

From Department of Microbiology, Tumor and Cell Biology,  
Karolinska Institute, Stockholm, Sweden

# **INNATE IMMUNITY TO INTRACELLULAR BACTERIAL INFECTIONS**

Christian Trumstedt



**Karolinska  
Institutet**

Stockholm 2008

Cover picture: © Joakim Larsson, 2008

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet. Printed by Larseries Digital Print AB.

© Christian Trumstedt, 2008

ISBN 978-91-7409-131-1

*Till minne av min morfar Bengt Barr*

## SUMMARY

Intracellular bacterial pathogens have developed mechanisms to enter and invade cells, to survive the immune response and to replicate inside the host. We studied the innate mechanisms that have evolved in the host to battle intracellular bacterial pathogens, such as the obligate intracellular *Chlamydia pneumoniae* and the facultative intracellular *Listeria monocytogenes*, which invade the respiratory and the gastrointestinal tracts in humans.

Infection of murine bone marrow-derived macrophages (BMM) with *C. pneumoniae* induces IFN- $\alpha/\beta$ -dependent IFN- $\gamma$  secretion leading to the control of intracellular bacterial growth. We studied the molecular details of chlamydial-induced IFN- $\alpha$  and IFN- $\gamma$  expression in BMM. We demonstrated that TLR4, but not TLR2, TLR6 or TLR9, is essential for the control of *C. pneumoniae* infection. We found that TLR4-MyD88-IRAK4-dependent signaling is necessary for IFN- $\alpha$  and IFN- $\gamma$  mRNA expression, and protection against infection of BMM with *C. pneumoniae*. In *C. pneumoniae*-infected BMM, IFN- $\alpha/\beta$ -dependent STAT1 was necessary for increased IFN- $\gamma$  mRNA accumulation and bacterial growth control. Enhancement of IFN- $\gamma$  mRNA levels and control of *C. pneumoniae* infection also required NF- $\kappa$ B activation. We showed that NF- $\kappa$ B activation is TRAF6-dependent, but independent of TLR4-MyD88-IFN- $\alpha/\beta$  signaling in intracellular bacterial infection. In *C. pneumoniae*-infected IRF3<sup>-/-</sup> BMM, IFN- $\alpha$  and IFN- $\gamma$  mRNA levels and bacterial levels were not altered compared to the WT. However, IFN- $\beta$ <sup>-/-</sup> BMM showed higher loads of *C. pneumoniae* and no expression of IFN- $\alpha$  and IFN- $\gamma$  mRNA in comparison to the WT BMM. In conclusion, we demonstrated that TLR4-MyD88-IFN- $\alpha/\beta$ -STAT1-dependent signaling, as well as TLR4-MyD88-independent but TRAF6-dependent NF- $\kappa$ B activation play a role in IFN- $\gamma$  expression and protection against *C. pneumoniae* infection in BMM.

We then studied the protective role of STAT1 in mice infected intranasally with *C. pneumoniae*. STAT1 mediated an IFN- $\alpha/\beta$ R- and IFN- $\gamma$ R-dependent protection against *C. pneumoniae* infection *in vivo*. STAT1 phosphorylation was detected after chlamydial infection in IFN- $\alpha/\beta$ R<sup>-/-</sup> and IFN- $\gamma$ R<sup>-/-</sup> mice, but not in IFN- $\alpha/\beta$ R<sup>-/-</sup>/IFN- $\gamma$ R<sup>-/-</sup> mice. T cells released IFN- $\gamma$  and conferred protection against *C. pneumoniae* in a STAT1-independent fashion. STAT1 mediated microbicidal mechanisms of non-hematopoietic cells, leading to control of intracellular infection *in vivo*. Thus, STAT1 mediates a cooperative effect of IFN- $\alpha/\beta$  and IFN- $\gamma$  on non-hematopoietic cells, resulting in protection against *C. pneumoniae* in pulmonary infection.

We next addressed the role of NOD1 in growth control of *L. monocytogenes*. NOD1 conferred protection to intraperitoneal and subcutaneous infection of *L. monocytogenes*, and controlled the dissemination of *L. monocytogenes* into the brain. NOD1 was not involved in the generation of adaptive immune responses or the recruitment of inflammatory cells. Non-hematopoietic cells accounted for the NOD1-mediated resistance to *L. monocytogenes*. Furthermore, *L. monocytogenes*-infected NOD1<sup>-/-</sup> BMM, fibroblasts and astrocytes showed increased bacterial load, and IFN- $\gamma$ -induced inhibition of bacterial growth was dampened in NOD1<sup>-/-</sup> BMM. Surprisingly, a number of important inflammatory cytokines, chemokines, growth factors and metalloproteases were increased in NOD1<sup>-/-</sup> compared to WT fibroblasts as determined by microarray analysis. In conclusion, NOD1 confers non-hematopoietic cell-mediated resistance to infection with *L. monocytogenes* *in vivo*. It plays a role in the control of infection in BMM, fibroblasts and astrocytes, and is required for IFN- $\gamma$ -mediated *L. monocytogenes* growth control in BMM.

## LIST OF PUBLICATIONS

- I** Rothfuchs A, **Trumstedt C**, Wigzell H and Rottenberg M E. Intracellular bacterial infection-induced IFN- $\gamma$  is critically but not solely dependent on toll-like receptor 4-myeloid differentiation factor 88-IFN- $\alpha/\beta$ -STAT1 signaling. *Journal of Immunology*, 2004, 172(10):6345-6353
- II** **Trumstedt C**, Eriksson E, Lundberg A M, Yang T, Yan Z, Wigzell H, Rottenberg M E. Role of IRAK4 and IRF3 in the control of intracellular infection with *Chlamydia pneumoniae*. *Journal of Leukocyte Biology*, 2007, 81(6):1591-1598
- III** Rothfuchs A, **Trumstedt C**, Mattei F, Schiavoni G, Hidmark Å, Wigzell H and Rottenberg M E. STAT1 regulates IFN- $\alpha/\beta$ - and IFN- $\gamma$ -dependent control of infection with *Chlamydia pneumoniae* by non-hematopoietic cells. *Journal of Immunology*, 2006, 176(11):6982-90
- IV** Mosa A\*, **Trumstedt C\***, Eriksson E, Soehnlein O, Janik K, Klos A, Dittrich-Breiholz, Kracht M, Hidmark Å, Wigzell H and Rottenberg M E. Non-hematopoietic cells control the outcome of infection with *Listeria monocytogenes* in a NOD1-dependent manner. Submitted manuscript.

\*Authors contributed equally.

# TABLE OF CONTENTS

INTRODUCTION.....	1
Intracellular bacterial pathogens .....	1
<i>Chlamydia</i> .....	1
Chlamydial life cycle .....	2
<i>Listeria</i> .....	4
Listerial life cycle.....	5
The immune response to intracellular bacterial infections .....	7
Innate immunity .....	7
The macrophage and intracellular infections .....	8
Adaptive immunity .....	8
Innate immune receptors.....	10
Toll-like receptors .....	10
TLR4 signaling.....	12
Role of TLRs in bacterial infections.....	14
TLRs and <i>Chlamydia</i> .....	14
TLRs and <i>Listeria</i> .....	15
NOD-like receptors.....	15
NOD.....	16
Role of NOD in bacterial infections .....	17
NOD1 and <i>Listeria</i> .....	17
Other receptors .....	18
Interferons .....	18
IFN- $\alpha/\beta$ .....	19
IFN- $\gamma$ .....	20
JAK-STAT .....	21
Regulation of IFN- $\gamma$ expression .....	23
Interferon regulatory factors.....	25
Interferon-mediated effector mechanisms .....	28
AIMS.....	31
RESULTS AND DISCUSSION .....	33
Paper I & II – Studies with <i>C. pneumoniae</i> -infected BMM.....	33
Paper III – Role of STAT1 and IFNs in the outcome of <i>C. pneumoniae</i> infection <i>in vivo</i> . ....	40
Paper IV –Role of NOD1 during infection with <i>L. monocytogenes</i> .....	45
CONCLUDING REMARKS .....	49
ACKNOWLEDGEMENTS .....	51
REFERENCES.....	52

## ABBREVIATIONS

AP	Activating protein
BMDC	Bone marrow-derived dendritic cell
BMM	Bone marrow-derived macrophage
DAI	DNA-dependent activator of interferon regulatory factors
DAP	Diaminopimelic acid
DC	Dendritic cell
CARD	Caspase-recruiting domain
CD	Cluster of differentiation
EB	Elementary body
ERK	Extracellular signal-regulated kinase
GAF	GAS-binding transcription factor
GAS	IFN- $\gamma$ -activated site
HSP	Heat shock protein
IBD	Inflammatory bowel disease
ICE	IL-1 $\beta$ -converting enzyme
IDO	Indoleamine 2,3-deoxygenase
IFN	Interferon
I $\kappa$ B	Inhibitor of $\kappa$ B
IKK	I $\kappa$ B kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
IRAK	IL-1R-associated kinase
IRF	Interferon regulator factor
ISGF	IFN-stimulated gene factor
ISRE	Interferon stimulatory response elements
i.v.	Intravenous
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LBP	LPS-binding protein
LLO	Listeriolysin
LPS	Lipopolysaccharide
Mal	MyD88 adaptor-like
MAP	Mitogen-activated protein
MDA	Melanoma differentiation-associated protein
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation factor 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NBD	Nucleotide-binding domain
NFAT	Nuclear factor of activated T cells
NF- $\kappa$ B	Nuclear factor $\kappa$ B

NK	Natural killer
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NLR	NOD-like receptor
NLS	Nuclear localization signal
NRAMP	Natural resistance-associated macrophage protein
OAS	2', 5'-oligoadenylate synthetase
PGN	Peptidoglycan
PKR	Protein kinase R
PRR	Pattern recognition receptor
R	Receptor
RAG	Recombination-activating gene
RIP	Receptor-interacting protein
RIG-I	Retinoic acid-inducible protein I
RB	Reticulate body
STAT	Signal transducer and activator of transcription
TAD	Transactivation domain
TBK	TRAF-associated NF- $\kappa$ B activator-binding kinase
T <sub>H</sub>	T helper cell
TIR	Toll/IL-1R domain
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF R-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor inducing IFN- $\beta$
WT	Wild type



# INTRODUCTION

## **Intracellular bacterial pathogens**

Intracellular bacterial pathogens have evolved mechanisms to enter and invade cells, to survive intracellular antimicrobial defenses, and to replicate and spread to other cells. The strategy is similar for most intracellular pathogens, but the bacterial mechanisms vary substantially in terms of preferred cell type and cell compartment, microbial molecules revealed to immune receptors and elicited downstream signaling by the bacteria. For example, *Listeria*, *Shigella* and *Rickettsia* species escape the phagosome into the cytosol and are able to invade adjacent cells by cell-to-cell spread. Other bacteria block the maturation of the phagolysosome in the endocytic pathway, such as *Chlamydia*, *Salmonella* and *Mycobacterium* species (1). From the host's perspective, a number of mechanisms have been developed during evolution to confront the threat for survival posed by different pathogens. These mechanisms are usually successful, but in some cases co-adaptation of the bacteria to the host gives rise to a delicate balance of causing a chronic disease. This thesis treats different aspects of the host immune defense to the intracellular bacterial pathogens *Chlamydia pneumoniae* and *Listeria monocytogenes*.

## ***Chlamydia***

Chlamydiaceae were once considered viruses due to their small dimensions. However, it is now classified as a family of obligate intracellular Gram-negative bacterial pathogens, which need to differentiate, replicate and re-differentiate within a host cell to carry out their life cycle (2). They possess inner and outer membranes similar to Gram-negative bacteria, and are susceptible to antibiotics. They have DNA, RNA and prokaryotic ribosomes, and synthesize their own proteins, nucleic acids and lipids. However, Chlamydiaceae have been designated “energy parasites” as it is believed that they must scavenge high-energy compounds, such as ATP from the host cell (3).

Chlamydiaceae once consisted of only four species in the genus *Chlamydia*. Since 1999, a new taxonomy has divided the family into two genera of totally nine species. The genus *Chlamydia* now comprises *Chlamydia trachomatis* (isolated only in humans) and *Chlamydia muridarum* (mice and hamsters). The former *Chlamydia pneumoniae* (only humans), *Chlamydia psittaci* (humans and birds) and *Chlamydia pecorum* (cattle) were introduced into a new genus, *Chlamydophila* (4). However, this reclassification is still controversial and debated. In this thesis, *Chlamydia* will be used for all Chlamydiaceae as in the articles.

*Chlamydia* causes a wide range of clinically important diseases in humans. *C. trachomatis* causes ocular trachoma, which is endemic in the Middle East, North Africa and India. An estimated 150 million people worldwide are infected, of whom six million are blinded as a result. *C. trachomatis* is also the most common bacterial cause of sexually transmitted disease worldwide, with 90 million new infections worldwide per year reported by WHO (5). In Sweden, a dramatic increase has occurred in the last 10 years with 47,000 reported cases of *C. trachomatis* infection in

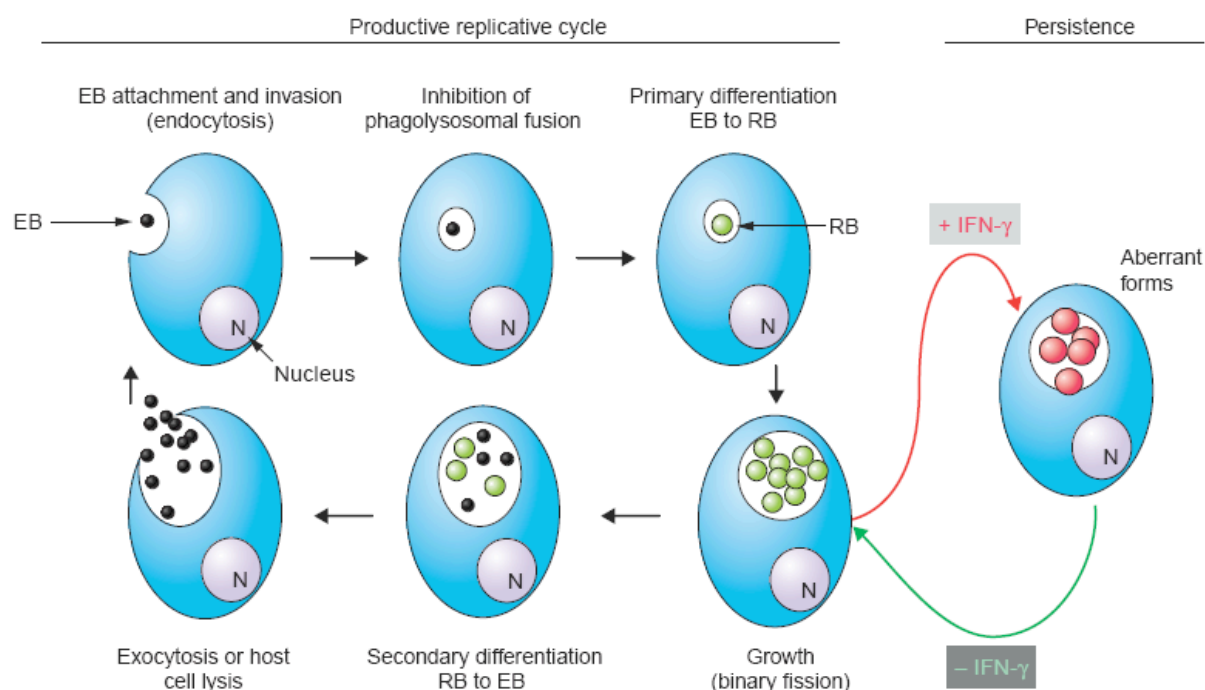
2007 (6). Acute infection with *C. trachomatis* can result in salpingitis and pelvic inflammatory disease, potentially leading to ectopic pregnancy and infertility primarily (5). Chlamydial genital tract infection has also been suggested to facilitate HIV transmission (7). The silent nature of the disease and people's reluctance to use condoms help the bacteria to spread, although antibiotics, such as azithromycin or doxycycline, are efficient in most cases to eradicate the bacteria. There is a high prevalence of *C. trachomatis* infection among young women, of which 70-75 % of have asymptomatic disease, which highlights the need for screening or a vaccine (8).

*C. pneumoniae* causes upper and lower respiratory diseases. In industrialized countries it accounts for approximately 10 % of community-acquired pneumonia and 5 % of cases of bronchitis and sinusitis (9). Infection may initially involve the upper respiratory tract and be later followed by cough and engagement of the lower respiratory tract. Chronic respiratory disease due to *C. pneumoniae* has been reported, as well as epidemics in school and military environments (10-15). More than 50 % of the world's population worldwide has been infected, as proven by serological evidence of past infections. There is only one serotype (TWAR) in humans, but *C. pneumoniae* infections in koalas, horses, frogs, reptiles and bandicoots have been reported (16). 70 % of infections with *C. pneumoniae* are asymptomatic or mild, and chlamydial pneumonias cannot be clinically differentiated from other atypical pneumonias, such as those caused by *Mycoplasma pneumoniae*, *Legionella pneumophila* and respiratory viruses (17, 18). Interestingly, *C. pneumoniae* has also been suggested to participate in the pathogenesis of atherosclerosis. *C. pneumoniae* can spread systemically to vascular tissue, where it can infect and grow in smooth muscle cells, endothelial cells and macrophages, and it has been found in atherosclerotic plaques (19, 20). Infection has been suggested to accelerate the progression of the experimental disease in some studies (21-23) but not in others (24, 25), and anti-chlamydial antibiotics can prevent or retard pathology (21). However, several extensive clinical intervention trials with antibiotics showed no evidence for treatment benefit in stable and acute coronary syndrome patients, and the exact role of *C. pneumoniae* in the development of atherosclerosis in human remains to be defined (reviewed in (26)).

### **Chlamydial life cycle**

*Chlamydia* has a unique biphasic developmental cycle, which occurs inside host cells. The infection is initiated by the extracellular elementary body (EB), which enters epithelial cells at mucosal surfaces. The EB is a small (0.3 to 0.4  $\mu\text{m}$ ), metabolically inactive, infectious and resistant spore-like form. The EB adheres to the host cell and enters by receptor-mediated endocytosis, pinocytosis or phagocytosis. It avoids the fusion of the phagosome with the lysosome and intracellular killing is hence inhibited. Within 8 to 12 hours after entry, the EB differentiates into a larger (0.8 to 1.0  $\mu\text{m}$ ), metabolically active reticulate body (RB). The RB is osmotically sensitive but protected by its intracellular location. It divides by binary fission and the phagosome with accumulated RBs is now called an "inclusion", which can be detected by

histological staining. The replication probably occurs by altering the host cell functions in order to establish and maintain a favorable environment. Thereafter the RB differentiates back into the EB form. Cell death at the end of the infection cycle allows *Chlamydiae* to exit the cell and reinitiate new rounds of infection after two to three days (figure 1) (2, 27). *Chlamydia* can differentiate into a persistent atypical form (aRB), which does not proliferate and causes the host cell to become resistant to apoptosis. This state is triggered by nutrient deprivation, elevated temperature or presence of interferon (IFN)- $\gamma$ . The aRB will redifferentiate into an active RB upon removal of the biological stress, giving rise to a reactivation of infection (figure 1). The persistent forms could account for chlamydial chronicity (28, 29).



**Figure 1.** Developmental cycle of *Chlamydia* (30).

The natural route of infection of *C. pneumoniae* is the respiratory tract, spreading from person to person via aerosols. It infects primarily bronchial and alveolar epithelial cells, but also endothelial cells and macrophages (17). In the lung, the bacteria establish a patchy interstitial pneumonia. Inflammatory infiltrates initially include neutrophils and later monocytes. The clinical manifestations of chlamydial infections are due to the direct destruction of cells during replication and the host inflammatory response (31).

Immunity to *C. pneumoniae* proceeds in two stages: 1) an early innate immune response requiring IFN- $\gamma$  to limit growth of bacteria, and 2) a later adaptive immune response that involves CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and IFN- $\gamma$  in protection (32-34). In contrast to infections with other invasive bacteria, epithelial cytokine response to *Chlamydia* is delayed until 20-24 hours after infection (35). This may be due to the weak immunostimulatory effect of chlamydial molecules, or to their low

concentration in the beginning of the life cycle. The adaptive immune responses are weak and often insufficient to resolve the infection (36). Immunity is unable to prevent reinfection, and chlamydial infections are often recurring. Instead the infection induces immune responses that chronically produce inflammatory cytokines, leading to tissue pathology associated with infection (37). Antibiotics are effective in curing acute chlamydial infections, but probably not in resolving chronic conditions. The persistent aRBs are refractory to antibiotic treatment, since they are mainly non-replicative and with low metabolic activity (29).

The production of a vaccine to *Chlamydia* would be an effective way to significantly reduce the prevalence of infection and use of antibiotics. When designing vaccines the innate immune mechanisms after infection determine the quality of the response, which should preferably be sterilizing and give long-term protection, but at least result in reduced pathology and shortened course of infection. Therefore, the details of innate immunity to *Chlamydia* need to be elucidated. We used *in vivo* and *in vitro* models for studying immunity to intracellular *C. pneumoniae* infections. Studies in mice are useful as a model for immunological studies of the immune response to *C. pneumoniae* in humans, as numerous knockout mice deficient in specific genes that participate at different levels in the innate and adaptive immune responses are available. Experimental infection models have been established to study acute *C. pneumoniae* infection in mice. Intranasal infection caused pneumonia in animals and no difference in susceptibility between several inbred mouse strains was seen (38). In our experiments with *C. pneumoniae*, a mouse model with the isolate Kajaani 6 was used (39). Kajaani 6 was obtained during an epidemic outbreak in military garrisons in northern Finland (40). *C. pneumoniae* mouse models seem to be similar to the human infection, in that mice acquire a mild, non-lethal lung inflammation with similar kinetics, development of partial protection and capacity to reinfect.

