined. LPS induced high amounts of IL-6 in C57BL/6, SPRET/Ei, and F_1 mice. However, the serum concentrations in SPRET/Ei and F_1 mice remained significantly lower than those in C57BL/6 mice, especially at later time points (Fig. 1*C*). Based on the dominant nature of the phenotype, F_1 mice were used to generate a set of 140 (C57BL/6 × SPRET)F_1 × C57BL/6 backcross mice (N2). The mice were injected with a high dose of LPS. All mice were also genotyped by using a set of 70 different polymorphic microsatellites, and a linkage study was performed. One-third of the N2 mice died, and significant linkage of the phenotype was found with five different chromosomes. So, inheritance is complex and non-Mendelian. After we had generated and studied consomic mice by isolating the relevant SPRET/Ei chromosomes in a C57BL/6 background, we had to conclude that the very strong phenotype depends entirely on epistatic interactions between different loci and that cloning of the responsible genes by a positional cloning strategy was virtually impossible.

Bone Marrow-Derived Cells Are Critically Involved in the LPS Resistance of F_1 Mice. Macrophages are the first targets of LPS (32). To study whether macrophages are relevant for LPS resistance, F_1 mice were lethally irradiated and reconstituted with bone marrow from C57BL/6 or F_1 mice. Unfortunately, lethally irradiated C57BL/6 mice could not be reconstituted with F_1 bone marrow because of transplant rejection. The mice where challenged with 250 μg of LPS 8 weeks after the transplant. All of the F_1 mice reconstituted with F_1 bone marrow survived (n = 5), whereas all of those reconstituted with C57BL/6 bone marrow (n = 4) died within <24 h (Fig. 1*D*). Furthermore, LPS induced high amounts of IL-6 in F_1 mice reconstituted with C57BL/6 bone marrow (3,852 ± 2,152 ng/ml), whereas IL-6 serum concentrations in F_1 mice reconstituted with F_1 bone marrow were significantly lower (812 ± 1,247 ng/ml) (P = 0.03). The data suggest that the LPS-resistant phenotype of F_1 depends critically on bone marrow-derived cells, probably macrophages.

The MyD88-Dependent and MyD88-Independent Pathways in SPRET/Ei Macrophages *in Vitro*. Bone marrow-derived macrophages (BMDM) from C57BL/6 and SPRET/Ei mice were stimulated with 100 ng/ml LPS. At different time points, total RNA was prepared from the cells for real-time quantitative PCR analysis, and cell lysates were prepared for Western blot. The phosphorylation of p38 MAPK and c-Jun N-terminal kinase (JNK) and the transient degradation of IκB were comparable in BMDM from SPRET/Ei and C57BL/6 mice (Fig. 2*A*). Hence, MyD88-dependent, early NF-κB and MAPK activation in SPRET/Ei is intact. This finding was confirmed by the absence of a significant difference between SPRET/Ei and C57BL/6 BMDM in LPS-induced TNF-α and IL-6 mRNA expression as measured 1 and 4 h after stimulation with LPS (data not shown). Furthermore, there were no significant differences between the two mouse strains in LPS-induced mRNA expression of other genes, e.g., *Il1a*, *Il1b*, *Il12a*, *Il12b*, *Bcl2*, *Birc4* (encoding XIAP), and *Tnfaip3* (encoding A20) (data not shown).

Induction of IFN-β mRNA by LPS was largely impaired in SPRET/Ei-derived BMDM (Fig. 2*B*). The same was observed when SPRET/Ei- and C57BL/6-derived BMDM were stimulated with 10 μg/ml polyinosine-polycytidylic acid (data not shown). As a consequence, LPS-induced STAT1 phosphorylation was impaired in SPRET/Ei BMDM (Fig. 2*C*). This reduced phosphorylation in turn impaired the induction of IRF-7 in LPS-stimulated SPRET/Ei BMDM (Fig. 2*D*). LPS-induced phosphorylation of IRF-3 was comparable in SPRET/Ei BMDM and C57BL/6 BMDM, and IRF-3 knockout BMDM had broader gene defects than SPRET/Ei macrophages (data not shown). Furthermore, there were no significant differences in LPS-mediated induction of *Cxcl10* and *Isg15* mRNA, two genes that are also induced by TRIF-mediated induction of IRF-3 (data not shown). Hence, the defect seems to be specific for IFN induction. Finally, the sequences of the IFN-β promoter

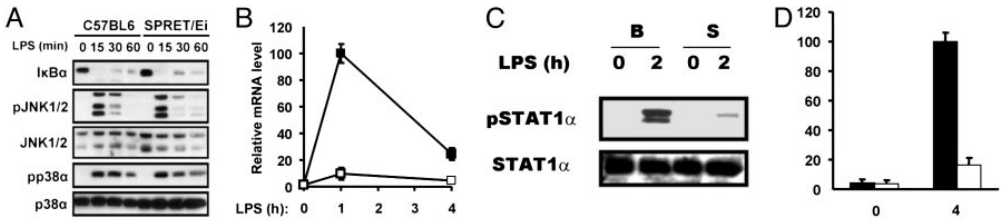

Fig. 2. The MyD88-dependent signaling pathway is intact in SPRET/Ei BMDM, and SPRET/Ei BMDM have a defect in IFN-β production *in vitro*. (*A*) BMDM were stimulated with 100 ng/ml LPS for the indicated time intervals. IκBα degradation, as well as JNK1/2 and p38 expression and phosphorylation, were analyzed by Western blot. (*B*) LPS-induced expression of the IFN-β gene in BMDM. BMDM from C57BL/6 and SPRET/Ei mice were stimulated with 100 ng/ml LPS for the indicated durations, and induction of IFN-β mRNA was analyzed by real-time quantitative PCR in triplicate cultures. ■, C57BL/6; □, SPRET/Ei. (*C*) Phosphorylation of STAT1 2 h after stimulation with 100 ng/ml LPS. (*D*) Induction of IRF-7 mRNA after stimulation with 100 ng/ml for 4 h. Black bars, C57BL/6; white bars, SPRET/Ei.

(150 bp before transcription start) in SPRET/Ei and C57BL/6 mice were identical. These data suggest that direct IRF-3-dependent IFN-β induction in SPRET/Ei is intact and that the defect must be in the second wave of IFN induction.

SPRET/Ei Has a Defect in IFN-β Production *in Vivo*. SPRET/Ei mice are extremely resistant to the lethal effects of LPS, and their macrophages are defective in IFN-β induction *in vitro*. We reasoned that pretreatment with IFN-β would restore LPS sensitivity to SPRET/Ei mice. Indeed, we found that F_1 mice can be sensitized to LPS by pretreatment with IFN-β. Two hours before injection of 200 μg of LPS, we pretreated F_1 mice (n = 4) with 20 μg of IFN-β. All of the pretreated F_1 mice died, but all of the control F_1 mice (n = 6) survived. All of the control C57BL/6 mice (n = 10) also died after injection of 200 μg of LPS. Furthermore, IFN (α and β) receptor 1 (IFNAR1) knockout mice (n = 4) resisted the dose of 200 μg of LPS, whereas 129/SvEv control mice (n = 4) died within 48 h after challenge (Fig. 3*A*). Thus, IFN-β is a critical factor in the resistance of SPRET/Ei mice to LPS.

To examine whether the impairment of LPS-induced IFN-β induction in SPRET/Ei was also evident *in vivo*, a dose of 500 μg of LPS was injected i.p. into C57BL/6 and F_1 mice, and lungs were collected at different times after challenge. High concentrations of biologically active IFN were found in lungs of C57BL/6 mice (n = 4), and concentrations were significantly lower in F_1 mice (n = 4) (Fig. 3*B*). Furthermore, C57BL/6, F_1, and SPRET/Ei mice were infected with influenza virus strain X47 and biologically active IFN measured in lung lysates 2 or 5 days after infection. Very large amounts of IFN were induced in C57BL/6 mice, but only minute amounts were detected in F_1 mice, and no IFN induction was found in SPRET/Ei (Fig. 3*C*).

Consequences of a Defective IFN-β Production. It is well known that IFN-β is an important factor in immunity, e.g., it can sensitize macrophages to cell death induced by *Listeria monocytogenes* (33) and can protect against progressive leishmaniasis (34). We therefore examined the responses to infection with *Listeria*

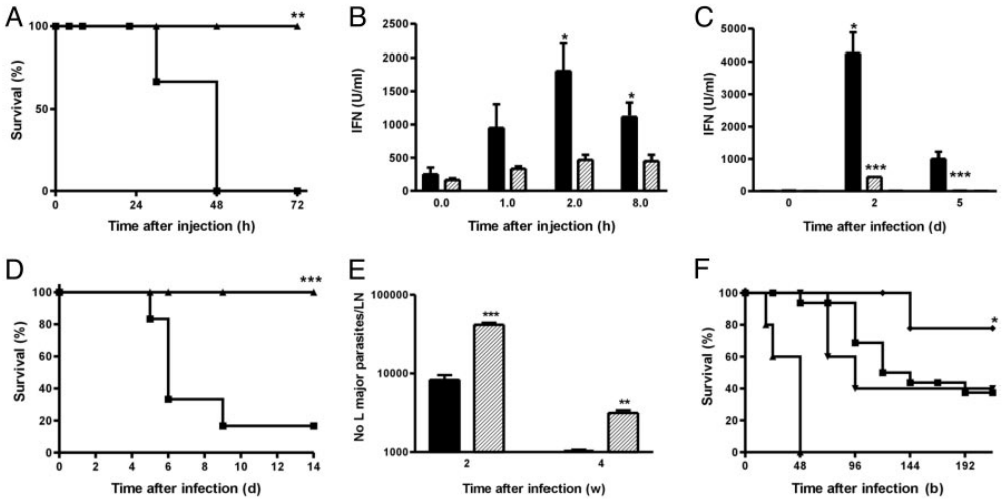

Fig. 3. SPRET/Ei has a defect in IFN-β production *in vivo*, shows IFN-β-deficient phenotypes, and resist a lethal challenge with *K. pneumoniae*. (*A*) Survival of IFNAR1$^{-/-}$ mice after injection of 200 μg of LPS. ▲, IFNAR1$^{-/-}$ mice (n = 4); ■, 129Sv mice (n = 4). (*B*) IFN biological activity in lung extracts at the indicated time points after injection of 500 μg of LPS. Black bars, C57BL/6 (n = 4); hatched bars, F_1 (n = 4). (*C*) IFN biological activity in lung lysates after influenza virus infection. Black bars, C57BL/6 (n = 12); hatched bars, F_1 (n = 10); white bars, SPRET/Ei (n = 2). Significance was calculated for difference from C57BL/6. (*D*) Mortality after i.v. injection with 8.5×10^3 colony-forming units of *L. monocytogenes*. ▲, SPRET/Ei (n = 12); ■, C57BL/6 (n = 6). (*E*) Response to infection with *Leishmania major*. Parasite burdens in the draining lymph nodes were determined by limiting-dilution assays (four mice per group per time point; dilutions tested in quadruplicate). Black bars, C57BL/6 mice; hatched bars, F_1 mice. (*F*) Lethal response of C57BL/6 (■; n = 16), F_1 (◆; n = 9), C3H/HeJ (▲; n = 5), and C3H/HeN (▼; n = 5) mice after infection intranasally with 10,000 colony-forming units of *K. pneumoniae*. Mortality was monitored for 8 days. No further deaths occurred. Significance was calculated for difference from C57BL/6.

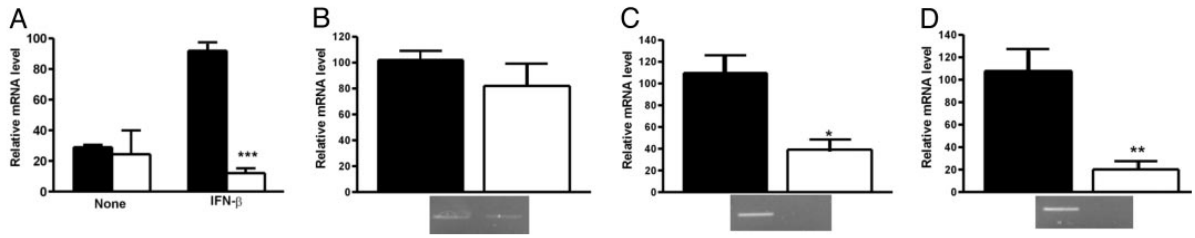

Fig. 4. SPRET/Ei has a defect in the autocrine/paracrine loop of IFN-β induction. (*A*) IFN-β-induced expression of the *Oas* gene in BMDM. Cells from C57BL/6 and SPRET/Ei mice were left unstimulated or were stimulated with 200 units/ml IFN-β for 1 h, and induction of OAS mRNA was analyzed by semiquantitative RT-PCR. Black bars, C57BL/6 mice; white bars, SPRET/Ei mice. (*B–D*) Relative IFNAR1 mRNA levels in unstimulated BMDM (*B*), 1 h after stimulation with 100 ng/ml LPS (*C*), and 1 h after treatment with 200 units/ml IFN-β (*D*). Black bars, C57BL/6 mice; white bars, SPRET/Ei mice ($n = 3$). Significance was calculated for difference from C57BL/6 mice. See ethidium bromide-stained gels below the panels.

monocytogenes. SPRET/Ei mice ($n = 12$) and C57BL/6 mice ($n = 6$) were infected with 8.5×10^3 colony-forming units of *Listeria monocytogenes*; SPRET/Ei mice were completely resistant to the infection (Fig. 3*D*). We also infected F_1 mice by injecting their footpads with 2×10^6 stationary-phase *Leishmania major* promastigotes. Parasite burdens in the draining lymph nodes were determined by limiting-dilution assays ($n = 6$). F_1 mice were significantly more sensitive to *Leishmania major* infection (Fig. 3*E*).

TLR4 deficiency leads to extreme sensitivity to Gram-negative sepsis. We tested the response of F_1 mice to infection with *Klebsiella pneumoniae*, a model of Gram-negative septic pneumonia. C3H/HeJ mice, which express a mutant *Tlr4* gene, are extremely sensitive to Gram-negative infections (5). F_1 ($n = 9$), C57BL/6 ($n = 16$), C3H/HeN ($n = 5$), and C3H/HeJ ($n = 5$) mice were infected intranasally with an LD_{50} (for C57BL/6) of *K. pneumoniae*, and mortality was monitored. Approximately 60% of the C57BL/6 and C3H/HeN mice succumbed to this infection, and C3H/HeJ mice were extremely sensitive and died very soon after infection, but F_1 mice were relatively resistant to Gram-negative infections (Fig. 3*F*).

IFN-β Production Is Reduced by a Defect in the Positive Feedback Loop of IFN-β Induction. To determine whether there is a defect in the intracellular signaling pathway activated by type I IFN, BMDM from SPRET/Ei and C57BL/6 were stimulated with LPS or IFN-β. We studied the mRNA induction of *Oas*, an IFN-dependent gene, using semiquantitative RT-PCR. Significantly less 2′–5′ oligoadenylate synthetase (OAS) mRNA was induced in SPRET/Ei BMDM than in C57BL/6 BMDM 1 h after LPS treatment (data not shown) or IFN-β stimulation (Fig. 4*A*). Thus, a defect in the positive feedback loop of IFN-β induction appears likely. In addition, after IFN-β stimulation, induction of IL-6 was significantly lower in SPRET/Ei BMDM than in C57BL/6 BMDM (data not shown).

We then used semiquantitative RT-PCR to study IFNAR1 mRNA expression in BMDM after stimulation with LPS or IFN-β. We found that the basal expression of this gene was identical in SPRET/Ei and C57BL/6 BMDM but that it was down-regulated in SPRET/Ei cells very soon after LPS or IFN-β stimulation. No similar effect was seen in C57BL/6 cells (Fig. 4 *B–D*). These data suggest that hyporesponsiveness to IFN-β in SPRET/Ei, as defined by inefficient induction of OAS and IL-6, may be due to rapid down-regulation of IFNAR1 expression by IFN-β itself.

Discussion

To identify pathogens, the innate immune system relies on the TLR family (1, 35), whose members recognize pathogen-associated molecular patterns. One of the pathogen-associated molecular patterns is LPS, an essential component of the outer membrane of Gram-negative bacteria (2). LPS causes many of the inflammatory effects observed in Gram-negative septic shock patients, and considerable research has been devoted to elucidating the mechanisms by which LPS functions. A major breakthrough was achieved by identification of two LPS-resistant mouse strains, C3H/HeJ and C57BL10/ScCr, and the identification of *Tlr4* as the gene encoding the major receptor for LPS, TLR4 (5).

We describe here an LPS-resistant mouse strain, SPRET/Ei, an inbred strain derived from *M. spretus*. We found that both SPRET/Ei and F_1 mice resist doses of LPS at least 10 times higher than those tolerated by C57BL/6 mice. We demonstrated by bone marrow transplantation that bone marrow-derived cells, most probably macrophages, are critical in the resistance of F_1 mice. In contrast to the resistance of two other LPS-resistant inbred lines, C3H/HeJ and C57BL10/ScCr, which is caused by mutations in the *Tlr4* gene encoding the LPS receptor TLR4, the LPS-resistant phenotype of SPRET/Ei is not a monogenic, simple, Mendelian trait, but is subject to polygenic, complex, non-Mendelian inheritance. Moreover, the loci involved in the phenotype interact with each other in a strictly epistatic way. We found that SPRET/Ei-derived macrophages have a defect in IFN-β production and in subsequent STAT1 phosphorylation and IRF-7 induction. The MyD88-dependent pathway leading to early NF-κB and MAPK activation seems to be intact. The defect in IFN-β production is also observed *in vivo* in SPRET/Ei mice treated with LPS or influenza virus. Furthermore, the resistance of SPRET/Ei mice can be reversed by exogenous IFN-β. IFNAR1-deficient mice proved resistant to LPS as well. As expected (33, 34), the defect in IFN-β production caused F_1 mice to be more sensitive to *Leishmania major* infection and SPRET/Ei mice to show complete resistance to *Listeria monocytogenes*.

The phenotype that we observed in SPRET/Ei and F_1 mice is very comparable to that observed in mice with induced mutations in signaling molecules of the Janus kinase–STAT-dependent pathway. Macrophages derived from TYK2-null mice show impaired expression of IFN-β mRNA 30 min to 2 h after LPS stimulation (30). Also, IRF-7 mRNA expression, which is positively regulated by IFNAR signaling, is reduced in TYK2-null cells. NF-κB and MAPK activation are normal in macrophages derived from TYK2-null mice. IFN-β-deficient mice are resistant to LPS, but they produce normal amounts of TNF, IFN-γ, and NO after LPS injection. In addition, STAT1-null mice are also resistant to LPS (30). Furthermore, IRF-$3^{-/-}$ mice are resistant to LPS and show defects in the induction of IFN-β and chemokine (C-X-C motif) ligand 10 (CXCL10) mRNA (36). SPRET/Ei seems to be unique in that the defect is specific to IFN-β induction, because other IRF-3-responsive genes, such as *Cxcl10* and *Isg15*, are induced with comparable kinetics in SPRET/Ei and C57BL/6, and phosphorylation of IRF-3 ap-

pears normal in SPRET/Ei macrophages. In this respect, the phenotype observed in SPRET/Ei closely resembles that of IFN-β-deficient mice. Also, induction of OAS mRNA expression (by LPS or IFN-β), which is positively regulated by IFNAR signaling, was found to be severely reduced in SPRET/Ei-derived macrophages. Thus, it appears that the defect is situated not in the TRIF-dependent signaling pathway, but in the autocrine/paracrine loop after IFN-β induction. We found that LPS and IFN-β caused rapid down-regulation of IFNAR1 mRNA expression in SPRET/Ei-derived macrophages but not in C57BL/6 macrophages. Mice lacking IFNAR1 are completely unresponsive to type I IFNs (37). Therefore, down-regulation of IFNAR1 expression is likely to be the major contributor to the inefficient induction of IL-6 by IFN-β and *Oas* gene expression in SPRET/Ei macrophages. Furthermore, just like SPRET/Ei mice, IFNAR1 knockout mice exhibit profound resistance to LPS and to infection by the Gram-positive intracellular bacterium *L. monocytogenes* (38). It has been reported that TYK2 slows down IFNAR1 degradation, a process that is at least partly due to inhibition of IFNAR1 endocytosis (39). Future work will need to address the extent to which TYK2 is directly responsible for IFNAR1 mRNA down-regulation in SPRET/Ei macrophages or whether another mechanism is responsible for this phenomenon. A thorough investigation of IFNAR1 mRNA turnover will be needed, along with a detailed expression profiling to identify other similarly affected mRNA species in SPRET/Ei macrophages.

We believe that the phenotypes that we describe here are very relevant in the search for new therapeutic interventions for sepsis. SPRET/Ei, in contrast to the TLR4-deficient LPS-resistant C3H/HeJ and C57BL10/ScCr strains, not only resists the toxic effects of LPS but can also resist infection by Gram-negative pathogens, such as *K. pneumoniae*. SPRET/Ei mice exhibit the characteristics of a perfect therapy for sepsis: unresponsiveness to the toxic effects of LPS and preservation of recognition and destruction of pathogens. Probably, the considerable reduction in IFN-β in SPRET/Ei mice enables them to resist endotoxic shock, but the small amount that is still produced ($\approx$10% of normal) is sufficient to stimulate innate and adaptive immunity. Therefore, detailed identification of the defect that leads to the down-regulation of the IFNAR1 could lead to a major breakthrough in sepsis research.

Materials and Methods

Mice. C57BL/6JIco mice, hereafter called C57BL/6, originated from The Jackson Laboratory and were bred further at Iffa Credo (Brussels), from which they were purchased. SPRET/Ei mice were obtained from The Jackson Laboratory. It should be noted that only $\approx$10 SPRET/Ei mice were available each year and that, in our animal house, SPRET/Ei mice were extremely poor breeders or did not breed at all. Hybrid F_1 mice obtained by crossing C57BL/6 female mice with SPRET/Ei males are designated as F_1. C3H/HeJOlaHsd-Lpsd and C3H/HeNHsd mice were purchased from Harlan (Oxon, U.K.). IFNAR1 KO mice and 129 SvEv control mice were purchased from B & K Universal (Aldbrough, Grimston, U.K.). Mice were housed in individually ventilated cages in an air-conditioned, temperature-controlled conventional animal house and received food and water ad libitum. They were used at the age of 8 weeks and had comparable body weights. All experiments were approved by the local ethics committee.

Agents. LPS from *Salmonella abortus equii* was purchased from Sigma–Aldrich. IFN-β (4.6×10^6 units/mg protein) was expressed in and purified from *E. coli* in our laboratory in Ghent. Polyinosine-polycytidylic acid was purchased from Amersham Pharmacia Biosciences. Antibodies directed against IκBα, phospho-JNK, phospho-p38α, p38α, STAT1, and NF-κB p65 were purchased from Santa Cruz Biotechnology. Antibody against phospho-STAT1 was from Upstate Biotechnology. Antibody against JNK was from Pharmingen.

Isolation of Mouse Embryonic Fibroblasts. Mouse embryonic fibroblasts were isolated from 18-day-old embryos and cultured in RPMI medium 1640 supplemented with 10% FCS, penicillin, streptomycin, gentamycin, and L-glutamine.

Immunoblot Analysis. Whole-cell, cytoplasmic, and nuclear extracts for immunoblot analysis were prepared and subjected to SDS/PAGE. Proteins transferred to nitrocellulose membrane were probed with rabbit antiserum against phospho-Stat1 and Stat1, and the immune complexes were visualized with the ECL Western blot reagent (Pierce).

Injection, Blood Collection, and Measurement of Body Temperature. LPS was diluted in endotoxin-free PBS immediately before injection. All injections were given i.p. in a volume of 250 μl. Blood was collected by retroorbital bleeding and allowed to clot for 1 h at 37°C and for another hour at 4°C. Serum was prepared and stored at −20°C. Rectal body temperatures were measured with an electronic thermometer (Comarks, Littlehampton, U.K.). To prepare lung extracts, mice were anesthetized with avertin (tribromoethanol), the thorax was opened, and the left and right lungs were isolated, washed, and kept in 1 ml of ice-cold, sterile PBS. Lungs were homogenized in a tissue homogenizer (Heidolph Homogenizer, model RZR 2020; Heidolph-Elektro, Kelheim, Germany) and centrifuged for 15 min at $15{,}000 \times g$. The clear supernatant was centrifuged once more and kept at −80°C until needed.

Infection Models. *K. pneumoniae* (ATCC 43816) was inoculated intranasally (50 μl) under light isoflurane anesthesia. Before each experiment the LD_{50} was determined in C57BL/6 mice, and this dose was used in the experiment. Mortality was monitored for at least 8 days. For infection with influenza type A virus strain X47, the LD_{100} dose was first determined in C57BL/6 mice. The virus was inoculated intranasally (50 μl) under light isoflurane anesthesia. Just before infection and on days 2 and 5 after infection mice were anesthetized with avertin, and lungs were isolated and processed as described. For infection with *L. monocytogenes*, EGD cells (serotype 1/2a) were washed with PBS, and viable bacteria were counted by plating serial dilutions. Bacteria were diluted in PBS to a final concentration of 2×10^5 colony-forming units per ml, and mice were infected with 100 μl in a lateral tail vein. For infection with *Leishmania major* (MHOM/TN/95/GLC94), the parasites were maintained by continuous passage in BALB/c mice. Each mouse was infected s.c. in one hind footpad with 2×10^6 stationary-phase promastigotes in a volume of 25 μl. Parasite burden in the draining lymph nodes was determined by a limiting dilution assay of single-cell suspensions made from individual lymph nodes. Each sample was plated in quadruplicate in 96-well flat-bottom microtiter plates. The number of viable parasites was determined from the reciprocal of the highest dilution at which promastigotes could be detected after 10 days of incubation at 26°C. Four mice were used for each parasite burden determination. The results presented are the averaged values ($\pm$ standard errors) of the parasite burdens found for each group.

Determination of IL-6 in the Serum. IL-6 was determined by using an IL-6-dependent 7TD1 hybridoma cell line as described in ref. 40.

Determination of IFN Biological Activity in Lung Lysates. IFN biological activity was determined by using an encephalomyocarditis virus-dependent biological assay. In the presence of serially

diluted lung lysates or IFN as a standard, 8×10^3 L929 cells were cultured in 96-well plates in DMEM supplemented with 2% heat-inactivated FCS. After overnight incubation at 37°C, encephalomyocarditis virus was added at a concentration of 10^3 per ml (50 pfu per well), and cell death was measured by a colorimetric technique (MTT assay), which is based on the reduction of a yellow tetrazolium salt to a purple formazan. The assay has a detection limit of 1 pg/ml.

Bone Marrow Transplantation. Mice were lethally irradiated (10 Gy) and then maintained under sterile conditions. Drinking water was supplemented with 2 mg/ml neomycin sulfate. Bone marrow cells were isolated from tibias and femurs. Bone marrow cells (7×10^6) were injected i.v. 1 day after lethal irradiation. As a control, several irradiated mice did not receive donor cells. These mice died within 2 weeks of irradiation. The bone marrow-reconstituted mice were challenged with 250 μg of LPS 8 weeks after the bone marrow transplant.

Real-Time Quantitative PCR Analysis. Total RNA was isolated from triplicate cultures of BMDM by using the RNAwiz reagent (Ambion). cDNAs were synthesized by using the SuperScript II reverse transcriptase system (Invitrogen). The cDNA equivalent of 0.2 μg of total RNA was amplified through 40 cycles of 15 sec at 95°C and 1 min at 60°C. Output was monitored by using SYBR Green core reagents and the ABI Prism 7700 System (Applied Biosystems). The results were normalized to the level of cyclophilin mRNA. Individual primer sequences are available upon request.

Semiquantitative RT-PCR. Total RNA was isolated from BMDM by using the RNeasy Mini Kit (Qiagen). cDNAs were synthesized by using the SuperScript II reverse transcriptase system (Invitrogen). The cDNA equivalent of 0.1 μg of total RNA was amplified by using primers for OAS and IFNAR1. The results were normalized to the level of β-actin.

Statistical Analysis. Mortality data were analyzed by using a χ^2 test. Kaplan–Meier survival curves were compared by means of a log-rank test, and all other data were analyzed by using a one-tailed Student t test. Significance levels in the figures are as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

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2. DESCRIPTION OF THE LPS RESISTANCE OF SPRET/Ei *IN VIVO*

2.1. Introduction

Compared to the commonly used laboratory strain C57BL/6, SPRET/Ei and (BxS)F_1 mice easily survive a dose of 500 μg, which also shows that the hypo-responsiveness to LPS is a dominant trait. C57BL/6 mice die from a dose of 50 μg LPS. Also, SPRET/Ei and (BxS)F_1 mice are significantly protected to LPS-induced hypothermia. In addition, IL-6 concentrations in serum of SPRET/Ei and (BxS)F_1 mice remain significantly lower than those in C57BL/6 mice, especially 3 and 9 hours after LPS (PNAS article). To describe the LPS-resistance phenotype of SPRET/Ei more in detail, we also looked at TNF-α induction in serum and inflammation after LPS.

2.2. Experimental procedures

Injections, preparation of serum, determination of IL-6 in the serum (7TD1 assay) and measurement of body temperature is as described in Mahieu *et al*. (PNAS article).

2.2.1. Determination of TNF in the serum

TNF was measured by using the sensitive cell line WEHI 164 cl 13 (Espevik and Nissen-Meyer, 1986). In the presence of serially diluted serum or recombinant TNF as a standard, 50.000 cells per well were cultured and stimulated with 1 μg/ml Actinomycin D in flat-bottom, 96-well microtiter plates. Next, the number of surviving cells was determined after 18 hours of incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Mosmann, 1983; Niks and Otto, 1990). The assay has a detection limit of about 0.5 pg/ml.