## **Listeria**

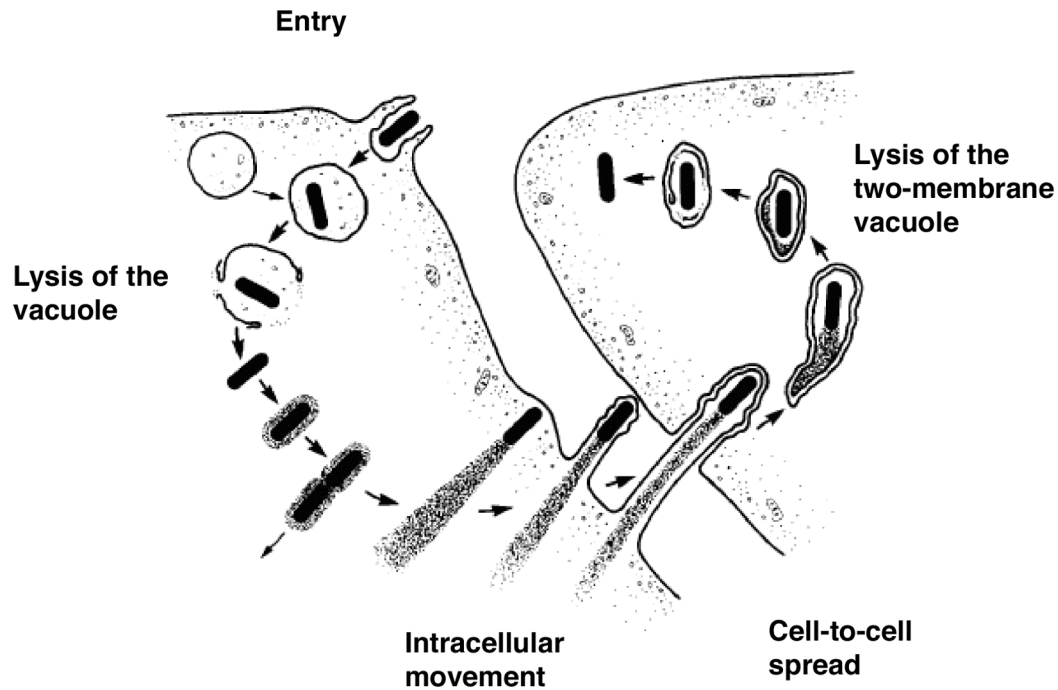
The genus *Listeria* consists of six species, of which *Listeria monocytogenes* is the only human pathogen. *L. monocytogenes* is a short (0.4 to 0.5 x 0.5 to 2.0  $\mu\text{m}$ ), Gram-positive, facultative intracellular anaerobic bacillus (41). It is found ubiquitously in soil and water, and can grow between 1° and 45° C, for example in refrigerated food. Focal epidemics or spontaneous cases of listeriosis often occur, spreading via contaminated foods, such as unpasteurized dairy products and undercooked foods (42). Listeriosis is uncommon, as only around 40-70 cases are reported each year in Sweden (6). However, the mortality rate of symptomatic listerial infection is higher than other food-borne diseases, being up to 30 % (41).

In healthy adults most listerial infections are asymptomatic or give a mild influenza-like illness. However, *L. monocytogenes* has the ability to cross three barriers: the intestinal, the placental and the blood-brain barriers (41). In a few cases in healthy adults, but more commonly in patients at higher risk, *L. monocytogenes* can cause clinical diseases, such as gastroenteritis, septicemia, meningitis and

meningoencephalitis (41). High-risk populations include neonates, elderly people, pregnant women and patients with suppressed cell-mediated immunity, e.g. AIDS and transplant patients (42). *Listeria* can spread from the pregnant woman to the fetus, which may cause abortion and stillbirth. It can also enter the brain in a hematogenous way via the blood-brain barrier or the choroid plexus (41). *Listeria* encephalitis can result from retrograde invasion of the brain stem via a neural route (43, 44).

### **Listerial life cycle**

*L. monocytogenes* is taken up by phagocytes, but also non-phagocytic cells, where the entry is facilitated by bacterial cell attachment proteins, called internalins, which are expressed on the surface of the bacteria (45). *L. monocytogenes* is phagocytosed by macrophages via a process thought to involve complement factors and scavenger receptors (46, 47). After entry into the cell, the low pH in the phagolysosome activates a bacterial cholesterol-dependent cytolysin, called listeriolysin O (LLO). This will result in the escape of the bacterium into the cytosol (41). LLO is encoded by the *hly* gene and binds as a monomer to membranes. It is diffused and oligomerized on the membrane, resulting in the formation of a pore. The activity of LLO is restricted to the intracellular space through an optimum at acidic pH and a rapid degradation (41). Once in the cytosol, the bacterium starts replicating at a doubling rate of around one hour, utilizing host nutrients. It then moves to the cell membrane, and penetrates into another host cell. This intracellular movement is driven by a bacterial motility protein, ActA, which is localized on the surface of one end of the bacterium. It assembles actin filaments into a tail, with the bacterium at the assembling end of the tail. This moves the bacteria through the cytoplasm to the cell membrane. By protruding the cell membrane a filopod is created, propelling the bacteria through the cytoplasm into an adjacent cell. The double membrane vacuole created is lysed by LLO and a lecithinase, and the cycle is thus completed (figure 2)(48).



**Figure 2.** Infectious cycle of *Listeria*. Figure is adapted from (49).

The natural route of infection is the gastrointestinal tract. A majority of the listerial organisms ingested with contaminated food are killed by the acidic pH in the stomach. The surviving *L. monocytogenes* infects intestinal epithelial cells and M cells in the Peyer's patches. They can spread to adjacent enterocytes, causing enteritis, or transigrate through the epithelial cell layer and disseminate through lymph or blood to mesenteric lymph nodes, spleen and liver, where they are primarily internalized by splenic and hepatic macrophages and normally killed in immunocompetent individuals (41). Innate immune responses are rapid and essential for host survival during infection with *L. monocytogenes*. It prevents growth and dissemination of bacteria into a systemic and lethal infection. Primarily neutrophils and then macrophages are main mediators of the initial killing of *L. monocytogenes*. The adaptive immune response is mostly important for long-term clearance of bacteria (50).

*L. monocytogenes* is a useful model for studying the innate immune responses to intracellular bacterial infections. There are mutants of *Listeria* that are defect in defined steps of the intracellular life cycle. Although other animals have been used, mice are the most useful model due to availability of knockout mice. Macrophages are the primary cells to be infected and the main reservoir of *L. monocytogenes* *in vivo*, and therefore the main focus of studies of the innate immunity to *L. monocytogenes*. Replication occurs primarily in macrophages and they are necessary for clearing bacterial infection (51). However, *L. monocytogenes* has been shown to grow also in hepatocytes, endothelial cells, epithelial cells, fibroblasts and various types of nerve cells (41). Unfortunately, mice are quite resistant to gastrointestinal invasion by *L.*

*monocytogenes*, due to the fact that listerial internalin A does not bind to mouse E-cadherin (52). Therefore, most laboratory studies are done using intravenous or intraperitoneal inoculations, hence focusing on the systemic infection.

## **The immune response to intracellular bacterial infections**

### **Innate immunity**

The innate immunity of the host is the front line of defense. It is an evolutionarily conserved, swift and ontogenetically fixed response, which does not require priming or memory (53). Multicellular organisms depend on a rapid innate response to successfully eradicate invading microbes. Innate immunity has multiple aspects: pathogen recognition, antimicrobial defense and instruction of the adaptive immune response (54). The innate immune system is composed of non-phagocytic and phagocytic cells in tissue and blood, circulating plasma-derived proteins, such as complement factors, and cell-derived proteins, such as cytokines. The recruitment of inflammatory cells to the site of infection and the subsequent release of proinflammatory cytokines, such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)- $\alpha$  and IL-12 is crucial for innate resistance to intracellular bacterial infections. Different classes of pathogens elicit different innate immune responses and the effect of this is dependent on the type and concentration of cytokines. However, cytokines have significant redundancy, since different cytokines seem to have similar effects (55).

During a bacterial infection, bacteria initially encounter the polarized epithelia of the mucosal surface, which act as a first chemical, mechanical and microbiological barrier to infection. The epithelial cells are not considered professional immune cells, but are nevertheless mediators and effectors of the innate immune responses after infection (35, 56). If bacteria cross the epithelial barrier, they are immediately recognized by macrophages that reside in tissues, such as Kupffer cells, alveolar and peritoneal macrophages. Resident macrophages are responsible for the initial killing of the majority of bacteria (51). Neutrophils account for 70 % of all leukocytes in blood, and are quickly recruited to the site of infection, where they engulf bacteria. They can also kill extracellular bacteria by releasing granule and antimicrobial peptides (55). Within 8-12 hours, inflammatory macrophages are attracted to the site of infection. Macrophages and other phagocytic cells recognize bacteria with a set of innate immune receptors, called pattern recognition receptors (PRRs), which can discriminate bacterial surface molecules. In response to innate immune receptor signaling, cells produce pro-inflammatory cytokines and chemokines, such as CXCL8 and CCL2, triggering a state of inflammation and attracting more cells to the site of infection. Inflammation facilitates both killing of the bacteria and initiation of repair of the injured tissue. Dendritic cells (DC) are activated by PRRs, leading to expression of MHC and costimulatory molecules, and cytokines. This enhances the ability of DC to stimulate T cells. DC migrate to lymphoid tissue where they interact

with T and B cells to initiate and shape the adaptive response (53). Innate immunity is therefore also necessary for triggering the adaptive immune responses.

### **The macrophage and intracellular infections**

Monocytes circulate in the bloodstream and then migrate to tissues upon chemotaxis, where they mature into different types of macrophages at different anatomical locations. Monocytes become resident macrophages under normal states and in response to inflammation signals they move to sites of infection in the tissues and differentiate into inflammatory macrophages to elicit an immune response. Macrophages have a double role in the immune response to intracellular bacterial infections, since they are both important effector cells of the innate immune system and one of the primary cells in which bacteria survive and proliferate. Intracellular bacteria can grow inside resident macrophages, as they have developed several particular and sophisticated immune escape mechanisms. *Chlamydia* inhibits the phagolysosomal fusion and directs the infected phagosome to the Golgi apparatus. *Listeria* escapes from the phagosome into the cytosol, where it proliferates. Although macrophages mount a potent innate response to infection required for effective bacterial clearance, such a response is not by itself sufficient for destruction of intracellular bacterial pathogens. The immunological dogma indicates that T cells activated in the peripheral lymphoid organs after cognate recognition of dendritic cells presenting bacterial antigens will be recruited into the inflammatory site, recognize the infected macrophage and secrete a variety of cytokines. Among them, IFN- $\gamma$  is the most important cytokine during early phase of infection with intracellular bacteria. IFN- $\gamma$  acts in synergy with signals transmitted from PRRs to further trigger the activation of macrophages and their phagocytosis of the bacteria. Once activated, macrophages block the escape into the cytosol in the case of *Listeria*, or increase dramatically bacteriocidal effector mechanisms, such as increased acidification, a more efficient maturation of the phagosome, increased levels of hydrolytic enzymes, production of reactive oxygen and nitrogen species, restriction of iron and nutrients, and other induced effector mechanisms, which have varying relevance on different infections. Furthermore, activated macrophages also produce proinflammatory cytokines and chemokines to further recruit and activate other cells to the site of inflammation in a feedback loop. On the other hand, destruction of the pathogen and the consequent decreased levels of bacterial innate immune receptor ligands can ultimately downregulate these responses (55).

### **Adaptive immunity**

The adaptive immune cells (T and B cells) have an exceptional diversity of antigenic specificity. Professional antigen-presenting cells (pAPCs), such as macrophages, DC and B cells, engulf bacteria or infected cells, and present antigen from degraded bacteria in the lysosome via the MHC class II pathway to CD4<sup>+</sup> T cells. Intracellular bacteria that enter epithelial cells, which lack the expression of MHC class II, will replicate inside the cell without being presented to CD4<sup>+</sup> T cells. However, proteins



from vesicle membranes and the cytosol are degraded into peptides by the host proteasome. These peptides are transported into the endoplasmic reticulum, loaded onto MHC class I molecules, displayed on the cell surface and presented to CD8<sup>+</sup> T cells. In addition to recognition of antigen on MHC by the T cell receptor, T cells need to be activated by costimulatory molecule signal and stimulation by multiple T cell growth factors, such as the cytokines IL-2, IL-4, IL-12, IL-15 and IFN- $\alpha/\beta$  (57). As epithelial cells lack costimulatory molecules, T cells need to be activated by pAPCs to respond to epithelial cells.

The cells of the adaptive immune system are necessary to limit infection and provide protection during infection with *Chlamydia* and *Listeria*. In intracellular infections, CD4<sup>+</sup> and CD8<sup>+</sup> T cells confer most of the adaptive immune response, whereas B cells only play a minor role. In fact, *Chlamydia* and *Listeria* have been found to be susceptible to T cell-mediated immunity, but B cells seem to be less important for resolving infection (36, 51, 58). *Chlamydia* persists inside cells and *Listeria* spreads cell-to-cell, without being detected by antibodies. B cells probably play a small role in secondary infection of *Listeria* and *Chlamydia*, as antibodies will bind extracellular bacteria and impede their ability to reinfect. T cells have a major role in clearance of infection with *C. pneumoniae* and *L. monocytogenes* (32, 59). T cells establish long-term protective immunity upon infection with *L. monocytogenes*. *Chlamydia*-specific memory T cells are able to mount a strong response after secondary infection, but not efficiently enough to prevent recurrent infections (32, 51, 60).

CD4<sup>+</sup> T cells activate and regulate B cells, CD8<sup>+</sup> T cells and inflammatory cells by contact- and cytokine-dependent processes. The type of cytokines that T cells produce reflects the nature of infection, the host genetics and the environment. CD4<sup>+</sup> T cells can differentiate into T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17 and regulatory T cell lineages. T<sub>H</sub>1 cells participate in cell-mediated immunity and inflammation, T<sub>H</sub>2 cells provide help for B cells of the humoral immunity, T<sub>H</sub>17 cells protect surfaces, such as the lining of the intestine against extracellular pathogens and regulatory T cells suppress the activity of the immune responses and thereby maintain immune system homeostasis and tolerance to self-antigens (61, 62). Intracellular bacteria induce a strong T<sub>H</sub>1 response, and via the secretion of IFN- $\gamma$ , T<sub>H</sub>1 cells will, for example, trigger the microbicidal activity of macrophages, directly limiting replication and also enhancing antigen presentation to T cells (36, 51, 55). T<sub>H</sub>2 cells do not seem to confer protection against intracellular infections, and could even enhance infection by inhibiting T<sub>H</sub>1 responses by secreting IL-4 (63). Activated CD8<sup>+</sup> T cells produce effector cytokines, such as IFN- $\gamma$  and TNF- $\alpha$ , but also mediate immunity through lysis activity by means of perforin and granzymes. Lysing activity leads to exposure of intracellular bacteria for killing by activated macrophages (64). However, perforin does not seem to be important for protection against primary infection with *C. pneumoniae* and *L. monocytogenes* (32, 65).

## **Innate immune receptors**

The innate immune system is able to recognize microorganisms through receptors, called PRRs. They recognize pathogen-specific conserved molecules that are vital for the survival of microbes, often carbohydrates or lipids, which are not present in the host itself. These receptors do not require gene arrangement and clonal expansion as T and B cell receptors do, and they are expressed on a wide range of cells. They include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain protein (NOD)-like receptors (NLRs), scavenger receptors, C-type lectin receptors, including the mannose and Dectin-1 receptors, and others, such as retinoic acid-inducible protein I (RIG-I), melanoma differentiation-associated protein 5 (MDA-5) and DNA-dependent activator of interferon regulatory factors (DAI). Some innate receptors are cell surface- or endosome-bound receptors that recognize microbes as they enter the cell, whereas cytoplasmic receptors function after escape or leakage of components from the phagolysosome. Circulating complement factors have the ability to recognize pathogens directly and are in turn recognized by complement receptors on macrophages (66). Pathogens can also be recognized by antibodies of the adaptive immune response and subsequently opsonized through binding of Fc receptor on macrophages. Innate receptors have been shown to have redundant and co-operative functions in the detection of particular pathogens (67).

## **Toll-like receptors**

TLRs are transmembrane receptors, present either on the cell surface or inside endosomes. They are highly conserved across species and so far twelve different TLRs have been identified in mice and ten in humans (68). They recognize an array of microbial products (as shown in table 1). However, the mechanism for recognition has not been well characterized and no clear evidence for direct interaction have been demonstrated.

**Table 1.** Some Toll-like receptors and their ligands in mice and humans (68).

RECEPTOR	LIGAND
TLR1	Triacyl lipopeptide
TLR2	Lipopeptide/lipoprotein, peptidoglycan, lipoteichoic acid, yeast zymosan and others
TLR3	dsRNA
TLR4	Lipopolysaccharide, heat shock proteins and others
TLR5	Flagellin
TLR6	Diacyl lipopeptide, lipoteichoic acid, yeast zymosan
TLR7	ssRNA
TLR8	ssRNA (not known in mice)
TLR9	CpG-containing DNA

TLRs have a leucine-rich repeat (LRR) extracellular domain involved in the recognition of its ligand, and a cytoplasmic Toll-IL-1R (TIR) domain. Highly conserved regions of the TIR domain recruit TIR-containing adaptor molecules, leading to a TIR-TIR heterophilic interaction and different intracellular signaling cascades depending on the adaptors involved. Each receptor binds to a specific combination of adaptors in the signal transduction, triggering appropriate and effective responses to pathogens (69). The TIR domain is also present on the IL-1 and IL-18 receptors, thus sharing the same signaling pathway as TLR. TLR3, 7 and 9 are endosomal and sense nucleic acid in particular. TLR2 (forming a heterodimer with either TLR1 or TLR6), TLR4 and TLR5 are localized on the plasma membrane (70). Most of the TLRs are known to be expressed on macrophages. TLR9 is almost exclusively expressed on pDC but in response to LPS it is also expressed in macrophages (71).

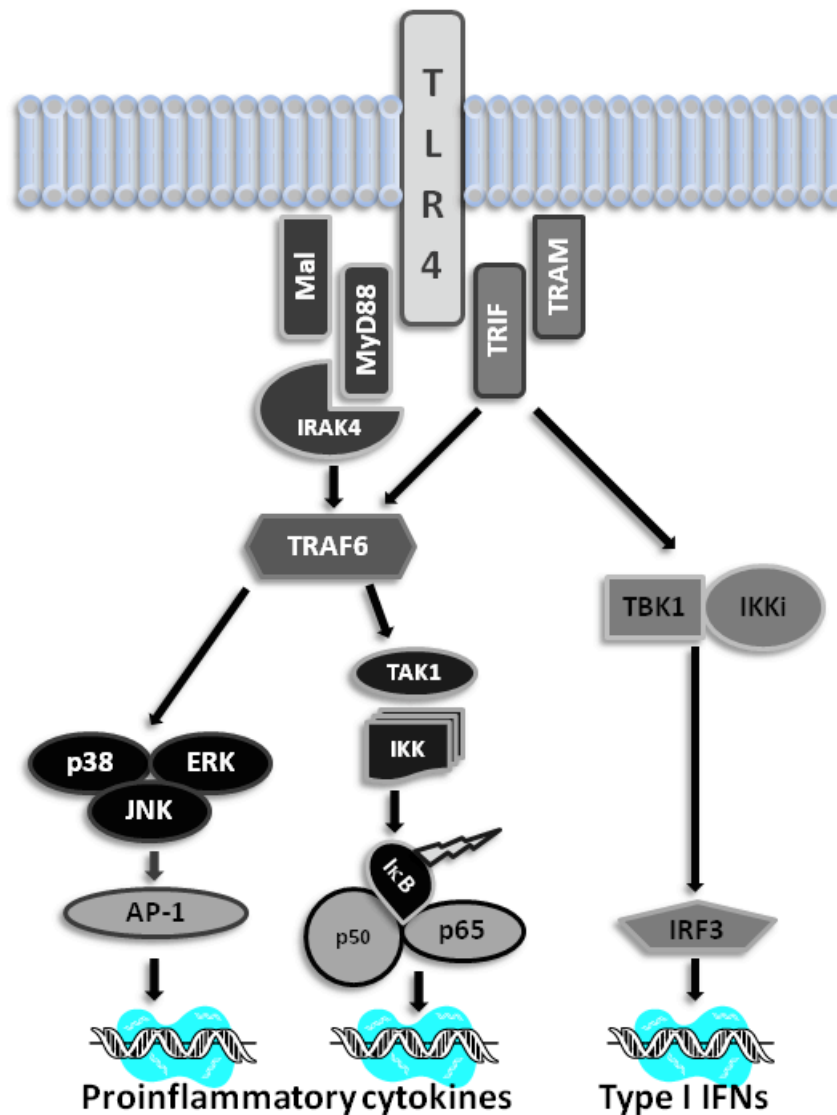
TLR4 recognizes the endotoxin lipopolysaccharide (LPS) from Gram-negative bacteria. LPS is known to bind to a serum protein, the LPS-binding protein (LBP) and this complex interacts with a soluble form of glycoprotein CD14 and the adaptor MD2 on the cell surface, which is associated with the extracellular portion of TLR4 (72). TLR4 has also been suggested to recognize heat shock protein (HSP) 60 and 70, fibrinogen and viral envelope proteins, but they could be questioned to be contaminated with LPS (68).

Upon binding of their ligand TLRs initiate a common signaling cascade leading to induction of inflammatory responses and importantly to the initiation of the adaptive immune response. All TLRs except TLR3 use the adaptor molecule myeloid differentiation factor 88 (MyD88), which consequently has an important role in the downstream transduction of the signal. TLR signaling is known to induce transcription of proinflammatory cytokines, chemokines and costimulatory molecules.

This occurs via activation of two downstream signaling pathways nuclear factor  $\kappa$  enhancer binding protein (NF- $\kappa$ B) and mitogen-activated protein (MAP) kinases. Several TLRs can induce also IFN- $\alpha/\beta$ , through the activation of interferon regulatory factors (IRFs) (69).

### **TLR4 signaling**

TLR4 signaling can be both MyD88-dependent and -independent (figure 3)(69, 72). The intracellular TIR domain of TLR4 associates to the TIR domain of the MyD88 (73), together with the MyD88-adaptor-like protein (Mal, also known as TIRAP), which is attached to the cell membrane. The death domain of MyD88 recruits the death domain of IL-1R-associated kinases (IRAK), IRAK1, -2, -4 and -M, to the receptor complex. IRAK4 is, in particular, required for TLR signaling (74, 75). The association with MyD88 triggers the phosphorylation and the dissociation of IRAK4 from the complex. IRAK4 will associate with tumor necrosis factor receptor-associated factor 6 (TRAF6), an E3 ubiquitin ligase (76, 77). TRAF6 forms a complex with Ubc13 and Uev1a, which are ubiquitin conjugating enzymes, to promote synthesis of polyubiquitin chains. These chains are required to activate the transformation growth factor- $\beta$ -activating kinase 1 (TAK1), which consequently leads to activation of I $\kappa$ B kinases (IKKs). IKKs (consisting of IKK $\alpha$ , IKK $\beta$  and IKK $\gamma$ ) phosphorylate the inhibitor I $\kappa$ B family, which normally sequesters NF- $\kappa$ B in the cytoplasm, masking the nuclear localization signals (NLS). NF- $\kappa$ B consists of a family of transcription factors, such as p50, p52, p65, RelB and c-Rel, which form homo- or heterodimers. Phosphorylation of I $\kappa$ B proteins at two serine residues leads to the subsequent ubiquitination and degradation in the proteasome and release of free dimers of NF- $\kappa$ B. These dimers are translocated into the nucleus, where they bind to cognate binding sites, to induce and regulate several target genes involved in cell survival, cell proliferation and importantly in host defense, such as proinflammatory cytokines, costimulatory molecules, adhesion molecules, chemokines, growth factors and inducible enzymes (reviewed in (78)).



**Figure 3.** MyD88-dependent and -independent TLR4 signaling.

TRAF6 signaling also leads to activation of MAP kinases, such as p38, c-Jun N-terminal kinases (JNK) and extracellular signal-regulated kinases (ERK), which are serine/threonine-specific kinases. An extracellular stimulus leads to activation of a MAP kinase in a cascade, where upstream MAP kinases activate other MAP kinases by phosphorylation. The activated MAP kinase phosphorylates different substrates, such as effector kinases or transcription factors. MAP kinases regulate various cellular activities, such as proliferation and differentiation, cell survival and also inflammation (79). For example, MAP kinases phosphorylate and activate the transcription factor activating protein 1 (AP-1). AP-1 has a role in expression of proinflammatory cytokines (72). Apart from the involvement in MyD88 and IL-1R signaling, TRAF6 also mediates TNFR and CD40 signaling (80).

The TIR domain of TLR4 can also bind to other adaptor proteins including the TIR domain-containing adaptor inducing IFN- $\beta$  (TRIF), as well as TRIF-related adaptor molecule (TRAM), which is associated with the plasma membrane. TRIF

interacts physically with TRAF6 (81, 82). In the absence of MyD88, TRIF cannot produce early proinflammatory cytokines in the LPS-induced response in macrophages, implicating other transcription factors or the requirement for both pathways in the induction of proinflammatory cytokines. However, TRIF can promote a delayed activation of NF- $\kappa$ B and MAP kinases, called a late-phase activation, and co-stimulatory molecule production (73, 83, 84). TRIF also associates with the TRAF family member-associated NF- $\kappa$ B activator (TANK)-binding kinase 1 (TBK1) and IKKi (also called IKK $\epsilon$ ) (85). These two kinases can in turn phosphorylate the transcription factor IRF3 (86), linking TLR4 signaling to induction of type I IFNs.

## **Role of TLRs in bacterial infections**

The recognition of specific molecular patterns by TLRs has been widely studied, but the numbers of reports on the relevance of TLRs in infection models are still limited. For example, TLR4 has been shown to protect against *Salmonella typhimurium* infection (87). As infection involves several TLR ligands, deficiency in one receptor can be redundant to the overall immune response. However, mice deficient in MyD88 and thereby in signaling by a number of TLRs, have been proved to be susceptible to several different bacterial infections, such as *Staphylococcus aureus* (88), *Streptococcus pneumoniae* (89), *Mycobacterium* species (90-93) and *Neisseria meningitidis* (94). Human patients with deficiencies in MyD88 and IRAK4 suffer from severe bacterial infections, but only to some agents, mostly *S. pneumoniae* and *S. aureus* (95, 96).

## **TLRs and *Chlamydia***

TLRs appear to have a role in innate protection to chlamydial infections, as MyD88<sup>-/-</sup> mice are more susceptible than WT mice at 14 days after intranasal infection with *C. pneumoniae* (97). *Chlamydia* has been reported to be recognized by TLR2 and TLR4 *in vitro* (98-103) and *in vivo* (104). However, TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice were not more susceptible than WT controls after intranasal infection with *C. pneumoniae* (97, 105). Rodriguez *et al.* suggested a partial role for TLR2 and TLR4 in protection since TLR2<sup>-/-</sup>/TLR4<sup>-/-</sup> mice showed higher mortality than TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup> and WT mice after *C. pneumoniae* infection (106). Conversely, MyD88<sup>-/-</sup> mice have also been shown to be more resistant during the early phases of chlamydial infection, as explained by increased bacterial replication in neutrophils recruited in a TLR-mediated way (107). Thus, although MyD88 plays a role in the outcome of infection, the signaling pathways activated and the precise regulation of immune responses that MyD88 signaling mediates during chlamydial infection is still unclear.

The relevance of specific TLRs during chlamydial infections is still questionable. *Chlamydia* synthesizes a modified LPS structure, with weaker endotoxin activity than enteric LPS and chlamydial DNA shows a low CpG frequency (108, 109). Unlike other bacteria *Chlamydia* is thought to lack a peptidoglycan (PGN) layer, as it has never been detected biochemically. However, the genome encodes all

the enzymes required for PGN synthesis, suggesting that it may produce small amounts of PGN (110).

Several studies have addressed the role of TLR pathways in the activation of NF- $\kappa$ B and MAP kinases, and the production of proinflammatory cytokines after infection with *C. pneumoniae*. In bone marrow-derived DC (BMDC) infected with *C. pneumoniae*, TNF- $\alpha$  production and NF- $\kappa$ B activation was reported to be controlled by TLR2 and not TLR4, but IL-12 secretion was dependent on both TLR2 and TLR4 (103). In peritoneal macrophages infected with *C. pneumoniae*, IL-1 $\beta$  and TNF- $\alpha$  secretion was MyD88- and TLR2-dependent, but independent of TLR4 (98). Chlamydial HSP60 stimulated TLR2- and TLR4-dependent TNF- $\alpha$  production, and activated MAP kinases and NF- $\kappa$ B in a MyD88- and TRAF6-dependent way in macrophages (102). The precise role of TLRs and the subsequent signaling involved in NF- $\kappa$ B and MAPK activation and induction of proinflammatory cytokines in macrophages after chlamydial infection is thus controversial.

Different reports indicate or rule out the participation of TRIF-dependent pathways in chlamydial infection. For example, MyD88<sup>-/-</sup>, but not TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> macrophages infected with *C. muridarum* showed reduced levels of IFN- $\beta$  compared to WT controls (111). Derbigny *et al.* suggested that TLR3 but not TLR4, could be implicated in the IRF3-dependent IFN- $\beta$  production by *C. muridarum*-infected oviduct epithelial cells (112). This implicates a potential role for a TRIF/ IRF3-dependent pathway in the immunobiology of chlamydial infection.

## **TLRs and *Listeria***

*L. monocytogenes* expresses several TLR ligands, such as PGN, flagellin and bacterial DNA. TLR signaling is involved in innate immune defense to *L. monocytogenes*, as MyD88<sup>-/-</sup> mice demonstrated higher bacterial load than WT controls during systemic infections (113, 114). TLR2 seems to be the most important TLR in recognition of *L. monocytogenes* (113, 115), however TLR2<sup>-/-</sup> mice are only slightly more susceptible to i.p. infection than WT mice (114).

Infection of macrophages with *L. monocytogenes* triggers distinct innate immune receptors at different time points. First, TLR signaling independent of the invasion of live bacteria is present, leading to expression of NF- $\kappa$ B-dependent genes. After invasion IFN target genes are triggered following bacterial escape from the phagosome. IFN- $\beta$  production requires the LLO-dependent escape into the cytoplasm, but not TLR signaling (116-121). However, it is unclear which cytosolic innate receptors are involved in such responses.

## **NOD-like receptors**

NLRs function as sensors of exogenous microbes and endogenous danger signals in the cytosol (some examples of NLRs are shown in table 2). They are structurally and functionally related to a plant disease-resistant protein family and therefore conserved during evolution. NLRs share a common structure : (1) a C-terminal LRR domain; which recognizes ligands, (2) a central nucleotide-binding domain (NBD), which

regulates self-oligomerization and the activity of the NLR and (3) a N-terminal effector domain, which is either a caspase-recruiting domain (CARD), a pyrin domain or a baculovirus inhibitory repeat (122).

Table 2. Ligands to some studied NLRs (122-124).

RECEPTOR	LIGAND
NOD1	Muropeptide (PGN)
NOD2	Muropeptide (PGN)
Nalp3	Bacterial and viral RNA, danger signals (extracellular ATP and K <sup>+</sup> )
Ipaf	Flagellin and other unknown ligand
Naip	Flagellin

The effector domain is responsible for recruiting and interacting with downstream molecules, containing the same effector domain. When a ligand binds to the LRR domain, the NLR molecule changes confirmation, leading to an oligomerization of the NBD. The effector domain of the NLR is then exposed and recruits downstream adaptors or effector proteins, leading to signal transduction. NLRs activate two major pathways leading to proinflammatory signaling: the activation of NF- $\kappa$ B and the caspase-1-mediated pathways (122). Caspase-1 (also called IL-1 $\beta$ -converting enzyme (ICE)), converts IL-1 and IL-18 to their active forms. Similar to TLRs, no direct interaction has been demonstrated between the NLRs and the ligands.

## NOD

NOD1 and NOD2 detect different structures derived from PGN. The continuous synthesis and degradation of the PGN layer in bacteria leads to release of muropeptides. NOD1 recognizes muropeptides GlcNAc-MurNAc-L-Ala-D-Glu-*meso*-diaminopimelic acid (DAP) (GM-triDAP) and GM-L-Ala-D-Glu-*meso*-DAP-D-Ala (GM-tetraDAP). The minimal motif recognized by NOD1 is suggested to be a dipeptide D-Glu-*meso*-DAP (iE-DAP) or only *meso*-DAP. Most Gram-negative and some Gram-positive bacteria, such as *L. monocytogenes*, but not eukaryotes, possess *meso*-DAP. NOD2 recognizes muramyl dipeptide, which is found in all PGNs (122, 125, 126).

Both NOD1 and NOD2 recruit the CARD-containing receptor-interacting protein 2 (RIP2) (also called RICK or Cardiak), resulting in the activation of the NF- $\kappa$ B and MAP kinase pathways (127). RIP2 interacts with and activates IKK $\gamma$ , a regulatory subunit of the IKK complex (128). NOD1 and NOD2 have an important role in innate immunity through their ability to stimulate the secretion of pro-inflammatory cytokines and chemokines (126, 127, 129, 130). Masumoto *et al.* demonstrated that i.p. injection of the synthetic NOD1-specific ligand iE-DAP



induced an increase of chemokine levels in serum and resulted in the recruitment of neutrophils to the site of injection (131).

However, NOD ligands by themselves trigger poor cytokine responses, but there is evidence that NOD1 and NOD2 act in synergy with TLRs in inducing maximal responses (132-134). Whilst RIP2 was first suggested to link NOD and TLR signaling systems (135), recent studies have been unable to confirm that RIP2 is involved in TLR signaling (127).

### **Role of NOD in bacterial infections**

NOD1 and NOD2 are believed to play a central role in the control of immune homeostasis and inflammation at mucosal surfaces. Mutations in NOD2 have been implicated in the development of inflammatory bowel disease (IBD) (122). The role of NOD as intracellular PRRs is, in fact, a paradox, since a mutation in a receptor triggering inflammation leads to increased IBD. In intestinal epithelial cells the expression of TLRs appears to be downregulated and/or compartmentalized which could be necessary to avoid continuous triggering of the immune system by commensal bacteria in the intestine (136). NOD receptors could then recognize invading bacteria. NOD1 is expressed in most cells (137) but NOD2 is mainly expressed in monocytes and intestinal epithelial cells (138).

NOD1 has mainly been suggested to be involved in the defense against Gram-negative bacteria, such as *Escherichia coli* (139), *Shigella flexneri* (140), *C. pneumoniae* (141) and *Pseudomonas aeruginosa* (142) and some Gram-positive bacteria, such as *L. monocytogenes* (127, 143) *in vitro*. Studies *in vivo* suggest a role for NOD1 and NOD2 in gastrointestinal disease. Gram-negative *Helicobacter pylori* appears to be detected by NOD1 in a non-invasive-dependent way, by injecting their muopeptides via the type IV secretion system (144). NOD2-deficient mice are more sensitive than WT to oral infection with *L. monocytogenes*, but not to i.v. and i.p. infection, suggesting that the effect of NOD2 is at the intestinal and not the systemic level (145).

### **NOD1 and *Listeria***

The involvement of NOD1 in sensing *L. monocytogenes* has been argued, partly due to the fact that products from *L. monocytogenes* did not stimulate NOD1 signaling (125). However, *L. monocytogenes* activated p38 and NF- $\kappa$ B, leading to induction of IL-8 in a NOD1-dependent manner in endothelial cells (143). RIP2<sup>-/-</sup> macrophages produced decreased levels of IL-6, TNF- $\alpha$  and CXCL1, compared to WT controls after infection with *L. monocytogenes* (127, 135, 146). RIP2<sup>-/-</sup> mice infected i.v. with *L. monocytogenes* showed higher titers of bacteria than WT controls in liver and spleen five days after infection, and succumbed after eight days (147). NOD1<sup>-/-</sup>/NOD2<sup>-/-</sup> mice showed slightly enhanced bacterial load in liver, but not in spleen, at 48 hours after i.p. infection *L. monocytogenes* (148).

Long term responses to LPS can be deleterious to the host, but responses are hampered by a transient state of tolerance (149). Both TLR and NOD ligands confer

self-tolerance, but not cross-tolerance (148). Kim *et al.* demonstrated that TLR-tolerized cells still mediate protection to infection with *L. monocytogenes* in a NOD1-, NOD2- and RIP2-dependent way. This was demonstrated by the fact that NOD1<sup>-/-</sup>/NOD2<sup>-/-</sup> mice pretreated with LPS or heat-killed *E. coli* were more susceptible to *Listeria* than WT mice. However, non-treated NOD1<sup>-/-</sup>/NOD2<sup>-/-</sup> mice were only slightly more sensitive to infection than WT, indicating that TLR tolerance affects responses to NOD ligands (148). Altogether, a protective role of NOD receptors seems to be beyond doubt for the case of gastrointestinal infections. In the case of systemic infections these receptors are known to synergize with TLR signaling. Whether NOD1 and NOD2 by themselves are redundant or required for defense is not clear and the protective mechanisms that they could activate are debated.

### **Other receptors**

Cytoplasmic RNA can be sensed by RIG-I and MDA-5, two structurally related cytoplasmic receptors. They activate IRF3, leading to type I IFN induction (150). Cytosolic DNA was shown to stimulate type I IFNs in a TLR- and RIG-I/MDA-5-independent way (146). DAI is a newly discovered cytosolic DNA sensor that can initiate innate immune responses independently of TLR9, including IRF3-dependent type I IFN production (151). In fact, DNA from *L. monocytogenes* was suggested to stimulate synthesis of type I IFNs and IL-6, but not NF-κB and MAP kinases, following the activation of the IRF3 pathway, but independently of TLRs and RIP2 (118, 152). IFN-β was induced independently of RIG-I and MDA-5 after *L. monocytogenes* infection in macrophages (153). Thus, DAI is a candidate for cytosolic recognition of listerial DNA.

### **Interferons**

The IFNs are a group of secreted cytokines that elicit distinct antiviral effects. They are grouped into three classes called type I, II and III IFNs, according to their amino acid sequence. Type I IFNs (discovered in 1957, (154)) comprise a large group of molecules. Mammals have multiple distinct IFN-α genes (13 in human), one to three IFN-β genes (one in human) and other genes, such as IFN-ω, -ε, -τ, -δ and -κ. The IFN-α and -β genes are induced directly in response to infection, whereas IFN-ω, -ε, -τ, -δ and -κ play less defined roles, such as regulators of maternal recognition in pregnancy (155). Thus, use of the term ‘type I IFN’ in this thesis refers to IFN-α/β. Type II IFN has a single member, also called IFN-γ or ‘immune IFN’, and its role and regulation in bacterial infections is described below. Type III IFNs have been described more recently and comprise IFN-λ1, -λ2 and -λ3, also referred to as IL-29, IL-28A and IL-28B, respectively (156). These cytokines are also induced in direct response to viral infection and appear to use the same pathway as the IFN-α/β genes to sense viral infection (157).

Clinically, IFNs are widely studied for use in immune therapies to treat for example cancer, multiple sclerosis and viral diseases (158). In addition to their

dramatic effect on immune responses, they modulate cell growth and viability via different mechanisms. In order to mediate such pleiotropic effects IFNs trigger numerous signaling events, leading to induction of different IFN target genes. Many of the upregulated genes in LPS-stimulated cells are part of a secondary response that requires IFN signaling (159). Due to the crucial functions that they regulate, the response to IFNs must be balanced and fine tuned. IFN-dependent signaling involves members of four protein families: (1) IFN receptors; (2) the receptor-associated Janus protein tyrosine kinases (JAKs); (3) the signal transducers and activators of transcription (STATs); and (4) members of the IRF family of transcription factors.

## IFN- $\alpha/\beta$

The thirteen members of IFN- $\alpha$  and the single form of IFN- $\beta$  all signal through the IFN- $\alpha/\beta$ R. Although the multigenic nature of IFN- $\alpha$  has been known for over 20 years the significance of this is still debated, i.e. whether these genes are expressed differentially in distinct cell types, whether they are inducible by different types of viruses or whether they are functionally specialized (160). The antiviral state of IFN- $\alpha/\beta$ -treated cells is characterized by inhibition of both viral replication and cell proliferation. IFN- $\alpha/\beta$  elicit innate immune responses and promote the transition from innate to acquired immunity, by activating macrophages, increasing cellular cytotoxicity in NK cells, stimulation of cytokine and chemokine production, expression of costimulatory molecules and differentiation and activation of DC. IFN- $\alpha/\beta$  can also enhance adaptive immune responses by stimulating the promotion of T<sub>H</sub>1 and antibody responses (161). Type I IFNs have been implicated as candidates for vaccine adjuvants (162).

In response to viruses IFN- $\alpha/\beta$  are secreted by most cell types, of which macrophages and plasmacytoid DC (pDC) are known to be a major source (163, 164). Some cell types show a selective expression pattern, only inducing IFN- $\beta$  or only some subtypes of IFN- $\alpha$ . In addition to viral infection, poly I:C, cytokines, mitogens, tumor cells and many microbes and microbial products can trigger IFN- $\alpha/\beta$  production (165). However, experiments with IFN- $\alpha/\beta$ R<sup>-/-</sup> mice have shown that these cytokines can protect against or increase the susceptibility to bacterial infections. IFN- $\alpha/\beta$ R<sup>-/-</sup> mice were more susceptible than WT to infection with Group B Streptococci, *S. pneumoniae* and *E. coli* (166). IFN- $\alpha/\beta$ -treated mice infected with *S. typhimurium* demonstrated increased protection (167). However, IFN- $\alpha/\beta$ R<sup>-/-</sup> mice were more resistant to pulmonary and genital infection with *C. muridarum* and i.v. infection with *L. monocytogenes*, than WT controls (168-173).

The increased resistance in mice lacking IFN- $\alpha/\beta$  has been suggested to be connected to the decreased cell death (117, 169, 172, 174). Carrero *et al.* suggested that *L. monocytogenes*-induced IFN- $\alpha/\beta$  activate T cells non-specifically and increase LLO-induced apoptosis in T cells, and they showed that lymphocytes could even be detrimental in early stages of infection with *L. monocytogenes* (172, 175). Other studies showed that *L. monocytogenes* infection induces death of macrophages with necrotic features by the action of IFN- $\beta$  (117, 118, 176). *C. muridarum*-infected WT

mice showed higher level of apoptosis of pulmonary macrophages than IFN- $\alpha/\beta$ <sup>-/-</sup> mice, and in the absence of IFN- $\alpha/\beta$ , mice depleted of pulmonary macrophages result in a higher bacterial load than non-depleted mice after infection with *C. muridarum*. This indicates that type I IFNs promote macrophage death and inhibit macrophage function during infection with *C. muridarum*, leading to increased susceptibility (169). However, the role of apoptosis during intracellular bacterial infection is controversial. During infection with *S. pneumoniae* and *M. tuberculosis* macrophage function is thought to be controlled by induction of apoptosis, which can contribute to bacterial clearance and resolution of the inflammatory response (177).

The pleiotropic roles of IFN- $\alpha/\beta$  in different bacterial infections could probably be explained by different cellular tropisms of the infectious agents or different levels of the IFN- $\alpha/\beta$  cytokines. For example, Reutterer *et al.* demonstrated that two strains of *L. monocytogenes* differed in their ability to trigger IFN- $\beta$  production, which determined the susceptibility to infection and cell death in macrophages (171). Thus, different strains or species seem to elicit distinct immune responses, determining the outcome of infection.

## IFN- $\gamma$

IFN- $\gamma$ , which signals through IFN- $\gamma$ R, is mostly known to activate a microbicidal state in macrophages and is a key cytokine of T<sub>H</sub>1 responses during infection with intracellular, non-viral pathogens, autoimmune diseases and antitumor defenses. Whereas many intracellular bacterial pathogens will grow in the cytosol or the phagosome of infected macrophages, incubation with IFN- $\gamma$  activates killing mechanisms of macrophages that will ultimately eliminate or control the pathogen growth. Many genes are known to be regulated by IFN- $\gamma$ , and most are involved directly or indirectly in the eradication of pathogens from host cells. IFN- $\gamma$  increases the production of potent antimicrobial molecules, such as superoxide radicals, nitric oxide (NO) and hydrogen peroxide. IFN- $\gamma$  plays a central role in phagocytosis by increasing the expression of Fc and complement receptors in macrophages and other cells, B cell switching to Ig-classes involved in opsonization by macrophages and regulation of the development of T<sub>H</sub>-cell subsets, downregulating the generation of T<sub>H</sub>2 cells. IFN- $\gamma$  upregulates antigen presentation to T cells in both APC and pAPC by increasing the expression of MHC I and II molecules, the antigen presentation mechanisms and the levels of co-stimulatory molecules (178).

NK and T cells are the major sources of IFN- $\gamma$ . Infected macrophages secrete IL-12, which induces NK and T cells to secrete IFN- $\gamma$ , in turn activating macrophages that will secrete more IL-12 in a positive feedback loop. However, several independent studies have shown that IFN- $\gamma$  can be secreted by myeloid cells including dendritic cells and macrophages (179).

IFN- $\gamma$  has a central role in limiting most experimental intracellular infections *in vivo* (180). In human patients, mutations in IFN- $\gamma$ R result in increased susceptibility to mycobacterial infections (181, 182). The importance of IFN- $\gamma$  in chlamydial infections *in vivo* has been shown by enhanced growth in IFN- $\gamma$ <sup>-/-</sup> or IFN-

$\gamma R^{-/-}$  mice or with mice treated with anti-IFN- $\gamma$  antibodies (32, 183-186). High concentrations of IFN- $\gamma$  inhibit the reproductive cycle of *Chlamydia*, while lower IFN- $\gamma$  concentrations promote the persistent stage, which means development of atypical, non-proliferating forms of *Chlamydia* that have previously been described. IFN- $\gamma$  derived from both innate immune cells and T cells can play important and complementary roles in the control of *C. pneumoniae* infection (34). The protective effect of non-lymphoid IFN- $\gamma$  is observed 7-14 days after infection, whereas the effect of protective T cell IFN- $\gamma$  is seen after 3 weeks (34). NK cells are a main source of “innate” IFN- $\gamma$ , but IFN- $\gamma$  from NK cells was not needed for innate immune protection *in vivo* (33, 34). Bone marrow-derived macrophages (BMM) secrete IFN- $\gamma$  in response to *C. pneumoniae* and IFN- $\gamma R^{-/-}$  BMM show higher levels of *C. pneumoniae*, confirming a role of infection-induced IFN- $\gamma$  in macrophages in the control of chlamydial infection (187). Furthermore, a protective role of IFN- $\gamma$  secretion by macrophages against *C. pneumoniae in vivo* has also been suggested (34). IFN- $\gamma$  secreted by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells is sufficient for protection against infection with *C. pneumoniae in vivo*, and it does not require “innate” IFN- $\gamma$  for its secretion. In line with this, in the absence of IFN- $\gamma R$  signaling T cells still secrete IFN- $\gamma$  (34). However, a B and T cell-dependent IFN- $\gamma$ -independent protection also plays a role in resistance to chlamydial infection, since mice lacking IFN- $\gamma$  or IFN- $\gamma R$ , but not those lacking IFN- $\gamma$  or IFN- $\gamma R$  and RAG1 (B and T cell-deficient) survive when infected with *C. pneumoniae* (33).