2.2.2. Histological analysis

Lungs and liver were fixed overnight in 4% formalin. Fixation is followed by dehydration with a mounting percentage of alcohol and by clearing with histo-clear. After clearing, the tissues are infiltrated with paraffin wax (making a paraffin block). A tissue paraffin block is cut in sections of 4-5 μm with a microtome. The advantage from paraffin embedding is that it is easy to cut a paraffin block into thin serial tissue sections: once embedded tissue sections can be preserved for a long time at room

temperature. Inflammation, as observed by the infiltration of inflammatory cells, was examined in haematoxylin/eosin stained lung and liver sections (5μm).

2.2.3. Gelatin zymography

A 10% gel was prepared according to the standard procedure. When preparing the running gel, 1 mg/ml gelatin was added. One part sample is mixed with one part sample buffer (2x) and the mix is incubated for 10 min at room temperature (RT). Samples were applied (typically 10-25 μl) and the gel is runned with 1x tris-glycine running buffer according to the standard running conditions (~125V, constant voltage). After running, the gel is incubated in zymogram renaturing buffer with gentle agitation for 30 min at RT. The zymogram renaturing buffer is decanted and replaced with 1x zymogram developing buffer. The gel is equilibrated for 30 min. at RT with gentle agitation and then replaced with fresh 1x zymogram developing buffer and incubated at 37°C for at least four hours. Next, the gel is stained with 0.5% coomassie blue R-250 for 30 min. Gels are destained with coomassie R-250 destaining solution. Areas where the protease has digested the substrate (protease activity) will appear as clear bands against a dark blue background.

2.3. Results

2.3.1. TNF-α induction after injection of a high dose LPS

LPS acts on myeloid and non-myeloid cells to express pro-inflammatory proteins such as IL-6 and TNF-α (Ulevitch and Tobias, 1995). We looked at TNF-α induction after an intraperitoneal (i.p.) injection of 250 μg *S.a.e.* LPS.

Besides a defective IL-6 gene induction, SPRET/Ei mice demonstrate also a defective TNF-α induction after LPS injection (Figure III.2.1.). So, SPRET/Ei mice have a defect in pro-inflammatory cytokine gene induction after LPS. However, it is possible that the SPRET/Ei TNF-α molecule is not active on the WEHI 164 cl 13 cell line. To exclude this possibility, we should test a serum sample in both an ELISA- and TNF-assay, and compare the data. In contrast to the defective gene induction in SPRET/Ei at early time points after LPS *in vivo*, we observe a normal IL-6 and TNF-α mRNA induction at early time points after LPS *in vitro* in BMDMs (PNAS article). It should be kept in mind that the *in vivo* situation is much more complex. LPS can activate myeloid (neutrophils, monocytes, macrophages, platelets) and/or non-

myeloid cells (fibroblasts) to produce pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6 and IL-1. These cytokines (e.g. IL-1) might activate other cells to produce also IL-6 and TNF-α.

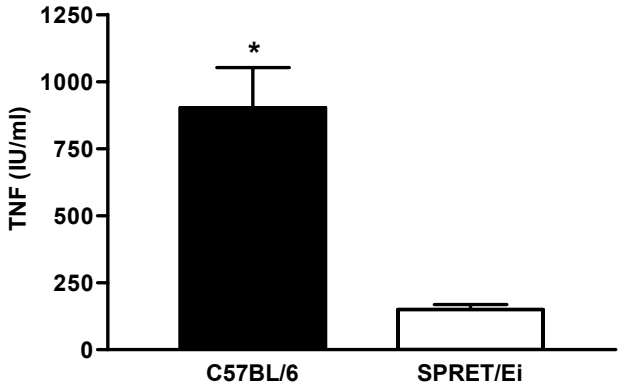

Figure III.2.1. – Concentration of TNF in serum after 250 μg *S.a.e.* LPS. A dose of 250 μg *S.a.e.* LPS was given i.p. to C57BL/6 (n = 10) and SPRET/Ei mice (n = 6). 1 hour after injection TNF was measured in serum (*p<0,05 C57BL/6 vs SPRET/Ei).

2.3.2. Inflammation after a high dose LPS

Sepsis is characterized by an acute systemic inflammatory response and frequently dysfunction of lungs, liver, kidneys and gastrointestinal channel are observed (Tracey et al., 1986). LPS can mimic much of the devastating effects of bacterial sepsis (Ulevitch and Tobias, 1995). Nine hours after an i.p. injection of 250 μg LPS, lungs and livers were removed from C57BL/6 and SPRET/Ei mice. Histological analysis showed that in the blood vessels of the liver of C57BL/6 mice leukocytes are attached to the endothelium. Also, in the lung of C57BL/6 we note that leukocytes are attached to the endothelium and that leukocytes migrate to the alveoli. In SPRET/Ei lungs and livers much less leukocytes attach to the endothelium and leukocytes do not migrate to the alveoli (Figure III.2.2.). Recently, it has been demonstrated that IFN-β can induce the expression of a large set of chemokines (Coelho et al., 2005). In addition, we have performed a differential expression study (in collaboration with Dr. J. Grooten), using macroarray filters containing about 500 macrophage specific genes, which demonstrated that different chemokines are not or less induced in SPRET/Ei BMDMs after LPS stimulation. However, these data are still preliminary because we have tested only one RNA sample of LPS-stimulated

BMDMs (C57BL/6 vs SPRET/Ei). Possibly, the defective IFN-β induction in SPRET/Ei causes a diminished chemokine expression leading to a defective migration of leukocytes.

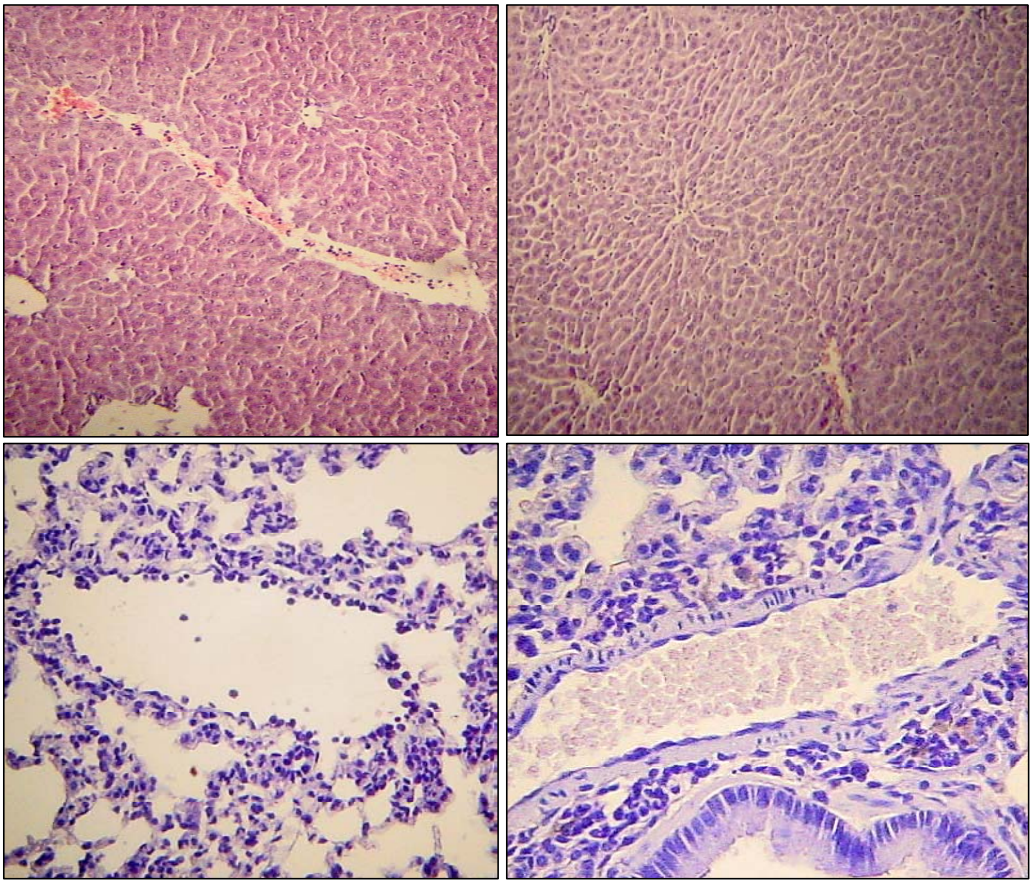

Figure III.2.2. – Liver and lung inflammation after LPS. C57BL/6 and SPRET/Ei mice were i.p. injected with 250 μg LPS. Inflammation (attachement of leukocytes to the endothelium of blood vessels) in the liver (upper panel, left) and lung (lower panel, left) of C57BL/6 mice, but not in the liver (upper panel, right) and lung (lower panel, right) of SPRET/Ei mice. In the lung of C57BL/6, leukocytes migrate to the alveoli.

Another marker for the progression of inflammation is the induction of matrix metalloproteinase (MMP)-9. Monocytes/macrophages are prominent at inflammation sites, and activation of these cells by LPS leads to the production of significant amounts of MMP-9 (Lu and Wahl, 2005). We looked at the induction of MMP-9 in serum after LPS by gelatin zymography.

MMP-9 expression in serum of SPRET/Ei and (BxS)F_1 mice is reduced compared to MMP-9 expression in serum of C57BL/6 mice after injection of 250 μg *S.a.e.* LPS (Figure III.2.3). To conclude, SPRET/Ei mice show a reduced LPS-induced inflammatory state as seen by less inflammatory cells in the lung and liver, and reduced expression of serum MMP-9, a marker for the progression of inflammation.

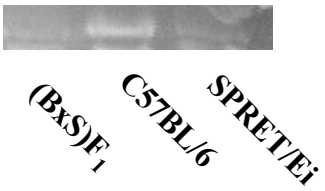

Figure III.2.3. – MMP-9 as a marker for inflammation. After an i.p. injection of 250 μg LPS, we examined the MMP-9 expression by gelatine zymography. MMP-9 expression is observed in serum of C57BL/6 mice, but much less in serum of (BxS)F_1 and SPRET/Ei mice.

2.3.3. Response of C57BL/6 and (BxS)F_1 mice to *E.coli* LPS

SPRET/Ei and (BxS)F_1 mice can resist high doses of *S.a.e.* LPS. In order to confirm that the LPS-resistance of SPRET/Ei is not specific for *S.a.e.* LPS, we tested the response of (BxS)F_1 mice to LPS derived from *Eschericiae coli*. We investigated the response of (BxS)F_1 mice to an i.p. injection of 500 μg *E.coli* LPS, a 100% lethal dose to C57BL/6 mice. (BxS)F_1 mice resist also *E.coli* LPS-induced lethal shock and hypothermia, in contrast to C57BL/6 mice. In addition, significantly less IL-6 was found 3 hours after LPS injection in serum of (BxS)F_1 mice compared to the IL-6 concentration in serum of C57BL/6 mice. Thus, hereby we confirmed the LPS hypo-responsiveness of SPRET/Ei (Figure III.2.4.).

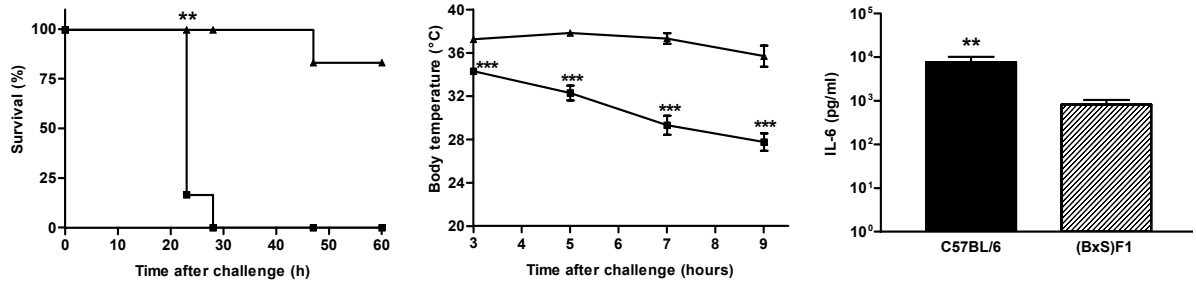

Figure III.2.4. - Response of C57BL/6 and (BxS)F_1 mice to 500 μg *E.coli* LPS. Lethality (left panel), body temperature (middle panel) and IL-6 induction (right panel) after an i.p. injection of 500 μg *E.coli* LPS in C57BL/6 and (BxS)F_1 mice. (Triangles: (BxS)F_1 (n = 6) and squares: C57BL/6 (n = 6); line bars: (BxS)F_1 (n = 9) and black bars: C57BL/6 (n = 4)) (**p<0,01 and ***p<0,001 C57BL/6 vs (BxS)F_1).

2.4. Conclusion

LPS is able to reproduce many of the features of a bacterial infection, including hypothermia, induction of circulating mediators such as pro-inflammatory cytokines and (lethal) shock (Glauser et al., 1991). We have identified a third LPS-resistant mouse strain, SPRET/Ei, next to the two known LPS-resistant

mouse strains C3H/HeJ and C57BL/10ScCr. SPRET/Ei exhibits an extreme resistance against LPS. In response to LPS, SPRET/Ei mice show a stable body temperature (in contrast to hypothermia observed in C57BL/6 mice), reduced production of pro-inflammatory cytokines such as IL-6 and TNF, reduced inflammation in the lungs and livers, and reduced expression of the inflammatory marker MMP-9 in serum. The trait is dominant, because (BxS)F_1 mice also resist a high dose of LPS. In addition, we have confirmed the hypo-responsiveness of SPRET/Ei to LPS by testing the response of (BxS)F_1 to LPS derived from *E.coli*.

We think that this extreme phenotype of SPRET/Ei mice could be very relevant in the search for new therapeutic interventions for sepsis. Therefore, it is first needed to identify the SPRET/Ei LPS-resistance genes.

3. *IN VITRO* DATA

3.1. Introduction

Macrophages and DCs are the first target cells of LPS and respond by expressing many inflammatory cytokines (Guha and Mackman, 2001). Mouse embryonic fibroblasts (MEFs) also express high levels of TLR4 mRNA. Fibroblast cells participate in inflammatory processes and non-specific immunity by producing cytokines and mediators in response to bacterial LPS (Kurt-Jones et al., 2004). Through bone marrow transplantation experiments (see PNAS article) we demonstrated that white blood cells (WBCs), and most likely macrophages, are the relevant cell type in the LPS-resistance of SPRET/Ei. Lethally irradiated (BxS)F_1 mice were reconstituted with bone marrow from C57BL/6 or (B x S)F_1 mice. Due to transplant rejection, we could not reconstitute lethally irradiated C57BL/6 mice with (BxS)F_1 bone marrow. (BxS)F_1 mice reconstituted with C57BL/6 bone marrow were sensitive to 250 μg LPS, a 100% lethal dose for C57BL/6 mice.

Next to the LPS-resistance *in vivo*, we could demonstrate the LPS hypo-responsiveness of SPRET/Ei *in vitro* in macrophages and MEFs. LPS activates both the MyD88-dependent pathway and the MyD88-independent TRIF-dependent pathway to induce pro-inflammatory cytokines. MyD88 induces early NF-κB activation, while TRIF induces late NF-κB activation. Recruitment of the adaptor protein TRIF leads also to induction of IFN-β and IFN-dependent genes (Akira and Takeda, 2004). In order to examine the MyD88-dependent pathway in SPRET/Ei we looked at the degradation of IκBα, which leads to release of the transcription factor NF-κB, and the phosphorylation of MAPK p38 and JNK, which activate the transcription factor AP-1. Pro-inflammatory gene induction depends on both NF-κB and AP-1. To investigate if the TRIF-dependent pathway is normal in SPRET/Ei, we measured the induction of IFN-β mRNA.

3.2. Experimental procedures

3.2.1. *In vitro* experiments: IL-6 concentration after LPS in supernatant

Thioglycolate-elicited peritoneal macrophages from C57BL/6 and SPRET/Ei mice were seeded at 50.000 cells per 24-well in RPMI medium 1640 containing 10% foetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 5 10^{-5} M β-mercaptoethanol, 1 mM sodium pyruvate and 2 mM L-

glutamine. LPS was added to the cultures to a final concentration of 1 μg/ml. After 24 hours of stimulation, supernatant was harvested and IL-6 was measured by using the 7TD1 assay.

MEFs were isolated from 18-day old embryos and cultured in RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin, gentamycin and L-glutamine. MEFs were seeded at 100.000 cells per 24-well in supplemented RPMI medium 1640. LPS was added to the cultures to a final concentration of 1 μg/ml. After stimulation, supernatant was harvested and IL-6 was measured by using the 7TD1 assay.

3.2.2. Derivation of macrophages from bone marrow cells

To derive macrophages from bone marrow cells we carried out the next protocol. Bone marrow cells were isolated from tibias and femurs of 3 month old C57BL/6 and SPRET/Ei mice. Cells from one mouse are collected in 50 ml DMEM medium (high glucose) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1mM sodium pyruvate and 20 ng/ml mouse recombinant M-CSF. M-CSF is a potent hematopoietic factor and is a key regulator of cellular proliferation, differentiation, and survival of blood monocytes, tissue macrophages and their progenitor cells. Bone marrow cells collected in 50 ml medium were grown in two bacterial Petri dishes. Cells were kept in culture for 7 days and every 2 days the medium, supplemented with growth factors, was changed. After 7 days cells could be used for experiments. For C57BL/6 up to 20 million bone marrow-derived cells could be obtained, while for SPRET/Ei a maximum of 10 million bone marrow-derived cells was counted.

3.2.3. Real-time Q-PCR analysis

The IL-6, TNF-α and IFN-β mRNA levels were analyzed by real-time Q-PCR in triplicate cultures (500.000 cells per 6-well) after stimulation of BMDMs with 100 ng/ml LPS. In addition, the IFN-β mRNA level was also analyzed after stimulation with 10 μg/ml poly (I:C), a TLR3 ligand. Total RNA was isolated using the RNAwiz reagent (Ambion). Complementary DNAs (cDNAs) were synthesized using the Superscript II reverse transcriptase system (Invitrogen). The cDNA equivalent of 0.2 μg of total RNA was amplified through 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. Output was monitored using SYBR Green core reagents and the ABI Prism 7700 System (PE Applied Biosystems). The results were normalized to the level of cyclophilin mRNA.

3.2.4. Immunoblot analysis

Whole cell, cytoplasmic, and nuclear extracts for immunoblot analysis were prepared and subjected to SDS-PAGE. Proteins transferred to nitrocellulose membrane were probed with rabbit antiserum against NF-κB p65, phospho-Stat1 and Stat1, and the immune complexes were visualized with the ECL Western blot reagent (Pierce).

3.3. Results

3.3.1. Response of peritoneal macrophages and MEFs to LPS

Bone marrow transplantation experiments revealed the importance of WBCs, and most likely macrophages, in the LPS-resistance of SPRET/Ei. To test the response of macrophages *in vitro* to LPS, we stimulated thioglycolate-elicited peritoneal macrophages with 1 μg/ml LPS and we measured IL-6 concentration in supernatant. In addition, the response of immortalized (with SV40 LargeT) MEFs to LPS was also tested.

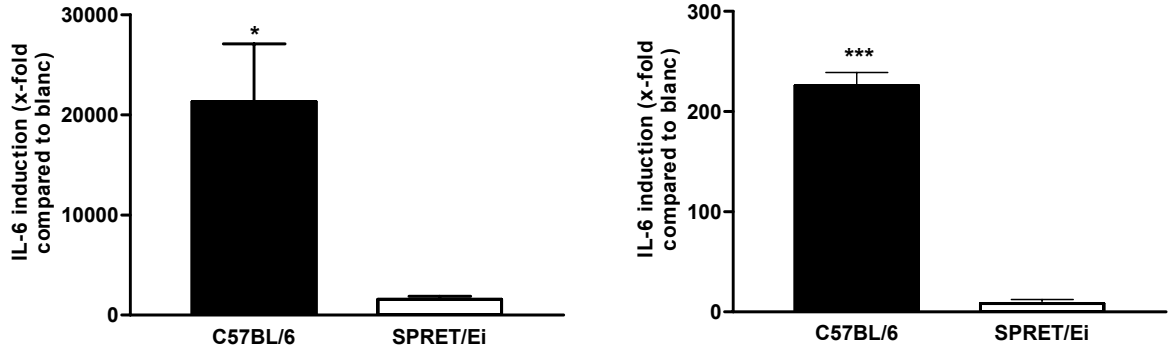

Figure III.3.1. – IL-6 induction *in vitro* after 24 hours of LPS stimulation. Thioglycolate-elicited peritoneal macrophages (left figure; n = 3) and MEFs (right figure; n = 5) from C57BL/6 and SPRET/Ei were seeded at respectively 50.000 and 100.000 cells/well and were stimulated with 1 μg/ml LPS. 24 hours after stimulation, supernatant was harvested and IL-6 was measured using the 7TD1 assay (*p<0,05 and ***p<0,001 C57BL/6 vs SPRET/Ei).

Both thioglycolate-elicited peritoneal macrophages and MEFs from SPRET/Ei induce significantly less IL-6 compared to thioglycolate-elicited peritoneal macrophages and MEFs from C57BL/6 (Figure III.3.1.). This reduced IL-6 induction is in line with the reduced IL-6 serum levels after injection of LPS in SPRET/Ei and with the elevated IL-6 levels after LPS injection in (BxS)F_1 mice reconstituted with

C57BL/6 bone marrow. However, relatively high basal levels of IL-6 were measured in thioglycolate-elicited macrophages and MEFs from both C57BL/6 and SPRET/Ei mice. Probably, the thioglycolate already stimulates macrophages to induce IL-6. In contrast, in non-stimulated BMDMs from C57BL/6 and SPRET/Ei no IL-6 was detectable. For that reason, and also the fact that it was very difficult to isolate thioglycolate-elicited macrophages from SPRET/Ei mice, we decided to use BMDMs for our *in vitro* experiments.

3.3.2. The MyD88-dependent pathway is normal in SPRET/Ei

To investigate the mechanism of the LPS-resistance, we used BMDMs as an *in vitro* model. LPS stimulation leads to recruitment of the adaptor molecule MyD88 to the TIR domain of TLR4 and is followed by the activation of NF-κB, which induces pro-inflammatory genes such as *Il6* and *Tnf*. To release NF-κB, first the IκB protein sequestering NF-κB in the cytoplasm has to be phosphorylated by the IKK complex and degraded (Akira and Takeda, 2004). LPS also induces MAPK p38 and JNK, which activate the transcription factor AP-1 (Akira and Takeda, 2004; Guha and Mackman, 2001). Both IL-6 and TNF induction are dependent on NF-κB, but also on other transcription factors such as nuclear factor IL-6 (NF-IL6) and AP-1 (Akira et al., 1990; Dendorfer et al., 1994; Guha et al., 2001).

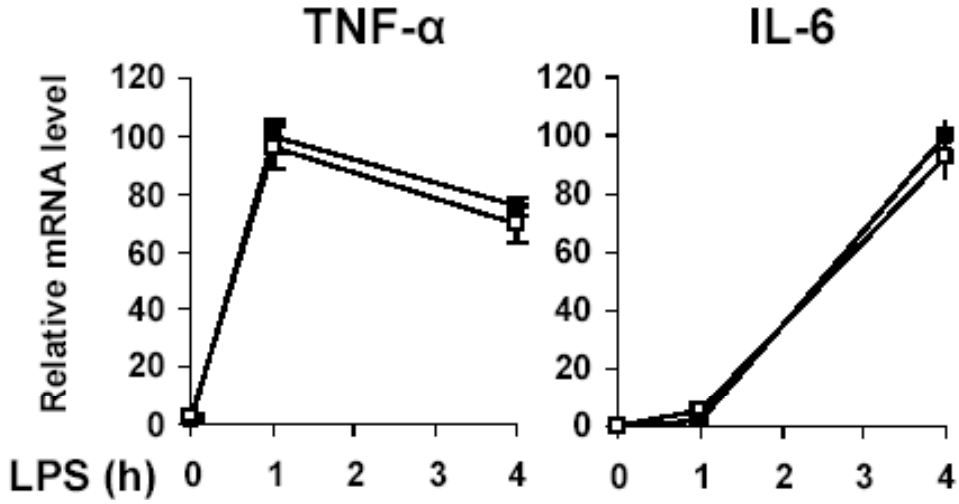

Figure III.3.2. – TNF-α and IL-6 mRNA induction in BMDMs after LPS stimulation. BMDMs of C57BL/6 and SPRET/Ei mice were stimulated with 100 ng/ml LPS for 1 and 4 hours. TNF-α and IL-6 mRNA levels were analyzed by real-time Q-PCR in triplicate cultures (Black squares, C57BL/6; white squares, SPRET/Ei).

IκBα degradation and phosphorylation of MAPKs p38 and JNK1/2 was shown to occur with approximately the same kinetics in SPRET/Ei BMDMs as in C57BL/6 BMDMs after stimulation with 100 ng/ml LPS (see PNAS article). Furthermore, the intact MyD88 signalling was confirmed by studying the induction of TNF-α and IL-6 mRNA. BMDMs from C57BL/6 and SPRET/Ei mice were stimulated with 100 ng/ml LPS for 1 and 4 hours. Induction of IL-6 and TNF-α mRNA after LPS was comparable between SPRET/Ei and C57BL/6 BMDMs (Figure III.3.2.). Also, no significant differences were observed between SPRET/Ei and C67BL/6 with respect to LPS-induced mRNA expression of *Il1a*, *Il1b*, *Il12a*, *Il12b*, *Bcl2*, *Birc4* (encoding XIAP) and *Tnfaip3* (encoding A20).

TLR4 can induce an early phase NF-κB induction through activation of the MyD88-dependent pathway, but it can also induce a late phase NF-κB activation by the TRIF-dependent pathway (Akira and Takeda, 2004). TRAF6 and TBK1/IKK-ε compete with each other for binding with the N-terminal portion of TRIF. When TRIF recruits TRAF6 this leads to a second wave of pro-inflammatory cytokine production. When TRIF recruits TBK1/IKK-ε this leads to expression of IFN-β and IFN-dependent genes (Sato et al., 2003). We also wanted to investigate the production of pro-inflammatory cytokines at an early (4 hours) and at a late (24 hours) time point after LPS stimulation. It has been demonstrated that TRIF knockout macrophages have diminished TNF-α, IL-6 and IL-12p40 production after 24 hours of LPS stimulation (Yamamoto et al., 2003).

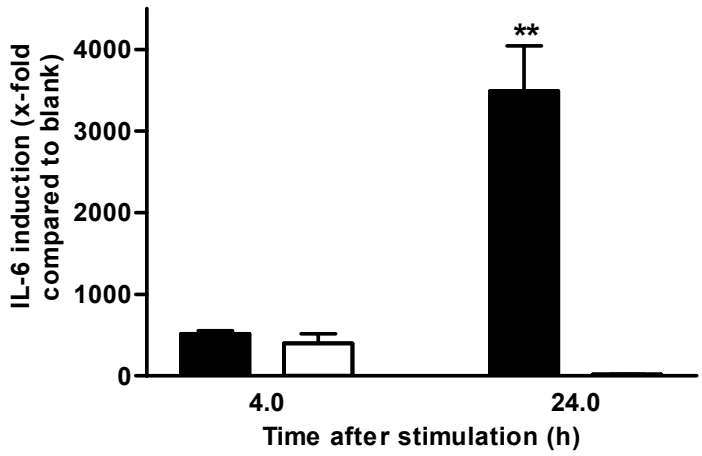

Figure III.3.3. – Early and late induction of IL-6 after LPS stimulation. MEFs from C57BL/6 and SPRET/Ei mice were stimulated with 1 μg/ml LPS for 4 and 24 hours. IL-6 induction was analyzed by a 7TD1-bioassay, and expressed as relative induction compared to non-stimulated control cells (black bars: C57BL/6 (n = 3) and white bars: SPRET/Ei (n = 3)) (**p<0,01 C57BL/6 vs SPRET/Ei).

MEFs were used instead of BMDMs because during my work SPRET/Ei mice were not easily available since they are very poor breeders. MEFs were stimulated with 1μg/ml LPS. IL-6 was measured in supernatant after 4 and 24 hours stimulation. As demonstrated before, IL-6 fold induction is significantly different between C57BL/6 and SPRETEi MEFs 24 hours after LPS stimulation. In contrast, early pro-inflammatory cytokine production is comparable between C57BL/6 and SPRET/Ei (Figure III.3.3.). Thus, the early MyD88-dependent pro-inflammatory cytokine production seems intact in SPRET/Ei, while late MyD88-independent, TRIF-dependent pro-inflammatory cytokine is defective. These results show that early IL-6 release is normal in SPRET/Ei, and indirectly confirm the data obtained from the BMDM.

3.3.3. The MyD88-independent, TRIF-dependent pathway is defective in SPRET/Ei

To investigate if the MyD88-independent, TRIF-dependent pathway in SPRET/Ei is intact, BMDMs from C57BL/6 and SPRET/Ei were stimulated with LPS for 1 and 4 hours to analyze IFN-β mRNA induction. IFN-β mRNA induction is defective in SPRET/Ei BMDMs (see PNAS article). In addition, the IFN-β mRNA level was also analyzed by real-time Q-PCR after stimulation of BMDMs with 100 ng/ml LPS and 10 μg/ml poly (I:C), a TLR3 ligand, for 4 hours. TLR3 is the only TLR, which does not use the adaptor protein MyD88, but only recruits TRIF to induce IFN-β and IFN-dependent genes (Moynagh, 2005). BMDMs of SPRET/Ei induce significantly less IFN-β mRNA in response to poly (I:C) compared to C57BL/6 BMDMs. IFN-β stimulation after poly (I:C) is stronger than after LPS stimulation (Figure III.3.4.).