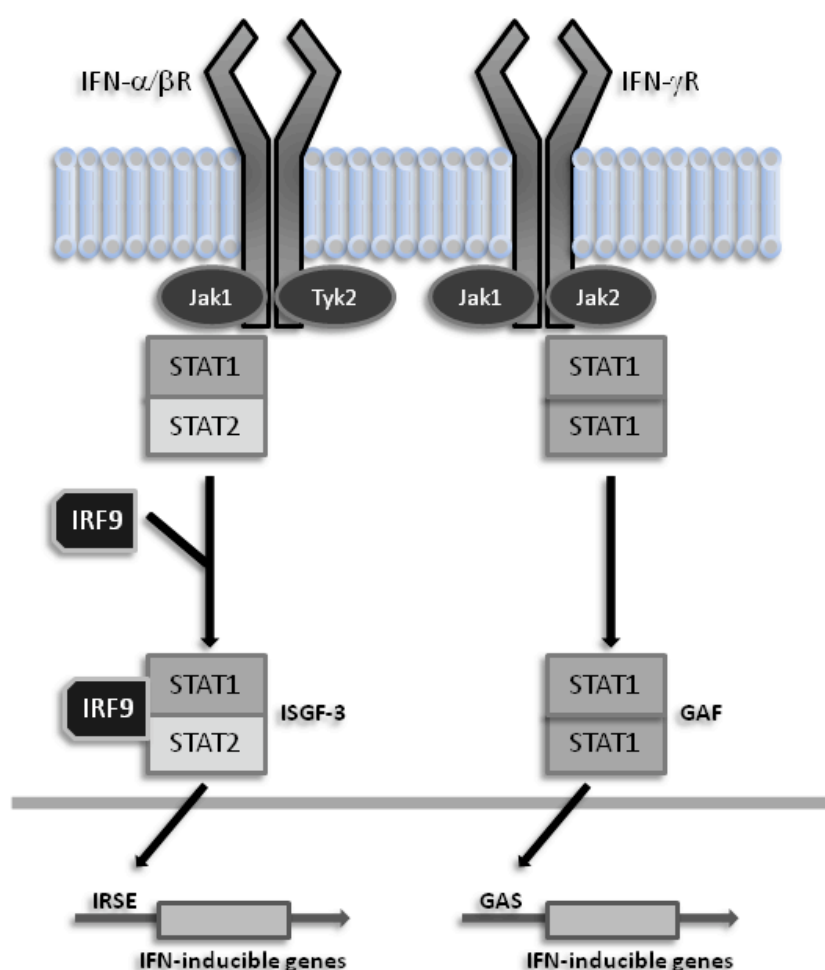
IFN- $\gamma$  is probably the most important cytokine for controlling a primary *L. monocytogenes* infection. The resistance of IFN- $\gamma^{-/-}$  mice to *L. monocytogenes* is severely impaired compared to WT mice (188). As is the case with other pathogens, *L. monocytogenes* is also killed in IFN- $\gamma$ -activated macrophages, but replicates in the cytoplasm of resting macrophages. Treatment with IFN- $\gamma$  prevents the escape of *L. monocytogenes* from the phagosome in macrophages (189). However, IFN- $\gamma$  plays a less crucial role for protection against reinfection (188). Production of IFN- $\gamma$  by CD4<sup>+</sup> T cells is required for protection, while IFN- $\gamma$  produced by *L. monocytogenes*-specific CD8<sup>+</sup> T cells is redundant (188, 190). *L. monocytogenes*-infected macrophages do not produce IFN- $\gamma$  (117).

## JAK-STAT

Interferons transduce signals that elicit responses in target cells by involving the signaling pathway JAK-STAT. Janus kinases (JAKs) are associated to the intracellular part of the IFN- $\alpha/\beta R$  and IFN- $\gamma R$ . Upon ligand binding to these receptors the receptors monomers are brought together and the associated JAKs are activated by transphosphorylation. Activated JAKs also phosphorylate tyrosine residues on the cytokine receptors, creating active docking sites for a set of transcription factors called signal transducers and activators of transcription (STATs). When bound to the receptors STATs are then activated by JAKs by phosphorylation, enabling them to release from the receptor and form complexes with each other and other proteins. These complexes are translocated into the nucleus where they bind specific DNA

sequences in the promoter regions of cytokine-responsive genes and activate gene transcription. Both IFN receptors use JAK-STAT signaling to induce hundreds of genes that can be specific or common to the type of IFN (figure 4)(191).

There are four different JAKs: Tyk2, Jak1, Jak2 and Jak3, and seven different STATs in mammals: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. STATs possess a DNA-binding domain that directs binding to enhancers, a SH2 domain is responsible for binding of STAT to the receptor, but also to a phosphorylated tyrosine residue on other STATs once it is released, a tyrosine activation domain that contains the tyrosine residue activated by JAKs and a transactivation domain (TAD) with a serine phosphorylation site that plays a role in regulation of transcriptional activity through the recruitment of co-activators and histone acetylases. Whilst tyrosine phosphorylation is a prerequisite for the role of STAT1, serine phosphorylation is important for increased efficiency of the IFN response (192, 193).



**Figure 4.** IFN-α/β- and IFN-γ-mediated STAT signaling.

Different receptors bind different types of JAKs and recruit specific STATs, which will form homo- or heterocomplexes. This confers the specificity of the

responses to different cytokines. IFN- $\gamma$  stimulation leads primarily to the formation of STAT1 homodimers, called GAF (IFN- $\gamma$ -activated site-binding transcription factor). IFN- $\alpha/\beta$  leads to formation of a STAT1-STAT2-IRF9 heterotrimer, called ISGF-3 (IFN-stimulated gene factor-3). GAF binds to IFN- $\gamma$ -activated sites (GAS), while ISGF-3 binds primarily to IFN-stimulated response elements (ISRE) (191). However, it has been reported that both factors can be activated by both types of IFNs, and other types of STAT complexes can be formed in response to IFN, partially explaining the overlapping effect of both cytokines (194). Moreover, depending on the cell type STATs other than STAT1 and STAT2 can also be activated by IFN. Type I IFNs are able to activate all known STATs in different cell types (195). IFN- $\gamma$  can activate STAT-1, STAT-3 and STAT-5 in different cells (196). This increases the complexity of their responses.

STAT1 is shared by both IFN- $\alpha/\beta$ R and IFN- $\gamma$ R signaling pathways, in which it has a non-redundant role. STAT1 is required for protection to both viral and bacterial pathogens (197). Human patients with mutations in STAT1 have demonstrated impaired responses to mycobacterial and viral infections (198, 199). Lad *et al.* demonstrated that human cell lines upregulate STAT1 to control growth of *C. trachomatis* (200). STAT1<sup>-/-</sup> mice were more susceptible to *L. monocytogenes* infection and showed reduced IFN-inducible gene expression when stimulated with IFN- $\alpha/\beta$  or IFN- $\gamma$ , compared to WT (193, 197). However, studies in which IFN- $\alpha/\beta$ R<sup>-/-</sup>/IFN- $\gamma$ R<sup>-/-</sup> mice were shown to be more susceptible to viral infections than STAT1<sup>-/-</sup> mice suggest a STAT1-independent IFN signaling (201). Both IFN- $\alpha/\beta$  and IFN- $\gamma$  can regulate gene expression independently of STAT1 (201-203). The transcription factors involved in STAT1-independent IFN signaling remain to be identified.

The phosphatidylinositol 3'-kinase and MAP kinases can be triggered by IFN signaling and they are also thought to be required for Ser727 phosphorylation of STAT1. These alternative kinases stimulate STAT1 or other transcription factors, such as NF- $\kappa$ B and AP-1. Priming with cytokines (IFN- $\gamma$ , IFN- $\alpha/\beta$ , IL-6) can positively (via receptor cross-talk or increasing levels of STAT1) or negatively (via suppressor of cytokine signaling 1 (SOCS1)) influence the activation of STAT and thereby the IFN response (196). SOCS1 has been shown to block tyrosine phosphorylation sites on JAKs (204).

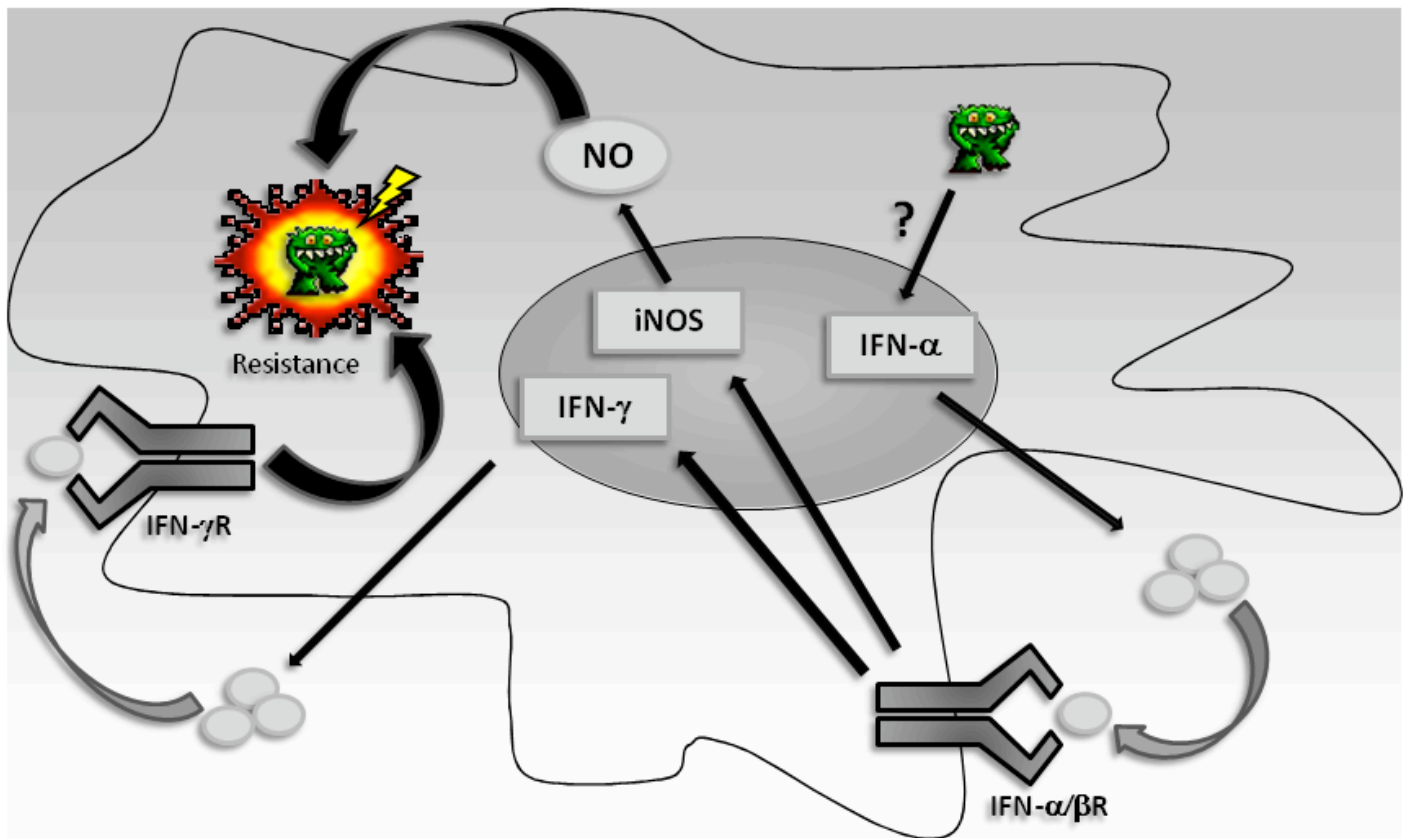
## **Regulation of IFN- $\gamma$ expression**

IFN- $\gamma$  expression is thought to be regulated primarily by the transcription factors NFAT, AP-1, NF- $\kappa$ B, STAT4 and T-bet (205). STAT4 is the main transcription factor for IL-12 signaling. However, studies suggest that IL-12 is not absolutely required for IFN- $\gamma$  expression. In NK and T cells, the production of IFN- $\gamma$  has been demonstrated to be enhanced by IFN- $\alpha/\beta$ , IL-18 and IFN- $\gamma$  itself. Production of IFN- $\gamma$  can also be promoted by IL-15 and IL-2 in NK cells and IL-27 in T cells (206). The signals and pathways that cause IFN- $\gamma$  production by macrophages are poorly understood. Macrophages stimulated with live bacteria, LPS, IL-12, a combination of IL-12 and

IL-18 or IFN- $\gamma$  itself can themselves produce IFN- $\gamma$  (179). Thus, IFN- $\gamma$  seems to act in an autocrine positive feedback loop to facilitate its own expression.

Nguyen *et al.* suggested that IFN- $\alpha/\beta$  was involved in IFN- $\gamma$  production by activating STAT4, which was needed for virus-induced IFN- $\gamma$  in splenocytes (207). STAT4-dependent induction of IFN- $\gamma$  in splenocytes by IFN- $\alpha/\beta$  in synergy with IL-18 was found to be crucial for the IFN- $\gamma$  response to bacteria (208). IFN- $\alpha/\beta$  signaling also seem to be required for the IFN- $\gamma$ -mediated response, since IFN- $\gamma$ -mediated antiviral activity is weaker in IFN- $\alpha/\beta$ R<sup>-/-</sup> murine embryonic fibroblasts (MEF) than WT cells (209) and the IFN- $\alpha/\beta$ R has been suggested to provide a docking site required for effective STAT1 dimerization in the cross-talk between IFN- $\alpha/\beta$  and IFN- $\gamma$  signaling (210). IFN- $\alpha/\beta$  have also been proposed to induce IFN- $\gamma$  in an IL-15-mediated way, since IL-15 expression depends on IFN- $\alpha/\beta$ R (211) and IL-15-treated macrophages can secrete IFN- $\gamma$  (212). However, the role of IFN- $\alpha/\beta$  in IFN- $\gamma$  expression is still controversial. IFN- $\alpha/\beta$  have been reported to inhibit IFN- $\gamma$  expression in splenocytes in a mechanism dependent on IFN- $\alpha/\beta$ R and STAT1 (213). This unexpected heterogeneity of the effects of IFN- $\alpha/\beta$  could explain why IFN- $\alpha/\beta$  can have both protective and counter-protective functions during different infectious diseases. In the infection with *C. pneumoniae*, human peripheral blood mononuclear cells secreted IFN- $\gamma$  48 hours after stimulation in an IL-12-, IL-18- and IL-1 $\beta$ -dependent manner (98). However, IFN- $\gamma$  secretion in BMM infected with *C. pneumoniae* was found to be dependent on IFN- $\alpha/\beta$ , but independent of IL-12 (187). Whether STAT1 is involved in the IFN- $\alpha/\beta$ -mediated IFN- $\gamma$  induction in *C. pneumoniae*-infected BMM is still unknown. It is neither known which pathways are involved in IFN- $\alpha/\beta$  and IFN- $\gamma$  induction in response to infection of BMM with *C. pneumoniae* (figure 5). In contrast to *in vitro* results in macrophages IFN- $\gamma$  expression is mediated by IL-12 during the infection with *C. pneumoniae in vivo*, but IL-12-independent IFN- $\gamma$ -induced protection can also be observed (33).





**Figure 5.** IFN- $\alpha/\beta$ -mediated iNOS and IFN- $\gamma$  is induced in *C. pneumoniae*-infected BMM and participates in the control of bacterial growth (187).

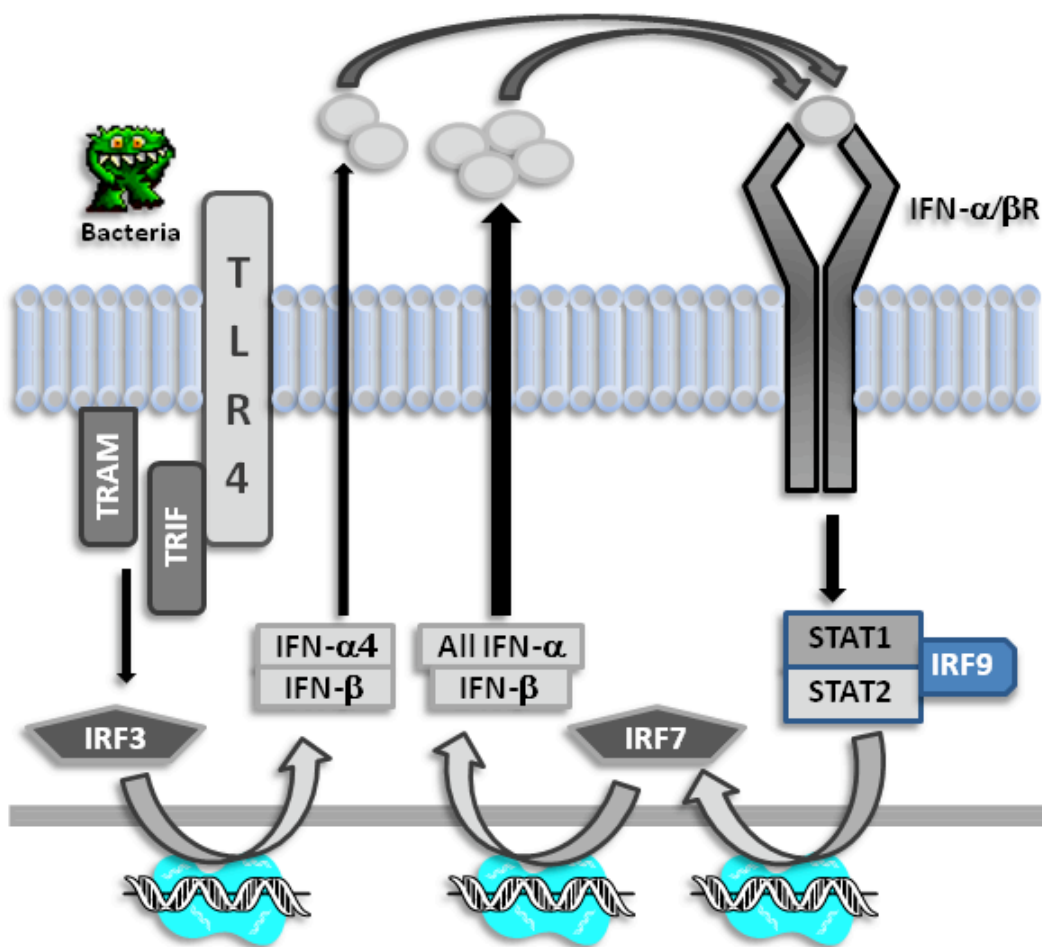
NF- $\kappa$ B-binding elements have been found on the IFN- $\gamma$  promoter (214, 215). Furthermore, NF- $\kappa$ B activation has been suggested to upregulate IFN- $\gamma$ R expression, which renders the cell more sensitive to IFN, requiring less IFN- $\gamma$  for gene activation (216). Furthermore, regulatory elements of the IFN- $\beta$  gene contain binding sites for NF- $\kappa$ B (217) and cells with attenuated NF- $\kappa$ B activity fail to induce early IFN- $\beta$  after LPS stimulation (218). Thus, NF- $\kappa$ B seems to be involved in the regulation of IFN production.

## Interferon regulatory factors

IRFs are a family of nine transcription factors that were first described to be involved in the induction of IFN- $\alpha/\beta$  genes and the response to IFNs. IRF members have later been shown to play central roles in the regulation of gene expression in response to pathogen-derived danger signals, in the cellular differentiation of hematopoietic cells and in the regulation of the cell cycle and apoptosis (219).

IRF3 and IRF7 have critical roles in the transcription of IFN- $\alpha/\beta$  genes (220, 221). IRF3 is expressed constitutively and resides in the cytosol. It is known to be activated in the signaling pathways of TLR3, TLR4, RIG-I/MDA5 and the recently discovered DAI. Activation occurs through phosphorylation on serine residues by

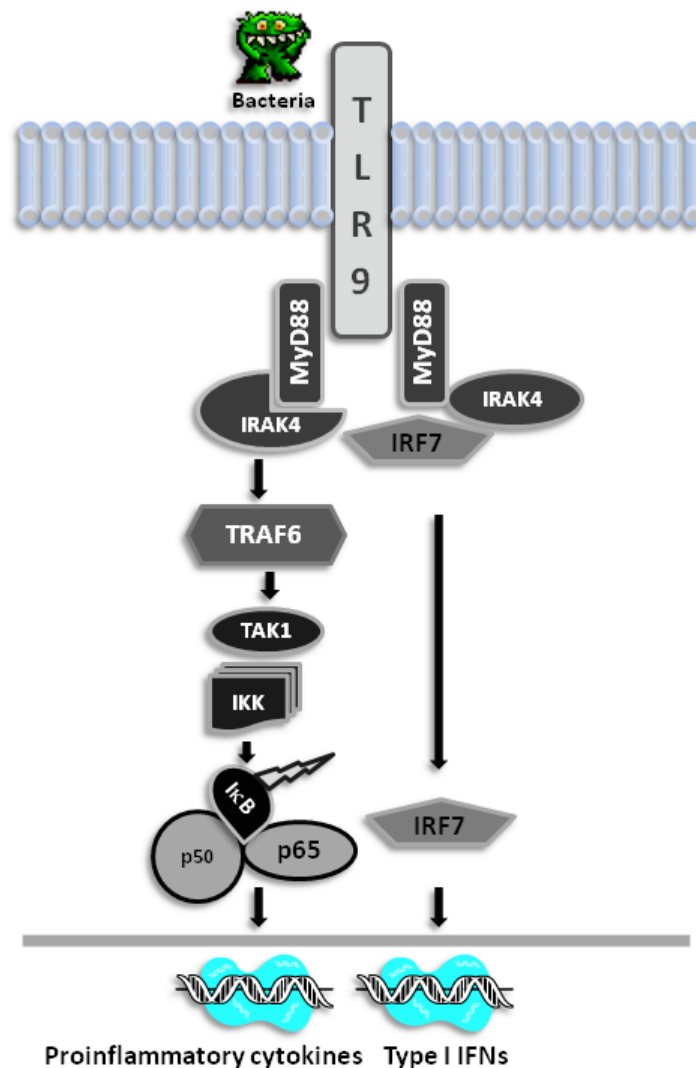
TBK1 and IKK-i, forming an IRF3 homodimer or a heterodimer with IRF7 (219). The dimer translocates to the nucleus where it associates with NF- $\kappa$ B and AP-1 to form an enhanceosome, which is recruited to interferon regulatory elements (219). IRF3 induces an early wave of IFN-independent induction of IFN- $\beta$  and in some cell types IFN- $\alpha$ 4 (222). For example, LPS stimulation of peritoneal macrophages results in IRF3 activation and synthesis of IFN- $\beta$  and IFN- $\alpha$ 4 (223). IFN- $\beta$  will activate the expression of a number of IFN-inducible genes, including IRF7, which is essential for expression of other subtypes than IFN- $\alpha$  (222, 224). In contrast to IRF3 the transcription of IRF7 needs to be induced. However, similar to IRF3 the activation of IRF7 occurs via phosphorylation by the kinases TBK1 and IKK-i (86). Once induced and activated IRF7 translocates into the nucleus and in turn activates the promoters for both IFN- $\alpha/\beta$  genes in an autocrine positive loop, that can occur independently of IRF3 (figure 6)(225, 226). IFN- $\alpha/\beta$  mRNA expression reaches the levels of WT controls in IRF3<sup>-/-</sup> mice infected with selected viruses (225), and IRF7<sup>-/-</sup> mice are more vulnerable to viral infection than WT and IRF3<sup>-/-</sup> mice (226).



**Figure 6.** IRF3- and IRF7-mediated IFN- $\alpha/\beta$  pathway.

Macrophages infected with *L. monocytogenes* produce an early wave of IFN- $\beta$  and a second wave of subtypes of IFN- $\alpha$ . IRF3 is activated in *L. monocytogenes*-infected macrophages, leading to IFN- $\beta$  mRNA expression and type I IFN signal transduction (117). *L. monocytogenes*-induced cell death was shown to be abolished in IRF3<sup>-/-</sup> macrophages, but IFN- $\beta$  treatment could restore the sensitivity to cell death (118). IFN- $\beta$  production after *L. monocytogenes* infection in BMM is independent of TLR, NOD1, NOD2 and RIP2 signaling (118, 130, 152). In line with this, NOD1 and NOD2 ligands do not trigger IFN- $\beta$  expression *in vitro* (130). Following the infection with *C. muridarum* IFN- $\beta$  production was reduced in TBK<sup>-/-</sup> MEF and in oviduct epithelial cells transfected with IRF3-specific siRNA, compared to WT or untreated controls (111, 112). Thus, IRF3 has a role in the production of type I IFNs during listerial and chlamydial infections.

IRF7 activation has been shown in pDC that express TLR9 and TLR7 but not TLR4 (164). After stimulation with CpG IRF7 is activated at an early stage, leading to high levels of IFN- $\alpha$  expression in pDC. IRF7 forms a complex with MyD88, TRAF6 and IRAK4 (figure 7)(226-228). IRF7 is thus suggested to have a role in bacterial induction of IFN- $\alpha$  in both MyD88-dependent and MyD88-independent TLR signaling pathways (224, 226).



**Figure 7.** MyD88-dependent TLR9 signaling, leading to NF-κB and IRF7 activation.

### Interferon-mediated effector mechanisms

The different signaling pathways of IFN-γR lead to induction of possibly more than 1000 inducible genes. They encode host proteins with a long-recognized antimicrobial activity, notably inducible nitric oxide synthase (iNOS), phagocyte oxidase (NADPH oxidase), the natural resistance-associated macrophage protein 1 (NRAMP1) and the tryptophan catalysing enzyme indoleamine 2,3-deoxygenase (IDO) and two families of small GTPases, the immunity-related GTPases (also termed p47 GTPases) and the p65 guanylate-binding proteins (p65 GBPs) (229). NADPH oxidase catalyzes a respiratory burst in macrophages and granulocytes through the production of  $O_2^-$ . iNOS produces nitric oxide (NO), another radical with anti-microbial activities. IFN-γ is over 100,000-fold more potent in aiding the oxidative burst of human mononuclear phagocytes than, for example, other macrophage-activating cytokines like TNF-α or type I IFNs (229). IDO limits tryptophan availability, which is essential for chlamydial survival and growth since it is not produced by the bacteria, by converting tryptophan to kynurenin. The p47 GTPase LRG-47 is thought to regulate maturation

of vacuoles, leading to acidification and fusion with lysosomes. It is also thought to regulate autophagy, resulting in elimination of bacteria (230, 231).

IFN- $\gamma$ -mediated resistance to *C. pneumoniae* is at least partially linked to iNOS. iNOS is induced in *C. pneumoniae*-infected WT mice, and iNOS<sup>-/-</sup> mice have been observed to be more susceptible to *C. pneumoniae* infection than WT, but less than IFN- $\gamma$ R<sup>-/-</sup> mice (32, 33). NO production is involved in the control of infection of BMM with *C. pneumoniae* and it has been shown to be dependent on IFN- $\alpha/\beta$  (figure 5)(187). IFN- $\gamma$  produced by innate cells was found to be necessary for the expression of iNOS, NADPH oxidase and IDO during *C. pneumoniae* infection *in vivo* (33). IDO<sup>-/-</sup> and WT mice have been demonstrated to have same susceptibility to *C. muridarum* and *C. trachomatis* genital infection (232), but the roles of IDO and NADPH in *C. pneumoniae* infections should be investigated in knockout mice. NADPH oxidase and iNOS have shown to contribute to clearance of *L. monocytogenes* *in vivo* (233, 234). LRG47 is required for host resistance to *L. monocytogenes* and *M. tuberculosis* *in vivo* (230, 235). Two studies have shown a role for p47 GTPases in chlamydial growth control *in vitro* (232, 236). p47 GTPases were suggested to limit lipid trafficking from Golgi to the inclusions of infecting *C. trachomatis* (232). However, in the infection of epithelial cells with *C. trachomatis*, LRG-47 mRNA was induced but a screen using siRNA ruled out a non-redundant role for LRG-47 in the IFN- $\gamma$ -mediated response (232).

The role for these effector mechanisms in humans is still debated. Mutations in NADPH oxidase cause a serious genetic disease called chronic granulomatous disease, characterized by susceptibility mostly to pyogenic bacteria, such as *S. aureus*, but also some intracellular bacteria (237). Alveolar macrophages from patients with tuberculosis express iNOS (238), and *in vitro* killing by NO has been demonstrated in human macrophages infected with *Mycobacterium avium* (239). However, Roshick *et al.* demonstrated that many human cell lines infected with *C. trachomatis* did not express iNOS, and IDO was only expressed after IFN- $\gamma$  stimulation. IDO seems to be the main mechanism against chlamydial growth in human epithelial cells (240). Interestingly, murine cells infected with *C. muridarum* expressed iNOS, but not IDO mRNA. The human pathogen *C. trachomatis* has probably adapted to survive tryptophan starvation by human cells, by developing a tryptophan synthase, whereas *C. muridarum* has not evolved this ability probably due to the lack of IDO expression in murine cells (232). The putative human LRG-47 homolog IRGM could limit mycobacterial infection in human macrophages, suggesting a role for p47 GTPases in humans (231). Thus, in the infection with *L. monocytogenes* it is clear that these mechanisms are required for protection, but how IFN- $\gamma$  protects against infection with *C. pneumoniae* requires further investigation.

Serine/threonine kinase protein R (PKR) and 2', 5'-oligoadenylate synthetase (OAS) are considered intracellular PRRs, as they recognize dsRNA in the cytoplasm. They are also known to be upregulated by IFN- $\alpha/\beta$  and participate in antiviral defense. PKR phosphorylates the initiation factor 2 (eIF-2 $\alpha$ ) and inhibits eukaryotic gene translation and thereby viral infections. PKR also sensitizes cells to apoptosis.

Furthermore, PKR is a component of the IKK complex and is thought to be required for the efficient activation of NF- $\kappa$ B. p38 and JNK are also regulated by PKR. The OAS pathway can inhibit viral replication through polymerization of ATP into 2', 5'-oligomers of adenosine, which in turn activate the endonuclease RNAase L (241, 242). The importance of PKR and OAS has mostly been described in viral infections and their roles in intracellular bacterial infections are not clear.

# AIMS

## The general aims

- to investigate the innate immune signaling pathways leading to IFN- $\alpha/\beta$  and IFN- $\gamma$  expression and control of bacterial growth during infection with *C. pneumoniae* in BMM.
- to understand the role of STAT1 in resistance to *C. pneumoniae* infection *in vivo*.
- to study the role of NOD1 in different cell populations and in the protection following infection with *L. monocytogenes* *in vivo*.

## The specific aims

### In the infection of BMM with *C. pneumoniae*

1. Which TLRs are required in sensing *C. pneumoniae* and for the production of protective IFN- $\alpha/\beta$ -dependent IFN- $\gamma$ ?  
Does this occur in a MyD88-dependent or -independent manner?  
What is the role of IRAK4 in defense and in IFN- $\alpha/\beta$  and IFN- $\gamma$  production?
2. How does IFN- $\alpha/\beta$  control the secretion of IFN- $\gamma$  during infection with *C. pneumoniae*?
3. What role does the transcription factor NF- $\kappa$ B play in protection *against C. pneumoniae* and in IFN- $\gamma$  expression?  
Which innate receptors and intracellular molecules are needed for NF- $\kappa$ B activation during the infection with *C. pneumoniae*?
4. What role does IRF3 play in secretion of IFN- $\alpha$  and IFN- $\gamma$  and in protection during chlamydial infection?

#### During infection with *C. pneumoniae*

1. What is the role of STAT1 signaling in protection against infection with *C. pneumoniae in vivo*?  
Is STAT1 phosphorylation dependent on both IFN- $\gamma$ R and IFN- $\alpha/\beta$ R?
2. What is the role of IFN- $\alpha/\beta$  signaling in the expression of IFN- $\gamma$  and the protection against infection with *C. pneumoniae in vivo*?
3. What role does STAT1 have in T cell-mediated IFN- $\gamma$  expression and protection against chlamydial infection?
4. Which cells mediate STAT1-dependent protection against infection with *C. pneumoniae in vivo*?

#### In the infection with *L. monocytogenes*

1. What is the role of NOD1 in intraperitoneal and snout infection with *L. monocytogenes*?  
Is NOD1 involved in resistance against re-infection?  
Is NOD1 involved in triggering adaptive responses and the recruitment of inflammatory cells after *L. monocytogenes* infection?  
Which cellular populations mediate NOD1-dependent protection in the systemic infection with *L. monocytogenes*?
2. What is the role of NOD1 in different cell populations?  
Is NOD1 important in the elimination of *L. monocytogenes* in IFN- $\gamma$ -activated macrophages?  
What genes are regulated by NOD1 in cells infected by *L. monocytogenes*?



## RESULTS AND DISCUSSION

### **Paper I & II – Studies with *C. pneumoniae*-infected BMM**

#### **Recognition of *C. pneumoniae* by TLR4 is essential for IFN- $\gamma$ expression in a MyD88- and IRAK4-dependent way (Paper I & II)**

When the cell first encounters the bacteria it triggers a response leading to innate immune responses. The cell recognizes surface molecules of pathogens by means of PRRs, such as the TLRs. Other studies have shown that *C. pneumoniae* activates an immune response in a TLR2- and TLR4-dependent way (98-104), but more details on innate activation and especially the induction of IFN- $\gamma$  need to be further studied. IFN- $\gamma$  was reported to be essential for protection against *C. pneumoniae* infection both *in vitro* in BMM and *in vivo* (32, 187). In *C. pneumoniae*-infected BMM, the levels of IFN- $\gamma$  mRNA or protein were independent of IL-12, but required IFN- $\alpha/\beta$ R signaling. We investigated if different TLRs were essential for the *C. pneumoniae*-induced response and the consequent downstream signaling leading to IFN- $\gamma$  expression and control of infection in BMM. TLR2, TLR4, TLR6 and TLR9 are candidates for recognition of *C. pneumoniae* and we therefore investigated their role in *C. pneumoniae* infection in BMM. TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup>, TLR6<sup>-/-</sup> and TLR9<sup>-/-</sup> BMM were infected with *C. pneumoniae*. Only TLR4<sup>-/-</sup> BMM showed higher levels of intracellular bacteria compared to the WT BMM. In addition to the increased bacterial load, mRNA levels of IFN- $\alpha$  and IFN- $\gamma$  in TLR4<sup>-/-</sup> BMM after infection with *C. pneumoniae* were decreased compared to the WT BMM, suggesting that TLR4 controls IFN- $\alpha$  and IFN- $\gamma$  expression. Thus, we found that TLR4 is crucial in protection to infection with *C. pneumoniae* in BMM (Paper I).

Next, we studied the signaling downstream of TLR4 in the control of infection and in IFN- $\alpha/\beta$ -mediated IFN- $\gamma$  induction in BMM, infected with *C. pneumoniae*. TLR4 signals at least partially via MyD88 (70). MyD88<sup>-/-</sup> BMM showed higher levels of *C. pneumoniae* compared to the WT controls. In accordance, MyD88 has been reported to be essential in the infection with *C. pneumoniae* *in vivo* (97). TLR ligands are known to induce IFN- $\alpha$  expression via TRIF or MyD88 (223, 243, 244). MyD88<sup>-/-</sup> BMM showed reduced mRNA levels of IFN- $\alpha$  and IFN- $\gamma$  compared to the WT control. In line with this, Nagarajan *et al.* have shown that peritoneal macrophages infected with *C. muridarum* induced IFN- $\beta$  in a MyD88-dependent way (111). However, Derbigny *et al.* have reported that TRIF, but not MyD88 signalling is implicated in the IRF3-dependent IFN- $\beta$  production by *C. muridarum*-infected oviduct epithelial cells (112), indicating differences in IFN signaling between different cellular populations or among infections with different chlamydial species. MyD88 also mediates signaling downstream of IL-1R and IL-18R. To ensure that differences between MyD88<sup>-/-</sup> and WT BMM were linked to TLR signaling, ICE<sup>-/-</sup>

BMM were infected with *C. pneumoniae*. The IL1- $\beta$ -converting enzyme (ICE) also called caspase 1, cleaves the inactive precursors from IL1- $\beta$  and IL-18 to their biologically active proinflammatory forms. The ICE<sup>-/-</sup> and WT BMM showed similar levels of *C. pneumoniae* and levels of IFN- $\gamma$  mRNA after infection. The higher bacterial load in MyD88<sup>-/-</sup> BMM was therefore caused by defects in TLR signaling. We showed here that during infection of BMM with *C. pneumoniae*, TLR signaling-mediated MyD88 is required for IFN- $\alpha$  secretion (Paper I).

The kinase IRAK4 is required for TLR4 signaling (74, 75). IRAK4 was shown to be required for secretion of IFN- $\gamma$  and proinflammatory cytokines in LPS-stimulated DC. IRAK4<sup>-/-</sup> macrophages showed diminished levels of IFN- $\beta$  mRNA after LPS treatment (75). In line with the results obtained using MyD88<sup>-/-</sup> BMM, IRAK4<sup>-/-</sup> BMM contained higher levels of *C. pneumoniae* than the WT controls. Levels of IFN- $\alpha$ , IFN- $\beta$  and IFN- $\gamma$  mRNA and IFN- $\gamma$  protein were diminished in IRAK4<sup>-/-</sup> BMM in comparison to WT BMM, indicating that IRAK4 is required for full expression of IFN genes and control of bacterial growth (Paper II). Thus, we demonstrated that IFN- $\alpha$ -mediated IFN- $\gamma$  expression and control of bacterial growth in *C. pneumoniae*-infected BMM is dependent on TLR4, MyD88 and IRAK4.

Further investigations are required to understand the mechanisms responsible for the protective action of IFN- $\gamma$  in BMM infected with *C. pneumoniae*. As mentioned before, iNOS participates in the control of bacterial load, but the accumulation of iNOS mRNA and NO protection in BMM was dependent on IFN- $\alpha/\beta$  and not IFN- $\gamma$ .

### **IFN- $\alpha/\beta$ R-dependent STAT1 signaling is necessary for protective IFN- $\gamma$ expression (Paper I)**

STAT1 is required for both IFN- $\alpha/\beta$ R and IFN- $\gamma$ R signaling (197) but STAT1-independent IFN signaling has also been reported (201). We studied the role of STAT1 in the control of macrophage infection with *C. pneumoniae*. STAT1<sup>-/-</sup> BMM and lung fibroblasts had higher levels of *C. pneumoniae* and expressed lower IFN- $\gamma$  mRNA titers during the infection with *C. pneumoniae* than the WT controls. We then investigated the role of IFN- $\alpha/\beta$ R and IFN- $\gamma$ R in the activation of STAT1. STAT1 activation was measured by analyzing the Tyr701 phosphorylation of STAT1 (pSTAT). STAT1 was shown to be phosphorylated only after infection with *C. pneumoniae* in WT BMM. We showed that in the absence of IFN- $\alpha/\beta$ R, STAT1 activation is completely abolished. pSTAT1 levels were decreased in infected IFN- $\gamma$ R<sup>-/-</sup> BMM compared to the WT control, suggesting that IFN- $\gamma$  played a role in STAT1 activation, but in the absence of IFN- $\gamma$  signaling STAT1 was still activated. Thus, IFN- $\alpha/\beta$ R-dependent STAT1 signaling in BMM is necessary for induction of IFN- $\gamma$  and control of bacterial growth in *C. pneumoniae* infected BMM. In line with our findings, it has been shown that STAT1 can bind to the IFN- $\gamma$  promoter (245). However, our finding is in contrast to other studies, which suggest that STAT4 mediates IFN- $\alpha/\beta$ -stimulated IFN- $\gamma$  induction (207, 208), and Nguyen *et al.* found that

STAT1 can even act as a negative regulator and absence of STAT1 results in higher IFN- $\gamma$  levels after viral infection or stimulation with IFN- $\alpha/\beta$  (213). These studies focused on the regulation of IFN- $\gamma$  synthesis in lymphoid populations, which might explain the different signaling mechanisms for IFN- $\gamma$  expression.

IFN- $\alpha/\beta$  have also been proposed to induce IFN- $\gamma$  in an IL-15-mediated way (211, 212). IL-15 is a cytokine with structural similarity to IL-2, but in contrast to IL-2, which is mainly secreted by T cells, IL-15 is secreted by mononuclear phagocytes. We studied the role of IL-15 in the induction of IFN- $\gamma$  and protection of bacterial growth. IL-15 mRNA expression was observed in WT BMM, was absent in IFN- $\alpha/\beta$ <sup>-/-</sup> and STAT1<sup>-/-</sup> BMM and was somewhat reduced in IFN- $\gamma$ R<sup>-/-</sup> BMM after *C. pneumoniae* infection. IL-15 signals through  $\gamma_c$ R. Signaling by  $\gamma_c$ R was required for bacterial control and expression of IFN- $\gamma$ , but not IFN- $\alpha$  mRNA. Thus, IL-15 is involved downstream of IFN- $\alpha/\beta$ R in the induction of IFN- $\gamma$  mRNA and chlamydial growth control.

As described in the introduction, PKR mediates IFN- $\alpha/\beta$ -dependent antiviral effects and signal transduction in the proinflammatory cytokine response (241). PKR mRNA induction was IFN- $\alpha/\beta$ R- and STAT1-dependent in *C. pneumoniae*-infected BMM. We used the specific inhibitor 2-aminopurine (2-AP) to block PKR. Treatment of BMM with 2-AP demonstrated that PKR has no effect on bacterial growth in the absence of IFN- $\alpha/\beta$ R but it is required for control of infection and intact IFN- $\gamma$ , but not IFN- $\alpha$  mRNA levels in WT BMM infected with *C. pneumoniae*. This suggests that *C. pneumoniae* activates PKR in an IFN- $\alpha/\beta$ R-dependent manner and that it plays a role in protection through mediating IFN- $\gamma$  expression.

In conclusion, BMM controls *C. pneumoniae* load by secreting IFN- $\gamma$ . IFN- $\gamma$  is expressed in a TLR4-MyD88-IRAK4-IFN- $\alpha/\beta$ -STAT1-dependent way. PKR and  $\gamma_c$ R signaling participate in downstream of IFN- $\alpha/\beta$ R in the *C. pneumoniae* infection-induced IFN- $\gamma$  expression, leading to bacterial growth control. Whether the PKR and IL-15 effects act in parallel or require synergy for their action is not known.

## **TRAF6-mediated NF- $\kappa$ B activation is necessary for protective IFN- $\gamma$ (Paper I & II)**

MyD88 forms a complex with IRAK4, which in turn induces ubiquitination of TRAF6. Oddly enough, ubiquitination of TRAF6 does not result in its targeting into and degradation in the proteasome, but instead is often associated with the activation of signaling molecules. Ubiquitinated TRAF6 may recruit ubiquitin-binding adapter proteins including TAK1-binding proteins that bind to TAK1, which in turn indirectly activate NF- $\kappa$ B, leading to induction of proinflammatory cytokines (246). Both MyD88- and IRAK4-deficient mice are resistant to septic shock, and cells from these mice show delayed NF- $\kappa$ B activation and no proinflammatory cytokine production after stimulation with TLR ligands (73-75, 247).

We studied the role of NF- $\kappa$ B regulation in control of chlamydial infection. NF- $\kappa$ B activation can be measured by analyzing the phosphorylation of I $\kappa$ B- $\alpha$  (pI $\kappa$ B).

Unless phosphorylated, I $\kappa$ B- $\alpha$  forms a complex with NF- $\kappa$ B and inhibits NF- $\kappa$ B translocation to the nucleus. NF- $\kappa$ B activation has been detected after infection with *C. pneumoniae* in macrophages, DC and epithelial cells (100, 103, 248). We confirmed that NF- $\kappa$ B is activated (pI $\kappa$ B is increased) after infecting BMM with *C. pneumoniae*. Thereafter we investigated the role of NF- $\kappa$ B in the expression of IFN- $\alpha$  and IFN- $\gamma$ , and control of infection in BMM, by using BAY 11-7082, an I $\kappa$ B kinase inhibitor. We found that resistance against *C. pneumoniae* infection was impaired and mRNA levels of IFN- $\gamma$  and NF- $\kappa$ B-dependent proinflammatory cytokines IL-1 $\alpha$ , IL-6 and TNF- $\alpha$  were decreased in BAY treated cells, compared to the non-treated controls. However, IFN- $\alpha$  mRNA levels were not reduced in comparison with the untreated controls. This suggests that NF- $\kappa$ B is required for IFN- $\gamma$  expression and infection growth control (Paper I). In support of this observation, NF- $\kappa$ B-binding elements have been found on the IFN- $\gamma$  promoter (214, 215).

NF- $\kappa$ B can be activated via MyD88-dependent and MyD88-independent pathways. For example, upon LPS stimulation a TRIF-dependent delayed NF- $\kappa$ B activation occurs in MyD88<sup>-/-</sup> macrophages (83). However, in the response to other TLR ligands, such as peptidoglycan, lipoprotein or CpG DNA, there is no NF- $\kappa$ B activation in MyD88<sup>-/-</sup> macrophages (118). TLR4<sup>-/-</sup> macrophages show no activation of NF- $\kappa$ B after incubation with LPS (249).