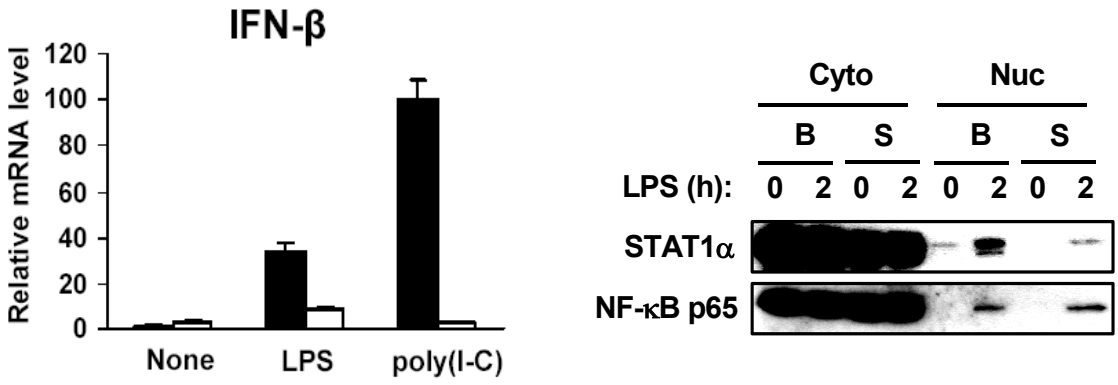

Figure III.3.4. – IFN-β mRNA induction after LPS and poly (I:C) stimulation and nuclear translocation of STAT1 after LPS. Left figure: The IFN-β mRNA level in C57BL/6 and SPRET/Ei BMDMs was analyzed by real-time Q-PCR in triplicate cultures after stimulation with 100 ng/ml LPS and 10 μg/ml poly (I:C) for 4 hours (Black bars, C57BL/6; white bars, SPRET/Ei). Right figure: Western Blot to look at STAT1α and NF-κB p65 translocation to the nucleus 2 hours after stimulation with 100 ng/ml LPS.

As a consequence of a defective IFN-β production in SPRET/Ei, less STAT1α is phosphorylated (PNAS article). The phosphorylation of STAT1 results in dimerization and translocation to the nucleus to activate gene transcription (Imada and Leonard, 2000). Thus, because of this lack of phosphorylated STAT1α, there is also a defect in nuclear translocation of STAT1α. Nuclear translocation of NF-κB p65 is normal in SPRET/Ei (Figure III.3.4).

3.4. Conclusion

The hypo-responsiveness of SPRET/Ei to LPS is not only evident *in vivo*, but also *in vitro* in macrophages and MEFs. By using BMDMs we demonstrated that the MyD88-dependent pathway leading to IκB degradation and phosphorylation of MAPK p38 and JNK1/2 is intact in SPRET/Ei. This was confirmed by comparable induction of IL-6 and TNF-α mRNA after LPS. However, the TRIF-dependent late IL-6 induction and the IFN-induction are defective in SPRET/Ei. Thus, early pro-inflammatory gene induction seems intact, while IFN-β and late pro-inflammatory gene induction is defective. The recruitment of TRAF6 to TRIF leads to activation of NF-κB and AP-1 and subsequently a second wave of pro-inflammatory cytokine production. On the other hand recruitment of TBK1/IKK-ε to TRIF leads to induction of IFN-β and IFN-dependent genes (Figure III.3.5.).

So, our results suggest a defect in the adaptor molecule TRIF. However, we have only demonstrated that degradation of IκB is normal in SPRET/Ei and this only at early time points after LPS stimulation. IκB degradation and phosphorylation of MAPK does not prove that NF-κB and AP-1 activation is intact. Therefore, we must do an electrophoretic mobility shift assay (EMSA). In addition, it is also still possible that other dimers, such as p50/p50 NF-κB homodimers lacking transactivation activity, are formed in SPRET/Ei instead of the transcriptionally active p50-p65 heterodimer. Formation of p50/p50 homodimers leads to less TNF and a decrease in the levels of NO after LPS (Goldring et al., 1998). The transcription factors that are known to be involved in the control of the IL-6 and TNF transcription rate are AP-1, nuclear factor IL-6 (NF-IL6) or CCAAT/enhancer binding protein (C/EBP), and NF-κB (Dendorfer et al., 1994). Thus, another possibility is a defect in the transcription factor NF-IL-6. In addition, at this stage there remain several candidate molecules for the defect in IFN-β production and in late phase NF-κB activation (see Figure III.3.5.).

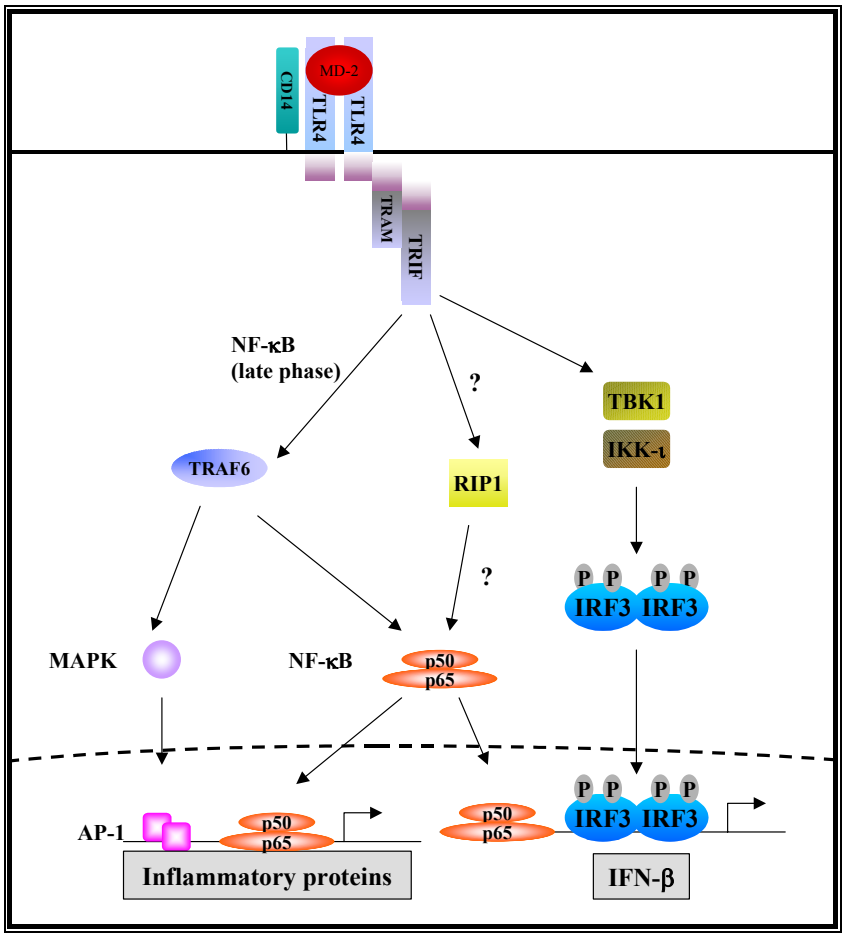

Figure III.3.5. – Candidate molecules at this stage. TLR4 activates the TRIF-dependent pathway to induce late induction of inflammatory proteins and to induce IFN-β. At this stage there are still multiple candidate molecules that can cause a defect in IFN-β induction and in late phase NF-κB activation.

4. IMPORTANCE OF IFN-β IN THE LPS RESISTANCE

4.1. Introduction

SPRET/Ei not only has a defective IFN-β induction *in vitro,* but also *in vivo* the induction of IFN-β by LPS or *Influenza* virus is very low. Probably due to the defective induction of IFN-β, SPRET/Ei mice are completely resistant to *Listeria monocytogenes* and highly sensitive to *Leishmania major* infection (see PNAS article). Deletion of the *Ifnb* gene (Karaghiosoff et al., 2003) or genes involved in the induction of (e.g. IRF-3) (Sakaguchi et al., 2003), or in the response to (e.g. Tyk2) (Karaghiosoff et al., 2003), type I IFNs can induce resistance to endotoxemia. These observations indicate that type I IFN is essential in LPS-induced endotoxemia. Next, we wanted to investigate the importance of IFN-β in the LPS-resistance of SPRET/Ei.

4.2. Results

4.2.1. The LPS-resistance can be reverted by administration of IFN-β

SPRET/Ei mice are extremely resistant to the lethal effects of LPS, and are defective in IFN-β induction *in vitro* and *in vivo*. We reasoned that pre-treatment with IFN-β would restore LPS sensitivity to SPRET/Ei mice. Indeed, we found that (BxS)F_1 mice can be sensitized for LPS by pre-treatment with IFN-β. Two hours before injection of a lethal dose of 200 μg LPS, we pre-treated (B x S)F_1 mice with 20 μg IFN-β. All of the pre-treated (B x S)F_1 mice died, but all of the control (B x S)F_1 mice survived. All of the control C57BL/6 mice also died after injection of 200 μg of LPS (Figure III.4.1.). So, this suggests that IFN-β is a critical factor in the resistance of SPRET/Ei mice to LPS.

4.3. Conclusion

Non-viral PAMPs, such as LPS, induce expression of the *Ifnb* gene through a TRIF-dependent pathway (Yamamoto et al., 2002). Although IFN-β has been known as a potent antiviral molecule for a long time, IFN-β also mediates a variety of immune regulatory effects and may be considered as an important link between innate and adaptive immune responses (Smith et al., 2005). In the last few years, a critical role for IFN-β has been observed in LPS-induced endotoxemia. The mechanism by which IFN-β mediates

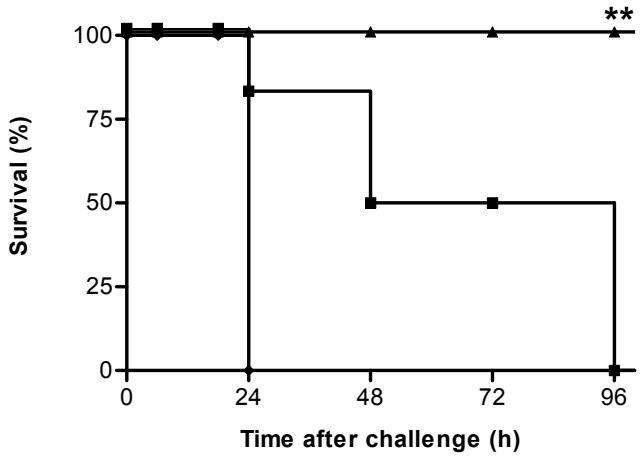

Figure III.4.1. – The LPS-resistance is reverted by exogenous IFN-β administration. Two hours before an i.p. injection of 200 μg LPS, we pre-treated $(BxS)F_1$ mice with 20 μg IFN-β. All of the pre-treated $(BxS)F_1$ mice died, but all of the control $(BxS)F_1$ mice survived. All of the control C57BL/6 mice also died after injection of 200 μg of LPS. (Squares: (C57BL/6 (n = 10), triangles: $(BxS)F_1$ (n = 6) and circles: $(BxS)F_1$/IFN (n = 4)) (**p<0,01 $(BxS)F_1$ vs $(BxS)F_1$/IFN).

LPS is still an open question, but several studies have clearly shown that IFN-β is able to induce the expression of a large set of chemokines and genes encoding other inflammatory molecules such as NF-IL6 (Coelho et al., 2005; Der et al., 1998). So, a possible mechanism by which IFN-β mediates LPS, is the induction of chemokines. Expression of chemokines attracts leukocytes to the inflammatory sites. The defective IFN-β induction in SPRET/Ei might cause a defect in chemokine production and subsequently in migration of leukocytes to the inflammatory sites. Indeed, in lungs of LPS-injected SPRET/Ei mice leukocytes do not migrate to the alveoli and in lungs and livers of LPS-injected SPRET/Ei mice less leukocytes are attached to the endothelium. Also, preliminary data from a differential mRNA expression study demonstrated that several chemokines are not or less induced in SPRET/Ei BMDMs compared to C57BL/6 BMDMs after LPS stimulation.

We found that the resistance of SPRET/Ei to LPS can be reverted by administration of exogenous IFN-β, suggesting that IFN-β is an important factor in the LPS-induced lethality. However, IFNAR1 knockout mice, protected against a lethal dose of LPS, show a drop in body temperature (less than 32°C 22 h after LPS injection). In contrast, SPRET/Ei and $(BxS)F_1$ mice are protected against LPS-induced hypothermia. Therefore, to explain the extreme LPS-resistance of SPRET/Ei we assume that other genes than the *Ifnb* gene play a role in the LPS-resistance of SPRET/Ei mice. These results confirm the data from the backcross demonstrating that the LPS-resistance of SPRET/Ei is a complex trait, involving multiple genes. To find these other LPS-resistance genes, we will first repeat the differential expression

study to investigate the differential expression of macrophage specific genes between C57BL/6 and SPRET/Ei after LPS stimulation. In addition, we will also perform a differential expression study after poly (I:C) stimulation since SPRET/Ei mice have also a defect in IFN-β production in response to poly (I:C). We will also study more than one time point after LPS and poly (I:C) stimulation. We hope to decrease the amount of candidate genes by combining the data from the differential expression studies. Also, we intend to use the bioinformatica program 'Difference Distance Matrix Analysis' to find a limited amount of transcription factors/genes that might be responsible for the differential expression of SPRET/Ei genes.

5. WHAT CAUSES THIS DEFECTIVE IFN-β PRODUCTION?

5.1. Introduction

IFN-β is induced upon TLR4 stimulation using the MyD88-independent, but TRIF-dependent pathway. Two kinases, TBK1 and IKK-ε, are recruited to TRIF to phosphorylate and activate the transcription factor IRF-3 (Sharma et al., 2003). IRF-3 can induce IFN-β expression, but need co-activators CBP/p300 to induce transcription (Taniguchi and Takaoka, 2002). On the other hand, IFN-β can stimulate, in an autocrine or paracrine way, the IFNAR receptor complex to produce massive IFN-β through a positive-feedback loop involving IRF-7 (Sato et al., 1998). In addition, the IFN-β promoter is post-transcriptionally regulated by two destabilizing sequences - AU-rich element in the 3' UTR and the coding region instability domain (CRID) - as well as regulated by various positive and negative regulators (Harada et al., 1989; Nourbakhsh and Hauser, 1997; Paste et al., 2003; Weill et al., 2003). The defective IFN-β induction can be situated at level of the IFN-β promoter or its regulators (1), the TRIF-dependent pathway (2) or the positive-feedback loop induced after IFN-β signalling (3) (see Figure III.5.1).

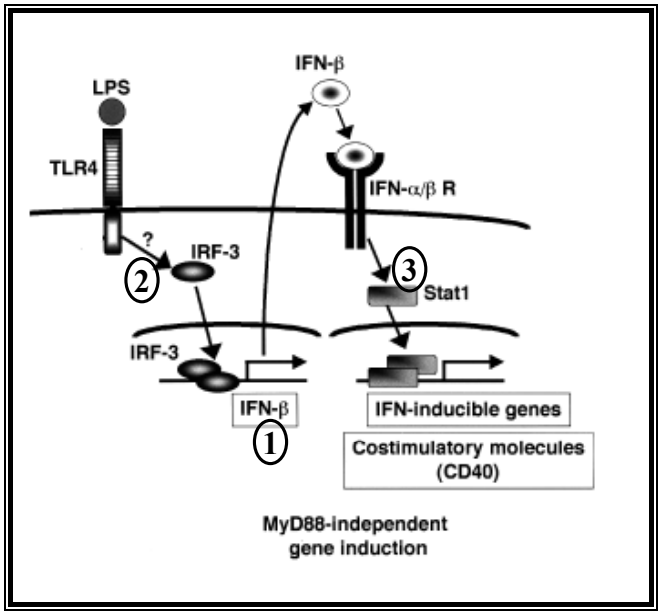

Figure III.5.1. – The defect in IFN-β production can be situated at three levels. TLR4 stimulation activates the TRIF-dependent pathway to activate the transcription factor IRF-3 which induces IFN-β. IFN-β stimulates, in an autocrine or paracrine way, the IFNAR receptor to induce IFN-dependent genes and co-stimulatory molecules (Reprinted from (Sato et al., 1998).

First, we wanted to sequence the IFN-β promoter of SPRET/Ei and C57BL/6 to find possible nucleotide changes between these two strains. If no nucleotide changes are found in the promoter of SPRET/Ei, the defect can be due to a defective activation of the IRF-3 transcription factor. IRF-3 is activated by the kinases TBK1 and IKK-ε, which are recruited to the adaptor molecules TRIF and TRAM after LPS stimulation. IFN-β mRNA induction by poly (I:C), a TLR3 ligand, is also compromised, indicating that TRIF-dependent, but not TRAM-dependent, responses can be defective in SPRET/Ei BMDMs. In case of a defective IRF-3 phosphorylation, molecules of the TRIF-dependent pathway such as TRIF, TBK1/IKK-ε and IRF-3 should be investigated at sequence and expression level. If the activation of IRF-3 is normal in SPRET/Ei, we wanted to investigate if the positive-feedback loop after IFN stimulation is still intact in SPRET/Ei by looking at the mRNA levels of the IFN-dependent gene *Oas* and the IFNAR1 receptor.

5.2. Experimental procedures

5.2.1. Sequencing

For the sequencing of the IFN-β promoter from SPRET/Ei and C57BL/6, genomic DNA was extracted from tail and the region encoding the IFN-β promoter was PCR amplified, isolated from gel using the 'QIAEX II Agarose Gel Extraction' kit (Qiagen, Germany) and ligated into pGEM-T vector for sequencing. The sequences were assembled and analyzed using the DNAstar software program.

For the sequencing of the serine/threonine cluster in IRF-3 of SPRET/Ei and C57BL/6, total RNA was extracted from snap-frozen livers using the 'Qiagen RNeasy® Mini' kit and was reverse transcribed using the 'Superscript first-strand synthesis for RT-PCR' kit (Invitrogen N.V.). The cDNA encoding the serine/threonine cluster of IRF-3 was PCR amplified, isolated from gel using the 'QIAEX II Agarose Gel Extraction' kit (Qiagen, Germany) and ligated into pGEM-T vector for sequencing. The sequences were assembled and analyzed using the DNAstar software program.

5.2.2. Immunoblot analysis

Cytoplasmic extracts for immunoblot analysis were prepared and subjected to SDS-PAGE. Proteins transferred to nitrocellulose membrane were probed with rabbit antiserum against IRF-3 (Zymed) and

the immune complexes were visualized with the ECL Western blot reagent (Pierce). To analyze the phosphorylation of IRF-3, BMDMs were stimulated with 100 ng/ml LPS for 30 min.

5.2.3. Real-time Q-PCR analysis

The TNF-α, IL-6, IFN-β, CXCL10, ISG15 and RANTES mRNA levels were analyzed by real-time Q-PCR in triplicate cultures (500.000 cells per 6-well) after stimulation of BMDMs with 100 ng/ml LPS. Total RNA was isolated using the RNAwiz reagent (Ambion). Complementary DNAs (cDNAs) were synthesized using the Superscript II reverse transcriptase system (Invitrogen). The cDNA equivalent of 0.2 μg of total RNA was amplified through 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. Output was monitored using SYBR Green core reagents and the ABI Prism 7700 System (PE Applied Biosystems). The results were normalized to the level of cyclophilin mRNA.

5.2.4. *In vitro*: determining IL-6 concentration after IFN

BMDMs were seeded at 100.000 cells per 24-well in supplemented DMEM medium. IFN was added to the cultures to a final concentration of 100 U/ml. The supernatant was harvested after 6 hour of stimulation and IL-6 was measured by using the 7TD1 assay.

5.2.5. Semi-quantitative RT-PCR

Total RNA was isolated from BMDMs using the RNeasy Mini Kit (Qiagen). cDNAs were synthesized using the Superscript II reverse transcriptase system (Invitrogen). The cDNA equivalent of 0.1 μg of total RNA was amplified using primers for OAS. The results were normalized to the level of β-actin.

5.3. Results

5.3.1. Defect in the IFN-β promoter?

The murine IFN-β promoter, spanning from the TATA box (at position –34) to position –210, contains four positive regulatory units binding NF-κB/Rel family members (PRDII), ATF-2 homodimers or c-Jun/ATF-2 heterodimers and IRF family members, and contains also two negative regulatory units (Du et al., 1993) (Figure III.5.2.). In addition to these negative regulatory units, two destabilizing sequences

are found in the IFN-β promoter: an AU-rich element (ARE) in the 3' UTR, and a second element called CRID in the 3' end of the coding region.

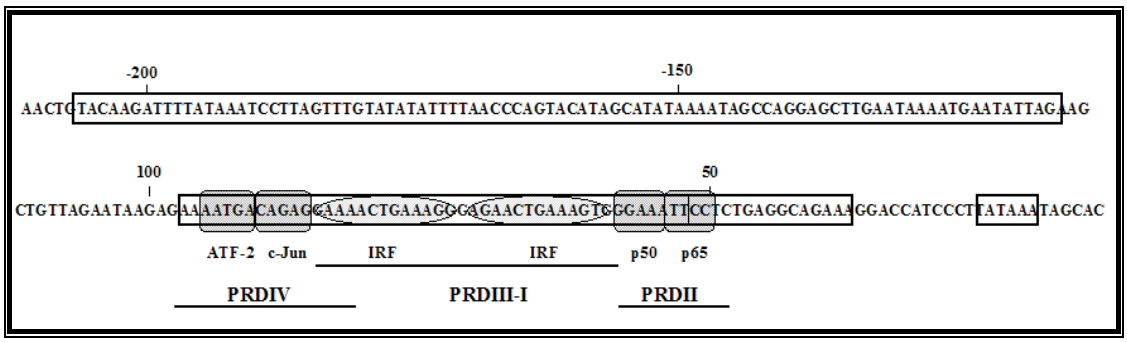

Figure III.5.2. – IFN-β promoter elements. The DNA sequence of the murine IFN-β promoter is spanning from the TATA box to position –210. The DNA binding sites of transcription factors NF-κB (p50 and p65), IRF proteins, and ATF-2/c-Jun are indicated.

We wanted to sequence the IFN-β promoter to find possible nucleotide changes between the C57BL/6 and SPRET/Ei IFN-β promoter. A lot of polymorphisms are found between SPRET/Ei and C57BL/6, because they diverged from each other about 1,5 million years ago (Guenet and Bonhomme, 2003). Therefore, frequently nucleotide changes are found between SPRET/Ei and C57BL/6.

We sequenced the 210 bp region of the IFN-β promoter and some additional nucleotides before and after this 210 bp region. The sequences do not show any nucleotide change between SPRET/Ei and C57BL/6 (Figure III.5.3). So, it demonstrates that the IFN-β promoter is a conserved part of the genome.

5.3.2. Defect in the TRIF-dependent pathway?

The defective IFN-β induction in SPRET/Ei can be caused by a defect in the TRIF-dependent pathway leading to activation of the transcription factor IRF-3. The IFN-β promoter contains IRF-binding sites and its induction is dependent on functional cooperation between IRF-3, NF-κB and ATF-2 homodimers or c-Jun/ATF-2 heterodimers (Du et al., 1993). The activation domains of all the activators are necessary to form a novel surface that constitutes a high-affinity binding site for the recruitment of a complex, namely GCN5/PCAF, important in the induction of the IFN-β promoter. IRF-3 is present in the cytoplasm of non-stimulated cells. Intramolecular association between the C terminus and the DNA-binding domain (DBD) maintains IRF-3 in a latent state by masking both DBD and (IRF association domains) IAD regions.

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B  TTTGAGAGTTCTTTTATCTTCAGGGCTGTCTCCTTTCTGTTCTTCTCTCCTGGATATTCT  60
S  TTTGAGAGTTCTTTTATCTTCAGGGCTGTCTCCTTTCTGTTCTTCTCTCCTGGATATTCT  60

B  CTTCCTTTGCTCCAGCAATTGGTGAAACTGTACAAGATTTTATAAATCCTTAGTTGTATA  120
S  CTTCCTTTGCTCCAGCAATTGGTGAAACTGTACAAGATTTTATAAATCCTTAGTTGTATA  120

B  TATTTTAACCCAGTACATAGCATATAAAATAGCCAGGAGCTTGAATAAAATGAATATTAG  180
S  TATTTTAACCCAGTACATAGCATATAAAATAGCCAGGAGCTTGAATAAAATGAATATTAG  180

B  AAGCTGTTAGAATAAGAGAAAATGACAGAGGAAAACTGAAAGGGAGAACTGAAAGTGG  238
S  AAGCTGTTAGAATAAGAGAAAATGACAGAGGAAAACTGAAAGGGAGAACTGAAAGTGG  238

B  GAAATTCCTCTGAGGCAGAAAGGACCATCCCTTATAAATAGCACAGGCCATGAAGGAA  296
S  GAAATTCCTCTGAGGCAGAAAGGACCATCCCTTATAAATAGCACAGGCCATGAAGGAA  296

B  GATCATTCTCACTGCAGCCTTTGACAGCCTTTGCCTCATCTTGCAGGTAGCAGCCGAC  354
S  GATCATTCTCACTGCAGCCTTTGACAGCCTTTGCCTCATCTTGCAGGTAGCAGCCGAC  354

B  ACCAGCCT  362
S  ACCAGCCT  362
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Figure III.5.3. – Sequences of the C57BL/6 and SPRET/Ei IFN-β promoter region. No nucleotide changes between the C57BL/6 and SPRET/Ei IFN-β promoters. In blue is the promoter region containing all the PRD and NRD elements of the IFN-β promoter.

Stimulation of TLR3 or TLR4 leads to phosphorylation of the serine/threonine cluster in the C-terminal region of IRF-3, which leads to conformational changes of IRF-3 that relieve C-terminal autoinhibition and exposes the DBD and IAD domains. IRF-3 can now homodimerize, translocate to the nucleus and bind to the IFN-β promoter. The presence of a nuclear export signal (NES) shuttles IRF-3 back to the cytoplasm and terminates the response (Moynagh, 2005). Recently, Ser386 was recognized as the primary phosphorylation-site. This study provides an explanation for the TRIF activation of IRF-3, because both TBK1 and IKK-ε can phosphorylate IRF-3 at Ser386. Stimulation of TLR3 and TLR4 by dsRNA and LPS eventually leads to phosphorylation of this residue. The first serine of the 2S-site is important in the recognition of the IRF-3 kinases TBK1 en IKK-ε, while the second serine is the critical phosphorylation-site of IRF3. The 5ST-site might have a helping role with the phosphorylation of IRF-3 (Mori et al., 2004). We sequenced the serine/threonine cluster in the C-terminal region of the *irf3*-gene.

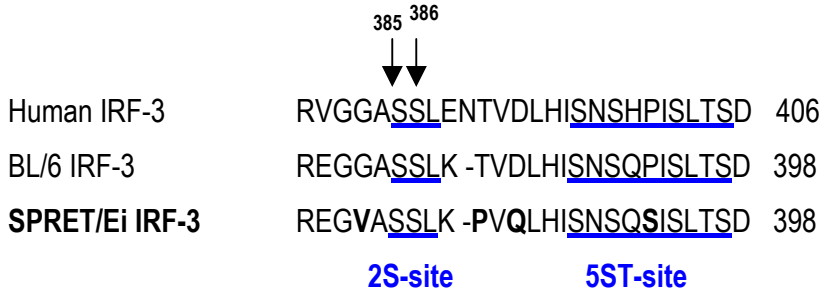

Figure III.5.4. – Amino acid changes in SPRET/Ei in the serine/threonine cluster of IRF-3. 4 unique AA changes are found in the serine/threonine cluster of IRF-3.

We found 4 unique amino acid changes in the serine/threonine cluster of IRF-3 in SPRET/Ei (see Figure III.5.4 and Table III.5.1.). The two serines of the 2S-site of IRF-3 are unchanged in the 2S-site of SPRET/Ei IRF-3. In the 5ST-site a proline to serine change is found (S392P). This might have an influence on the phosphorylation of the 2S-site. The serines and the threonine of the 5ST-site are intact. Before the 2ST-site and between the 2ST- and 5ST-site three amino acid changes were found (V376G, P382T and Q384D) (Table III.5.1.). The importance of these mutations is not known yet. However, all 4 amino acid changes are unique for SPRET/Ei, when comparing the sequences across different species. Possibly, these amino acid changes can have an influence on the accessibility of the serine/threonine cluster for its kinases. It should be kept in mind that the serine/threonine cluster was sequenced only once, so first the 4 AA changes in SPRET/Ei need to be confirmed.

Table III.5.1. – Overview of amino acid changes found in the SPRET/Ei serine/threonine cluster of IRF-3. Sequences are compared across different species. All 4 amino acids are unique for SPRET/Ei.

Position	C57BL/6	SPRET/Ei	*Mus musculus*	*Homo sapiens*	*Canis familiaris*	*Bos taurus*
376	G	V	G	G	G	G
382	T	P	T	T	T	T
384	D	Q	D	D	D	D
392	P	S	P	P	P	P

At the same moment we had data demonstrating that IRF-3 activation was intact in SPRET/Ei. We tried to visualize IRF-3 phosphorylation after 30 minutes 100 ng/ml LPS stimulation in SPRET/Ei and C57BL/6 BMDMs through Western Blot. IRF-3 phosphorylation after LPS stimulation in BL6 BMDMs is not so clear due to a blot artefact, but IRF-3 becomes phosphorylated in SPRET/Ei BMDMs after LPS as seen by the higher band (Figure III.5.5.). In the future, we will surely repeat this experiment.

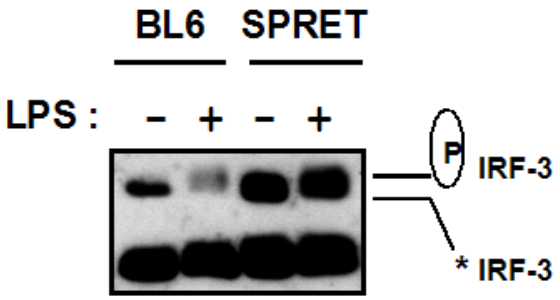

Figure III.5.5. – Phosphorylation of IRF-3 in BMDMs after LPS stimulation. Western Blot to look at IRF-3 phosphorylation in C57BL/6 and SPRET/Ei BMDMs 30 minutes after stimulation with 100 ng/ml LPS.