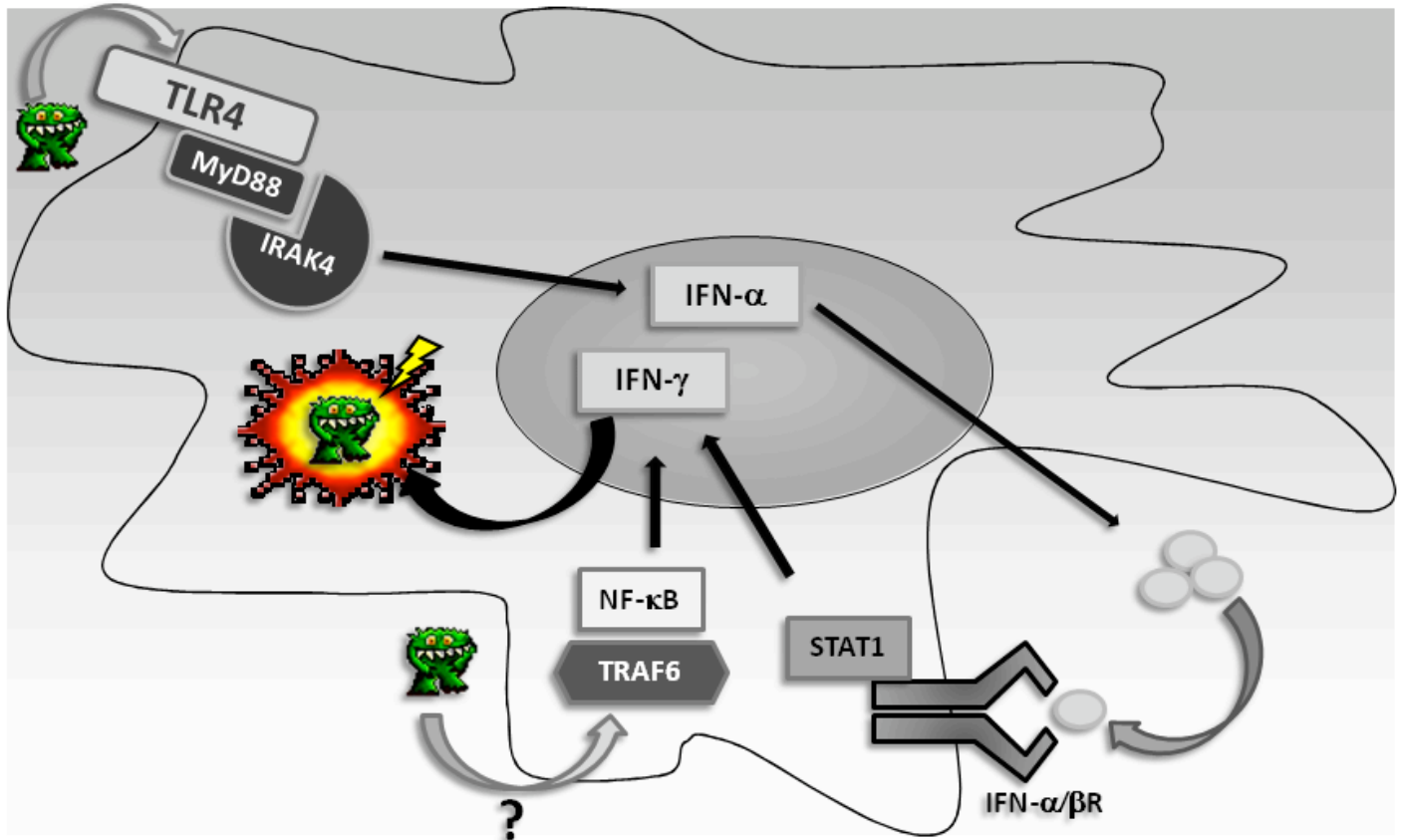
We investigated the regulation of NF- $\kappa$ B activation after infection with *C. pneumoniae*. Surprisingly, there was no difference in the phosphorylation of I $\kappa$ B- $\alpha$  between TLR4<sup>-/-</sup>, MyD88<sup>-/-</sup>, IRAK4<sup>-/-</sup>, IRF3<sup>-/-</sup>, IFN- $\beta$ <sup>-/-</sup> and WT BMM during the infection with *C. pneumoniae*. Furthermore, levels of IL-1 $\alpha$ , IL-6 and TNF- $\alpha$  were similar in *C. pneumoniae*-infected TLR4<sup>-/-</sup>, MyD88<sup>-/-</sup> and WT BMM. IRAK4<sup>-/-</sup> BMM showed similar levels of IL-1 $\beta$  and IL-6 mRNA, IL-6 protein and NF- $\kappa$ B DNA-binding ability, as compared to WT. Furthermore, IRF3<sup>-/-</sup>, IFN- $\beta$ <sup>-/-</sup> and WT BMM also showed similar levels of IL-1 $\beta$  and IL-6 mRNA expression. We also studied the role of IFN- $\alpha/\beta$ -dependent PKR-mediated signal transduction, leading to NF- $\kappa$ B activation. Neither inhibition of PKR, nor deficiency of IFN- $\alpha/\beta$ R<sup>-/-</sup> in *C. pneumoniae*-infected BMM had an impact on the NF- $\kappa$ B activation, as compared to the WT (Paper I & II). Together, this suggests that bacterial recognition by TLR4 and signaling by MyD88, IRAK4, IRF3, IFN- $\beta$ , IFN- $\alpha/\beta$ R and PKR are redundant in the activation of NF- $\kappa$ B during the infection of BMM with *C. pneumoniae*. In line with this, proinflammatory cytokine production in BMDC and peritoneal macrophages infected with *C. pneumoniae* is independent of TLR4 (98, 103).

TRAF6 seems to be involved both in MyD88-dependent and MyD88-independent NF- $\kappa$ B activation after TLR4-stimulation in MEF, as NF- $\kappa$ B activation was shown to be completely abolished after LPS stimulation (250). However, in spleen macrophages TRAF6 was required for early NF- $\kappa$ B activation and secretion of proinflammatory cytokines (77). Furthermore, both TLR-dependent and -independent signals converge to TRAF6 (80). Activation of TRAF6 can be mediated by TRIF, MyD88, NOD1 and TNFR signaling (80, 251, 252). We next studied the role of TRAF6 in activation of NF- $\kappa$ B after infection with *C. pneumoniae*. TRAF6 is

essential for peri- and postnatal survival (76), and therefore no TRAF6<sup>-/-</sup> BMM could be obtained. Due to this we instead isolated MEF and used them to examine the regulation of NF-κB after *C. pneumoniae* infection. *C. pneumoniae*-infected MEF showed high levels of proinflammatory cytokine mRNA and protein, and phosphorylation of IκB-α. Also, TRAF6<sup>-/-</sup> MEF demonstrated impaired phosphorylation of IκB-α and nuclear translocation of NF-κB and reduced levels of IL-1β, IL-6 and TNF-α mRNA and IL-6 protein in comparison to the WT controls. Low levels of IFN-β, IFN-α and IFN-γ mRNA were expressed in MEF infected with *C. pneumoniae*. However, no difference in IFN-β mRNA accumulation was found between TRAF6<sup>-/-</sup> and WT MEF (Paper II). Thus, we demonstrate that TRAF6 is required for NF-κB activation in *C. pneumoniae*-infected MEF. TRAF6 is probably a converging point for different innate signaling pathways leading to NF-κB activation, but TLR4, MyD88, IRAK4, IFN-α/βR and PKR are redundant in NF-κB activation and induction of proinflammatory genes after infection with *C. pneumoniae*. Whether these molecules mediate the control of infection with *C. pneumoniae* via activation of MAP kinases is unknown. Furthermore, the relevance of TRIF and TNFR signaling pathways in the outcome of chlamydial infection is still unclear.

It is unlikely that TLR1, TLR2, TLR6 and TLR9 are required for the induction of proinflammatory cytokines in *C. pneumoniae*-infected BMM, since we showed that MyD88 was redundant in the induction of proinflammatory cytokines. However, TLR2 has been suggested to have a role in the secretion of proinflammatory cytokines in BMDC and peritoneal macrophages infected with *C. pneumoniae* (98, 103). In our experimental model the redundancy of TLR4 and MyD88 signaling in *C. pneumoniae*-induced NF-κB activation in BMM implicates a TLR-independent sensing and signaling pathway. Other PRRs, such as the NOD proteins, could be candidates. NOD1 and NOD2 were in fact suggested to mediate NF-κB activation by *C. pneumoniae* in HEK293 cells (141).

In conclusion, we show that *C. pneumoniae* can induce TLR4- and MyD88-dependent and independent pathways and that these pathways are needed and complementary for IFN-γ induction and protection in BMM (figure 8).



**Figure 8.** IFN- $\alpha$  is induced in a TLR4-MyD88-IRAK4-dependent way in BMM infected with *C. pneumoniae*. In turn IFN- $\alpha/\beta$ -dependent, STAT1-mediated IFN- $\gamma$  protects against bacterial growth. TRAF6-mediated NF- $\kappa$ B activation is independent of TLR4-MyD88-dependent IFN- $\alpha/\beta$  signaling, but critical to IFN- $\gamma$  release in the infection of BMM with *C. pneumoniae* (Adapted from paper I).

### **IRF3 is redundant in protection against infection with *C. pneumoniae* in BMM (Paper II)**

The role of IRF3 in protection and regulation of IFN- $\alpha$  and IFN- $\gamma$  expression was then studied in BMM infected with *C. pneumoniae*. *C. trachomatis* has been shown to induce translocation of IRF3 into the nucleus (111). We used phosphorylation of IRF3 (pIRF3) as a measurement of activation, since it is needed for nuclear translocation. Surprisingly, we found similar levels of phosphorylation in both non-infected and *C. pneumoniae*-infected WT BMM. Levels of pIRF3 were similar in IRAK4<sup>-/-</sup> and WT BMM, even though IRAK4<sup>-/-</sup> BMM express reduced levels of IFN- $\alpha$  and IFN- $\gamma$  mRNA, as described before. In accordance, IRAK4 was dispensable for IRF3 activation in macrophages after LPS stimulation (75). IFN- $\alpha$  and IFN- $\gamma$  mRNA levels in IRF3<sup>-/-</sup> and WT BMM were similar. A similar bacterial load was also seen in IRF3<sup>-/-</sup> and WT BMM after infection with *C. pneumoniae*. In line with our results, IRF3<sup>-/-</sup> MEF have been shown to have a normal IFN-mediated antiviral response to vesicular stomatitis virus and HSV (224, 226, 253). The lack of IRF3 activation

further questions its role in BMM after *C. pneumoniae* infection. We showed earlier that MyD88-independent signaling was not sufficient for enhanced IFN- $\alpha$  mRNA accumulation in BMM infected with *C. pneumoniae*. In agreement with this, we showed that IRF3 was not required for IFN- $\alpha$  and IFN- $\gamma$  induction following infection with *C. pneumoniae* in BMM, and hence further ruled out a pivotal role for TRIF-dependent IFN induction.

IFN- $\beta$  turns on IFN-inducible genes, including IRF7, leading to second wave of induction of IFN- $\beta$  and many IFN- $\alpha$  subtypes. IRF7 has also been implicated in MyD88-dependent IFN- $\alpha/\beta$  expression in TLR9 signaling (227, 228). In the absence of IRF3, IFN- $\alpha/\beta$  induction is thought to be dependent of presence of constitutive levels of IFN- $\beta$  and expression of IRF7 (253). For example, both IFN- $\alpha$  and IRF7 induction depend on IFN- $\alpha/\beta$ R signaling in response to CpG in BMDC (243), and in IFN- $\beta^{-/-}$  fibroblasts, constitutive IFN- $\alpha$  mRNA is hardly detectable (210). We then studied the requirement for IFN- $\beta$  in IFN- $\alpha$  mRNA expression and control of infection of BMM with *C. pneumoniae*. Interestingly, we demonstrated that IFN- $\beta^{-/-}$  BMM show reduced expression of IFN- $\alpha$  and IFN- $\gamma$  mRNA, and higher loads of *C. pneumoniae*, in comparison to WT. IRF7 mRNA expression was slightly reduced in IFN- $\beta^{-/-}$  BMM as compared to WT. Thus, the presence of IFN- $\beta$  was required for IFN- $\alpha$  and IRF7. We hypothesized that *C. pneumoniae* infection in BMM will activate IRF7 in a MyD88-IRAK4-dependent manner, resulting in a second wave of IFN- $\alpha$  expression. The presence of IFN- $\beta$  probably explains the normal levels of IFN- $\alpha$  expression seen in the absence of IRF3 in BMM infected with *C. pneumoniae*.

In conclusion, IFN- $\beta$ , but not IRF3, is required for IFN- $\alpha$  and IFN- $\gamma$  mRNA expression, and the control of *C. pneumoniae* infection of BMM. We suggest that *C. pneumoniae* infection triggers a MyD88-IRAK4-dependent induction of IFN- $\alpha$  and IFN- $\beta$ . The presence of IFN- $\beta$  is needed for *C. pneumoniae*-induced IFN- $\alpha$ , probably through the activation of IRF7. The details on the role of IRF7 in IFN- $\alpha$  expression after chlamydial infection needs to be further studied. Infection with *C. pneumoniae* activated NF- $\kappa$ B in a TRAF6-dependent manner. We hypothesize that MyD88-IRAK4, TRIF and possibly other pathways lead to NF- $\kappa$ B activation (figure 9).





macrophages was similar. First, we confirmed the protective role of IFN- $\gamma$  by infecting IFN- $\gamma^{-/-}$  and IFN- $\gamma$ R $^{-/-}$  mice, which showed higher levels of *C. pneumoniae* than the WT mice. We then addressed the role of STAT1 in infection of mice with *C. pneumoniae*. STAT1 $^{-/-}$  mice infected intranasally with *C. pneumoniae* showed even higher susceptibility and mortality than IFN- $\gamma$ R or IFN- $\gamma$  knockout mouse strains. Thus, STAT1 is important for control of infection with *C. pneumoniae in vivo*. *In vitro* STAT1 activation by *C. pneumoniae* was strictly regulated by IFN- $\alpha/\beta$ R. We studied the role of IFN- $\alpha/\beta$ R and IFN- $\gamma$ R in the activation of STAT1 *in vivo*, by analyzing levels of pSTAT1 in lysates of lung tissue and lung mononuclear cells from IFN- $\alpha/\beta$ R $^{-/-}$ , IFN- $\gamma$ R $^{-/-}$  and IFN- $\alpha/\beta$ R $^{-/-}$ /IFN- $\gamma$ R $^{-/-}$  mice. In contrast to the BMM infection, STAT1 activation was only abolished in the absence of both IFN- $\alpha/\beta$ R and IFN- $\gamma$ R. Interestingly, IFN- $\alpha/\beta$  and IFN- $\gamma$  are required for the optimal expression of total STAT1 as well. The effects of crosstalk between IFN- $\alpha/\beta$ R and IFN- $\gamma$ R signaling on STAT1 activation are well documented. For example, cells pretreated with IFN- $\alpha/\beta$  show an increased response to IFN- $\gamma$  and *vice versa* (254). We suggest that IFN- $\alpha/\beta$  and IFN- $\gamma$  cooperate in control of STAT1 phosphorylation and expression *in vivo*. STAT1 can be phosphorylated at Ser727, but the role and the regulation of serine phosphorylation during *C. pneumoniae* infection are not known. In conclusion, we show that STAT1 is essential for resistance to intranasal infection of mice with *C. pneumoniae*, and IFN- $\alpha/\beta$ R and IFN- $\gamma$ R signaling cooperate in STAT1 activation, but are individually redundant. STAT1 signaling can also be negatively regulated by for example SOCS1. Later studies have shown that *C. pneumoniae* induces a STAT1-, IFN- $\alpha/\beta$ -dependent and IFN- $\gamma$ -independent SOCS1 production in mice, which controls infection-induced lethal inflammatory disease, but impairs the bacterial control (255).

### **IFN- $\alpha/\beta$ protects against intranasal chlamydial infection**

We investigated the role of IFN- $\alpha/\beta$  in the outcome of intranasal infection with *C. pneumoniae*. Surprisingly, we found that IFN- $\alpha$  mRNA levels in lung lysates were not increased after infection and no IFN- $\alpha/\beta$  could be detected in serum. Furthermore, IFN- $\alpha/\beta$ R $^{-/-}$  mice showed no enhanced bacterial load in the lungs after infection with *C. pneumoniae*, compared to WT. Thus, IFN- $\alpha/\beta$  signaling was redundant in mice infected with *C. pneumoniae*. The role of IFN- $\alpha/\beta$  in protection against bacterial infection is controversial and poorly documented. IFN- $\alpha/\beta$ R $^{-/-}$  mice demonstrated less resistance to streptococcal infection (166), but more resistance to infection with *C. muridarum* and *L. monocytogenes* than the WT controls (168-173).

We studied the impact of IFN- $\alpha/\beta$  deficiency on the expression of IFN- $\gamma$  and IFN- $\gamma$ -inducible genes. In lungs from infected IFN- $\alpha/\beta$ R $^{-/-}$  and WT mice mRNA levels of IFN- $\gamma$ , iNOS, IL-12p40 and IL-12R $\beta$ 1 were similar. This was in contrast to with the previous studies in *C. pneumoniae*-infected BMM, which demonstrated that IFN- $\alpha/\beta$ R was required for IFN- $\gamma$  and iNOS expression levels (187). During viral infection IFN- $\alpha/\beta$  have been demonstrated to inhibit IL-12 and IFN- $\gamma$  production (256). The

low levels of IFN- $\alpha$  in *C. pneumoniae*-infected mice are probably not sufficient to inhibit IL-12. However, the low constitutive expression of IFN- $\alpha$  in lung was involved in STAT1 activation. Indeed, steady state levels of IFN- $\alpha/\beta$  and IFN- $\gamma$  have been demonstrated to be important for STAT1 activation (257). IFN- $\alpha/\beta$ R components have been suggested to be involved in the assembly of IFN- $\gamma$ -activated transcription factors, and constitutive sub-threshold levels of IFN- $\alpha/\beta$  signaling were required for this crosstalk (210).

In opposition to our results, IFN- $\gamma$  and proinflammatory cytokine levels in lungs of *C. muridarum*-infected mice were decreased in the absence of IFN- $\alpha/\beta$ . In this model however, more severe inflammation was seen in the WT controls, compared to the less susceptible IFN- $\alpha/\beta$ R<sup>-/-</sup> mice. In contrast to our model, increased IFN- $\alpha$  mRNA expression was observed in lungs after infection with *C. muridarum* (169), which was proposed to lead to increased apoptosis of macrophages and subsequent promotion of bacterial growth. This could be explained by the distinct immune responses triggered by the two pathogens, and the presence of low levels of IFN- $\alpha/\beta$  mRNA in *C. pneumoniae*-infected mice might result in lower and similar levels of apoptosis in WT and IFN- $\alpha/\beta$ R<sup>-/-</sup> mice. We observed no indications of cell death in BMM cultures 10 days after infection. We need to understand better whether macrophage cell death is beneficial for *Chlamydia* and if there is a causal relationship of apoptotic cells to the detrimental effect of type I IFNs after bacterial infections. *Chlamydia* can modulate apoptosis of the infected host cell at different stages of the infectious cycle. *Chlamydia* is thought to inhibit cell death during early stages and trigger apoptosis at the end of the cycle to spread (258). Thus, whether apoptosis or other forms of programmed cell death actually are involved in the IFN- $\alpha/\beta$ -mediated response to *C. pneumoniae* infection is not known.

IFN- $\alpha/\beta$  did not seem to have a role in protection to *C. pneumoniae* *in vivo*. However, we observed that STAT1<sup>-/-</sup> mice were more susceptible than IFN- $\gamma$ R<sup>-/-</sup> mice to infection, and both IFN- $\alpha/\beta$  and IFN- $\gamma$  participate in STAT1 activation. We further investigated whether IFN- $\alpha/\beta$ R signaling was responsible for differences between STAT1<sup>-/-</sup> and IFN- $\gamma$ R<sup>-/-</sup> in the susceptibility to *C. pneumoniae*. For this purpose, we infected IFN- $\alpha/\beta$ R<sup>-/-</sup>/IFN- $\gamma$ R<sup>-/-</sup> mice and found that these were more susceptible to *C. pneumoniae* infection than IFN- $\gamma$ R<sup>-/-</sup> mice and showed similar bacterial titers to STAT1<sup>-/-</sup> mice. Thus, IFN- $\alpha/\beta$ R-dependent signaling participates in protection to *C. pneumoniae* *in vivo*. In conclusion, STAT1 mediates both IFN- $\alpha/\beta$ R- and IFN- $\gamma$ R-dependent protection against *C. pneumoniae* infection. IFN signaling can also be mediated by other kinases and transcription factors, such as phosphatidylinositol 3'-kinase and MAP kinases, and NF- $\kappa$ B and AP-1. However, whether they are involved in IFN signaling during infection with *C. pneumoniae* *in vivo* requires further investigation.

IFN- $\alpha/\beta$  secretion results in STAT1 activation and subsequent IFN- $\gamma$  gene induction during the infection of BMM infection with *C. pneumoniae*. IFN- $\gamma$  expression occurs in the absence of IFN- $\alpha/\beta$  signaling *in vivo*. Indeed, apart from IFN- $\alpha/\beta$ , IL-12 and IL-18 are also known to induce IFN- $\gamma$  in a STAT4-dependent way

(206). Since the regulation of IFN- $\gamma$  *in vitro* and *in vivo* varied, we then investigated whether the IFN- $\alpha/\beta$ - and STAT1-dependent activation of IFN- $\gamma$  expression observed in BMM has the same role in other cell populations *in vitro*. We infected BMM and BMDC from WT and IFN- $\alpha/\beta$ R<sup>-/-</sup> mice in parallel with *C. pneumoniae*. In the infection of BMDC the expression of IFN- $\gamma$  was similar in WT and IFN- $\alpha/\beta$ R<sup>-/-</sup> cells. In contrast, the expression of IFN- $\gamma$  in *C. pneumoniae*-infected BMM was IFN- $\alpha/\beta$ R-dependent, confirming our previous data. We showed that there is a distinct mechanism in regulating IFN- $\gamma$  in different myeloid cell populations, which can explain the redundancy of IFN- $\alpha/\beta$ R signaling in IFN- $\gamma$  induction *in vivo*. In conclusion, the ability of IFN- $\alpha/\beta$  to regulate myeloid IFN- $\gamma$  depends on the particular cell population.