In addition, Jin Mo Park, with whom we closely collaborated, showed that IRF-3 knockout BMDMs have a broader gene defect than SPRET/Ei BMDMs. IRF-3-knockout macrophages are defective in their production of IFN-β, CCL5/RANTES, CXCL10/IP10 and ISG15 mRNA, while SPRET/Ei BMDMs are only defective in their IFN-β and RANTES/CCL5 mRNA production (Figure III.5.6.). Thus, the gene induction in SPRET/Ei macrophages after LPS does not reflect the gene induction in the IRF-3 knockout macrophages.

However, an intact IRF-3 phosphorylation does not provide the certainty that IRF-3 dimers are formed and that the nuclear translocation and DNA-binding capacity of IRF-3 is intact. Also, IRF-3 needs co-activators such as CBP and p300 to activate the IFN-β promoter (Taniguchi and Takaoka, 2002). In addition, trans-activators CITED1 and CITED2 can bind with CBP/p300 through their conserved C-terminal acidic domain (Shioda et al., 1997; Yahata et al., 2000). CITED proteins do not appear to bind to DNA directly but function as transcriptional co-activators (Yahata et al., 2000). *Cited1* and *Cited2* lie respectively on chromosome X and chromosome 10, which are linked with the LPS-resistance of SPRET/Ei. Since their expression is regulated by cytokines and stress, they are predicted to have a possible role in modifying CBP/p300-dependent transcription in a variety of biological events. So, further research is needed to clarify the role of the IRF-3 complex in IFN-β activation. We have the intention to sequence the IRF-3 gene, but also to look at dimerization of IRF-3 molecules after stimulation with LPS

or poly (I:C). CBP/p300, CITED1 and CITED2 co-activators will also be sequenced and their expression will be investigated at mRNA and/or protein level.

Also, RANTES and IFN-β contain both an NF-κB and an IRF-3 element in their promoter region. IRF-3 and NF-κB need to cooperate to induce IFN-β, so possibly IRF-3 and NF-κB fail to cooperate with each other in SPRET/Ei to induce the *Ifnb* and *Rantes* genes. Using a bioinformatica tool we will identify other genes with both an NF-κB and an IRF element in their promoter region. These genes will be investigated at their expression after LPS and/or poly (I:C).

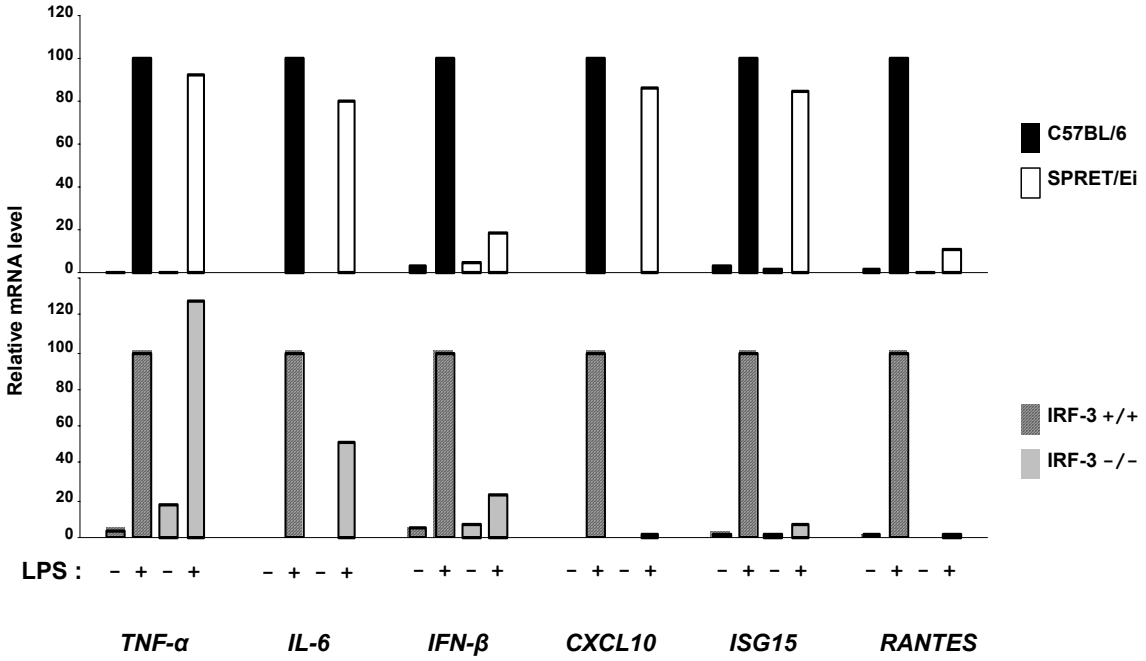

Figure III.5.6. – Gene induction in IRF-3-knockout and SPRET/Ei macrophages after LPS stimulation. BMDMs from C57BL/6, SPRET/Ei, IRF-3-knockout and IRF-3 wild-type mice were stimulated with 100 ng/ml LPS (triplicate cultures, 6-well). Via Q-PCR, mRNA induction of TNF-α, IL-6, IFN-β, CXCL10, ISG15 and RANTES was investigated.

5.3.3. Defect in the JAK-STAT pathway?

Preliminary data of a differential mRNA expression study, using BMDMs stimulated with LPS for 6 hours, demonstrated that SPRET/Ei IFNAR1 mRNA was relatively low compared to the C57BL/6 IFNAR1 mRNA after LPS stimulation. This interesting result led us to the investigation of the IFNAR1 mRNA expression by semi-quantitative RT-PCR. Via RT-PCR we were able show that the basal expression of IFNAR1 mRNA was identical in SPRET/Ei and C57BL/6 BMDM, but that it was down-regulated in SPRET/Ei cells very soon after LPS or IFN-β stimulation. In addition, we could demonstrate

that induction of OAS mRNA, which is dependent on ISGF3 complex formation after IFNAR stimulation, is significantly lower in SPRET/Ei BMDMs after LPS or IFN stimulation (see Figure III.5.7).

In addition, significantly less IL-6 is induced upon IFN stimulation in SPRET/Ei BMDMs compared to C57BL/6 BMDMs (Figure III.5.7.). The mechanisms of IFN-induced IL-6 expression is not known yet, but these results confirm the defect in the positive-feedback loop after IFN stimulation. Also in another experiment using BMDMs from SPRET/Ei and C57BL/6 (in triplicate cultures, 100.000 cells per 24-well), IL-6 concentration was significantly lower in SPRET/Ei BMDMs compared to C57BL/6 BMDMs after stimulation with 250 and 125 U/ml IFN-β.

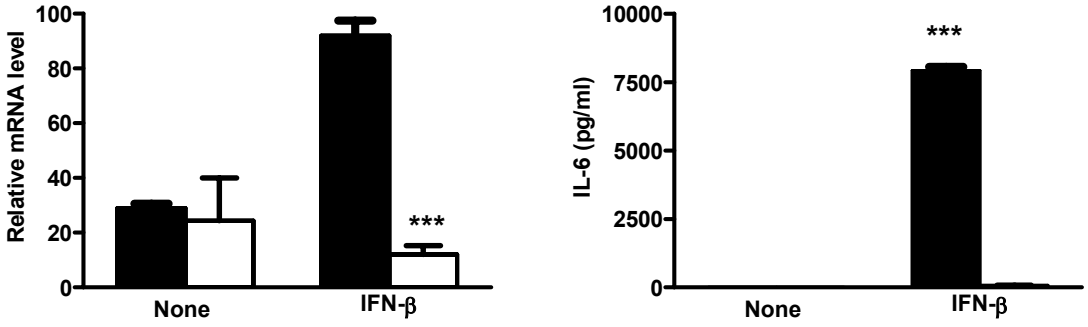

Figure III.5.7. – IFN-induced OAS mRNA expression and IL-6 induction in BMDMs. BMDMs from C57BL/6 and SPRET/Ei were seeded at 100.000 cells/well and were stimulated with 100 U/ml IFN-β for 1 hour (Oas mRNA, left figure) and 6 hours (IL-6 induction, right figure). RNA was isolated 1 hour after IFN-β stimulation and supernatant was collected 6 hours after IFN-β stimulation. IL-6 was measured using the 7TD1 assay. (black bars: C57BL/6 (n = 3) and white bars: SPRET/Ei; n = 3) (***p<0,001 C57BL/6 vs SPRET/Ei). In non-stimulated BMDMs, expression of IL-6 is not detectable.

These data suggest that hypo-responsiveness to IFN-β in SPRET/Ei may be due to rapid downregulation of the IFNAR1 by IFN-β or LPS. Nevertheless, IFNAR1 knockout mice, resistant to a lethal dose LPS, show a huge drop in body temperature after LPS injection. In contrast, SPRET/Ei mice are protected against LPS-induced hypothermia. This indicates that besides IFNAR1, other genes are involved in the LPS-resistance of SPRET/Ei. In the differential expression study using BMDMs stimulated with LPS for 6 hours, several MMPs, chemokines and interleukines (or their receptors) were differentially expressed between SPRET/Ei and C57BL/6 after LPS. The data of this experiment need to be confirmed, and by using the bioinformatica program 'Difference Distance Matrix Analysis' we hope to find a limited amount of transcription factors/genes that might be responsible for the differential expression of SPRET/Ei genes.

5.4. Conclusion

No nucleotide changes are found in the IFN-β promoter sequence of SPRET/Ei. In addition, IRF-3 activation seems fine in SPRET/Ei as demonstrated by its phosphorylation after LPS and the broader gene defect of IRF-3-knockout mice. However, we did not look yet at its dimerization after LPS and neither at the expression of its co-activators CBP/p300, or at the trans-activators of CBP/p300 CITED1 and CITED2. Also, IFN-β mRNA stability is regulated at the post-transcriptional level by 2 elements: an AU-rich element in the 3' UTR and the coding region instability domain (CRID) in the 3' end of the coding region. Thus, first the *Ifnb* gene itself should be sequenced. We must also investigate if IFN-β mRNA of SPRET/Ei mice is more rapidly degraded compared to IFN-β mRNA of C57BL/6 after LPS stimulation. We will do this by adding Actinomycin D to LPS-stimulated cell cultures to inhibit RNA synthesis and subsequently measure IFN-β mRNA expression at certain time points. Also, regulators such as YY1, IRF-1 and IRF-2 are known to be involved in IFN-β activation or shutdown and need to be investigated.

Furthermore, it is interesting to know that the *Ifnb* and *Ccl5* genes have both an NF-κB and IRF-3 PRD element. So, perhaps the induction of the *Ifnb* gene is defective because of a bad transactivation between the NF-κB and IRF-3 transcription factors. It is also still possible that p50/p50 homodimers, lacking transcriptional activity, are formed instead of p50/p65 dimers and that NF-κB or AP-1 activation is defect. We demonstrated that the early MyD88-dependent IκBα degradation and MAPK phosphorylation after LPS stimulation is intact in BMDMs from SPRET/Ei. This was confirmed by comparable induction of IL-6 and TNF-α mRNA after LPS in SPRET/Ei and C57BL/6 BMDMs. In contrast, late phase pro-inflammatory gene induction is defective in SPRET/Ei. So, we should look at all these elements (IRF-3 dimerization; NF-κB and AP-1 activation, NF-κB dimers) both at early and at late time points after LPS stimulation.
We have demonstrated that IFNAR1 mRNA becomes rapidly downregulated in SPRET/Ei after IFN and LPS stimulation. The mechanism and significance of this phenomenon needs further investigation. It would also be interesting to look at the other molecules of the JAK-STAT pathway. Since the LPS-resistance of SPRET/Ei is a dominant phenotype, the over-expression of a negative regulator would correlate with the dominant phenotype. Therefore, it would also be interesting to investigate the negative regulators of the JAK-STAT pathway such as SOCS, SHP and PIAS molecules.

6. RESPONSE OF SPRET/Ei ON DIFFERENT TLR/IL-1R LIGANDS

6.1. Introduction

The Toll/IL-1R family signals to downstream transcription factors using common signalling molecules and signalling pathways (Moynagh, 2005). We already demonstrated that SPRET/Ei is hypo-responsive to LPS *in vivo* and *in vitro* and that significantly less IFN-β is produced after LPS (*in vivo* and *in vitro*) and poly (I:C) (*in vitro*) stimulation. TLR4 is the only TLR using both TRIF and MyD88 to induce a variety of molecules, which makes it a very complicated pathway to analyze. TLR3 only recruits the adaptor molecule TRIF for signalling. We already knew that significantly less IFN-β is produced after poly (I:C) stimulation. We also tested the response of SPRET/Ei and (BxS)F_1 mice to poly (I:C)/d-GalN-induced lethality. If we could use poly (I:C) instead of LPS to investigate the mechanism of the IFN-β defect, we only have to take the TRIF-dependent pathway into consideration. We also tested the response of SPRET/Ei to ligands for the IL-1R and TLR2. The IL-1R recruits only the adaptor molecule MyD88, while TLR2 recruits the adaptor molecules MyD88 and TIRAP. The Myd88-dependent pathway leading to early pro-inflammatory gene induction is intact in SPRET/Ei. Thus, we assumed a normal response of SPRET/Ei to IL-1R and TLR2 ligands.

6.2. Experimental procedures

6.2.1. Injections

IL-1 was diluted in endotoxin-free PBS immediately before injection. All injections were given i.p. in a volume of 250 μl and with a with a dose of 1μg/25g bodyweight. Zymosan was injected in the footpad (20 μl). Swelling was measured and was compared to the PBS- injected foot. Poly (I:C) and d-GalN were diluted in endotoxin-free PBS immediately before injection**.** Injections were given i.p. in a volume of 500 μg and with a dose of 10 μg poly (I:C) and 20 mg d-GalN per mouse.

6.2.2. *In vitro* experiments

Thioglycolate-elicited macrophages and MEFs were seeded at 100.000 cells per well in supplemented RPMI medium 1640. IL-1 was added to the cultures to a final concentration of 1 μg/ml. After 24 hours of stimulation, supernatant was harvested and IL-6 was measured using the 7TD1 assay.

BMDMs were seeded at 100.000 cells per well in supplemented DMEM medium. PGN was added to the cultures to a final concentration of 2 μg/ml. Poly (I:C) was added to a final concentration of 25 μg/ml**.** After 6 hours of stimulation, supernatant was harvested and TNF was measured by using the sensitive cell line WEHI 164 cl 13 and IL-6 was measured using the 7TD1 assay.

6.3. Results

6.3.1. Response of SPRET/Ei, *in vivo* and *in vitro* to IL-1

IL-1 binds to the IL-1R complex to stimulate the MyD88-dependent pathway to activate NF-κB and MAPK inducing pro-inflammatory cytokines such as IL-6. The adaptor molecule MyD88 is the only adaptor molecule which is recruited by the IL-1R complex (Janssens and Beyaert, 2002).

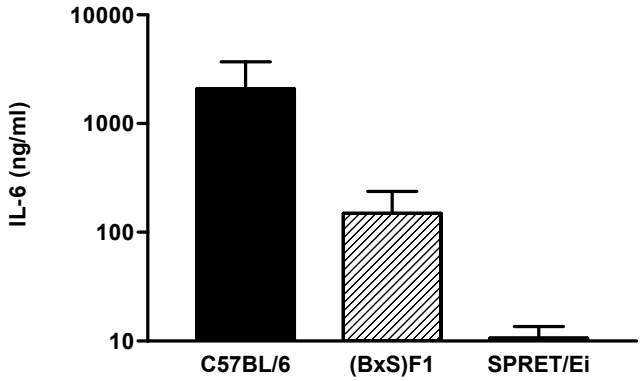

Figure III.6.1. – IL-6 concentration after an i.p. IL-1 injection. C57BL/6 (n = 3), (BxS)F_1 (n = 2) an SPRET/Ei mice (n = 3) were i.p. injected with a dose of 1 μg IL-1. 3 hours after injection, IL-6 concentration was measured in serum using the 7TD1 assay.

To investigate the response to a ligand activating only the MyD88-dependent pathway SPRET/Ei, (BxS)F_1 and C57BL/6 mice were i.p. injected with a dose of 1μg/25g bodyweight IL-1. IL-6 was measured in serum 3 hours after injection using the 7TD1 assay (Figure III.6.1). IL-1-induced IL-6 expression is lower in SPRET/Ei mice compared to C57BL/6 mice, while (BxS)F_1 mice have an intermediate phenotype.

This phenotype was also evident *in vitro*. Significantly less IL-6 was induced in SPRET/Ei macrophages and fibroblasts compared to C57BL/6 macrophages and fibroblasts (Figure III.6.2.). These data indicate that the MyD88-dependent pathway leading to IL-6 gene induction is defective.

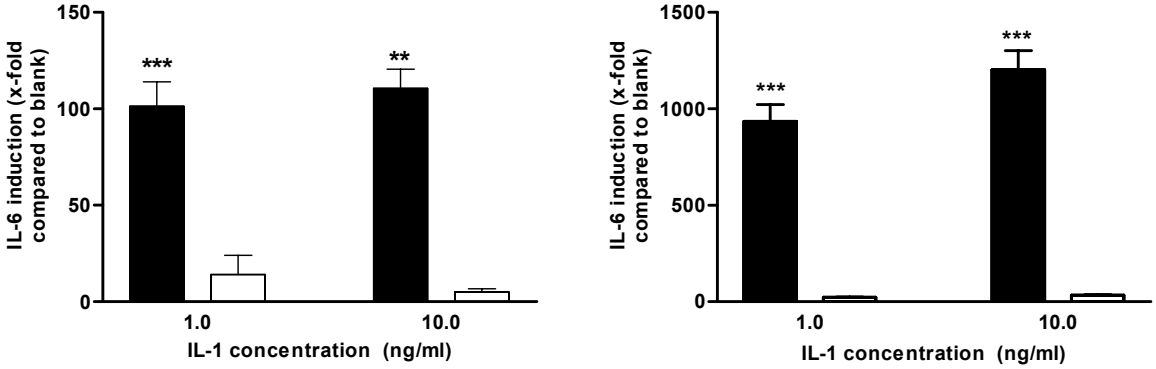

Figure III.6.2. – IL-6 induction *in vitro* after 24 hours of IL-1 stimulation. Thioglycolate-elicited peritoneal macrophages (left picture) and MEFs (right picture) from C57BL/6 and SPRET/Ei were seeded at 100.000 cells/well and were stimulated with 1 or 10 ng/ml IL-1. 24 hours after stimulation, supernatant was harvested and IL-6 was measured using the 7TD1 assay (black bars: C57BL/6 (n = 3) and white bars: SPRET/Ei; n = 3) (**p<0,01; ***p<0,001 C57BL/6 vs SPRET/Ei).

6.3.2. Response of SPRET/Ei, *in vivo* and *in vitro* to TLR2

TLR2 activates the MyD88-dependent pathway, but not the TRIF-dependent pathway, to activate NF-κB and MAPK to produce pro-inflammatory cytokines such as IL-6 and TNF. The IL-1R and TLR2 use many, but not all, of the same signalling components. For example, the adaptor molecule TIRAP is used by TLR2, but not by the IL-1R (Fitzgerald et al., 2001). TLR2 and TLR6 physically interact in the cell and both receptors cooperate in the response to zymosan derived from the cell-wall of yeast (Ozinsky et al., 2000). Injection of zymosan in the footpad of mice induces inflammation, which can be seen as a swelling of the foot. When injected in the footpad of C57BL/6 mice, zymosan induced swelling of the foot, while in SPRET/Ei mice there was no swelling of the foot at all (Figure III.6.3.). In (BxS)F_1 mice some swelling of the foot was seen.

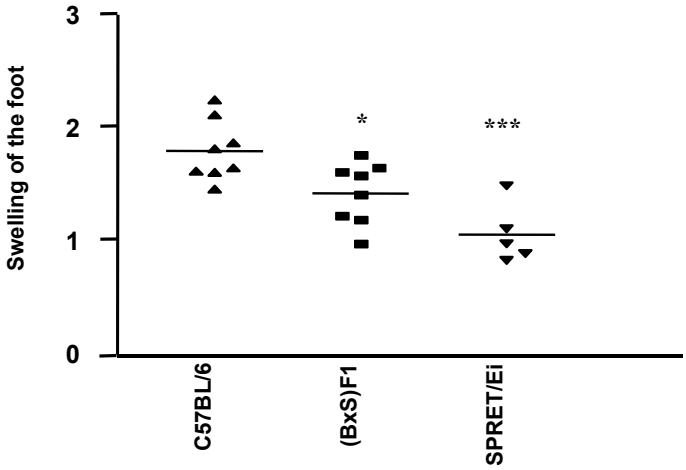

Figure III.6.3. – Swelling of the foot after injection of zymosan. C57BL/6 (n = 8), (BxS)F_1 (n = 8) and SPRET/Ei mice (n = 5) were injected in the footpad with 20 μl zymosan. Swelling was measured and was compared to the PBS- injected foot (*p<0,05; ***p<0,001).

In addition, we looked *in vitro* to stimulation with the TLR ligand peptidoglycan (PGN), a component of Gram-positive bacteria. PGN is recognized by the functional cooperation between TLR2 and TLR6. TLR2 needs a partner, demonstrated to be TLR6, to activate cytokines like TNF-α in macrophages (Ozinsky et al., 2000). PGN-induced TNF-α production is significantly less in C57BL/6 BMDMs compared to SPRET/Ei BMDMs (figure III.6.4.). Hence, also these data indicate a defect in the MyD88-dependent pathway. After stimulation with PGN, IL-6 concentration in supernatants of C57BL/6 and SPRET/Ei BMDMs was very low.

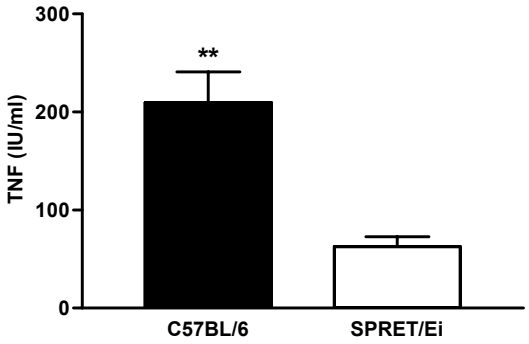

Figure III.6.4. – TNF-α concentration in BMDMs after stimulation with PGN. BMDMs from C57BL/6 and SPRET/Ei were seeded at 100.000 cells/well and were stimulated with 2 μg/ml PGN for 6 hours (triplicate cultures). Concentration of TNF-α in supernatant was measured using the sensitive cell line WEHI 164 cl 13 (**p<0,01 C57BL/6 vs SPRET/Ei).

6.3.3. Response of SPRET/Ei, *in vivo* and *in vitro* to TLR3

TLR3 is the only TLR that does not activate the MyD88-dependent pathway (Moynagh, 2005). Instead it uses the TRIF-dependent pathway, mainly to induce IFN-β and IFN-dependent genes, but also to induce pro-inflammatory cytokines (Sato et al., 2003). TLR3 recognizes dsRNA and some synthetic dsRNA molecules, such as poly (I:C) (Alexopoulou et al., 2001).

We decided to investigate pro-inflammatory gene induction after stimulation with poly (I:C). Significantly less IL-6 is produced upon poly (I:C) stimulation in SPRET/Ei BMDMs compared to C57BL/6 BMDMs (Figure III.6.5.). After poly (I:C) stimulation, TNF-α concentration in supernatants of C57BL/6 and SPRET/Ei BMDMs was very low.

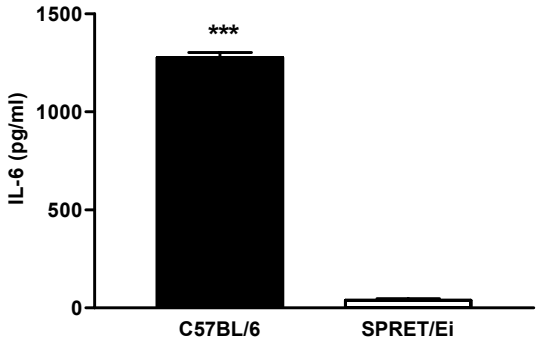

Figure III.6.5. – IL-6 concentration in BMDMs after poly (I:C) stimulation. BMDMs from C57BL/6 and SPRET/Ei were seeded at 100.000 cells/well and were stimulated with 25 μg/ml poly (I:C) for 6 hours (triplicate cultures). Concentration of IL-6 in supernatant was measured using the 7TD1 assay (***p<0,001 C57BL/6 vs SPRET/Ei).

TLR3 knockout mice show a reduced response to poly (I:C). In addition, they are resistant to the lethal effects of poly (I:C) when sensitized with d-GalN and show reduced production of inflammatory cytokines (Alexopoulou et al., 2001). To test the response of SPRET/Ei mice to a TLR3 ligand, we injected mice with poly (I:C)/d-GalN and looked at survival. There is a significant difference in survival between (BxS)F_1 or SPRET/Ei and C57BL/6 mice (Figure III.6.6). So, SPRET/Ei is hypo-responsive to poly (I:C) *in vivo*. In addition, SPRET/Ei has a defect in IFN-β mRNA induction and in IL-6 gene induction after poly (I:C) *in vitro*.

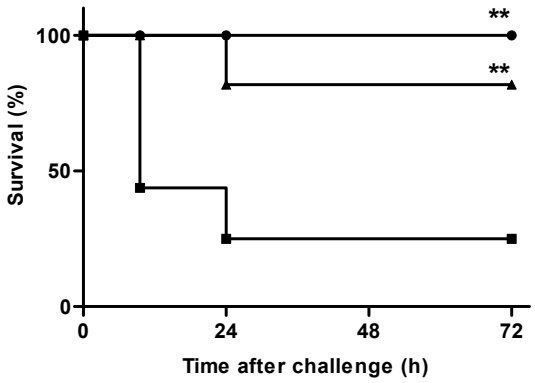

Figure III.6.6. – Survival after a lethal injection of poly (I:C) and d-GalN. C57BL/6, (BxS)F_1 and SPRET/Ei mice were i.p. injected with 10 μg poly (I:C) and 20 mg d-GalN. Survival was monitored for 5 days. (Squares: C57BL/6 (n = 16), triangles: (BxS)F_1 (n = 11) and circles: SPRET/Ei (n = 3)) (**p<0,01).

6.4. Conclusion

Next to a defective pro-inflammatory cytokine induction *in vivo* and *in vitro* after LPS stimulation, also after stimulation with ligands for IL-1R, TLR2 and TLR3 a defective pro-inflammatory cytokine induction in SPRET/Ei *in vitro* is observed. SPRET/Ei mice are also hypo-responsive to IL-1 induced IL-6 induction, zymosan-induced swelling of the foot and poly (I:C)/dGalN-induced lethality. We have shown that IκB degradation and MAPK p38 and JNK phosphorylation after LPS is intact at early time points, suggesting that the MyD88-dependent pathway in SPRET/Ei is intact. The intact MyD88 signalling was confirmed by studying the induction of TNF-α and IL-6 mRNA at early time points after LPS stimulation. However, MyD88, the adaptor molecule recruited by IL-1R and TLR2, is responsible for the activation of NF-κB and MAPK leading to pro-inflammatory cytokine production. Nevertheless, MyD88-dependent pro-inflammatory cytokine induction after IL-1R and TLR2 induction is defective in SPRET/Ei. Possible explanations could be the defective activation of NF-κB and MAPK or the formation of other NF-κB dimers, such as the p50-p50 homodimers lacking transactivation activity, instead of the transcriptionally active p50/p65 heterodimers. Also, CBP and p300 are identified as co-activators of the NF-κB component p65 (Gerritsen et al., 1997). Perhaps a defect in CBP/p300 is responsible for the defective pro-inflammatory cytokine induction. Recently, it was also shown that all TLRs use IRF-5, next to NF-κB, to induce production of pro-inflammatory cytokines such as TNF, IL-6 and IL-12 (Napolitani et al., 2005). There are still contradictory results about the induction of IRF-5 in TLR3 signalling. So, the

defective pro-inflammatory cytokine (IL-6 and TNF) induction in SPRET/Ei can still be caused by multiple factors and requires further investigation.

SPRET/Ei is hypo-responsive to poly (I:C) *in vitro*, as seen by defective IFN-β mRNA and IL-6 induction in BMDMs, and can resist the poly (I:C)/dGalN-induced lethality. For future work, we can use poly (I:C) instead of LPS to search for the mechanism causing the defective IFN-β expression. By using poly (I:C) we only have to take the TRIF-dependent pathway into consideration but not the MyD88-dependent pathway, which makes the response more complicated. A differential expression study of macrophage specific genes already showed us that about 50 genes are ≥ 2 times higher expressed in SPRET/Ei and about 50 genes are ≥ 2 times less expressed in SPRET/Ei BMDMs compared to C57BL/6 BMDMs after 6 hours LPS stimulation. However, data are still preliminary because only one RNA sample of LPS-stimulated BMDMs of SPRET/Ei and C57BL/6 was tested. The experiment will be repeated and we will also investigate the differential expression of macrophage specific genes in BMDMs of SPRET/Ei and C57BL/6 after poly (I:C) stimulation. By comparing the genes that are differentially expressed after both LPS and poly (I:C) stimulation we can reduce the amount of candidate genes, which might be responsible for the differential expression of SPRET/Ei genes.