### **STAT1 is not needed for protection and IFN- $\gamma$ production by T cells after chlamydial infection**

After infection, activated macrophages secrete IL-12 and promote differentiation of naïve CD4<sup>+</sup> T cells into T<sub>H</sub>1 cells, which produce IFN- $\gamma$  (259). During *C. pneumoniae* infection *in vivo* IL-12 is necessary for resistance, probably by regulating protective IFN- $\gamma$  levels. IFN- $\gamma$  also upregulates IL-12 production, suggesting a positive feedback mechanism (33). IFN- $\gamma$  secreted by both T cells and non-lymphoid cells is important in the infection with *C. pneumoniae* (34). We previously showed that STAT1 is important *in vivo*, and we also noted that STAT1<sup>-/-</sup> and IFN- $\gamma$ R<sup>-/-</sup> mice had enhanced numbers of bacteria after 60 days of infection, in contrast to the WT mice, which cleared the infection. STAT1 has been reported to be involved in differentiation of T cells, through the expression of the transcription factor T-bet, which is supposedly involved in T<sub>H</sub>1 development, by upregulating IL-12R (260). STAT1<sup>-/-</sup> mice have also shown impaired development of regulatory T cells (261). We investigated the role of STAT1 in T cell signaling leading to IFN- $\gamma$  secretion and protection. For this purpose, we intravenously inoculated RAG1<sup>-/-</sup>/IFN- $\gamma$ <sup>-/-</sup> mice with WT and STAT1<sup>-/-</sup> CD4<sup>+</sup> and CD8<sup>+</sup> naïve spleen cells and infected these animals after 24 days. We measured bacterial load and IFN- $\gamma$  levels in their lungs 21 days after infection. In these animals the inoculated T cells were the only source of IFN- $\gamma$ . Reconstitution with either STAT1<sup>-/-</sup> or WT T cells protected the RAG1<sup>-/-</sup>/IFN- $\gamma$ <sup>-/-</sup> mice against *C. pneumoniae* infection. No differences in levels of IFN- $\gamma$  mRNA and intracellular bacteria between lungs from STAT1<sup>-/-</sup> and WT T cell inoculated mice were observed. In agreement, T cell activation and IFN- $\gamma$  expression did not require STAT1 during infection with *Toxoplasma gondii* (262). Thus, STAT1 is not required for T cell activation and IFN- $\gamma$  mRNA expression.

In line with this, T cells do not require IFN- $\gamma$ R to confer protection to *C. pneumoniae* infection (34). The fact that STAT1<sup>-/-</sup> and WT T cells expressed similar levels of IFN- $\gamma$  might also explain the similar bacterial load, since all other cells could respond to the IFN- $\gamma$  secretion. The similar mRNA levels of IFN- $\gamma$  in lungs from mice reconstituted with WT and STAT1<sup>-/-</sup> T cells might also result from STAT1-

independent T cell-mediated IFN- $\gamma$  production. Whether STAT4 or other transcription factors are involved in IFN- $\gamma$  production in T cells during infection with *C. pneumoniae* is not known. Thus, BMM but not T cells, required STAT1 to express IFN- $\gamma$ .

### **Non-hematopoietic cells require STAT1 for resistance to *C. pneumoniae***

*C. pneumoniae* can infect a vast array of cells. Both phagocytic and non-professional phagocytes primed with IFN- $\gamma$  showed enhanced protection to chlamydial pathogens (263-265). STAT1 is critical for protection to *C. pneumoniae* infection in lung fibroblasts and BMM. We then investigated whether hematopoietic or non-hematopoietic cells confer STAT1-dependent protection to mice. For this reason reciprocal bone marrow radiation chimeras were generated by irradiating WT and STAT1<sup>-/-</sup> mice and reconstituted with bone marrow (hematopoietic) cells from WT and STAT1<sup>-/-</sup> mice. The validity of the model was confirmed by showing that total STAT1 protein was detected in WT  $\rightarrow$  STAT1<sup>-/-</sup> mice 9 weeks after bone marrow transfer. Six weeks after reconstitution they were infected with *C. pneumoniae*.

The bacterial load of the different groups was measured 21 days after infection. Lungs from positive control sham chimeric mice (WT  $\rightarrow$  WT) showed a lower bacterial load in comparison with the negative mock controls (STAT1<sup>-/-</sup>  $\rightarrow$  STAT1<sup>-/-</sup>), confirming that STAT1 is needed for protection. The positive control mice (WT  $\rightarrow$  WT) demonstrated similar bacterial load to WT mice reconstituted with hematopoietic STAT1<sup>-/-</sup> type cells (STAT1<sup>-/-</sup>  $\rightarrow$  WT). Furthermore, STAT1<sup>-/-</sup>  $\rightarrow$  STAT1<sup>-/-</sup> showed similar susceptibility to the STAT1<sup>-/-</sup> mice reconstituted with hematopoietic WT cells (WT  $\rightarrow$  STAT1<sup>-/-</sup> mice), confirming that STAT1-mediated mechanisms by hematopoietic cells are not required for bacterial control. To test the role of STAT1 in non-hematopoietic cells, we compared STAT1<sup>-/-</sup>  $\rightarrow$  WT and WT  $\rightarrow$  WT mice with STAT1<sup>-/-</sup>  $\rightarrow$  STAT1<sup>-/-</sup> and WT  $\rightarrow$  STAT1<sup>-/-</sup> mice, respectively. STAT1<sup>-/-</sup>  $\rightarrow$  STAT1<sup>-/-</sup> and WT  $\rightarrow$  STAT1<sup>-/-</sup> mice were more susceptible to infection with *C. pneumoniae* than STAT1<sup>-/-</sup>  $\rightarrow$  WT and WT  $\rightarrow$  WT mice, indicating that somatic cells are involved in the STAT1-mediated protection. Thus, STAT1 is necessary for protection against chlamydial infection, but mainly non-hematopoietic cells account for this protection.

Non-hematopoietic cells are needed for STAT1-mediated protection to *C. pneumoniae*. We next investigated the expression of STAT1-regulated antimicrobial effector enzymes (197, 266). The mRNA levels for IDO, LRG47 and iNOS were measured in lungs of the bone marrow chimeras. IDO, LRG-47 and iNOS mRNA is expressed in a STAT1-dependent way. Non-hematopoietic cells are required for IDO expression, hematopoietic cells for iNOS expression and both hematopoietic and non-hematopoietic cells are needed for LRG-47 expression. iNOS is therefore less relevant in protection, compared to the other mechanisms. However, whether IDO and LRG-47 actually contribute to protection during the infection with *C. pneumoniae* is not known. Together, we demonstrated that T cells still express IFN- $\gamma$  in the absence of STAT1 after infection with *C. pneumoniae* *in vivo* and that the secretion of IFNs by T

cells is needed for STAT1-dependent protection mainly mediated by non-hematopoietic cells.

## **Paper IV –Role of NOD1 during infection with *L. monocytogenes***

### **NOD1 protects against infection with *L. monocytogenes***

NOD1 senses the cytosolic presence of muropeptides containing meso-DAP from Gram-negative and some Gram-positive bacteria. NOD1 signaling can lead to the activation of NF- $\kappa$ B and MAP kinases, involving the downstream molecule RIP2. These signals can induce proinflammatory cytokines and chemokines. We addressed the role of NOD1 in the control of infection with *L. monocytogenes* *in vivo*. NOD1<sup>-/-</sup> mice infected i.p. with 10<sup>5</sup> CFU *L. monocytogenes* showed enhanced bacterial load in liver and spleen 3 days and 5 days after infection. 85 % of NOD1<sup>-/-</sup> mice succumbed between 6 and 15 days after infection. NOD1<sup>-/-</sup> mice showed increased levels of bacteria in liver and spleen and diminished survival compared to WT, even when infecting with a 50 fold lower dose of *L. monocytogenes* (2 x 10<sup>3</sup> CFU). In accordance with these results RIP2<sup>-/-</sup> mice were more susceptible to i.p infection with *L. monocytogenes* (147). Kim et al showed that NOD1<sup>-/-</sup>/NOD2<sup>-/-</sup> mice pretreated with LPS or *E. coli* were more susceptible than WT to infection with *L. monocytogenes*. In contrast to our result, however, untreated NOD1<sup>-/-</sup>/NOD2<sup>-/-</sup> mice demonstrated only slightly decreased survival and the bacterial load was only enhanced in liver, but not in spleen at 48 hours after i.p. infection with 10<sup>4</sup> *L. monocytogenes*, compared to WT mice (148).

*L. monocytogenes* can cause encephalitis by spreading via a neural route along the trigeminal nerve (43, 44). We then investigated if NOD1 is required for control of disseminated listerial infection into the brain after snout infection. Levels of *L. monocytogenes* in the snout were similar in WT and NOD1<sup>-/-</sup> mice. However, listerial levels in the brain and in the trigeminal nerve were higher in NOD1<sup>-/-</sup> mice than WT. This suggests that neural cells in the trigeminal nerve and the brain stem are capable of controlling listerial infection and inhibiting dissemination. *L. monocytogenes* can also spread to CNS via a hematogenous route through the blood brain barrier or the choroid plexus. After i.p. infection levels of *L. monocytogenes* were also higher in brains of NOD1<sup>-/-</sup> mice than WT. Taken together, this indicates that NOD1 protects against dissemination into the brain.

Levels of IFN- $\gamma$ , IL-1 $\beta$  and IL-6 mRNA in spleen were increased 5 days after infection with *L. monocytogenes* but none of these transcripts were reduced in spleen from NOD1<sup>-/-</sup> mice, compared to WT.

Next, we addressed the role of NOD1 in a protective memory immune response. NOD1, RIP2 and NOD2 have been reported to regulate adaptive immune responses (135, 145, 147, 267). WT and NOD1<sup>-/-</sup> mice were infected i.p. with 2 x 10<sup>3</sup> CFU *L. monocytogenes* and surviving mice were reinfected 20 days after the primary

infection with  $10^5$  CFU *L. monocytogenes*. NOD1<sup>-/-</sup> re-infected mice showed lower bacterial levels than the naïve animals and all survived, but showed a higher bacterial load in the spleen than WT mice 4 days after reinfection. WT and NOD1<sup>-/-</sup> reinfected mice contained similar numbers of IFN- $\gamma$ -secreting spleen T cells after stimulation with MHC class I and II-restricted listerial peptides or heat killed *Listeria*. In line with this, splenic CD11c<sup>+</sup> DC from WT and NOD1<sup>-/-</sup> mice expressed similar levels of the costimulatory molecules CD40, CD80 and CD86, and MHC class II molecule 4 days after infection. However, NOD1<sup>-/-</sup> BMDC showed lower levels of IL-6 mRNA expression after infection compared to WT cells. Taken together, this suggests that NOD1 is redundant in generating an adaptive immune response, but a role for NOD1 cannot be ruled out at other time points or on other immune parameters.

NOD1 stimulates the recruitment of cells to the inflammatory site and secretion of chemokines (129-131). We then investigated whether NOD1 controls the recruitment of inflammatory cells to the site of infection with *L. monocytogenes* using an air pouch model. A massive influx of monocytes and neutrophils was observed after inoculation of *L. monocytogenes* into the air pouch. However, similar numbers of granulocytes, inflammatory and resident monocytes were seen in WT and NOD1<sup>-/-</sup> mice. In the air pouch lavage fluid similar protein levels of the chemokines CCL2 and CCL7 were observed in WT and NOD1<sup>-/-</sup> mice. Hence, NOD1 is not required for chemokine secretion and recruitment of inflammatory cells after infection.

To understand the role of NOD1 in different cell populations in the infection with *L. monocytogenes*, we generated reciprocal bone marrow radiation chimeras between WT and NOD1<sup>-/-</sup> mice. NOD1 mRNA was detected in WT  $\rightarrow$  NOD1<sup>-/-</sup> mice, confirming the validity of the model. Six weeks after reconstitution mice were infected with *L. monocytogenes*. This experiment showed that the protective role for NOD1 against *L. monocytogenes* depended on non-hematopoietic cells.

NOD2<sup>-/-</sup> mice were more susceptible than WT following intragastric infection, but not after i.v. infection. Thus, NOD2 is not important in systemic infections (145). On the contrary, we demonstrated here that NOD1 confers protection to systemic infection with *L. monocytogenes*. The discrepancy might result from the restricted cell type expression by NOD2, compared to NOD1. NOD2 is primarily expressed in intestinal epithelial cells and monocytes, whereas NOD1 is expressed in most cells (137, 138). In conclusion, NOD1 confers protection to systemic infection with *L. monocytogenes*, mediated by non-hematopoietic cells, but it is redundant for adaptive immune responses and recruitment of inflammatory cells.

### **NOD1 controls intracellular growth of *L. monocytogenes* in vitro**

Whether NOD1 controls infection with *L. monocytogenes* in BMM was investigated. The levels of *L. monocytogenes* were increased in NOD1<sup>-/-</sup> BMM in comparison to WT. Such differences were not due to increased bacterial uptake by NOD1<sup>-/-</sup> BMM. IL-1 $\beta$  and IL-6 mRNA levels were lower in NOD1<sup>-/-</sup> compared to WT BMM. IFN- $\beta$  mRNA expression was not dependent on NOD1 in *L. monocytogenes*-infected BMM.

This is in line with experiments showing that induction of IFN- $\beta$  is independent of RIP2 and NOD1 (130, 152).

It has recently been suggested that NOD2 signaling is also triggered by ligands generated by degradation in the phagosome of IFN- $\gamma$ -activated macrophages (268). We investigated the role of NOD1 in the killing of intracellular *L. monocytogenes* of IFN- $\gamma$ -activated BMM. WT BMM pretreated with IFN- $\gamma$  clearly showed a reduced bacterial load compared to untreated cells. Pretreated NOD1<sup>-/-</sup> BMM showed similar levels of bacteria as nontreated cells at 24 hours after infection. Thus, IFN- $\gamma$ -mediated killing of intracellular *L. monocytogenes* is at least partially impaired in NOD1<sup>-/-</sup> BMM. We suggest that NOD1 signaling is triggered by degraded bacterial material from the phagosome of IFN- $\gamma$  activated macrophages. Cytosolic intracellular bacteria trigger signaling pathways distinct to non-invasive bacteria. Several groups have reported that cytosolic invasion by *L. monocytogenes* is needed for IFN- $\beta$  and CCL2 gene expression (117, 119, 120, 269). We then studied the requirement of the cytosolic invasion by *L. monocytogenes* in cytokine production by incubating BMM with an LLO-deficient *L. monocytogenes* strain mutant  $\Delta hly$  or heat killed *L. monocytogenes*. NOD1<sup>-/-</sup> and WT BMM showed similar IL-6 mRNA levels when infected with  $\Delta hly$  *L. monocytogenes* or incubated with heat killed *Listeria*, suggesting that the NOD1-mediated enhanced expression of cytokine mRNA requires cytosolic invasion of *L. monocytogenes*.

We addressed the role of NOD1 in the control of *L. monocytogenes* infection in other cell populations *in vitro*. Both NOD1<sup>-/-</sup> MEF and astrocytes showed enhanced bacterial load in WT cells after infection. However, at certain time points, NOD1<sup>-/-</sup> MEF showed up to 100-fold higher titers of *L. monocytogenes* whereas NOD1<sup>-/-</sup> astrocytes, like BMM, demonstrated up to 10-fold difference to WT cells. We then studied the gene expression pattern in fibroblasts in a low density microarray. Out of 82 inflammatory genes, 14 genes were induced in WT fibroblasts after infection. 13 of these 14 genes were induced in NOD1<sup>-/-</sup> cells together with 32 other genes, including important inflammatory cytokines, chemokines, growth factors and metalloproteases. The *tlr2* gene was induced in WT, but not in NOD1<sup>-/-</sup> fibroblasts. The *il6* and *il1b* gene expression was not diminished in NOD1<sup>-/-</sup> fibroblast, in contrast to BMM. The increased number of genes induced in NOD1<sup>-/-</sup> fibroblasts could result from a stronger host response to the higher bacterial load. The lack of reduced gene response in NOD1<sup>-/-</sup> fibroblasts could be explained by 1) the role of NOD1 in other important genes that were not included in the array and/or 2) the importance of NOD1 for expression of protective genes at another time point than 24 hours after infection. Interestingly, in uninfected NOD1<sup>-/-</sup> cells, 15 genes were upregulated (e.g. *tlr2* and *cxcl5*) and 6 were downregulated in comparison to WT cells. This suggests that NOD1 plays a role in gene expression unrelated to stimuli.

We then analyzed the NOD expression in BMM and fibroblasts after infection with *L. monocytogenes*. NOD1 mRNA levels were increased in both BMM and fibroblasts after infection with *L. monocytogenes* compared to uninfected cells. In line with this, lung tissue and epithelial cells showed enhanced mRNA levels of NOD1

after infection with *S. pneumoniae* (251). This suggests that the cells are sensitized to NOD1 recognition after infection. NOD ligands trigger a weak cytokine response but they are suggested to be positively regulated by TLR signaling (132). MyD88<sup>-/-</sup> macrophages showed an abolished response to *L. monocytogenes* in macrophages (113), supporting a synergistic role for NOD1 in TLR signaling, rather than a parallel role for NOD1 and TLRs in cytokine expression. We showed that the expression levels of NOD1 mRNA were increased in BMM stimulated with TLR ligands CpG, poly I:C and LPS, and to a lesser extent Pam<sub>3</sub> and MALP-2, compared to untreated cells. We observed that WT and NOD1<sup>-/-</sup> BMM stimulated with CpG, poly I:C and Pam<sub>3</sub> demonstrated similar levels of IL-1 $\beta$ , IL-6 and IFN- $\beta$  after infection with *L. monocytogenes*. Thus, TLR signaling probably enhances cell sensitivity to NOD1 recognition. However, further investigation is required to determine the details on the relation of NOD proteins to TLRs in triggering innate immune responses. In conclusion, we observed that NOD1 controls the infection with *L. monocytogenes* in both hematopoietic and non-hematopoietic cells, such as macrophages, astrocytes and particularly in fibroblasts. NOD1 is also required for IFN- $\gamma$ -mediated *L. monocytogenes* growth in macrophages.



## CONCLUDING REMARKS

Certain bacteria have evolved mechanisms to replicate and spread in a biological niche inside the cells of different hosts. The host has, in turn, developed an immune system to protect itself. Innate receptors are the first sensors of these bacteria and they trigger different responses, which induce the production of cytokines that mediate the immune response. IFN- $\gamma$  is probably the most important cytokine for the immune response to intracellular bacteria, the production of which ultimately results in the eradication of the bacteria through several effector mechanisms.

In this thesis I investigated the pathways that initiate innate immune responses and lead to protection against intracellular bacterial infection with *C. pneumoniae* and *L. monocytogenes*. Earlier studies have shown that *C. pneumoniae* infection of BMM induces IFN- $\alpha/\beta$ -dependent IFN- $\gamma$  secretion, leading to control of bacterial growth. In paper I and II we described that *C. pneumoniae* acts via TLR4 to trigger a pathway through MyD88 and IRAK4, which results in IFN- $\alpha$  secretion. In addition, STAT1 mediates IFN- $\alpha/\beta$ -dependent IFN- $\gamma$  production, which controls bacterial growth in *C. pneumoniae*. We discovered that IRF3 is redundant for IFN- $\alpha$  and IFN- $\gamma$  expression and control of bacterial growth in the infection of BMM with *C. pneumoniae*. However, IFN- $\beta$  regulates IFN- $\alpha$ , IFN- $\gamma$  and IRF7 induction and is required for protection against *C. pneumoniae* infection in BMM.

TLR signaling has been shown to lead to activation of both NF- $\kappa$ B and MAP kinases, both leading to the induction of proinflammatory cytokines. We described that NF- $\kappa$ B activation is critical to IFN- $\gamma$  release and that this pathway is TRAF6-mediated, but MyD88 and IRF3 are redundant for NF- $\kappa$ B activation. We believe that MyD88-IRAK4, TRIF and possibly other signaling pathways, such as NOD signaling, contribute together to NF- $\kappa$ B activation.

IFN- $\gamma$  derived from both macrophages and T cells can play a central and complementary role in protection against *C. pneumoniae* infection *in vivo*. In paper III we demonstrated that during intranasal infection with *C. pneumoniae* STAT1 is essential for resistance. STAT1 mediates IFN- $\alpha/\beta$  and IFN- $\gamma$  signaling, which are both required for protection. Several reports have described a detrimental role for type I IFNs in bacterial infection due to increased cell death. We observed that infection with *C. pneumoniae* triggers low levels of IFN- $\alpha$ , which together with differences in cell tropism or in immune responses to distinct pathogens, could explain the beneficial role for IFN- $\alpha/\beta$  signaling during infection with *C. pneumoniae*. T cell-secreted IFN- $\gamma$  is sufficient to confer protection against *C. pneumoniae*, but STAT1 signaling in T cells is not required for protection. We found that non-hematopoietic cells are important for protection against chlamydial infection *in vivo* in a STAT1-dependent manner. STAT1 mediates the microbicidal mechanisms IDO and LRG-47 in non-hematopoietic cells. However, it is not clear which effector mechanisms are responsible for IFN- $\gamma$ -mediated protection to infection with *C. pneumoniae*.

Infection of macrophages with *L. monocytogenes* triggers distinct innate immune receptors at different time points. Other groups have described that TLR signaling is involved in the early response to *L. monocytogenes*, leading to induction of NF- $\kappa$ B-regulated genes, independently of the invasion of live bacteria. NOD proteins recognize muropeptides in the cytosol, leading to activation of NF- $\kappa$ B and MAP kinases. In paper IV we described for the first time an important protective role for NOD1 in the infection with *L. monocytogenes* *in vivo* and *in vitro*. We showed that non-hematopoietic cells mediate NOD1 protection against systemic infection with *L. monocytogenes*. No major defects in triggering of specific adaptive immune responses and in the recruitment of inflammatory cells could be detected in NOD1<sup>-/-</sup> *L. monocytogenes*-infected mice. NOD1 plays a role in control of *L. monocytogenes* infection in macrophages, fibroblasts and astrocytes. It is required for IFN- $\gamma$ -mediated growth control in macrophages. We observed that IL-6 production was decreased in NOD1<sup>-/-</sup> macrophages compared to WT after infection with WT *L. monocytogenes*, but not with non-invasive *L. monocytogenes*. Surprisingly, a number of important inflammatory cytokines, chemokines, growth factors and metalloproteases were increased in NOD1<sup>-/-</sup> compared to WT fibroblasts, which we suggested to be an effect of higher numbers of bacteria. The major molecular mechanisms accounting for susceptibility of NOD1<sup>-/-</sup> mice to *L. monocytogenes* remain to be determined.

The field of innate immunity is subject to intense studies conducted by researchers from all over the world. The pathways I described here contribute to the comprehension of innate immunity to intracellular bacteria. Whether these pathways occur in response to other pathogens remains to be settled. However, many details of these pathways are still unknown and we cannot exclude that other pathways might function in parallel or in synergy. Thus, research is only scratching on the surface of innate immunity to intracellular bacteria and we can expect many important findings in the future. Whether these findings made in mice also apply to the human infections remain to be determined. If this is the case these and other research studies might contribute to the development of immunoprophylaxis or immunotherapy against these infections.