7. GENETIC ANALYSIS OF THE LPS RESPONSE OF SPRET/EI

7.1. Introduction

To identify and isolate the LPS protection genes in SPRET/Ei causing this LPS-resistance phenotype, we started a positional cloning experiment. To map a gene, two inbred strains are needed that are significantly different for a phenotype and that show significant genetic polymorphism. Firstly, SPRET/Ei and C57BL/6 are significantly different in their response to LPS. Secondly, *Mus spretus* diverged from *Mus musculus* about 1,5 million years ago. Because of this long period of evolutionary divergence, strains belonging to *Mus musculus* (C57BL/6) and *Mus spretus* (SPRET/Ei) demonstrate a high degree of genetic polymorphisms (Guenet and Bonhomme, 2003). It has been shown that 70-90% of the microsatellites between *Mus spretus* and common laboratory strains show size variations (Cornall et al., 1991; Hearne et al., 1991). In addition, a dense genetic microsatellite-map is known. A backcross between *Mus spretus* and C57BL/6 of about 1000 progeny can achieve a resolution of 0.1 cM (approximately 200 kb). This made SPRET/Ei very popular for genetic mapping experiments (Group., 1994; Rhodes et al., 1998). Also, SPRET/Ei can generate fertile female F1 animals with *Mus musculus*. These F1's can be used to cross back to one of the parentals. Thus, SPRET/Ei and C57BL/6 are ideal partners for mapping of LPS-resistance genes.

Nevertheless, the use of SPRET/Ei in mapping experiments has also some disadvantages. First, the degree of polymorphism between SPRET/Ei and C57BL/6 can be a disadvantage in gene identification. Due to this high degree of polymorphisms, almost in any gene a variation is found between SPRET/Ei and C57BL/6, making it difficult to identify the gene(s) causing the LPS-resistance of SPRET/Ei. Second, SPRET/Ei has very poor breeding performances. Third, since only fertile female F1 animals can be generated it is only possible to set up a backcross, but not an F2 intercross. In an F2 intercross 25% of the genome of the F2 progeny will be BB, 25% SS and 50% BS. In contrast, in a backcross 50% of the genome will be BB and 50% will be BS. Hence, backcross mice have no homozygous SPRET/Ei loci. However, since the LPS-resistance of SPRET/Ei is a dominant trait this is not a real problem.

7.2. Experimental procedures

7.2.1. Setting up an interspecific backcross

To map the genes responsible for the LPS-resistance of SPRET/Ei, an interspecific backcross between female (BxS)F_1 mice and male C57BL/6 mice (BSB backcross) was performed. Backcross mice were injected with 250 μg LPS at the age of 8 weeks. A genome scan was conducted by using microsatellite markers evenly spread across the genome. Primer sequences were obtained from the Massachusetts Institute of Technology (Cambridge) and primers were custom made by Invitrogen Life Technologies (Paisley, UK). The PCR reactions were performed on 100 ng of genomic tail DNA, as followed: DNA was denatured at 94°C for 4 minutes; 35 cycles of 30 seconds denaturation at 94°C, annealing of primers for 30 seconds at 55°C and elongation for 30 seconds at 72°C; elongation for 6 minutes at 72°C. PCR products are separated by electrophoresis on 3% Nusieve agarose and visualized by ethidium bromide staining. Survival data and genotyping data were analyzed by using MapManager QTX B17 (Manly et al., 2001).

7.2.2. Generation of consomic strains

To make a consomic strain homozygous for all loci and 1 chromosome from SPRET/Ei it is necessary to do brother-sister matings for different generations. Consomic strains were generated by first making a backcross between a fertile female (BxS)F_1 mouse and a C57BL/6 male. The progenies are screened for a specific chromosome (chromosome 2, 10, 13 and X) using microsatellite markers. The mice that contain an entire chromosome 2, 10, 13 or X from SPRET/Ei are selected to cross back to C57BL/6. For different generations, a selection is made for the mice containing the entire chromosome of interest. Each generation 50% of the donor genome is lost. After 7 generations mice are generated that contain less than 1% SPRET/Ei genes (called passenger DNA), except for the chromosome one performed a selection for. Males and females with the non-recombinant chromosome from SPRET/Ei can be intercrossed to create progeny homozygous for the chromosome of interest. It is not possible to make homozygous consomic strains for chromosome X because of the hybrid sterility of males having a C57BL/6 Y chromosome and a SPRET/Ei X chromosome.

Consomic strains were injected with high dose of LPS (100 μg/25g bodyweight) or low dose of LPS (10 μg/25g bodyweight). PCR reactions on genomic tail DNA was done like in section 7.2.1. Survival data and genotyping data were analyzed by using MapManager QTX B17 (Manly et al., 2001).

7.3. Results

7.3.1. Mapping to subchromosomal level

An interspecific backcross between C57BL/6 and SPRET/Ei was set up to identify the genes conferring resistance to LPS. Female (BxS)F_1 mice were crossed back with C57BL/6 males. A total of 141 BSB backcross mice were generated. At the age of 8 weeks the offspring was injected with a dose of 250 μg LPS, a dose of at least 5 times the LD_{100} for C57BL/6, but not lethal for SPRET/Ei. Out of 141 mice tested, 90 mice (64%) survived the injection of 250 μg LPS, while 51 mice (36%) died. When the trait follows a simple Mendelian inheritance pattern, 50% of the mice should be resistant and 50% should be sensitive for LPS. However, in the N_2 backcross population only 36% of the mice is sensitive, which suggests that the LPS-resistance is a complex trait, controlled by multiple loci.
A genome scan was performed by using microsatellite markers evenly spread across the genome. Genomic DNA was prepared from BSB mice and mice were PCR genotyped at marker loci. The genome scan was performed on 90 BSB mice. Afterwards, an additional 51 BSB mice were generated. These mice were only genotyped at marker loci on the 4 chromosomes found to be linked with the LPS-resistance (see further). Also, some additional marker loci on these 4 chromosomes were genotyped.
Subsequently, survival data and genotyping data were inserted into MapManager QTX B17 (Manly et al., 2001). To detect and localize QTLs the following steps need to be performed. First, putative QTLs are detected with tests like the 'single-locus association' test or simple interval mapping (SIM). Next, possible quantitative trait loci (QTLs) are more accurately localized with SIM. The significance of the putative QTLs is established with permutation tests. When multiple QTLs are present, also composite interval mapping (CIM) can be performed (Fig. III.7.1.)

First a linkage analysis was performed using the simple 'single-locus association' test of Map Manager QTX. This test is used to evaluate the association of trait values with the genotypes of single loci (Table III.7.1). A lethal response to LPS was fixed as zero and a surviving response as hundred. A permutation test was used to determine empirically the likelihood ratio statistic (LRS) values corresponding to suggestive linkage (genome-wide type I error probability of 0.63), significant linkage (genome-wide type I error probability of 0.05) and highly significant linkage (genome wide type I error probability of 0.001). At the suggestive level on average one false positive is found per genome scan. At this level, the detected QTLs are not strong QTLs. At the significant level moderate QTLs and at the highly significant

level strong QTLs will be detected. The suggestive level corresponded to an LRS value of 6.6, the significant level to an LRS value of 12.9, and the highly significant level to an LRS value of 21.9.

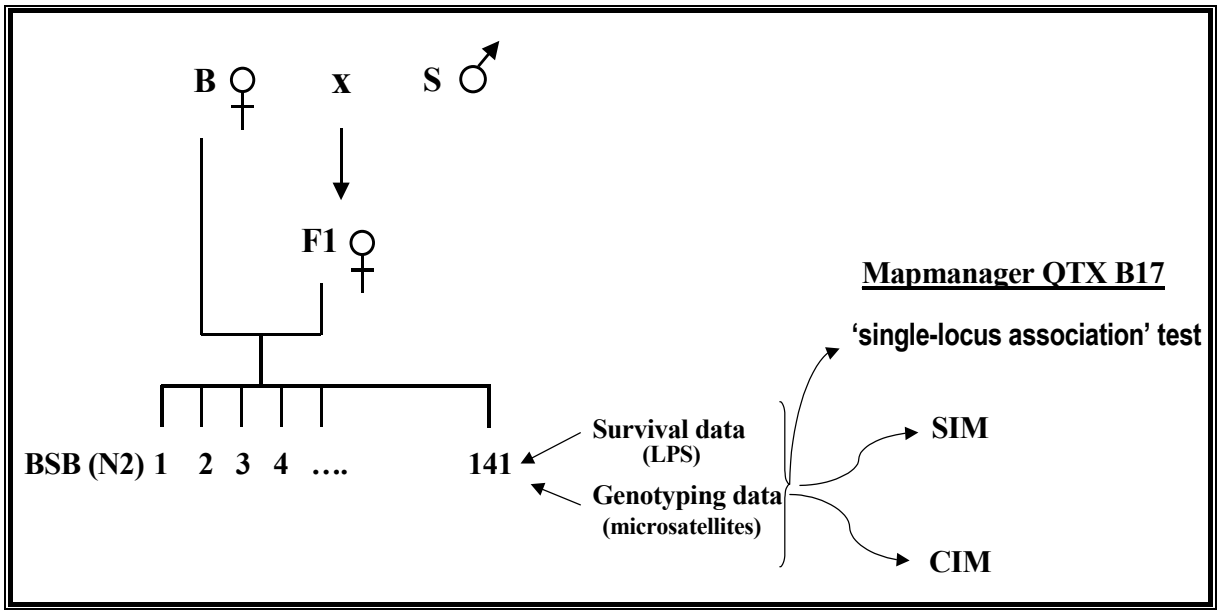

Figure III.7.1. – Steps to detect and localize QTL. First homozygous mutant mice (S/S) are crossed with the homozygous wild-type mice (B/B). The (BxS)F1 progeny will be heterozygous for all loci (B/S). Next, the (BxS)F_1 progeny is crossed back to the wild-type strain (B). Following this steps, we generated 141 BSB mice. Survival data (lethality after LPS) and genotyping data (microsatellite markers) were introduced into Mapmanager QTX B17. Next, the following tests are performed to detect and localize QTL: 'Single-locus association test', SIM and CIM.

These LRS values are less stringent than those of the genome-wide significance from Lander and Kruglyak determining the chance that a deviation will be found in a whole genome scan. The equivalent LRS needed for significance is about 20 for an F2 and about 15 for a backcross (Lander and Kruglyak, 1995). The p-value is the probability of obtaining the observed LRS value by chance, the probability of a 'false positive'. This p-value is calculated by interpreting the LRS as a χ^2 statistic. This p-value applies to one point in the genetic map. However, the probability of a false positive anywhere in the genome is much greater (Lander and Kruglyak, 1995). The p-value for a single point must be far below 0.001 to obtain a genome-wide p-value of 0.05 (Table III.7.1.)

The best method to determine genome-wide significance is a permutation test to estimate an empirical genome-wide probability for observing a given LRS score by chance (Churchill and Doerge, 1994) (Table III.7.1.).

Following the empirical determined significance levels, loci on chromosome 10 and 13 reach suggestive linkage to LPS-resistance. A locus on chromosome 2 reaches significant linkage and a locus on chromosome X is significantly linked with the LPS-resistance (Table III.7.1.). In addition, a locus on chromosome 6, containing a sensitivity gene for LPS, was approaching the suggestive level (Table III.7.1.).

Table III.7.1. - Linkage of genetic markers to the LPS-resistance.

Chr.	Marker^	Position*	N°	LRS	%§	P value#	95% CI†
	D2Mit89	32.0	119	7.9	5	0.00507	69
2	D2Mit510	65.0	134	10.0	7	0.00155	55
	D2Mit52	87.4	115	6.1	5	0.01324	89
6	D6Mit311	40.0	121	6.2	4	0.01251	87
10	D10Mit253	23.0	113	7.4	5	0.00658	74
	D13Mit24	43.0	124	8.9	7	0.00286	62
13	D13Mit145	52.0	117	8.9	7	0.00279	61
	D13Mit76	61.0	122	7.4	6	0.00649	74
	DXMit225	16.1	133	9.5	7	0.00205	58
	DXMit146	29.75	90	11.4	8	0.00072	48
X	DXMit60	30.8	95	13.2	9	0.00028	42
	DXMit130	55.0	127	10.2	7	0.00144	54
	DXMit135	69.0	123	13.4	10	0.00025	42

^ Microsatellites
* Expressed in cM
° Population size
§ Amount of total trait variance, explained by a QTL at this locus, as a percent
# Point-wise P-values: The probability of obtaining the observed LRS value by chance
† Confidence interval according to (Darvasi and Soller, 1997)

When we look at the corresponding 95% confidence intervals in Table III.7.1., which are data obtained by the simple 'single-locus association' test, we can see that the localizations of QTL (expressed in cM) are rather poor. The confidence interval for a QTL location determined by interval mapping is inversely proportional to the strength of the QTL and the square number of progeny. Even with a strong QTL, the confidence interval is unlikely to be less than 10 cM. A weak QTL may have a confidence interval, which covers the entire chromosome. For example, microsatellite DXMit135 on chromosome X is strongly linked with the LPS-resistance (high LRS value) and has a corresponding CI of 42 cM. In contrast, microsatellite D2Mit52 only reaches the suggestive level (low LRS value) and has a CI of 89 cM, which covers almost the entire chromosome.

To obtain a more detailed location of a putative QTL another test is needed, namely SIM. SIM analysis calculates the association between phenotype and the expected contribution of hypothetical QTL at multiple analysis points between each pair of adjacent marker loci. The location of the putative QTL is given by that analysis point yielding the most significant association (Manly et al., 2001). A SIM analysis was performed using Map Manager QTX B17. Using this test, we observed: suggestive linkage of the LPS-resistance with loci on chromosome 10 and 13, a locus on chromosome 2 reaching the significant level and a locus on chromosome X highly significantly linked with the LPS-resistance (Figure III.7.2.) A locus on chromosome 6 (Figure III.7.3.) reaches the suggestive level. Suggestive (*), significant (**) and highly significant levels (***) are empirically determined using a permutation test as described before.

We reanalyzed some of the data after our experiments with the consomic mice (see point 4.3). In Table III.7.1. we can learn that the effect of an individual locus on the overall trait variation ranges from 4 to 10%, which is rather small. The combined effects of the detected loci on chromosome 2, 10, 13 and X account for approximately 30% of the total trait variation (LPS-resistance trait). This suggests that epistatic interactions exist between the detected QTLs or that additional QTLs were not detected. The power to detect QTL, defined as the probability of detecting a QTL at a given level of statistical significance, depends upon the strength of the QTL and the number of progeny in the population and is inversely proportional to the square of the strength of the QTL. If the strength of the QTL is considered in terms of the fraction of the total trait variation, three categories of QTLs can be defined. A strong QTL, is a QTL which explains 20% of the variance. At the other extreme are weak QTLs which only explain 1% or less of the trait variance. Weak QTLs require at least a thousand progeny to detect them and it is not routinely feasible. Between these extremes are moderate QTLs, which can be detected with crosses of reasonable size. This means that probably additional weak QTLs were not detected and a lot more mice are needed to detect all of the loci determining the LPS-resistance trait.

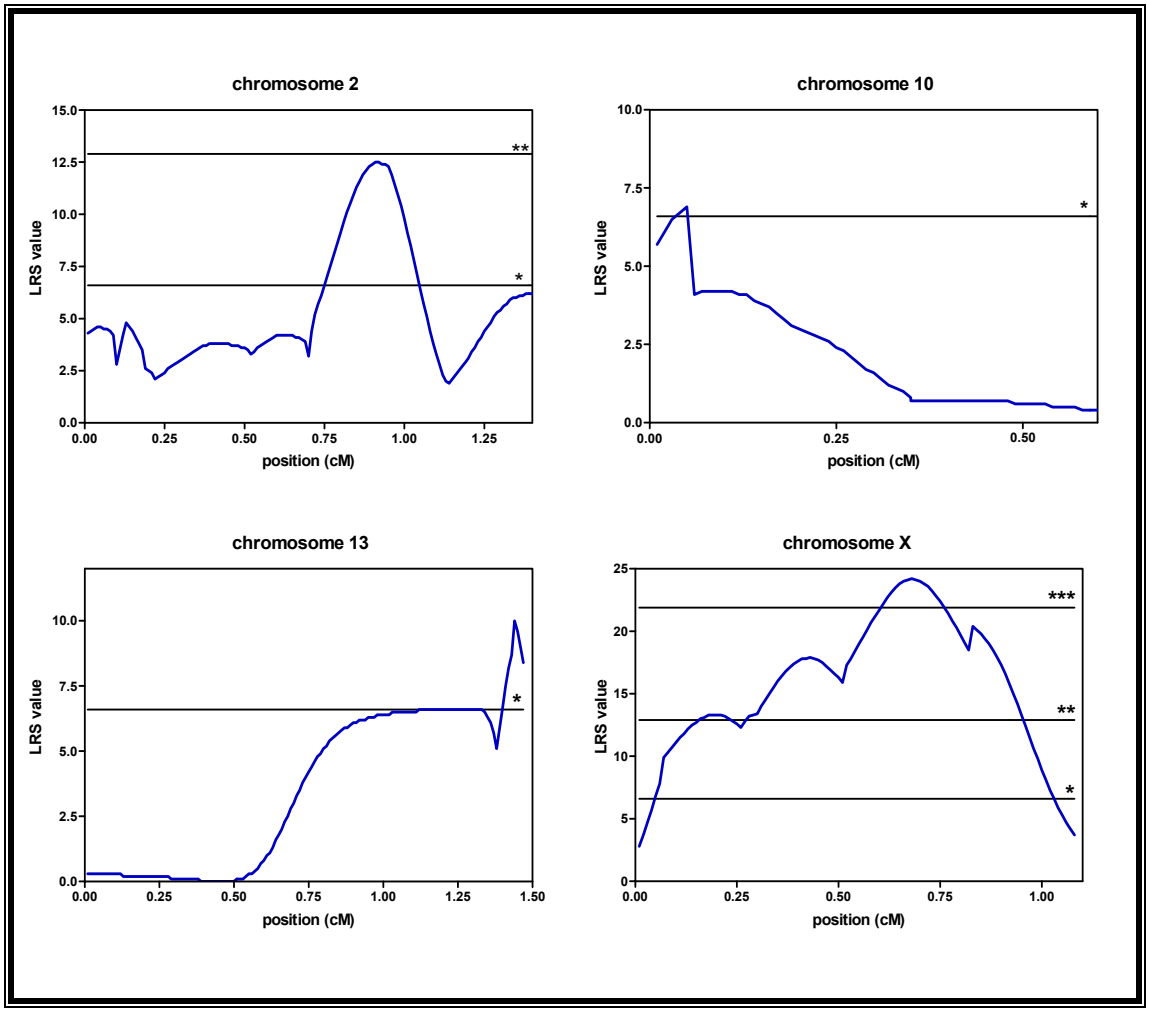

Figure III.7.2. – Simple interval mapping analysis of BSB backcross. *, suggestive level (genome wide type I error probability of 0.63); **, significant level (genome wide type I error probability of 0.05); ***, highly significant level (genome wide type I error probability of 0.001). Loci on chromosome 10 and 13 show suggestive linkage with LPS-resistance. A locus on chromosome 2 reaches significant linkage and a locus on chromosome X shows highly significant linkage with the LPS-resistance.

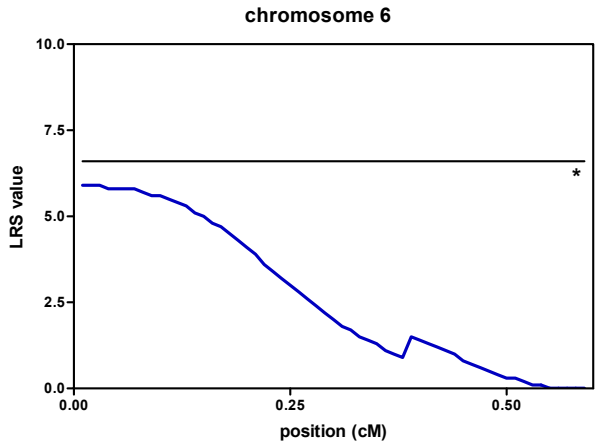

Figure III.7.3. - Simple interval mapping analysis of BSB backcross: a sensitivity gene on chromosome 6. *, suggestive level (genome wide type I error probability of 0.63). In addition to the loci, which contain possible resistance genes, on chromosome 2, 10, 13 and X, a locus on chromosome 6 was found containing a sensitivity gene for LPS.

We tested the possible epistatic interactions by analyzing the genome scan using an algorithm specifically aimed at detecting epistatic interactions. This algorithm is the 'interactions' function of Map Manager QTX (Manly et al., 2001). Multiple loci are possibly involved in epistatic interactions (Table III.7.2.). In Table III.7.2. the LRS values for the individual effect of locus 1 (Main 1) and locus 2 (Main 2), and the LRS values for the interaction between locus 1 and locus 2 are given. Above all, significant interactions between chromosomes 13 and X and between chromosomes 2 and X are detected. Some suggestive interactions are also detected. Most notably, DXMit135 lying on the distal part of chromosome X is involved in epistatic interactions with chromosome 2 and 13, detected using the SIM analysis. Thus, probably there is key role for a locus on chromosome X in the overall phenotype. This is easily comprehensible, since chromosome X and 2 were strongest linked with the LPS-resistance.

Table III. 7.2 – Epistatic interactions in the LPS response of SPRET/Ei.

Locus1	Locus2	LRS		Main1	Main2
D13Mit145	DXMit135	32.3	**	7.8	13.7
D2Mit472	DXMit135	27.0	**	4.4	12.8
D13Mit24	DXMit225	21.6	*	3.9	9.2
D12Mit88	D13Mit285	21.2	*	3.5	5.2
D12Mit118	D15Mit255	20.3	*	0.7	1.7

LRS, likelihood ratio statistic for the interaction; Main 1 and Main 2: individual effects of locus 1, respectively locus 2 (* suggestive, ** significant).

If more than one QTL is detected, another test, called composite interval mapping (CIM), can be performed (Jansen and Stam, 1994). SIM localizes a single QTL without regard to other QTLs affecting the trait, while CIM uses a marker near the first QTL to explore the effect of QTL combinations. Based on the assumption of epistatic interactions between QTLs, we performed CIM using markers DXMit135 and D2Mit510, the two markers with the highest LRS values for chromosome X and chromosome 2, to control for background QTL. By doing so, linkage at the suggestive level became apparent on proximal chromosome 12 (Figure III.7.4.). However, this region was negatively linked with the LPS-resistance, meaning that a sensitivity gene might be located around D12Mit88, lying on proximal chromosome 12. D12Mit88 was also found as a marker involved in epistatic interaction with D13Mit285.

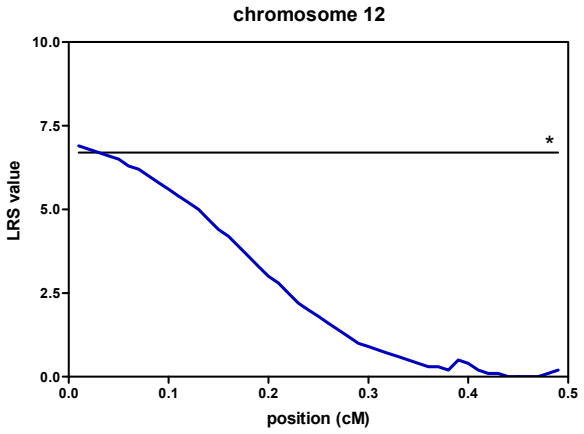

Figure III.7.4. – Composite interval mapping. Composite interval mapping was performed using DXMit135 and D2Mit510 to control for background QTL. Linkage at the suggestive level with proximal chromosome 12 was found. This locus might contain a sensitivity gene for LPS. (*, suggestive level).

7.3.2. Chromosome substitution strains (CSS) or consomic mice

To confirm the importance of the loci found in the initial backcross we started making consomic mice, also called chromosome substitution strains (CSS strains). At the 'Institut Pasteur' in Paris (Dr. J.-L. Guénet) consomic strains derived from *Mus spretus* are available. However, these consomic strains are derived from STF/Pas en SEG/Pas, 2 other inbred strains derived from *Mus spretus* individuals. STF/Pas and SEG/Pas were tested for their response to LPS and it was demonstrated that both strains are not resistant to LPS-induced lethal shock. We wanted to make consomic strains that are totally C57BL/6, except for one (pair of) chromosome(s) (in our case chromosome 2, 10, 13 or X) derived from SPRET/Ei (Nadeau et al., 2000). The complex trait has been turned into a monogenic trait if the trait is still (partially) evident in such consomic mice.

SPRET/Ei mice are also hyporesponsive to lethal shock induced by TNF (Staelens et al., 2002). By testing chromosome 2 or 6 consomic mice for their response to TNF, it became clear that the extreme TNF-resistance phenotype of SPRET/Ei cannot be reproduced by isolating loci lying on chromosome 2 or 6 into a C57BL/6 background. Consomic mice heterozygous for the region between D6Mit104 and D6Mit194 are very significantly protected to TNF compared to their littermate controls. However, the protection conferred by the chromosome 6 locus is only to a very low dose of TNF (12.5 μg/25g mouse) compared to the dose which SPRET/Ei and (BxS)F_1 resist (500 μg/25g mouse). Since the LPS-resistance phenotype is - as the TNF-resistance - a complex trait involving multiple genes, we wanted to test if we could screen with a low dose of LPS. One advantage of working with a low dose of LPS is the more subtle approach meaning that we can look at quantitative traits such as body temperature and IL-6 concentration. In contrast, after a high dose LPS (as in the backcross) mice are scored as either dead or alive. Bleeding of mice to measure IL-6 concentration in serum and measuring body temperature can stress them and influence lethality. So, a mouse with a dramatically drop in body temperature that survives the high dose LPS injection is scored as alive. Another advantage is that most mice survive a low dose of LPS. So, they can be used to cross back to C57BL/6. In addition, a high dose of LPS induces not only lethality but also causes sterility problems. However, a disadvantage of this approach is the possibility that the IL-6 concentration and the LPS response in terms of lethality might not be linked to each other. C57BL/6 and (BxS)F_1 mice were injected with a low dose (10 μg/25g mouse) LPS and IL-6 was measured 3 hours after injection. (BxS)F_1 mice barely produce IL-6, while C57BL/6 mice still produce high amounts of IL-6 (Figure III.7.5.). Thus, we can also use a low dose of LPS to screen consomic mice for their response to LPS.

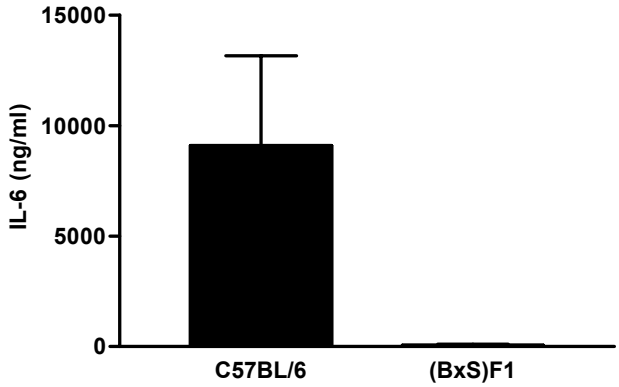

Figure III.7.5. – IL-6 concentration after a low dose LPS. C57BL/6 and (BxS)F_1 mice (n = 2) were injected i.p. with a low dose of 10 μg/25 g bodyweight LPS and IL-6 was measured in serum 3 hours after LPS injection.

7.3.3. Chromosome 2 semi-consomic mice

For chromosome 2, mice heterozygous for marker loci D2Mit32, D2Mit295, D2Mit472, D2Mit160, D2Mit510 and D2Mit109 (Figure III.7.7.) were selected for backcrossing to C57BL/6. In our initial interspecies backcross experiment, we found that a LPS-resistance locus is located on distal chromosome 2 between markers D2Mit516 and D2Mit109. Some chromosome 2 semi-consomic mice were injected with a high dose of LPS to look at lethality, while others were injected with a low dose LPS to look at body temperature and IL-6 in serum.

In total, we injected 54 semi-consomic offspring of intercrossed consomic mice at generation N7 (see Figure III.7.6.) with a high dose of LPS (100 μg/25g bodyweight). Mice were typed and can be heterozygous (B/S) or homozygous (B/B or S/S) for marker loci op chromosome 2. For mice at generation N7, the remaining heterozygous SPRET/Ei fraction - except for chromosome 2 - is less than 1%. Out of 54 mice, 13 mice survived. There was no linkage between a locus on chromosome 2 and lethality after LPS. For example, a mouse homozygous B/B for all the marker loci survived the LPS injection, while a mouse S/S or B/S for all the marker loci died.

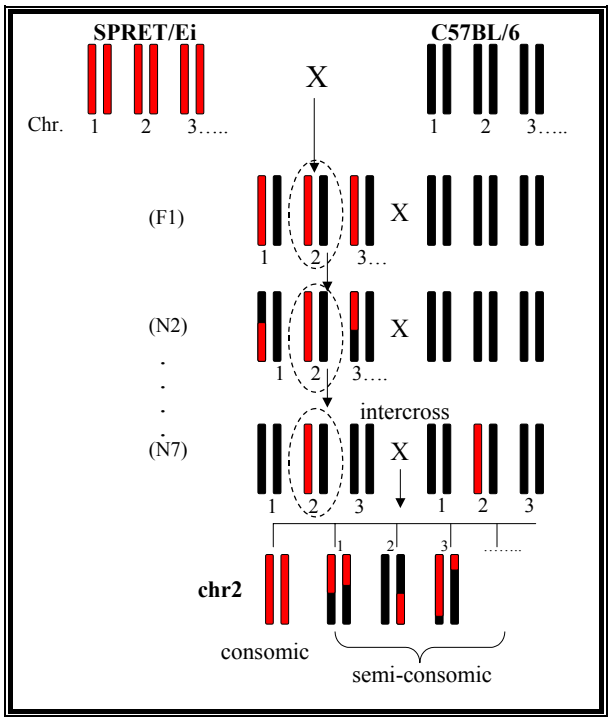

Figure III.7.6. – Difference between consomic and semi-consomic mice. Chromosome 2 is used as an example. An intercross between two chromosome 2 consomic mice (N7) generates consomic mice (homozygous (S/S) for all marker loci) or semi-consomic mice (heterozygous (B/S) or homozygous (B/B or S/S) for marker loci).

Also, 37 semi-consomic offspring of intercrossed consomic mice at generation N7 were injected with a low dose LPS (10 μg/25g bodyweight). Concerning the IL-6 concentration, no linkage at all is found with chromosome 2. Also, no significant linkage can be found between body temperature 2, 6 and 24 hours after the LPS injection and chromosome 2 (Figure III.7.7.). Most semi-consomic mice show low body temperature even after a low dose of 10 μg/25g mouse.

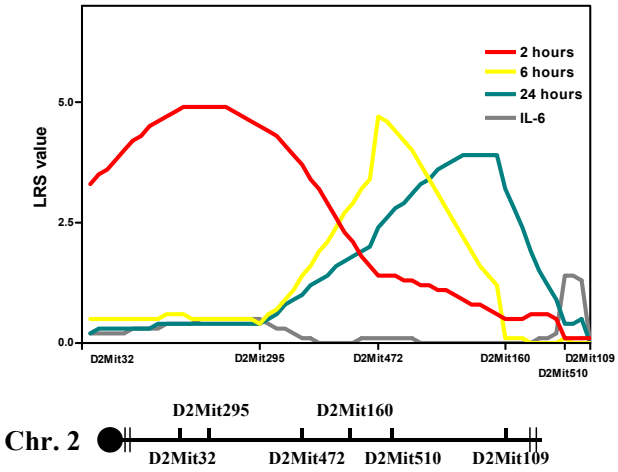

Figure III.7.7. – Interval mapping analysis of chromosome 2 semi-consomic mice. Interval mapping analysis using body temperatures and IL-6 concentration after a low dose LPS as trait.

7.3.4. Chromosome 10 (semi)-consomic mice

We decided to generate consomic mice for chromosome 10 (and 13), despite the fact that only suggestive linkage is found with the LPS-resistance. In each QTL experiment, a QTL can be underestimated and a QTL can be overestimated. For chromosome 10, mice heterozygous for marker loci D10Mit16, D10Mit186, D10Mit309, D10Mit233 and D10Mit238 were selected for backcrossing to C57BL/6 (Fig III.7.8.). In our initial interspecies backcross experiment, we found that a LPS-resistance locus is located on proximal chromosome 10 between markers D10Mit 16 and D10Mit253.

Chromosome 10 consomic mice were injected with a high dose of LPS to look at lethality, while chromosome 10 semi-consomic mice were injected with a low dose LPS to look at body temperature and IL-6 in serum. In total, 12 consomic mice at the N5 and N6 generation, heterozygous for all marker loci, were injected with a high dose of LPS (100 μg/25g bodyweight). At generations N5 and N6, the remaining heterozygous SPRET/Ei fraction - except for chromosome 10 - was 6.25% and 3.125%. All mice died, except one. There was no linkage between a locus on chromosome 10 and lethality. The

impressive resistance that SPRET/Ei mice display towards the lethal effects of LPS is lost in the chromosome 10 consomic mice.

Also, 66 chromosome 10 semi-consomic mice were injected with LPS at generations N4 and N5. At generations N4 and N5, the remaining heterozygous SPRET/Ei fraction - except for chromosome 10 - was 12.5% and 6.25%. Semi-consomic mice were injected with a low dose LPS (10 μg/25g bodyweight) and we looked at body temperature and IL-6 induction. We did find significant linkage between the IL-6 concentration and a region between markers D10Mit16 and D10Mit186 (significant level; LRS = 6.9). Thus, the peak LRS score comprises not the same region as identified by the initial backcross. Also, significant linkage was found between body temperature 3 hours after LPS and a region between markers D10Mit186 and D10Mit309 (significant level; LRS = 7.4). Nevertheless, there was no significant linkage anymore between body temperature 7 hours after LPS and genotype (Figure III.7.8.). Thus, chromosome 10 semi-consomic mice show linkage with a region between D10Mit16 and D10Mit186 and the IL-6 concentration after a low dose LPS.

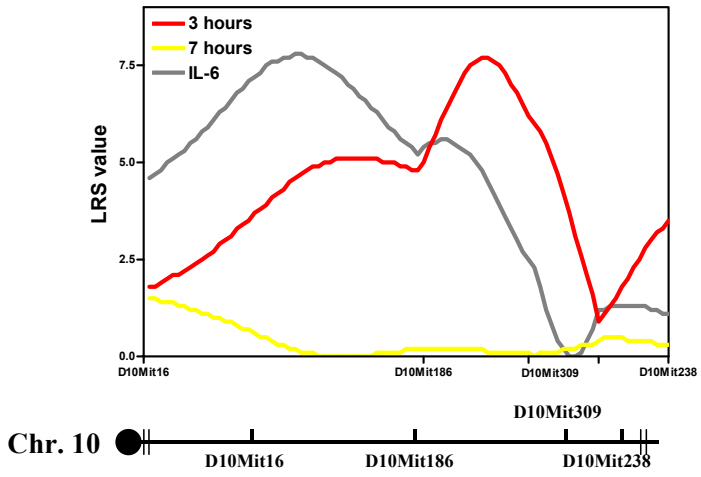

Figure III.7.8. –– Interval mapping analysis of chromosome 10 semi-consomic mice. Interval mapping analysis using body temperatures and IL-6 concentration after LPS as trait.

7.3.5. Chromosome 13 semi-consomic mice

For chromosome 13, mice heterozygous for marker loci D13Mit198, D13Mit24, D13Mit285, D13Mit145 and D13Mit76 were selected for backcrossing to C57BL/6. In total, 33 semi-consomic mice for chromosome 13 were used at generations N3, N4 and N5. At generations N3, N4 and N5, the remaining heterozygous SPRET/Ei fraction, except for the selected chromosome, was 25%, 12.5%, 6.25%. In our initial interspecies backcross experiment, we found that a possible LPS-resistance locus is located on

distal chromosome 13. At different generations, semi-consomic mice were injected with a low dose LPS (10 μg/25g bodyweight) and we looked at body temperature. We did not find significant linkage between body temperature after LPS and chromosome 13 (Figure III.7.9.).

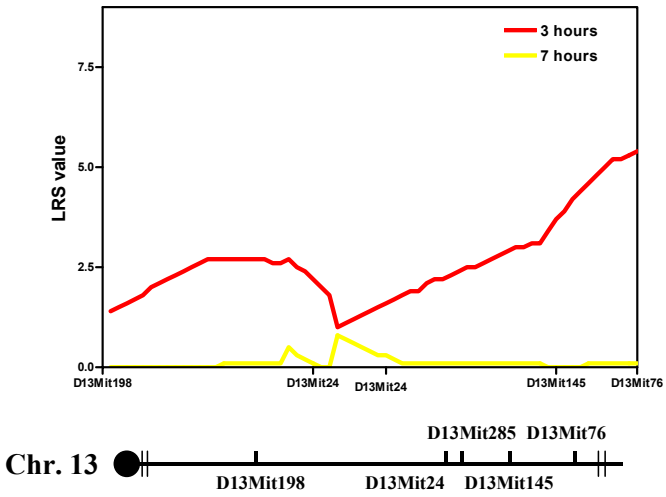

Figure III. 7.9 - Interval mapping analysis of chromosome 13 semi-consomic mice. Interval mapping analysis using body temperatures after LPS as trait.

7.3.6. Chromosome X semi-consomic mice

Mice heterozygous for marker loci DXMit50, DXMit60, DXMit130, DXMit135 and DXMit160 were selected for backcrossing to C57BL/6.

Only 15 chromosome X semi-consomic mice at generations N4 and N5 could be generated. At generations N3, N4 and N5, the remaining heterozygous SPRET/Ei fraction, except for chromosome 13, was 25%, 12.5% and 6.25%. In our initial interspecies backcross experiment, we found very significant linkage with chromosome X with a peak between markers DXMit130 and DXMit135. Almost the entire chromosome X was significantly linked with the LPS-resistance. At different generations, semi-consomic mice were injected with a high dose LPS (100 μg/25g bodyweight) to investigate body temperature and IL-6 induction. No significant linkage between body temperature, lethality and IL-6 after LPS was found. 6 mice out of 15 survived an injection of 100 μg LPS. Because we had breeding problems, only few consomic mice for chromosome X were generated. We did not have a group large enough to confirm the role for chromosome X in the LPS-resistance.

7.4. Conclusion

In an initial backcross experiment using 'the single-locus association' and SIM tests we found that loci on chromosome 2, 10, 13 and X are linked with the LPS-resistance of SPRET/Ei mice. On chromosome 6 we found a locus containing a sensitivity gene for LPS. Chromosome X is highly significantly linked with the LPS-resistance. However, because of the breeding problems that we encountered we were not able to generate a high amount of consomic mice, especially chromosome X consomic mice. By using (semi-) consomic mice we could not turn the LPS-resistance phenotype into a monogenic trait. We only found a region in the proximal part of chromosome 10 with some linkage with the IL-6 concentration after injection of a low dose LPS. However, this region is not the same region as found in the initial backcross and consomic mice for chromosome 10 do not survive a dose of 100 μg LPS, which is a dose 5 times less than the 500 μg dose that SPRET/Ei and (BxS)F_1 mice can resist (Table III.7.3.). Also, although the use of a low dose LPS (score for quantitative traits such as IL-6 and body temperature) is a more subtle approach than the use of a high dose LPS (score for dead or alive), the IL-6 concentration and the LPS response in terms of lethality might not be linked to each other. Hence, it looks that we are dealing with epistatic interactions between the different loci.

Indeed, we reanalyzed the data of the backcross and found that the cumulative effect of the detected QTLs is about 30% of the total trait variance. This suggests that epistatic interactions between the detected QTLs or additional QTLs were not detected. We found possible interactions, using the 'interactions' function of Map Manager QTX, between chromosomes 13 and X and between chromosomes 2 and X. This is not surprising since these are the most powerful QTL identified in the genome scan. So, a lack in this work is the generation of double consomic mice for chromosome 2 and chromosome X. However, after 3,5 years of trying to generate chromosome X consomic mice we decided to abandon this strategy and use an *in vitro* approach to identify the LPS-resistance genes of SPRET/Ei. Also, during this work we demonstrated that SPRET/Ei mice are defective in IFN-β induction after LPS and that this defective IFN-β production is a critical factor in the resistance of SPRET/Ei mice to LPS. So, probably it would have been better to measure the IFN-β concentration in serum of consomic mice instead of measuring IL-6 and body temperature. However, most of our experiments with consomic mice were already done and no serum was left.

Table III.7.3. – Overview of linkage found between different traits and chromosome 2, 10, 13 or X.

(Semi-) consomic	High dose LPS	Low dose LPS	
	Lethality	Body temperature	IL-6
Chr. 2	-	-	-
Chr.10	-	+ (Δ3h)	+
Chr.13	ND	-	-
Chr.X	-	ND	ND

We think that the loss of the LPS-resistance phenotype in the consomic mice is due to the absence of epistatic interactions (the function of one gene is dependent on the function of another gene) between different QTL or due to the absence of cooperation between the different loci (a threshold has to be crossed before becoming fully resistant to LPS). Also, additional QTL might be missed in the backcross experiment. The power of a QTL detection experiment is dependent on the strength of the QTL and the amount of mice. Thus, a backcross of 90 mice is not sufficient to detect weak QTL. A minimum of 1000 backcross mice are needed to detect very weak QTL, suggesting that loci contributing only a small effect are not detected in a simple genome scan. Only the loci having the strongest effects, in our case on chromosome 2, 10, 13 and X, are detected in this way.

To reduce complexity and to identify in this way the less powerful QTLs, we also tried to generate 'blind congenic' mice. Therefore, (BxS)F_1 mice (LPS resistant) are crossed back to C57BL/6 mice (LPS sensitive) and offspring are injected with a high dose LPS. Depending on the number of resistance genes present, a certain number of offspring will survive a high dose of LPS. This is repeated for a number of generations. After 5 generations C57BL/6 mice are generated containing only a fraction (6.25%) SPRET/Ei genome. However, because of sterility after a high dose LPS we were not able to generate 'blind congenic' mice.

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IV. SUMMARY OF THE RESEARCH

1. SUMMARY OF THE RESEARCH AND DISCUSSION

A first part of this work involved the detailed description of the LPS resistance of SPRET/Ei mice. Therefore we injected mice with high doses LPS. Compared to the commonly used laboratory strain C57BL/6, SPRET/Ei mice resist 10 times the LD_{100} for C57BL/6. C57BL/6 mice die from a dose of 50 μg LPS. SPRET/Ei and (BxS)F1 mice easily survive a dose of 500 μg, which shows that the hypo-responsiveness to LPS is a dominant trait. Even at this high dose, SPRET/Ei mice do not show any sign of illness. A number of pathological parameters were examined. In C57BL/6 mice, LPS induced hypothermia, liver and lung inflammation (as observed by attachment of leukocytes to the endothelium of blood vessels and migration of leukocytes to the alveoli) and MMP9 expression (a marker for the progression of inflammation). In SPRET/Ei and (BxS)F1 mice, LPS induced no hypothermia and expression of MMP9 in serum was lower than MMP9 expression in serum of C57BL/6 mice. In SPRET/Ei lungs and livers much less leukocytes attach to the endothelium and leukocytes do not migrate to the alveoli. By bone marrow transplantation experiments we could demonstrate that the LPS-resistant phenotype of (BxS)F1 mice depends critically on bone marrow-derived cells, likely macrophages.

In vivo gene induction was measured by studying the IL-6 and TNF-α concentrations in the serum after LPS. SPRET/Ei induces IL-6 and TNF-α after LPS injection, but at significantly lower levels than in C57BL/6. Especially 3 and 9 hours after LPS injection IL-6 levels are significantly different between SPRET/Ei and C57BL/6. IL-6 induction in $(BxS)F_1$ mice was as low as in SPRET/Ei mice. In addition, to confirm that the LPS resistance of SPRET/Ei is not specific for *S.a.e.* LPS, we also tested the response of $(BxS)F_1$ mice to LPS derived from *E.coli*. $(BxS)F_1$ mice resist 500 μg *E.coli* LPS, a dose 100% lethal to C57BL/6 mice. In $(BxS)F_1$ mice, LPS did not induce hypothermia or high levels of IL-6.
In vitro gene induction was determined in thioglycolate-elicited macrophages and in mouse embryonic fibroblast (MEF) cells. Again, we used IL-6 expression to look at gene induction. SPRET/Ei macrophages and immortalized MEFs produced significantly lower amounts of IL-6 compared to C57BL/6 macrophages and MEFs. These reduced IL-6 levels in SPRET/Ei are in line with the reduced IL-6 serum levels after injection of LPS. However, we measured high basal levels of IL-6 in both C57BL/6 and SPRET/Ei thioglycolate-elicited macrophages and in MEFs. So, probably due to the thioglycolate and the immortalization with SV40 Large T, respectively macrophages and MEFs are already activated to express IL-6. In contrast, in BMDMs obtained from both C57BL/6 and SPRET/Ei we did not detect basal IL-6 levels. Hence, BMDMs are far more reliable to use as an *in vitro* model.

Since we were pleasantly surprised of the spectacular nature of the LPS resistance of SPRET/Ei, we wanted to identify the genes conferring resistance to LPS. We have followed two different approaches to identify the LPS resistance genes: a genetic approach and an *in vitro* approach using BMDMs. Considering this extreme resistance of SPRET/Ei, very strong protective genes are supposed to be present. SPRET/Ei is not only known for its extreme and dominant resistance to LPS, but also for its resistance against arthritis, partial resistance against asthma and extreme and dominant resistance to TNF (Staelens et al., 2004; Staelens et al., 2002). Nevertheless, the LPS resistance is probably not due to the same genes causing the TNF resistance. This assumption is based on following arguments:

- TNF-deficient mice and TNF-receptor-deficient mice show increased susceptibility to high-dose LPS lethality (Marino et al., 1997; Rothe et al., 1993).
- The TNF-resistant mouse strain DBA/2 is not resistant to LPS (Dr. Ben Wiellockx and Dr. Claude Libert, unpublished data).
- LPS-resistant C3H/HeJ mice are totally responsive to TNF.
- The loci linked with the TNF resistance are different from those linked with the LPS resistance.
- 30% of the TNF-resistant backcross mice are sensitive to LPS (Dr. Jan Staelens, unpublished data).

These arguments demonstrate that probably other genes than those genes providing hypo-responsiveness to TNF account for the extreme resistance to LPS.

We first performed an interspecific backcross (n = 90). After analysis of the data from the backcross we generated an additional 51 BSB mice and these mice were genotyped only at marker loci lying on the 4 chromosomes detected in the 'genome scan' (see further). At the age of 8 weeks BSB backcross mice were injected with 250 μg LPS, a dose 100% lethal for C57BL/6, but not lethal for SPRET/Ei. Of the 141 mice challenged, 90 mice (64%) survived and 51 mice (36%) died. In case of a monogenic trait, 50% of the mice should die and 50% should survive. However, only 36% of the BSB mice are sensitive to LPS suggesting that the LPS resistance of SPRET/Ei is a complex trait, controlled by multiple loci. Survival data and genotyping data were inserted into MapManager QTX B17 and a 'genome scan' was performed. Using 'the single-locus association' and 'simple interval mapping' tests of MapManager we found that the LPS resistance of SPRET/Ei is linked to loci on chromosomes 2, 10, 13 and X. Suggestive linkage was found with loci on chromosome 10 and 13, almost significant linkage with a locus on chromosome 2 and highly significant linkage with a locus on chromosome X. On chromosome 6 we found a locus containing an LPS sensitivity gene.

By using (semi-) consomic mice we tried to confirm the importance of the detected loci in the LPS resistance of SPRET/Ei. We used both a low and a high dose LPS to screen the consomic mice for their response to LPS. One advantage of using a low dose LPS is that quantitative traits such as IL-6 and body temperature can be measured. The use of a low dose LPS is a more subtle approach than the use of a high dose LPS. Using a high dose of LPS, mice are scored as either dead or alive. For example, a mouse with a significant drop in body temperature that survives the high dose LPS injection will be scored as alive. However, a disadvantage of using a low dose LPS is that the IL-6 concentration and the LPS response in terms of lethality might not be linked to each other. It was necessary to make our own consomic strains, because STF/Pas and SEG/Pas - 2 other inbred strains derived from *Mus spretus* individuals and from which consomic strains are available in the Institut Pasteur, Paris, France - are not resistant to LPS-induced lethality. So, our own consomic strains were generated. However, we were not able to turn the LPS resistance phenotype into a monogenic trait by isolating the individual QTLs: using (semi-) consomic mice we only found a region between markers D10Mit16 and D10Mit186 - a region that is different from the region found on chromosome 10 in the initial backcross – which showed some linkage with the IL-6 concentration after injection of a low dose LPS. The chromosome 10 consomic mice did not survive a dose of 100 μg LPS, a dose 5 times smaller than the 500 μg dose that SPRET/Ei and (BxS)F_1 mice can resist. So, maybe a gene is present in the proximal region of chromosome 10 positively influencing the response to LPS. This region is still very large and by looking at the public Mouse Genome we could not find genes known to be involved in the LPS response. Taken the data of the consomic mice together, it looks that the presence of only one of the resistance QTLs is not sufficient to explain the extreme resistance of SPRET/Ei to LPS and hence that we are dealing with a case of complex genetics, on top of that depending on epistatic interactions between the different loci. I have to stress that, because of the breeding problems that we encountered, we were not able to generate a high amount of consomic mice. Especially the generation of chromosome X consomic mice went very bad, which was frustrating because this was precisely the chromosome we wanted to investigate in detail as much as possible using consomic mice.

By further analyzing the linkage data of the backcross, it became clear that the combined effects of chromosome 2, 10, 13 and X account for ± 30% of the total variation (effect of individual loci ranges from 4 to 10%). This suggests that epistatic interactions between the QTL - found in the backcross - or a number of additional QTLs, were not detected. Possibly, 2 or more loci are acting together in an additive fashion. This means that the one QTL present has only a sub-phenotype of the original complex phenotype because of the absence of additive effects from the other QTL(s) and that a threshold has to be reached to obtain full LPS resistance. Another explanation could be that QTLs are working in an epistatic fashion, meaning that interactions between QTLs result in an effect on the trait that would not

be predicted from the sum of the individual QTL effects. It is also possible that other loci involved in the resistance were not detected since a backcross of minimum 1000 mice is needed to detect a very weak QTL. Given the poor breading performance we had to deal with, expansion of the backcross was not considered as a valid option. By using the 'interactions' function of Map Manager QTX, we tested possible epistatic interactions (Manly et al., 2001). Multiple loci are possibly involved in epistatic interactions, but most notably DXMit135, located on the distal part of chromosome X, is involved in epistatic interactions with loci on chromosomes 2 and 13, both also detected using the SIM analysis. Knowing that there are probably epistatic interactions between QTLs, we performed composite interval mapping (CIM) to control for background QTLs. We found suggestive linkage with a locus on chromosome 12. The locus on chromosome 12 seems to contain a sensitivity gene and is also found as a locus involved in epistatic interaction with a locus on chromosome 13. A shortage in this work is the generation of double consomic mice for chromosome 2 and X. These two chromosomes are the strongest ones which are linked with the LPS resistance phenotype and possible epistatic interactions between loci on chromosome 2 and chromosome X indeed were clearly detected by MapManager QTX. However, after 3,5 years of problematic generation of consomic mice we decided to abandon this genetic approach to identify the LPS resistance genes and not to generate chromosome 2-X-double consomic mice and to focus on another strategy instead.

Because of the spectacular resistance of SPRET/Ei to LPS and the great amount of genetic polymorphisms between C57BL/6 and SPRET/Ei, we thought that the identification of the LPS resistance genes would be quite simple. We believed that based on the backcross data, we would easily find the QTL linked with the LPS resistance and that this extreme phenotype of SPRET/Ei would be (partly) present in consomic mice. Indeed, when this would have been the case, it would have been possible to fine map the region by setting up a second and larger backcross between an LPS-resistant consomic mouse and C57BL/6, to end up with a small critical region with a limited amount of candidate genes. Nevertheless, the backcross experiment was an interesting experience and at least learned that extreme phenotypes may be entirely depending on interaction between loci, just as was found earlier by Jan Staelens during his attempts to clone the SPRET/Ei TNF resistance genes.

In a second approach, I tried to identify the molecular basis of the LPS resistance using an *in vitro* system. The SPRET/Ei LPS hypo-responsiveness *in vitro* in macrophages was used to investigate the mechanism of the LPS resistance. The LPS-induced response involves a complex network of signalling molecules, which end up in inducing expression of a variety of genes. The binding of LPS to TLR4 causes the activation of the MyD88-dependent pathway leading to early NF-κB and MAPK activation

and the TRIF-dependent pathway leading to late NF-κB and MAPK activation and IRF-3 activation (Kawai et al., 1999; Yamamoto et al., 2003). LPS-induced pro-inflammatory gene induction (e.g. IL-6 and TNF) is dependent on the NF-κB pathway, but also on the activation of other transcription factors such as AP-1, which is dependent on the activation by MAPK p38 and JNK (Akira et al., 1990; Dendorfer et al., 1994; Guha and Mackman, 2001; Guha et al., 2001). A key event in the activation of NF-κB is the nuclear translocation of NF-κB after phosphorylation and subsequent degradation of the IκB protein (Akira and Takeda, 2004). We showed that the MyD88-dependent pathway leading to transient degradation of IκBα was normal in SPRET/Ei BMDMs at early time points. We also demonstrated that phosphorylation of p38 MAPK and JNK1/2 was intact. Furthermore, the intact MyD88 signalling was confirmed by the absence of a significant difference between SPRET/Ei and C57BL/6 BMDMs in LPS-induced early TNF-α and IL-6 mRNA expression. Furthermore, LPS-induced mRNA expression of, e.g. *Il1a*, *Il1b*, *Il12a*, *Il12b*, *Bcl2*, *Birc4* (encoding XIAP) and *Tnfaip3* (encoding A20*)* was not significantly different between C57BL/6 and SPRET/Ei.

So, IL-6 and TNF-α mRNA expression *in vitro* is normal in SPRET/Ei 1 hour and 4 hours after LPS. In contrast, IL-6 levels (3 hours after LPS) and TNF-α levels (1 hour after LPS) in serum of SPRET/Ei *in vivo* are significantly lower compared to IL-6 and TNF-α levels in serum of C57BL/6 mice. However, an *in vivo* situation is much more complex than an *in vitro* system. LPS can activate various cell types such as macrophages, DCs, neutrophils and fibroblasts to produce pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6 and IL-1. Subsequently, these cytokines (e.g. IL-1) might activate other cells to produce also IL-6 and TNF-α.

Next to an early phase NF-κB induction, TLR4 can also induce a late phase NF-κB and AP-1 activation by the TRIF-dependent pathway (Akira and Takeda, 2004). The adaptor molecule TRIF can recruit both TRAF6 and TBK1/IKK-ε to its N-terminal domain and recruitment of TRAF6 leads to a second wave of pro-inflammatory cytokine production. TRIF -/- macrophages are defective in their TNF-α, IL-6, and IL-12p40 production 24 hours after LPS stimulation (Yamamoto et al., 2003). We found that 24 hours after LPS stimulation IL-6 expression in SPRET/Ei MEFs was strongly impaired compared to IL-6 expression in C57BL/6 MEFs. In the same experiment it was confirmed that IL-6 induction was normal at an early time point, namely 4 hours after LPS stimulation. Thus, the (TRIF-dependent) late pro-inflammatory gene induction is defective in SPRET/Ei cells, while the early pro-inflammatory gene induction is intact.

However, it should be stressed that normal, transient IκB degradation and normal phosphorylation of MAPK p38 and JNK does not mean that NF-κB and AP-1 activation is intact in SPRET/Ei, but it demonstrates that the signalling components upstream are intact. In the future, *in vitro* experiments should be performed to analyze activation of these transcription factors by means of electrophoretic

mobility shift assays. In addition, a possible explanation for the defect in late IL-6 induction might be that an anti-inflammatory mechanism is overactive in SPRET/Ei. In endotoxin tolerance, which is a transient state of hypo-responsiveness towards a second stimulation by LPS after a preceding stimulation, one mechanism is the induction of specific anti-inflammatory mechanisms such as accumulation of p50-p50 NF-κB homodimers and the induction of signalling pathways such as PI3K. It is still possible that in SPRET/Ei p50-p50 NF-κB homodimers, lacking transactivation activity, are formed instead of the transcriptionaly active p50-p65 heterodimers. It has been observed that in endotoxin tolerance, formation of p50-p50 homodimers leads to a decrease in TNF levels (Goldring et al., 1998). Another mechanism to regulate the endotoxin tolerant phenomenon is the induction of PI3K signaling (Bowling et al., 1995). PI3K, found on mouse chromosome 13, can inhibit TLR signalling. The underlying mechanism remains unknown, but seems to involve the suppression of p38, JNK, ERK1/2 and NF-κB (Fukao et al., 2002). Thus, maybe an anti-inflammatory mechanism, such as the formation of p50-p50 homodimers or the induction of the signalling pathway PI3K, that blocks pro-inflammatory gene induction is overactive in SPRET/Ei. This anti-inflammatory mechanism could be induced by NF-κB or AP-1 themselves since at early time points IL-6 and TNF-α are perfectly induced in SPRET/Ei.

LPS induces expression of the *Ifnb* gene through a MyD88-independent TRIF-dependent pathway (Yamamoto et al., 2002). Poly (I:C), a TLR3 ligand, also induces the TRIF-dependent pathway to induce IFN-β. The TRIF-dependent pathway leads to activation of the transcription factor IRF-3, resulting in IFN-β induction, STAT1 phosphorylation and translocation, and the induction of STAT1 dependent genes, such as IRF-7 (Yamamoto et al., 2003). We showed that the induction of IFN-β mRNA by LPS, but also by poly (I:C), was largely impaired in SPRET/Ei-derived BMDMs. As a consequence of the defective IFN-β induction, LPS-induced STAT1 phosphorylation and subsequent nuclear translocation were impaired, and this in turn impaired the induction of IRF-7. The defect in IFN-β production is also observed *in vivo* in SPRET/Ei mice treated with LPS or *Influenza* virus.

IFN-β was discovered as a potent antiviral molecule (Isaacs and Lindenmann, 1957). For a long time, it was believed that this was the only function of type I IFNs, but in the last few years a more extensive role for IFNs in immunity has been demonstrated. We determined the role of IFN-β in the LPS resistance of SPRET/Ei and also the consequences of this defective IFN-β induction. Since the resistance of SPRET/Ei mice can be reversed by administration of exogenous IFN-β, this suggests that IFN-β is an important factor in the LPS-induced lethality. Deletion of genes encoding IFN-β or genes involved in the induction of or in the response to IFN-β can induce resistance to endotoxemia (Karaghiosoff et al., 2003; Sakaguchi et al., 2003). In our hands, IFNAR1 knockout mice are resistant

against a lethal dose LPS (we are the first group to publish this), but they do show a serious drop in body temperature. In contrast, SPRET/Ei mice have a stable body temperature after injection of the same dose of LPS. Therefore, it seems that other genes besides *Ifnb* determine the complete LPS-resistant phenotype of SPRET/Ei. Hereby the data of the genome scan demonstrating that the LPS resistance is a complex trait is confirmed. In collaboration with Dr. J. Grooten, we can use macro-array filters containing about 500 macrophage specific genes to study the differential mRNA expression in BMDMs of SPRET/Ei and C57BL/6. We have demonstrated that macrophages are the relevant cell type in the LPS resistance of SPRET/Ei. We already did a first differential expression study using these filters and observed that 6 hours after LPS stimulation about 50 genes are $\geq$ 2 times higher expressed in SPRET/Ei BMDMs and about 50 genes are $\geq$ 2 times less expressed in SPRET/Ei BMDMS compared to C57BL/6 BMDMs. This differential expression analysis has to be repeated, and we hope to lower the high number of differentially expressed genes by comparing data after LPS and poly (I:C) stimulation and studying more than one time point. Using the bioinformatics program 'Difference Distance Matrix Analysis' we hope to find transcription factors/genes that might be responsible for the differential expression of SPRET/Ei genes.

The defect in IFN-β has its consequences. It has been demonstrated that IFN-β can sensitize macrophages for cell death induced by *Listeria monocytogenes* and that it can protect against progressive leishmaniasis (Mattner et al., 2004; Stockinger et al., 2002). Probably due to the defective induction of IFN-β, SPRET/Ei mice indeed are completely resistant to *Listeria monocytogenes* and (BxS)F_1 mice are highly sensitive to *Leishmania major* infection. We also plan to study the response of SPRET/Ei mice to viral infections. Since IFN-β is a well-known potent antiviral cytokine, we assume that SPRET/Ei mice will be very vulnerable for viral infections. Several studies suggest that IFN-β is essential in LPS-induced endotoxemia, but its importance in Gram-negative sepsis has yet to be investigated. The advantages and disadvantages of blocking IFN-β or its receptors in the treatment of septic patients are discussed in depth in the review: 'Must we inhibit type I IFNs in endotoxemia and sepsis?' (Mahieu and Libert).

Another part of this work was to unravel the mechanism of the defective IFN-β induction. The defect can be situated at different levels: there can be a defect in the IFN-β promoter or in one of its regulators, in one of the molecules of the TRIF-dependent pathway or in the positive feedback loop induced after IFN-β signaling. First, we studied the sequence of the IFN-β promoter region but found no variations between C57BL/6 and SPRET/Ei. Second, we investigated the activation of IRF-3 as an endpoint of the

TRIF-dependent pathway. IFN-β induction is dependent on the phosphorylation/activation of the transcription factor IRF-3. Therefore, we sequenced the serine/threonine cluster in the C-terminal region of IRF-3. No variations were found in the important serines of this region, but we did find some other unique amino acid changes, which might have an influence on the phosphorylation of IRF-3. However, about the same time we demonstrated that IRF-3 becomes phosphorylated in SPRET/Ei BMDMs, our collaborator Dr. Jin Mo Park could show that IRF-3 BMDMs had a broader gene defect (*Ifnb*, *Rantes*, *Isg15* and *Cxcl10*) compared to SPRET/Ei BMDMs (*Ifnb* and *Rantes*) after LPS stimulation. Of course, normal phosphorylation of IRF-3 does not necessarily mean that the IRF-3 molecules dimerize or that its co-activators CBP/p300, or the trans-activators of CBP/p300 CITED1 and CITED2, found on mouse chromosome X and 10, are normal in SPRET/Ei. Interestingly, these two chromosomes are linked with the LPS resistance phenotype. Possibly, abolished or changed activation of these co-factors can change the IRF-3 dependent induction of IFN-β. In addition, CBP/p300 are co-activators of the NF-κB component p65 (Gerritsen et al., 1997). Thus, perhaps a defect in CBP/p300 or its trans-activators CITED1 and CITED2 can be responsible for both defective IL-6 induction and defective IFN-β induction. In addition, we will also look at IRF-3 dimerization, NF-κB and AP-1 activation, and NF-κB dimers at early and late time points after LPS stimulation.

Another interesting fact is that the IFN-β mRNA stability is regulated at the posttranscriptional level by an AU-rich element in the 3' UTR, and a second element called CRID in the 3' end of the coding region. So far, we have not sequenced the entire *Ifnb* gene and we have not looked at the sequence and expression of positive/negative regulators of the IFN-β promoter such as YY1, IRF-1 and IRF-2 (NRF-knockout mice are susceptible to LPS-induced lethality). Clearly, these elements require further investigation. Furthermore, it is interesting to know that the promoters of the *Ifnb* and *Ccl5* (encoding RANTES) genes both contain an NF-κB and an IRF-3 PRD element. In that case, the defective induction of both the *Ifnb* gene and the *ccl5* gene can be caused by a defective transactivation between NF-κB and IRF-3 transcription factors. So, an anti-inflammatory mechanism inhibiting NF-κB activation, such as formation of p50-p50 homodimers, or a defect in co-factors of IRF-3, such as CPB/p300 and CITED1/2, can be responsible for both defective late pro-inflammatory gene induction and defective IFN-β induction. Using a bioinformatics tools we will search for other genes that contain both an NF-κB and IRF element in their promoter and investigate their expression after LPS.

The defect can also be situated in the positive feedback loop after IFN-β signaling. We demonstrated that OAS mRNA induction, which is dependent on the formation of a STAT1/STAT2/IRF9 complex after IFNAR stimulation, is significantly lower in SPRET/Ei BMDMs after IFN or LPS stimulation We also showed that IFNAR1 mRNA becomes rapidly down-regulated in SPRET/Ei after IFN-β stimulation. The

mechanism and significance of this event is not yet known. The same was demonstrated after LPS stimulation. In addition, significantly less IL-6 is induced upon IFN stimulation in SPRET/Ei BMDMs. If these defective gene inductions after IFN are mediated by the down-regulation of the IFNAR1 has yet to be shown. Future work will need to address which mechanism exactly is responsible for the phenomenon of IFNAR1 down-regulation. Furthermore, sequence and expression analysis of several other molecules of the JAK-STAT pathway should be performed. The over-expression of a negative regulator would correlate with the dominance of the LPS resistance phenotype. Therefore, it would also be interesting to investigate the negative regulators of the JAK-STAT pathway such as SOCS, SHP and PIAS molecules.

As suggested clearly other genes are involved in the LPS resistance of SPRET/Ei. It has been demonstrated that IFN-β is able to induce the expression of a large set of chemokines (Coelho et al., 2005). Expression of chemokines leads to migration of leukocytes to the inflammatory sites. Interestingly, we have seen that in LPS-injected SPRET/Ei mice leukocytes do not migrate to the alveoli and in lungs and livers of LPS-injected SPRET/Ei mice fewer leukocytes were found attached to the endothelium. In addition, preliminary data from a differential mRNA expression study using blots with macrophage specific genes taught us that several chemokines are not or much less induced in SPRET/Ei BMDMs compared to C57BL/6 BMDMs after LPS stimulation. So, a possible mechanism to explain the importance of IFN-β to the LPS resistance is the following (see also Figure IV.1.1.). The defective IFN-β induction in SPRET/Ei might lead to a defect in chemokine production. The defective chemokine induction leads to a defect in migration of leukocytes to the inflammatory sites and a diminished tissue destruction. Also, IL-6 is produced upon stimulation of the IFNAR1 receptor by IFN-β. The mechanism of IFN-induced IL-6 is also not known at this moment. Hence, a combination of a defect in the MyD88-independent late IL-6 gene induction and a defect in the IFN-β induced IL-6 gene induction leads to significantly diminished IL-6 levels. Pro-inflammatory cytokines such as IL-6 can also act directly on tissue organs. So, a defective IL-6 induction may also lead to diminished tissue destruction.

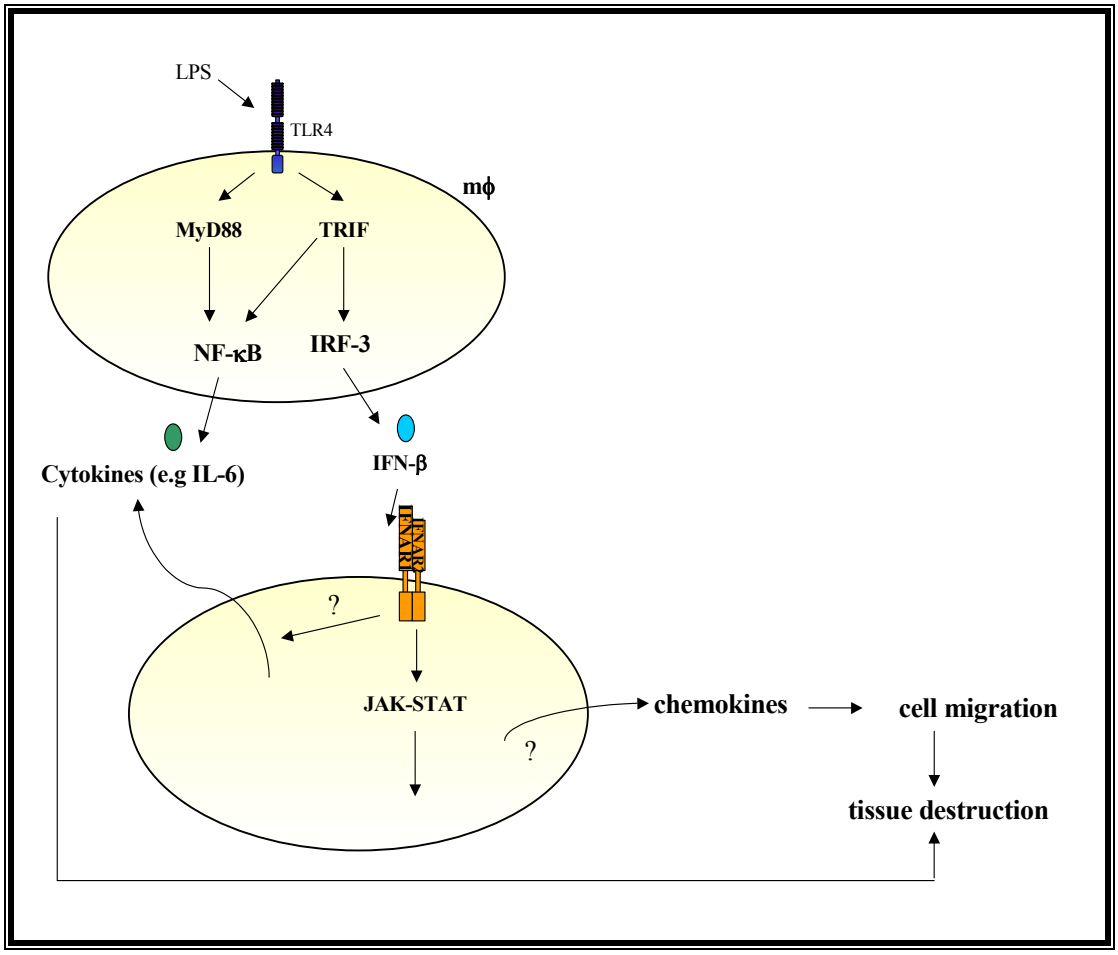


Figure IV.1.1.- Possible mechanism to explain the role of IFN-β in LPS-induced lethality.

Finally, we also tested the response of SPRET/Ei to other TLR ligands. The response to LPS involves the induction of two main signaling pathways, the MyD88-dependent and the MyD88-independent pathway. Most TLRs use only one signaling pathway to induce responses to pathogens, which makes the response not as complex compared to the LPS response (Moynagh, 2005). We demonstrated that the LPS-induced MyD88-dependent signaling events upstream of IκB degradation and MAPK phosphorylation are intact, suggesting a normal MyD88-dependent pathway in SPRET/Ei. LPS-induced IL-6 gene induction at late time points is TRIF-dependent and was impaired *in vivo* and in SPRET/Ei MEFs. MyD88 is the only adaptor molecule that is recruited after IL-1R and is also recruited by TLR2, besides TIRAP. We showed that stimulation with ligands for the IL-1R and TLR2 leads also to a defective pro-inflammatory cytokine induction in SPRET/Ei *in vitro.* In addition, SPRET/Ei mice are also hypo-responsive to IL-1 induced IL-6 induction and zymosan-induced swelling of the foot. The defective induction of pro-inflammatory genes after different TLR ligands can be explained by a common mechanism. As described above, we did not show activation of transcription factors NF-κB and AP-1,

and neither did we look at the composition of the NF-κB dimers formed after TLR stimulation or at co-activators such as CBP/p300 and CITED1/CITED2. Another putative explanation is a defect in IRF-5 activation. It has recently been demonstrated that all TLRs use not only NF-κB but also IRF-5 to induce pro-inflammatory cytokine production, such as TNF, IL-6 and IL-12 (Napolitani et al., 2005). SPRET/Ei is also hypo-responsive to poly (I:C) *in vitro* and can resist poly (I:C)/dGalN-induced lethality. Poly (I:C) only activates the TRIF-dependent pathway, which makes the response less complex compared to the LPS response. This phenotype makes it possible to use poly (I:C) instead of LPS in future experiments to determine the mechanism of the defective IFN-β induction.

In summary, in this work we wanted to map and identify the genes that confer hypo-responsiveness to LPS in SPRET/Ei. After describing the LPS resistance of SPRET/Ei in detail we aimed at mapping the LPS-resistance genes by a genetic strategy. Using this strategy we were not able to identify regions small enough to contain a limited number of candidate genes. Another strategy was to find the mechanism of the SPRET/Ei LPS resistance *in vitro*. Since about half of my time was invested in the typing and screening of mice to enlarge the initial backcross and to make consomic mice, the *in vitro* data are not as extensive as we would like them to be. So, the work presented in my thesis, showing that IFN-β plays a role in the LPS resistance of SPRET/Ei, also still raises a lot of questions about the exact cause of this defect in IFN-β production. Finally, we realize that also other genes than IFN-β are involved in the hypo-responsiveness of SPRET/Ei to LPS and other TLR ligands. Future work will have to identify these genes and their mechanism of action.

Perspectives

In this work we have presented some interesting data concerning the importance of IFN-β in LPS-induced shock. We have demonstrated that SPRET/Ei mice, which have a defective IFN-β production, are resistant against LPS-induced shock, and that (BxS)F_1 mice are partially protected against a Gram-negative infection. However, through our genetic studies and experiments with the IFNAR1-knockout mice, we know that IFN-β is not the only gene involved in the LPS-resistance of SPRET/Ei. Thus, the project concerning the LPS-resistance of SPRET/Ei will continue at two levels:

1. LPS-resistance genes of SPRET/Ei

We have set the following perspectives:

- Mechanism of the defective IFN-β production?
  We will investigate if IRF-3 is the cause of the defective IFN-β production in SPRET/Ei mice by looking at the sequence, dimerization and phosphorylation of IRF-3. We will also investigate other mechanisms or molecules involved in the IFN-β regulation in more detail, namely the degradation of IFN-β mRNA and molecules like SHIP, RIP1, CITED1 and CITED2. In addition, we will look at the relevance of the 'down-regulation' of IFNAR1.

- Are other genes important in the SPRET/Ei LPS-resistance?
  We will investigate if other genes then IFN-β are important in the SPRET/Ei LPS-resistance. For this, we will do a differential expression study of macrophage specific genes after LPS and poly (I:C).

- Gram–negative sepsis?
  SPRET/Ei and/or (BxS)F_1 mice will be used in different models of Gram-negative sepsis (Cecal Ligation and Puncture' (CLP), *Klebsiella pneumoniae and Salmonella typhimurium, …)*. However, the problem is that not much SPRET/Ei mice are available. So, mostly (BxS)F_1 mice will be used.

- Based on preliminary experiments we have seen that the SPRET/Ei phenotype 'resembles' a constitutive endotoxin tolerant phenotype: will this give us new information?

2. SPRET/Ei has a defective IFN-β production

We have set the following perspectives:

- Does IFN-β play an important role in sepsis?

  We will investigate this by using different models: CLP, *K.pneumiae*, *S. typhimurium*, … In addition, Jo et al. showed indeed that blocking type I IFN signalling could have therapeutic application in the treatment of endotoxemia.

- Can we protect against LPS and Gram-negative sepsis by inhibiting type I IFNs or their up-stream or down-stream mediators?

  We will investigate this by using an IFN-β neutralizing Ab (we are developing this in our lab) in C57BL/6 mice. Can we protect C57BL/6 mice against LPS-induced shock and sepsis by administration of an IFN-β neutralizing Ab? Another possibility is the administration of an antagonist of IFNAR1.

  However, it is possible that blocking IFN-β will lead to susceptibility to viral infections (IFNAR1 -/- mice are highly susceptible to viral infection).

Thus, we think that molecules of the type I IFN signalling pathway, like IFN-β and IFNAR1, may be new therapeutic targets in endotoxemia and sepsis, providing that septic patients are not hypersensitive for viral infections and that the activation of the immune system is not compromised. It may be possible to treat septic patients with IFN-blocking agents or agents that block type I IFN signalling over a short time interval in strictly contained conditions to prevent viral infection.

2. SAMENVATTING VAN HET ONDERZOEK EN BESPREKING

Een eerste deel van dit werk beschrijft gedetailleerd de LPS-resistentie van SPRET/Ei muizen. Hiervoor werden muizen geïnjecteerd met hoge doses LPS. In vergelijking met de veelgebruikte laboratoriumstam C57BL/6 kunnen SPRET/Ei muizen 10 keer de LD_{100} voor C57BL/6 verdragen. C57BL/6 muizen sterven aan een dosis van 50 μg LPS. SPRET/Ei en $(BxS)F_1$ muizen overleven zonder problemen een dosis van 500 μg LPS, wat ook het dominante karakter van de LPS resistentie aantoont. Zelfs met zo een hoge dosis vertonen SPRET/Ei muizen geen enkel teken van ziekte. Een aantal pathologische parameters werd onderzocht. LPS induceert in C57BL/6 muizen hypothermie, lever- en longinflammatie (aanhechting van leukocyten aan het endotheel in de bloedvaten en migratie van leukocyten naar de alveoli) en MMP-9 expressie (progressie van inflammatie). In SPRET/Ei en $(BxS)F_1$ muizen ontwikkelde zich geen hypothermie en was de expressie van MMP-9 lager dan in C57BL/6 muizen. In de longen en lever van de SPRET/Ei muizen hechten er zich minder leukocyten vast aan het endotheel van de bloedvaten en migreren er geen leukocyten naar de alveoli. Via beenmergtransplantaties konden we aantonen dat de LPS resistentie van SPRET/Ei muizen afhangt van beenmergafgeleide cellen en meer waarschijnlijk macrofagen.

In vivo gen-inductie werd gemeten door kwantificatie van IL-6 en TNF-α in het serum na LPS injectie. Zowel IL-6 als TNF-α werden inderdaad geïnduceerd in SPRET/Ei, maar in significant mindere mate dan in C57BL/6. 3 uur en 9 uur na LPS injectie waren IL-6 niveaus significant verschillend tussen C57BL/6 en SPRET/Ei. De IL-6 inductie in $(BxS)F_1$ muizen was even laag als in SPRET/Ei muizen. Om aan te tonen dat de LPS resistentie van SPRET/Ei niet specifiek is voor *S.a.e.* LPS, hebben we ook de respons getest op LPS afkomstig van *E.coli*. $(BxS)F_1$ muizen overleven een dosis van 500 μg *E.coli* LPS, een dosis die 100% letaal is voor C57BL/6 muizen. In $(BxS)F_1$ muizen ontwikkelde zich geen hypothermie en werd er significant minder IL-6 geïnduceerd in vergelijking met C57BL/6 muizen.

In vitro gen inductie werd bepaald in thioglycolaat-gestimuleerde macrofagen en in geïmmortaliseerde muis embryonaire fibroblasten (MEFs). Ook hier werd IL-6 gekozen om gen-inductie te bestuderen. SPRET/Ei macrofagen en MEFs induceren IL-6 in significant mindere mate dan C57BL/6 macrofagen en MEFs. Deze gereduceerde IL-6 niveaus komen overeen met de lagere IL-6 niveaus *in vitro* na LPS. Echter, zowel in peritoneale macrofagen als in MEFs van C57BL/6 en SPRET/Ei werden hoge basale IL-6 niveaus gemeten. Waarschijnlijk worden macrofagen en MEFs al aangezet tot de expressie van IL-6 door respectievelijk thioglycolaat en SV40 LargeT. In BMDMs van SPRET/Ei en C57BL/6 muizen werden echter geen basale IL-6 niveaus gemeten. BMDMs zijn dus veel betrouwbaarder om als een in *vitro* model gebruikt te worden.

Door de extreme aard van LPS-resistentie van SPRET/Ei muizen, wilden we de LPS-resistentiegenen identificeren. We hebben dit op twee verschillende manieren aangepakt: een genetische en een *in vitro* benadering. Gezien de extreme resistentie van SPRET/Ei muizen zouden zeer sterke beschermende genen aanwezig moeten zijn. SPRET/Ei is niet alleen gekend voor zijn extreme resistentie tegen LPS maar ook voor zijn resistentie tegen artritis, gedeeltelijke resistentie tegen astma en zijn extreme resistentie tegen TNF (Staelens et al., 2004; Staelens et al., 2002). Nochtans wordt de LPS-resistentie waarschijnlijk niet veroorzaakt door dezelfde genen die TNF resistentie beïnvloeden. Volgende argumenten staven deze redenering:

- TNF-deficiënte en TNFR-deficiënte muizen vertonen een verhoogde gevoeligheid voor letaliteit geïnduceerd door hoge doses LPS (Marino et al., 1997; Rothe et al., 1993).
- De TNF-resistente muisstam DBA/2 is niet resistent tegen LPS (Dr. Ben Wiellockx en Dr. Claude Libert, niet gepubliceerde data).
- LPS-resistente C3H/HeJ muizen zijn gevoelig voor TNF.
- De loci die gelinkt worden met de LPS resistentie zijn verschillend van de loci die gelinkt worden met de TNF resistentie.
- 30% van de TNF-resistente backcross muizen zijn gevoelig voor LPS (Dr. Jan Staelens, niet gepubliceerde data).

Deze bevindingen laten vermoeden dat andere genen dan de genen die de TNF-resistentie veroorzaken, verantwoordelijk zijn voor de extreme resistentie tegen LPS.

In een eerste fase werd een interspecifieke backcross uitgevoerd (n = 90). Na analyse van de backcross data werden er nog 51 extra nakomelingen gegenereerd. Deze muizen werden enkel getypeerd voor microsatelliet merkers op de 4 chromosomen die gelinkt werden met de LPS-resistentie in de 'genome scan' (zie later). Op een leeftijd van 8 weken werden alle nakomelingen uit deze kruising geïnjecteerd met 250 μg LPS, een dosis die 100% letaal is voor C57BL/6 muizen maar niet letaal is voor SPRET/Ei muizen. Van de 141 muizen die geïnjecteerd werden overleefden er 90 muizen (64%) en stierven er 51 muizen (36%). Indien we met een monogeen kenmerk te maken zouden hebben, zou 50% van de muizen moeten overleven en 50% moeten sterven. Echter, slechts 36% van de nakomelingen is gevoelig voor LPS. De LPS- resistentie van SPRET/Ei muizen lijkt dus een complex gegeven te zijn, dat door meerdere genen beïnvloed wordt. Overlevingsdata en genotyperingsdata werden ingevoerd in het computerprogramma MapManager QTX B17 en een 'genome scan' werd uitgevoerd. Na het analyseren van de data via de 'single-locus association' test en 'simple interval mapping' test van MapManager konden we besluiten dat de LPS-resistentie gelinkt is met loci op chromosoom 2, 10, 13 en X. Er werd

een suggestieve linkage gevonden tussen de LPS-resistentie en eenloci op chromosoom 10 en 13, bijna significante linkage met een locus op chromosoom 2 en zeer significante linkage met een locus op chromosoom X. Op chromosoom 6 werd er een sensitiserende locus gevonden.

Via (semi)-consome muizen hebben we getracht de belangrijkheid van de QTLs in de LPS-resistentie van SPRET/Ei muizen te bevestigen. Om consome muizen te screenen voor hun respons op LPS, hebben we zowel een hoge als een lage dosis LPS gebruikt. Het voordeel van een lage dosis LPS is de meer subtiele aanpak dan bij het gebruik van een hoge dosis LPS: kwantitatieve kenmerken, zoals IL-6 en lichaamstemperatuur, kunnen gemeten worden. Bij een hoge dosis LPS worden muizen enkel als dood of levend beschouwd. Een muis die bijvoorbeeld een enorme daling in lichaamstemperatuur vertoont na de LPS-injectie maar die toch blijft leven zal als LPS-resistent (levend) beschouwd worden. Een nadeel van het gebruik van een lage dosis LPS is dat de IL-6 concentratie en de LPS-respons wat betreft letaliteit misschien niet met elkaar gelinkt kunnen worden.

Het was nodig om onze eigen consome muizen te genereren, omdat STF/Pas en STG/Pas - 2 andere inteeltstammen afgeleid van *Mus spretus* individuen en waarvan er consome muizen beschikbaar zijn in het Institut Pasteur, Paris, France -niet resistent zijn tegen LPS-geïnduceerde letaliteit. Met onze eigen consome stammen waren we niet in staat om de LPS-resistentie om te zetten naar een monogeen kenmerk door het isoleren van individuele QTLs: we hebben enkel een gebied gevonden rond microsatelliet D10Mit186 – een ander gebied dan het gebied op chromosoom 10 gevonden in de initiële backcross – dat enigszins gelinkt kan worden met de IL-6 concentratie na injectie van een lage dosis LPS. De chromosoom 10 consome muizen overleefden echter niet een hoge dosis van 100 μg LPS , een dosis die 5 keer lager ligt dan de dosis die kan verdragen worden door SPRET/Ei en (BxS)F_1 muizen. Misschien ligt er een gen op proximaal chromosoom 10 dat de IL-6 concentratie na LPS positief beïnvloedt. Deze regio is nog altijd zeer groot en we vonden geen genen die direct gelinkt worden met LPS na het bekijken van het publieke muisgenoom. Als we data van de consome muizen bekijken, lijkt het er op dat de aanwezigheid van een enkel resistentiegen niet voldoende is om de extreme LPS-resistentie van SPRET/Ei te verklaren. We hebben dus te maken hebben met een geval van complexe genetica en bovendien treden er epistatische interacties op tussen verschillende loci. We moet en wel bemerken dat we, door de moeilijkheden die we hadden om te kweken, niet in staat waren om een groot aantal consome muizen te genereren. In het bijzonder de generatie van chromosoom X consome muizen liep niet volgens plan, wat nefast was voor het onderzoek omdat dit precies het chromosoom was dat we zoveel mogelijk in detail wilden bestuderen.

Na het heranalyseren van de 'genome scan' werd het duidelijk dat de gecombineerde effecten van chromosoom 2, 10, 13 en X oplopen tot ongeveer 30% van de totale variatie (het effect van de individuele loci bereikt 4 tot 10%). Dit suggereert dat er epistatische interacties werkzaam zijn tussen de

verschillende QTLs, of dat we een aantal QTLs gemist hebben. Het is mogelijk dat 2 of meer loci samenwerken op een additieve manier. Dit betekent dat iedere locus bijdraagt aan de graad van resistentie en dat een bepaalde drempel moet bereikt worden om volledige LPS-resistentie te bekomen. Een andere verklaring zou kunnen zijn dat er epistatische interacties optreden tussen QTLs, wat betekent dat de interacties tussen QTLs resulteert in een effect dat niet voorspeld kan worden door de som van de individuele effecten. Het is ook mogelijk dat we niet alle QTLs geïdentificeerd hebben in de 'genome scan', aangezien een backcross van minimum 1000 muizen nodig is om een zwak QTL te ontdekken. Een uitbreiding van de backcross was echter geen optie, gezien de moeilijkheden die we hadden om nakomelingen te genereren.

Via de 'interacties' functie van MapManager QTX hebben we mogelijke epistatische interacties opgespoord (Manly et al., 2001) en verschillende loci lijken betrokken te zijn bij epistatische interacties. In het bijzonder DXMit135, gelegen op het distale deel van chromosoom X, bleek betrokken te zijn in epistatische interacties met loci op chromosoom 2 and 13, die allebei eveneens gedetecteerd werden via SIM analyse. Aangezien er waarschijnlijk epistatische interacties zijn tussen loci, voerden we een test uit om achtergrond QTLs te detecteren. Deze test heet 'composite interval mapping' (CIM). We vonden suggestieve linkage met een locus op chromosoom 12. De locus op chromosoom 12 zou een sensitiserende locus zijn. De locus op chromosoom 12 zou ook betrokken zijn in epistatische interacties met een locus op chromosoom 13. Een tekortkoming in dit werk is het genereren van dubbele consome muizen voor chromosoom 2 en chromosoom X. Chromosoom 2 en X zijn immers het sterkst gelinkt met de LPS-resistentie en er werden mogelijke epistatische effecten tussen deze chromosomen gedetecteerd via MapManager QTX. Echter, na 3,5 jaar te hebben getracht chromosoom X consome muizen te genereren, hebben we beslist om het identificeren van de LPS resistentiegenen via deze strategie te stoppen en geen 2-X-dubbel consome muizen te genereren. In plaats daarvan hebben we ons gefocust op een andere strategie.

Door de spectaculaire resistentie van SPRET/Ei muizen tegen LPS en de hoge graad van genetisch polymorfisme tussen C57BL/6 en SPRET/Ei muizen, waren we van mening dat het identificeren van de LPS resistentiegenen redelijk gemakkelijk zou gaan. Op basis van de backcross data veronderstelden we dat de QTLs (gelinkt met de LPS-resistentie) gemakkelijk detecteerbaar zouden zijn en dat we tenminste gedeeltelijk het LPS-resistente fenotype zouden terugvinden in de consome muizen. In dit geval zou het vrij eenvoudig geweest zijn om tot een kritische regio komen met een beperkt aantal kandidaat-genen, door een tweede en grotere backcross op te starten tussen een LPS-resistente consome muis en een C57BL/6 muis. Desalniettemin was het backcross experiment een interessant werk en heeft het ons geleerd dat extreme fenotypes misschien helemaal afhankelijk zijn van interacties

tussen loci, zoals ook vroeger al was waargenomen door Dr. Jan Staelens tijdens zijn poging om de SPRET/Ei TNF resistentiegenen te klonen.

Via een tweede aanpak trachtten we de moleculaire basis van de LPS-resistentie te identificeren door een *in vitro* model te gebruiken. De verminderde respons van SPRET/Ei muizen op LPS in *vitro* in macrofagen werd gebruikt om het mechanisme van de LPS-resistentie te bestuderen. LPS induceert een complex netwerk van signalisatie-moleculen wat leidt tot de inductie van verschillende genen. TLR4-stimulatie activeert de MyD88-afhankelijke pathway, wat tot vroege NF-κB and MAPK activatie leidt, en de TRIF-afhankelijke pathway, wat tot late NF-κB- en MAPK-activatie leidt alsook tot activatie van IRF-3 . LPS-geïnduceerde pro-inflammatoire gen-inductie (bvb. IL-6 en TNF) is afhankelijk van NF-κB, maar ook van andere transcriptiefactoren zoals AP-1. AP-1 wordt geactiveerd door MAPK p38 en JNK. Fosforylatie en daaropvolgende degradatie van IκB resulteert in translocatie van NF-κB naar de nucleus. We hebben aangetoond dat de MyD88-afhankelijke pathway leidend tot IκB-degradatie intact is in SPRET/Ei BMDMs op vroege tijdstippen. We hebben ook aangetoond dat fosforylatie van de MAPK p38 en JNK normaal is. Daarenboven werd de intacte Myd88-afhankelijke signalisatie bevestigd doordat er geen significant verschil is tussen SPRET/Ei and C57BL/6 BMDMs in vroege IL-6 and TNF-α mRNA expressie. Ook de LPS-geïnduceerde mRNA expressie van bvb. *Il1a*, *Il1b*, *Il12a*, *Il12b*, *Bcl2*, *Birc4* (coderend voor XIAP) en *Tnfaip3* (coderend voor A20) is niet significant verschillend tussen SPRET/Ei and C57BL/6 muizen. Dus, na 1 uur en na 4 uur van LPS-stimulatie is de IL-6 en TNF-α mRNA expressie normaal in SPRET/Ei muizen. In tegenstelling tot de normale vroege IL-6 en TNF-α mRNA expressie in SPRET/Ei BMDMs, zijn de *in vivo* niveaus van TNF-α (1 uur na LPS-injectie) en IL-6 (3 uur na LPS-injectie) significant lager in SPRET/Ei muizen in vergelijking met C57BL/6 muizen. We moeten echter benadrukken dat een *in vivo* model veel complexer is dan een *in vitro* model. LPS kan verschillende celtypes activeren (bvb. macrofagen, DCs, neutrofielen en fibroblasten) om pro-inflammatoire cytokines zoals TNF-α en IL-6 te produceren. Deze cytokines (bvb. IL-1) kunnen dan op hun beurt andere cellen activeren om ook IL-6 en TNF-α te produceren.

Naast een vroege NF-κB activatie, kan TLR4 ook een late NF-κB en MAPK activatie induceren via de TRIF-afhankelijke pathway. De adaptor-molecule TRIF kan zowel TRAF6 als TBK1/IKK-ε naar zijn N-terminaal domein recruteren. Het recruteren van TRAF6 leidt tot een tweede golf van pro-inflammatoire cytokines. TRIF -/- macrofagen vertonen een defecte TNF-α, IL-6 en IL-12p40 productie 24 uur na LPS-stimulatie. Wij toonden aan dat de IL-6 expressie 24 uur na LPS-stimulatie defect is in SPRET/Ei MEFs in vergelijking met de IL-6 expressie in C57BL/6 MEFs. In hetzelfde experiment werd bevestigd dat de vroege IL-6 inductie (4 uur na LPS-stimulatie) normaal is. We kunnen dus concluderen dat de vroege

pro-inflammatoire gen-inductie intact is in SPRET/Ei cellen, terwijl de late (TRIF-afhankelijke) gen-inductie defect is.

We moeten er echter op wijzen dat IκB degradatie en fosforylatie van MAPK p38 en JNK niet noodzakelijk betekent dat de NF-κB en AP-1 activatie intact is, maar wel dat de stroomopwaartse signalisatiemoleculen in orde zijn. We zijn van plan de activatie van deze moleculen te bekijken via EMSA. Een andere mogelijke verklaring voor het defect in late IL-6 inductie zou kunnen zijn dat een anti-inflammatoir mechanisme overactief is in SPRET/Ei. In endotoxine-tolerantie, een transiënte staat van een verminderde respons op een tweede stimulatie van LPS, worden specifieke anti-inflammatoire mechanismen gebruikt, zoals de accumulatie van p50-p50 homodimeren of de inductie van signalisatie pathways zoals PI3K. Het zou bijvoorbeeld mogelijk kunnen zijn dat p50-p50 homodimeren worden gevormd, die transcriptioneel inactief zijn, in plaats van de transcriptioneel actieve p65-p50 heterodimeren. Men heeft gezien dat in endotoxine-tolerantie de formatie van p50-p50 homodimeren leidt tot een daling van TNF-α niveaus. Een ander mechanisme om endotoxine-tolerantie te reguleren is de inductie van PI3K signalisatie. PI3K ligt op chromosoom 13 en kan TLR signalisatie inhiberen. Het onderliggende mechanisme hiervan is tot op heden nog niet gekend, maar de onderdrukking van p38, JNK1/2 en NF-κB signalisatie blijkt een rol te spelen. Dus kan misschien een of ander anti-inflammatoir mechanisme dat de pro-inflammatoire gen-expressie blokkeert, zoals de vorming van p50-p50 homodimeren of de inductie van de PI3K signalisatie pathway, overactief zijn in SPRET/Ei muizen. Dit anti-inflammatoir mechanisme kan door NF-κB of AP-1 zelf geïnduceerd worden, aangezien IL-6 en TNF-α perfect wordt geïnduceerd in SPRET/Ei op vroege tijdstippen.

LPS induceert de expressie van het *Ifnb* gen door middel van een MyD88-onafhankelijke, TRIF-afhankelijke pathway (Yamamoto et al., 2002). Poly (I:C), een TLR3 ligand, activeert ook de TRIF-afhankelijke pathway om IFN-β te induceren. Activatie van de TRIF-afhankelijke pathway leidt tot activatie van de transcriptie factor IRF-3, wat vervolgens resulteert in IFN-β inductie, STAT1 fosforylatie en translocatie, en inductie van STAT1-afhankelijke genen zoals IRF-7 (Yamamoto et al., 2003). We hebben aangetoond dat IFN-β mRNA inductie door LPS, maar ook door poly (I:C), grotendeels verhinderd was in SPRET/Ei BMDMs. Ten gevolge van deze defectieve IFN-β inductie, werd LPS-geïnduceerde STAT1 fosforylatie en daaropvolgende nucleaire translocatie verhinderd. Een defect in de translocatie van STAT1 verhinderde op zijn beurt IRF-7 expressie. Het defect in IFN-β productie werd ook *in vivo* aangetoond na LPS en *Influenza* virus. IFN-β is ontdekt als een krachtige antivirale molecule (Isaacs and Lindenmann, 1957). Voor een lange tijd werd gedacht dat dit de enige functie was van type I IFN, maar in de laatste jaren is er een meer uitgebreide rol voor IFNs in immuniteit aangetoond. We

hebben de rol van IFN-β in de SPRET/Ei LPS-resistentie proberen aan te tonen alsook de gevolgen van de defectieve IFN-β inductie. Aangezien de LPS-resistentie van SPRET/Ei muizen ongedaan kan worden gemaakt door exogeen IFN-β toe te voegen, suggereert dit dat IFN-β een belangrijke rol speelt in de LPS-geïnduceerde letaliteit. Verwijdering van het *Ifnb* gen of van genen betrokken in de inductie van of in de respons op IFN-β leidt tot resistentie tegen endotoxemie (Karaghiosoff et al., 2003; Sakaguchi et al., 2003). Wijzelf hebben kunnen aantonen dat IFNAR1 knock-out muizen resistent zijn tegen een letale dosis LPS (wij zijn de eerste groep die dit konden publiceren), maar dat ze wel wel een serieuze daling in lichaamstemperatuur vertonen. In tegenstelling tot IFNAR1 knock-out muizen, vertonen SPRET/Ei muizen geen daling in lichaamstemperatuur na eenzelfde dosis LPS. Het lijkt erop dat andere genen dan het *Ifnb* gen nodig zijn om het volledige LPS-resistente fenotype te bekomen. Dit bevestigt de data van de 'genome scan' analysis die aantoonden dat de LPS-resistentie een complex kenmerk is. In samenwerking met Dr. Johan Grooten kunnen we macro-array filters, die ongeveer 500 macrofaag-specifieke genen bevatten, gebruiken om de differentiële mRNA expressie te bestuderen tussen SPRET/Ei BMDMs en C57BL/6 BMDMs. We hebben al in een eerste experiment, gebruikmakend van deze filters met macrofaag-specifieke genen, aangetoond dat 6 uur na LPS-stimulatie ongeveer 50 genen $\geq$ 2 maal sterker tot expressie komen en dat ongeveer 50 genen $\geq$ 2 minder sterk tot expressie komen in SPRET/Ei BMDMs in vergelijking met C57BL/6 BMDMs. Deze differentiële expressie-studie zal herhaald worden met LPS, maar zal ook uitgevoerd worden met poly (I:C). We hopen tot een kleiner aantal kandidaat genen te komen (genen die differentieel tot expressie komen tussen SPRET/Ei en C57BL/6) door de data na LPS- en poly (I:C)-stimulatie te vergelijken en door meerdere tijdspunten te bestuderen Via het Bioinformatica programma 'Difference Distance Matrix Analysis' hopen we transcriptiefactoren/genen te vinden die verantwoordelijk zouden kunnen zijn voor de differentiële expressie van SPRET/Ei genen.

Het defect in IFN-β heeft zo zijn consequenties. Het is aangetoond dat IFN-β macrofagen kan sensitizeren voor celdood geïnduceerd door *Listeria monocytogenes* en dat het kan beschermen tegen progressieve leishmaniasis (Mattner et al., 2004; Stockinger et al., 2002). Waarschijnlijk door het defect in IFN-β zijn SPRET/Ei muizen inderdaad compleet resistent tegen *Listeria monocytogenes* en (BxS)F_1 muizen zijn erg gevoelig voor een *Leishmania major* infectie. We plannen ook om de respons van SPRET/Ei na te gaan voor virale infecties. Aangezien IFN-β een krachtige antivirale molecule is, verwachten we dat SPRET/Ei muizen zeer gevoelig zullen zijn voor virale infecties. Verschillende studies hebben een rol voor IFN-β gesuggereerd in LPS-geïnduceerde endotoxemie, maar zijn belangrijkheid in Gram-negatieve sepsis moet nog worden aangetoond. De eventuele voor- en nadelen

van het blokkeren van IFN-β of zijn receptoren in het behandelen van sepsis-patiënten wordt uitvoerig besproken in de review: 'Must we inhibit type I IFNs in endotoxemia and sepsis?' (Mahieu and Libert).

Een belangrijk deel in dit werk was het ontrafelen van het mechanisme van de defectieve IFN-β inductie. Het defect kan op verschillende niveaus gelegen zijn: er kan een defect in de IFN-β promotor of in een van zijn regulatoren zijn, in een van de moleculen van de TRIF-afhankelijke pathway of in de positieve feedback lus na IFN-β signalisatie. Ten eerste hebben we de sequentie van de IFN-β promotor bestudeerd. We vonden echter geen variaties tussen C57BL/6 en SPRET/Ei muizen. Ten tweede zijn we de activatie van IRF-3 gaan bestuderen als een eindpunt van de TRIF-afhankelijke pathway. IFN-β inductie is afhankelijk van de fosforylatie/activatie van de transcriptie factor IRF-3. Daarom hebben we de sekwentie van de serine/threonine-cluster in het C-terminaal domein van IRF-3 bepaald. Er werden geen variaties gevonden in de belangrijke serines van deze regio, maar we hebben wel enkele unieke aminozuur- veranderingen gevonden in SPRET/Ei muizen welke een invloed zouden kunnen hebben op de fosforylatie van IRF-3. Echter, ongeveer op hetzelfde moment toonden we aan dat IRF-3 gefosforyleerd wordt in SPRET/Ei BMDMs. Meer nog, Jin Mo Park, met wie we samenwerken, kon aantonen dat IRF-3 knock-out macrofagen na LPS stimulatie een breder gendefect hebben (*Ifnb*, *Rantes*, *Isg15* en *Cxcl10*) dan SPRET/Ei BMDMs (*Ifnb* en *Ccl5*). Natuurlijk betekent een normale fosforylatie van IRF-3 niet noodzakelijk dat er IRF-3 dimeren worden gevormd of dat zijn co-factoren CBP/p300, of de trans-activatoren van CBP/p300, CITED1 en CITED2, normaal zijn in SPRET/Ei muizen. Een interessant gegeven is dat CITED1 en CITED2 respectievelijk op chromosoom X en 10 liggen, twee chromosomen die gelinkt worden met de LPS-resistentie. Het is mogelijk dat een defecte of veranderde activatie van deze co-factoren de IRF-3 afhankelijke inductie van IFN-β kunnen veranderen. Meer nog, CBP/p300 blijkt ook een co-factor te zijn van de NF-κB component p65 (Gerritsen et al., 1997). Misschien veroorzaakt een defect in CBP/p300 of in een van zijn trans-activatoren CITED1 en CITED2 zowel een defectieve IL-6 als IFN-β inductie. We zullen dus zeker kijken of er IRF-3 dimeren worden gevormd, of NF-κB en AP-1 activatie doorgaat en welke NF-κB dimeren gevormd worden op een vroeg en laat tijdstip na LPS-stimulatie.

Een ander interessant gegeven is dat IFN-β mRNA stabiliteit post-transcriptioneel gereguleerd wordt door een AU-rijk element in de 3'UTR, en een tweede element (CRID element) in het 3' eind van de coderende regio. Dusver hebben we de volledige sekwentie van het gehele *Ifnb* gen nog niet bepaald. We hebben ook nog niet zijn positieve/negatieve regulatoren bestudeerd zoals YY1, IRF-1 en IRF-2. Het spreekt vanzelf dat deze factoren verder onderzocht moeten worden. Verder is het ook interessant

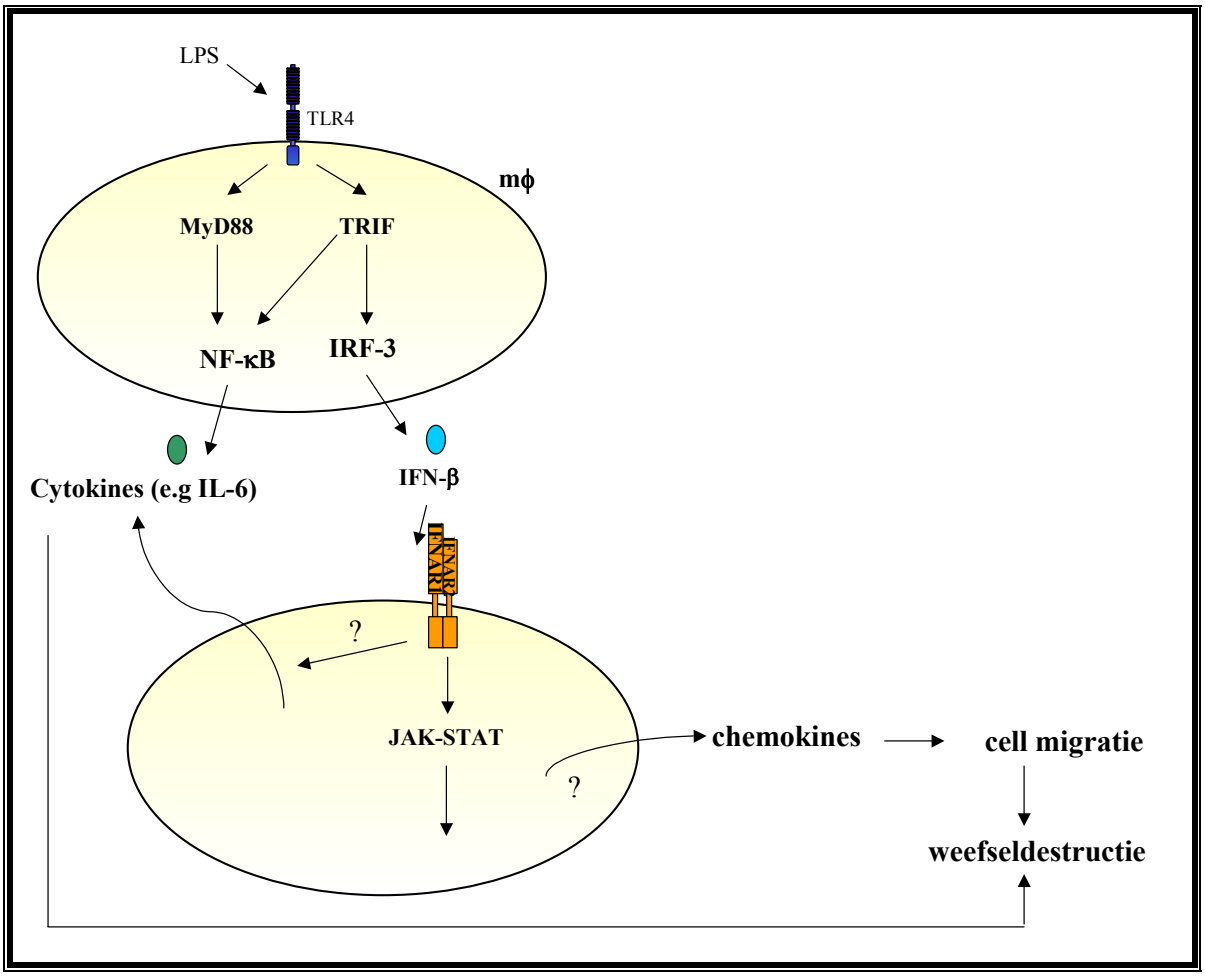
om te weten dat de promotors van *Ifnb* en *Ccl5* (coderend voor RANTES) zowel een NF-κB als een IRF-3 PRD element bevatten. In dat geval kan de defectieve inductie van zowel het *Ifnb* gen als het *Ccl5* gen te wijten zijn aan een defecte transactivatie van de transcriptiefactoren NF-κB en IRF-3. Dus, een anti-inflammatoir mechanisme dat NF-κB activatie verhindert, zoals de formatie van p50-p50 homodimeren, of een defect in een van de co-factoren zoals CBP/p300 of CITED1/2, kan verantwoordelijk zijn voor zowel late pro-inflammatoire gen-inductie als voor defectieve IFN-β inductie. Door gebruik te maken van een specifiek Bioinformatica programma, zullen we op zoek gaan naar andere genen die ook zowel een NF-κB als een IRF-3 element in hun promotor hebben. Vervolgens zullen we hun expressie na LPS-stimulatie onderzoeken.

De verminderde IFN-β inductie kan ook te wijten zijn aan een defect in de positieve feedback lus na IFN-β signalisatie. We hebben aangetoond dat OAS mRNA inductie, afhankelijk van de vorming van een STAT1/STAT2/IRF-9 complex na IFNAR stimulatie, significant lager is in SPRET/Ei BMDMs na IFN- of LPS-stimulatie. We hebben ook aangetoond dat IFNAR1 mRNA snel naar beneden gereguleerd wordt in SPRET/Ei muizen na IFN-β stimulatie. Het mechanisme en de significantie van dit fenomeen is nog niet gekend. Hetzelfde werd aangetoond na LPS-stimulatie. Bovendien wordt significant minder IL-6 geïnduceerd na IFN-stimulatie in SPRET/Ei BMDMs. Of het defect in OAS mRNA en IL-6 inductie na stimulatie met IFN-β gemedieerd wordt door regulatie naar beneden van IFNAR1 mRNA moet nog worden aangetoond. In de toekomst zullen we onderzoeken welk mechanisme instaat voor de down-regulatie van IFNAR1. Ook zal de sekwentie en de expressie onderzocht worden van verschillende moleculen van de JAK-STAT pathway. Overexpressie van een negatieve regulator zou correleren met de dominantie van de LPS-resistentie. Daarom zou het ook interessant zijn om de negatieve regulatoren van de JAK-STAT pathway te bestuderen zoals SOCS, SHP en PIAS moleculen.

Zoals gesuggereerd, zijn duidelijk andere genen dan *Ifnb* betrokken in de LPS-resistentie van SPRET/Ei muizen. Het is aangetoond dat IFN-β een brede set van chemokines kan induceren (Coelho et al., 2005). Expressie van chemokines leidt tot migratie van leukocyten naar de inflammatoire locaties. Interessant genoeg hebben we gezien dat in LPS-geïnjecteerde SPRET/Ei muizen geen leukocyten migreren naar de alveoli en dat in longen en lever van LPS-geïnjecteerde SPRET/Ei muizen er zich minder leukocyten aan het endotheel van de bloedvaten hechten. Tevens hebben we ook in een preliminaire differentiële expressie- studie kunnen zien dat chemokines niet of veel minder worden geïnduceerd in SPRET/Ei BMDMs in vergelijking met C57BL/6 BMDMs na LPS-stimulatie. Dus, een mogelijke hypothese om de rol van IFN-β in LPS-geïnduceerde letaliteit te verklaren is de volgende (zie

figuur IV.1.1): Het defect in IFN-β inductie in SPRET/Ei muizen zou kunnen leiden tot een defectieve chemokine-productie. Dit defect in chemokine-productie leidt vervolgens tot een defectieve migratie van leukocyten naar de inflammatoire sites en tot een verminderde weefseldestructie. Na stimulatie van de IFNAR1 receptor door IFN-β wordt ook IL-6 geproduceerd. Het mechanisme van IFN-β-geïnduceerde IL-6 productie is nog niet gekend op dit moment. Dus, een combinatie van een defect in de late TRIF-afhankelijke IL-6 geninductie en een defect in IFN-β-geïnduceerde IL-6 gen inductie leidt tot verminderde IL-6 niveaus. Pro-inflammatoire cytokines, zoals IL-6, kunnen ook direct op organen inwerken. Een defectieve IL-6 inductie zou dus ook kunnen leiden tot een verminderde weefseldestructie.

Tenslotte hebben we ook de respons van SPRET/Ei muizen op andere TLR liganden getest. De LPS respons leidt hoofdzakelijk tot activatie van 2 pathways: de MyD88-afhankelijke en de MyD88-onafhankelijke pathway. De meeste TLRs activeren slechts één pathway om een respons op pathogenen op te wekken, wat de respons minder complex maakt als de LPS-respons (Moynagh, 2005). We hebben aangetoond dat LPS-geïnduceerde MyD88-afhankelijke signalisatie stroomopwaarts van IκB degradatie en MAPK activatie intact is. Dit suggereert dat er een normale MyD88-afhankelijke pathway is in SPRET/Ei muizen. LPS-geïnduceerde IL-6 gen-inductie op late tijdspunten is TRIF-afhankelijk en gaat niet door *in vitro* in SPRET/Ei MEFs. MyD88 is de enige adaptor die door de IL-1R gerekruteerd wordt. TLR2 rekruteert de adaptor moleculen MyD88 en TIRAP. We hebben aangetoond dat stimulatie met liganden voor de IL-1R en TLR2 ook leidt tot een defectieve pro-inflammatoire gen-inductie *in vitro* in SPRET/Ei muizen. Bovendien reageren SPRET/Ei muizen ook minder op IL-1-geïnduceerde IL-6 inductie en op zymosan-geïnduceerde zwelling van de voetzool.



Figuur V.1.1. - Mogelijk mechanisme om de rol van IFN-β in LPS-geïnduceerde letaliteit te verklaren.

De defectieve inductie van pro-inflammatoire genen na verschillende TLR-liganden kan verklaard worden door een gemeenschappelijk mechanisme. Zoals hierboven beschreven, hebben we nog niet gekeken naar NF-κB en AP-1 activatie noch naar de compositie van de NF-κB dimeren na TLR-stimulatie of naar co-activatoren zoals CBP/p300 en CITED1 en CITED2. Een andere mogelijke verklaring zou misschien een defect in de IRF-5 activatie kunnen zijn. Recent werd aangetoond dat alle TLRs niet enkel NF-κB, maar ook IRF-5 nodig hebben om pro-inflammatoire cytokines (bvb. TNF, IL-6 en IL-12) te induceren (Napolitani et al., 2005). SPRET/Ei muizen reageren ook minder op poly (I:C) *in vitro* en zijn resistent tegen poly (I:C)/dGalN-geïnduceerde letaliteit. Poly (I:C) activeert enkel de TRIF-afhankelijke pathway, wat het een minder complexe pathway maakt om te analyseren in vergelijking met de LPS respons. Hierdoor kunnen we in de toekomst ook poly (I:C) in plaats van LPS gebruiken om het mechanisme van de defectieve IFN-β inductie te onderzoeken.

Tot besluit wilden we in dit werk de LPS-resistentiegenen van SPRET/Ei muizen mappen en identificeren. Na beschrijving van de LPS-resistentie van SPRET/Ei muizen in detail hebben we geprobeerd de LPS-resistentiegenen te mappen via een genetische aanpak. Via deze strategie zijn we er niet in geslaagd regios te identificeren die klein genoeg zijn om tot een beperkt aantal kandidaatgenen te komen. Een andere strategie om het mechanisme van de LPS resistentie te onderzoeken was het gebruik van een *in vitro* model. Aangezien gedurende het onderzoek ongeveer de helft van de tijd geïnvesteerd werd in het typeren en screenen van muizen om de initiële backcross te vergroten en om consome muizen te maken, zijn de *in vitro* data in dit werk niet zo uitgebreid als we zouden willen. Het onderzoek wat in deze thesis gepresenteerd wordt, suggererend dat IFN-β een rol zou spelen in de LPS-resistentie van SPRET/Ei muizen, roept dus ook nog vele vragen op over de precieze oorzaak van de defecte IFN-β productie in SPRET/Ei muizen. Tenslotte realiseren we ons ook dat andere genen dan IFN-β betrokken zijn bij de verminderde respons van SPRET/Ei muizen op LPS en andere TLR-liganden. Het identificeren van deze genen en hun actiemechanisme is werk voor de toekomst.

Perspectieven

In dit werk hebben we enkele interessante resultaten behaald betreffende de rol van IFN-β in LPS-geïnduceerde shock. We hebben aangetoond dat SPRET/Ei muizen (met een defecte IFN-β productie) resistent zijn tegen LPS-geïnduceerde shock, en dat (BxS)F_1 muizen gedeeltelijk beschermd zijn tegen een Gram-negatieve infectie. Echter, uit gegevens van onze genetische studies en van de experimenten met de IFNAR1 -/- muizen, weten we dat nog andere genen dan IFN-β betrokken zijn in de LPS-resistentie van SPRET/Ei. Dus, het project over de LPS-resistentie van SPRET/Ei zal op twee niveaus worden voortgezet:

1. LPS-resistentie genen van SPRET/Ei

Volgende perspectieven werden bepaald:

- Mechanisme van de defectieve IFN-β productie?
  We zullen onderzoeken of het defect in IFN-β productie veroorzaakt wordt door een defect in IRF-3 door te kijken naar de sekwentie, dimerisatie en fosforylatie van IRF-3. We zullen ook andere mechanismen of moleculen meer in detail gaan onderzoeken, namelijk de afbraak van IFN-β mRNA en moleculen zoals SHIP, RIP1, CITED1 en CITED2. Een laatste item in deze context is het onderzoek naar de relevantie van de 'down-regulation' van IFNAR1.

- Zijn andere genen belangrijk in de SPRET/Ei LPS-resistentie?
  We zullen onderzoeken of andere genen belangrijk zijn in de SPRET/Ei LPS-resistentie. Hiervoor zullen we zullen een differentiële expressie-studie van macrofaag specifieke genen na LPS en poly (I:C) doen

- Gram–negatieve sepsis?
  We zullen SPRET/Ei en/of (BxS)F_1 muizen testen op hun response op verschillende modellen van Gram-negatieve sepsis (Cecal Ligation and Puncture' (CLP), *Klebsiella pneumoniae en Salmonella typhimurium)*. Het grote probleem is de beperkte hoeveelheid beschikbare SPRET/Ei muizen, waardoor we dus vooral (BxS)F_1 muizen zullen gebruiken.

- Preliminaire experimenten doen ons vermoeden dat het SPRET/Ei fenotype 'vergelijkbaar' is met het constitutieve LPS-tolerante fenotype: kan dit ons nieuwe informatie opleveren?

2. SPRET/Ei heeft een defect in de IFN-β productie

Volgende perspectieven werden bepaald:

- Speelt IFN-β een belangrijke rol in sepsis?

  Dit zullen we onderzoeken door het gebruik van verschillende modellen: CLP, *K.pneumiae*, *S. typhimurium*, … Bovendien toonden Jo et al. aan dat het blokkeren van de type I IFN signalisatie therapeutische toekomst heeft voor de behandeling van endotoxemie.

- Kan het blokkeren van type I IFN, of zijn stroomopwaartse of stroomneerwaartse mediatoren, bescherming bieden tegen LPS of Gram- sepsis?

  Dit zullen we onderzoeken via een IFN-β neutraliserend antilichaam in C57BL/6 muizen. Momenteel zijn we zo'n antilichaam in ons laboratorium aan het ontwikkelen. De vraag die zich hierbij opdringt is: 'Kunnen we C57BL/6 muizen beschermen tegen LPS-geïnduceerde shock en sepis dmv toediening van dit IFN-β neutraliserend antilichaam?'. Een andere mogelijkheid is het toedienen van een antagonist van IFNAR1.

  Een nadeel zou echter kunnen zijn dat het blokkeren van IFN-β zal leiden tot een verhoogde vatbaarheid voor virale infecties (IFNAR1 -/- muizen bijvoorbeeld zijn heel vatbaar voor virale infecties).

We denken dus dat moleculen betrokken in type I IFN signalisatie, zoals IFN-β en IFNAR1, mogelijks nieuwe therapeutische doelwitten zijn in de behandeling van endotoxemie en sepsis. Dit kan echter alleen wanneer patiënten met sepsis niet supergevoelig worden voor virale infecties en wanneer de activatie van het immuunsysteem niet onderdrukt is. Misschien is het mogelijk om patiënten met sepsis te behandelen met agentia die IFN blokkeren of die type I IFN-signalisatie blokkeren gedurende een korte tijd onder gelimiteerde omstandigheden om virale infecties te verhinderen.

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V. ADDENDUM

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