## ACKNOWLEDGEMENTS

I started this journey in February 2003 doing my degree project and the road has not been straight. Two years after the registration I started medical school and I was not sure what would happen to my project. I'm very grateful to have reached my destination and I would like to thank the people who helped me there. Thanks in particular to:

My supervisor Martin Rottenberg for his passion for science in general and the projects in particular. I would not have succeeded without your help in methodology, analysis of the results, knowledge and new ideas.

My co-supervisor Hans Wigzell - an inspiration for everyone in science.

All co-authors of the papers: Tony, Ahmed, Emma, Åsa, Anna, Oliver *et al.*

Berit for taking care of my cells and keeping the order in the group.

My closest colleague Emma, especially for one of the great moments as a PhD student at the conference in Joachimstal and in Berlin in June 2005.

Laura and Frank for correcting the thesis. Jocke for the cover picture.

The animal house staff: Maggan, Mabbe, Torunn, Annelie, Emma *et al.* for all help with cages and mice and the girls for all fun at the MTC pub!

Danika, Alberto, Elio and Emma as the MTC pub muppet crew, but mille grazie are not enough for what you have done for MTC!

Other friends at MTC; It has been great to be back over summer and I realized how much I missed it here, especially the international atmosphere. Especially André, Alex, Emelie, Elisabeth, Nicolas, Hanna, Ing-Marie, Nancy, Stefano, Pia, Ying, Sofia, Ami, Frida, Eva, Kaloyan, Ulrika, Victoria, Lisette, Caroline and Andreas.

Thanks to MTC, KI, SU and Sweden for free higher education.

The journal club: Anders Örn, Michael Rehn, Gunilla Karlsson Hedestam *et al.*

Staff at MTC: Håkan, Birgitta, Torbjörn, Greger, Hanna, Milina *et al.*

Stefan, Lisa, Berit and Tangbin as former members of the group.

The new members Ahmed, Frank, Suman and Yeh for keeping up the good work.

All the undergraduate students we took care of (mostly me and Berit), and who vitalized our little group: Elizabeth, Camilla, Ingrid, Katja, Mira, Daniel, Simrat, Ulrika, Lisa and Corinna.

Friends from: medical school, the choir Stockholms Universitetskör, Stockholm University and Tourist office.

My friends outside KI, especially Albert, Fia, Jessica, Josef, Martin, Mattias, Docent Niclas B, Niclas C, Peter S, Sara, Peter F, Reine, Rocco, Ulrika, Karin och Sole. I utlandet: Anna, Kirre, Kristina, Doktor Anto och Nicola. Jag hälsar på snart!

My relatives and grandparents: Bonjour mon coeur, Farmor.

My family: Mina föräldrar Lars och Kerstin, för att ni alltid skämmer bort mig med kärlek, omtanke och goda söndagsmiddagar.

Min bror Kalle och familjen Johan & Lisa + Elsa.

Jonas, för att du vill dela ditt liv med mig.

## REFERENCES

1. Meresse, S., O. Steele-Mortimer, E. Moreno, M. Desjardins, B. Finlay, and J. P. Gorvel. 1999. Controlling the maturation of pathogen-containing vacuoles: a matter of life and death. *Nat Cell Biol* 1:E183-188.
2. Abdelrahman, Y. M., and R. J. Belland. 2005. The chlamydial developmental cycle. *FEMS Microbiol Rev* 29:949-959.
3. Bavoil, P. M., R. Hsia, and D. M. Ojcius. 2000. Closing in on Chlamydia and its intracellular bag of tricks. *Microbiology* 146 ( Pt 11):2723-2731.
4. Everett, K. D., R. M. Bush, and A. A. Andersen. 1999. Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms. *Int J Syst Bacteriol* 49 Pt 2:415-440.
5. Belland, R., D. M. Ojcius, and G. I. Byrne. 2004. Chlamydia. *Nat Rev Microbiol* 2:530-531.
6. Smittskyddsinstitutet. 2008. Epidemiologisk årsrapport 2007. Smittskyddsinstitutet, Stockholm.
7. Joyee, A. G., S. P. Thyagarajan, E. V. Reddy, C. Venkatesan, and M. Ganapathy. 2005. Genital chlamydial infection in STD patients: its relation to HIV infection. *Indian J Med Microbiol* 23:37-40.
8. Bunnell, R. E., L. Dahlberg, R. Rolfs, R. Ransom, K. Gershman, C. Farshy, W. J. Newhall, S. Schmid, K. Stone, and M. St Louis. 1999. High prevalence and incidence of sexually transmitted diseases in urban adolescent females despite moderate risk behaviors. *J Infect Dis* 180:1624-1631.
9. Kuo, C. C., L. A. Jackson, L. A. Campbell, and J. T. Grayston. 1995. Chlamydia pneumoniae (TWAR). *Clinical Microbiology Reviews* 8:451-461.
10. Hammerschlag, M. R., K. Chirgwin, P. M. Roblin, M. Gelling, W. Dumornay, L. Mandel, P. Smith, and J. Schachter. 1992. Persistent infection with Chlamydia pneumoniae following acute respiratory illness. *Clin Infect Dis* 14:178-182.
11. Falck, G., L. Heyman, J. Gnärpe, and H. Gnärpe. 1995. Chlamydia pneumoniae and chronic pharyngitis. *Scand J Infect Dis* 27:179-182.
12. Pether, J. V., S. P. Wang, and J. T. Grayston. 1989. Chlamydia pneumoniae, strain TWAR, as the cause of an outbreak in a boys' school previously called psittacosis. *Epidemiol Infect* 103:395-400.
13. Hagiwara, K., K. Ouchi, N. Tashiro, M. Azuma, and K. Kobayashi. 1999. An epidemic of a pertussis-like illness caused by Chlamydia pneumoniae. *Pediatr Infect Dis J* 18:271-275.
14. Kleemola, M., P. Saikku, R. Visakorpi, S. P. Wang, and J. T. Grayston. 1988. Epidemics of pneumonia caused by TWAR, a new Chlamydia organism, in military trainees in Finland. *J Infect Dis* 157:230-236.
15. Csango, P. A., S. Haraldstad, J. E. Pedersen, G. Jagars, and I. Foreland. 1997. Respiratory tract infection due to Chlamydia pneumoniae in military personnel. *Scand J Infect Dis Suppl* 104:26-29.
16. Hammerschlag, M. R. 2007. Chlamydia and Chlamydiales: beyond Chlamydia trachomatis. *Pediatr Infect Dis J* 26:639-640.

17. Hahn, D. L., A. A. Azenabor, W. L. Beatty, and G. I. Byrne. 2002. Chlamydia pneumoniae as a respiratory pathogen. *Front Biosci* 7:e66-76.
18. File, T. M., Jr., J. S. Tan, and J. F. Plouffe. 1998. The role of atypical pathogens: Mycoplasma pneumoniae, Chlamydia pneumoniae, and Legionella pneumophila in respiratory infection. *Infect Dis Clin North Am* 12:569-592, vii.
19. Jackson, L. A., L. A. Campbell, C. C. Kuo, D. I. Rodriguez, A. Lee, and J. T. Grayston. 1997. Isolation of Chlamydia pneumoniae from a carotid endarterectomy specimen. *J Infect Dis* 176:292-295.
20. Kuo, C. C., A. Shor, L. A. Campbell, H. Fukushi, D. L. Patton, and J. T. Grayston. 1993. Demonstration of Chlamydia pneumoniae in atherosclerotic lesions of coronary arteries. *Journal of Infectious Diseases* 167:841-849.
21. Muhlestein, J. B., J. L. Anderson, E. H. Hammond, L. Zhao, S. Trehan, E. P. Schwobe, and J. F. Carlquist. 1998. Infection with Chlamydia pneumoniae accelerates the development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model. *Circulation* 97:633-636.
22. Moazed, T. C., L. A. Campbell, M. E. Rosenfeld, J. T. Grayston, and C. C. Kuo. 1999. Chlamydia pneumoniae infection accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. *J Infect Dis* 180:238-241.
23. Hu, H., G. N. Pierce, and G. Zhong. 1999. The atherogenic effects of chlamydia are dependent on serum cholesterol and specific to Chlamydia pneumoniae. *J Clin Invest* 103:747-753.
24. Caligiuri, G., M. Rottenberg, A. Nicoletti, H. Wigzell, and G. K. Hansson. 2001. Chlamydia pneumoniae infection does not induce or modify atherosclerosis in mice. *Circulation* 103:2834-2838.
25. Aalto-Setälä, K., K. Laitinen, L. Erkkilä, M. Leinonen, M. Jauhiainen, C. Ehnholm, M. Tamminen, M. Puolakkainen, I. Penttilä, and P. Saikku. 2001. Chlamydia pneumoniae does not increase atherosclerosis in the aortic root of apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 21:578-584.
26. Watson, C., and N. J. Alp. 2008. Role of Chlamydia pneumoniae in atherosclerosis. *Clin Sci (Lond)* 114:509-531.
27. Wyrick, P. B. 2000. Intracellular survival by Chlamydia. *Cellular Microbiology* 2:275-282.
28. Morrison, R. P. 2003. New insights into a persistent problem -- chlamydial infections. *J Clin Invest* 111:1647-1649.
29. Beatty, W., R. Morrison, and G. Byrne. 1994. Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis. *Microbiol. Rev.* 58:686-699.
30. Rottenberg, M. E., A. Gigliotti-Rothfuchs, and H. Wigzell. 2002. The role of IFN-gamma in the outcome of chlamydial infection. *Curr Opin Immunol* 14:444-451.
31. Kuo, C. C. 1999. Pathologic manifestation of Chlamydial infection. *Am Heart J* 138:S496-499.
32. Rottenberg, M. E., A. C. Gigliotti Rothfuchs, D. Gigliotti, C. Svanholm, L. Bandholtz, and H. Wigzell. 1999. Role of innate and adaptive immunity in the outcome of primary infection with Chlamydia pneumoniae, as analyzed in genetically modified mice. *J Immunol* 162:2829-2836.
33. Rottenberg, M. E., A. Gigliotti Rothfuchs, D. Gigliotti, M. Ceausu, C. Une, V. Levitsky, and H. Wigzell. 2000. Regulation and role of IFN-gamma in the

- innate resistance to infection with *Chlamydia pneumoniae*. *J Immunol* 164:4812-4818.
34. Rothfuchs, A. G., M. R. Kreuger, H. Wigzell, and M. E. Rottenberg. 2004. Macrophages, CD4+ or CD8+ cells are each sufficient for protection against *Chlamydia pneumoniae* infection through their ability to secrete IFN-gamma. *J Immunol* 172:2407-2415.
  35. Rasmussen, S. J., L. Eckmann, A. J. Quayle, L. Shen, Y. X. Zhang, D. J. Anderson, J. Fierer, R. S. Stephens, and M. F. Kagnoff. 1997. Secretion of proinflammatory cytokines by epithelial cells in response to *Chlamydia* infection suggests a central role for epithelial cells in chlamydial pathogenesis. *J Clin Invest* 99:77-87.
  36. Loomis, W. P., and M. N. Starnbach. 2002. T cell responses to *Chlamydia trachomatis*. *Curr Opin Microbiol* 5:87-91.
  37. Bain, D. L., T. Lietman, S. Rasmussen, S. Kalman, J. Fan, C. Lammel, J. Z. Zhang, C. R. Dawson, J. Schachter, and R. S. Stephens. 2001. Chlamydial genovar distribution after community wide antibiotic treatment. *J Infect Dis* 184:1581-1588.
  38. Yang, Z. P., C. C. Kuo, and J. T. Grayston. 1993. A mouse model of *Chlamydia pneumoniae* strain TWAR pneumonitis. *Infection & Immunity* 61:2037-2040.
  39. Kaukoranta-Tolvanen, S. S., A. L. Laurila, P. Saikku, M. Leinonen, L. Liesirova, and K. Laitinen. 1993. Experimental infection of *Chlamydia pneumoniae* in mice. *Microb Pathog* 15:293-302.
  40. Ekman, M. R., J. T. Grayston, R. Visakorpi, M. Kleemola, C. C. Kuo, and P. Saikku. 1993. An epidemic of infections due to *Chlamydia pneumoniae* in military conscripts. *Clin Infect Dis* 17:420-425.
  41. Vazquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft. 2001. *Listeria* pathogenesis and molecular virulence determinants. *Clin Microbiol Rev* 14:584-640.
  42. Wing, E. J., and S. H. Gregory. 2002. *Listeria monocytogenes*: clinical and experimental update. *J Infect Dis* 185 Suppl 1:S18-24.
  43. Jin, Y., L. Dons, K. Kristensson, and M. E. Rottenberg. 2001. Neural route of cerebral *Listeria monocytogenes* murine infection: role of immune response mechanisms in controlling bacterial neuroinvasion. *Infect Immun* 69:1093-1100.
  44. Dons, L., Y. Jin, K. Kristensson, and M. E. Rottenberg. 2007. Axonal transport of *Listeria monocytogenes* and nerve-cell-induced bacterial killing. *J Neurosci Res* 85:2529-2537.
  45. Cossart, P., J. Pizarro-Cerda, and M. Lecuit. 2003. Invasion of mammalian cells by *Listeria monocytogenes*: functional mimicry to subvert cellular functions. *Trends Cell Biol* 13:23-31.
  46. Kuhn, M., and W. Goebel. 2000. Internalization of *Listeria monocytogenes* by nonprofessional and professional phagocytes. *Subcell Biochem* 33:411-436.
  47. Ishiguro, T., M. Naito, T. Yamamoto, G. Hasegawa, F. Gejyo, M. Mitsuyama, H. Suzuki, and T. Kodama. 2001. Role of macrophage scavenger receptors in response to *Listeria monocytogenes* infection in mice. *Am J Pathol* 158:179-188.
  48. Cossart, P., and H. Bierne. 2001. The use of host cell machinery in the pathogenesis of *Listeria monocytogenes*. *Curr Opin Immunol* 13:96-103.

49. Tilney, L. G., and D. A. Portnoy. 1989. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. *J Cell Biol* 109:1597-1608.
50. Unanue, E. R. 1997. Studies in listeriosis show the strong symbiosis between the innate cellular system and the T-cell response. *Immunol Rev* 158:11-25.
51. Zenewicz, L. A., and H. Shen. 2007. Innate and adaptive immune responses to *Listeria monocytogenes*: a short overview. *Microbes Infect* 9:1208-1215.
52. Lecuit, M., S. Dramsi, C. Gottardi, M. Fedor-Chaiken, B. Gumbiner, and P. Cossart. 1999. A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes*. *Embo J* 18:3956-3963.
53. Janeway, C. A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu Rev Immunol* 20:197-216.
54. Delbridge, L. M., and M. X. O'Riordan. 2007. Innate recognition of intracellular bacteria. *Curr Opin Immunol* 19:10-16.
55. Ismail, N., J. P. Olano, H. M. Feng, and D. H. Walker. 2002. Current status of immune mechanisms of killing of intracellular microorganisms. *FEMS Microbiol Lett* 207:111-120.
56. Hiemstra, P. S. 2007. The role of epithelial beta-defensins and cathelicidins in host defense of the lung. *Exp Lung Res* 33:537-542.
57. Mescher, M. F., J. M. Curtsinger, P. Agarwal, K. A. Casey, M. Gerner, C. D. Hammerbeck, F. Popescu, and Z. Xiao. 2006. Signals required for programming effector and memory development by CD8<sup>+</sup> T cells. *Immunol Rev* 211:81-92.
58. Rodriguez, A., M. Rottenberg, A. Tjarnlund, and C. Fernandez. 2006. Immunoglobulin A and CD8 T-cell mucosal immune defenses protect against intranasal infection with *Chlamydia pneumoniae*. *Scand J Immunol* 63:177-183.
59. Bhardwaj, V., O. Kanagawa, P. E. Swanson, and E. R. Unanue. 1998. Chronic *Listeria* infection in SCID mice: requirements for the carrier state and the dual role of T cells in transferring protection or suppression. *J Immunol* 160:376-384.
60. Loomis, W. P., and M. N. Starnbach. 2006. *Chlamydia trachomatis* infection alters the development of memory CD8<sup>+</sup> T cells. *J Immunol* 177:4021-4027.
61. Belkaid, Y., and B. T. Rouse. 2005. Natural regulatory T cells in infectious disease. *Nat Immunol* 6:353-360.
62. Matsuzaki, G., and M. Umemura. 2007. Interleukin-17 as an effector molecule of innate and acquired immunity against infections. *Microbiol Immunol* 51:1139-1147.
63. Wang, S., Y. Fan, R. C. Brunham, and X. Yang. 1999. IFN-gamma knockout mice show Th2-associated delayed-type hypersensitivity and the inflammatory cells fail to localize and control chlamydial infection. *Eur J Immunol* 29:3782-3792.
64. Harty, J. T., and V. P. Badovinac. 2002. Influence of effector molecules on the CD8(+) T cell response to infection. *Curr Opin Immunol* 14:360-365.
65. Kagi, D., B. Ledermann, K. Burki, H. Hengartner, and R. M. Zinkernagel. 1994. CD8<sup>+</sup> T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity. *Eur J Immunol* 24:3068-3072.
66. Pluddemann, A., S. Mukhopadhyay, and S. Gordon. 2006. The interaction of macrophage receptors with bacterial ligands. *Expert Rev Mol Med* 8:1-25.

67. Trinchieri, G., and A. Sher. 2007. Cooperation of Toll-like receptor signals in innate immune defence. *Nat Rev Immunol* 7:179-190.
68. Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124:783-801.
69. Akira, S., and K. Takeda. 2004. Toll-like receptor signalling. *Nat Rev Immunol* 4:499-511.
70. Kawai, T., and S. Akira. 2007. TLR signaling. *Semin Immunol* 19:24-32.
71. McCoy, C. E., and L. A. O'Neill. 2008. The role of toll-like receptors in macrophages. *Front Biosci* 13:62-70.
72. Lu, Y. C., W. C. Yeh, and P. S. Ohashi. 2008. LPS/TLR4 signal transduction pathway. *Cytokine* 42:145-151.
73. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11:115-122.
74. Suzuki, N., S. Suzuki, G. S. Duncan, D. G. Millar, T. Wada, C. Mirtsos, H. Takada, A. Wakeham, A. Itie, S. Li, J. M. Penninger, H. Wesche, P. S. Ohashi, T. W. Mak, and W. C. Yeh. 2002. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. *Nature* 416:750-756.
75. Suzuki, N., S. Suzuki, U. Eriksson, H. Hara, C. Mirtosis, N. J. Chen, T. Wada, D. Bouchard, I. Hwang, K. Takeda, T. Fujita, S. Der, J. M. Penninger, S. Akira, T. Saito, and W. C. Yeh. 2003. IL-1R-associated kinase 4 is required for lipopolysaccharide-induced activation of APC. *J Immunol* 171:6065-6071.
76. Lomaga, M. A., W. C. Yeh, I. Sarosi, G. S. Duncan, C. Furlonger, A. Ho, S. Morony, C. Capparelli, G. Van, S. Kaufman, A. van der Heiden, A. Itie, A. Wakeham, W. Khoo, T. Sasaki, Z. Cao, J. M. Penninger, C. J. Paige, D. L. Lacey, C. R. Dunstan, W. J. Boyle, D. V. Goeddel, and T. W. Mak. 1999. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev* 13:1015-1024.
77. Gohda, J., T. Matsumura, and J. Inoue. 2004. Cutting edge: TNFR-associated factor (TRAF) 6 is essential for MyD88-dependent pathway but not toll/IL-1 receptor domain-containing adaptor-inducing IFN-beta (TRIF)-dependent pathway in TLR signaling. *J Immunol* 173:2913-2917.
78. Li, Q., and I. M. Verma. 2002. NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2:725-734.
79. Chang, L., and M. Karin. 2001. Mammalian MAP kinase signalling cascades. *Nature* 410:37-40.
80. Kobayashi, T., M. C. Walsh, and Y. Choi. 2004. The role of TRAF6 in signal transduction and the immune response. *Microbes Infect* 6:1333-1338.
81. Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003. Role of Adaptor TRIF in the MyD88-Independent Toll-Like Receptor Signaling Pathway. *Science* 301:640-643.
82. Sato, S., M. Sugiyama, M. Yamamoto, Y. Watanabe, T. Kawai, K. Takeda, and S. Akira. 2003. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. *J Immunol* 171:4304-4310.



83. Kawai, T., O. Takeuchi, T. Fujita, J. Inoue, P. F. Muhlrad, S. Sato, K. Hoshino, and S. Akira. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol* 167:5887-5894.
84. Kaisho, T., O. Takeuchi, T. Kawai, K. Hoshino, and S. Akira. 2001. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J Immunol* 166:5688-5694.
85. Hemmi, H., O. Takeuchi, S. Sato, M. Yamamoto, T. Kaisho, H. Sanjo, T. Kawai, K. Hoshino, K. Takeda, and S. Akira. 2004. The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. *J Exp Med* 199:1641-1650.
86. Sharma, S., B. R. tenOever, N. Grandvaux, G. P. Zhou, R. Lin, and J. Hiscott. 2003. Triggering the interferon antiviral response through an IKK-related pathway. *Science* 300:1148-1151.
87. Weiss, D. S., B. Raupach, K. Takeda, S. Akira, and A. Zychlinsky. 2004. Toll-like receptors are temporally involved in host defense. *J Immunol* 172:4463-4469.
88. Takeuchi, O., K. Hoshino, and S. Akira. 2000. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. *J Immunol* 165:5392-5396.
89. Albiger, B., A. Sandgren, H. Katsuragi, U. Meyer-Hoffert, K. Beiter, F. Wartha, M. Hornef, S. Normark, and B. H. Normark. 2005. Myeloid differentiation factor 88-dependent signalling controls bacterial growth during colonization and systemic pneumococcal disease in mice. *Cell Microbiol* 7:1603-1615.
90. Feng, C. G., C. A. Scanga, C. M. Collazo-Custodio, A. W. Cheever, S. Hieny, P. Caspar, and A. Sher. 2003. Mice lacking myeloid differentiation factor 88 display profound defects in host resistance and immune responses to Mycobacterium avium infection not exhibited by Toll-like receptor 2 (TLR2)- and TLR4-deficient animals. *J Immunol* 171:4758-4764.
91. Nicolle, D. M., X. Pichon, A. Bouchot, I. Maillet, F. Erard, S. Akira, B. Ryffel, and V. F. Quesniaux. 2004. Chronic pneumonia despite adaptive immune response to Mycobacterium bovis BCG in MyD88-deficient mice. *Lab Invest* 84:1305-1321.
92. Scanga, C. A., A. Bafica, C. G. Feng, A. W. Cheever, S. Hieny, and A. Sher. 2004. MyD88-deficient mice display a profound loss in resistance to Mycobacterium tuberculosis associated with partially impaired Th1 cytokine and nitric oxide synthase 2 expression. *Infect Immun* 72:2400-2404.
93. Fremont, C. M., V. Yermeev, D. M. Nicolle, M. Jacobs, V. F. Quesniaux, and B. Ryffel. 2004. Fatal Mycobacterium tuberculosis infection despite adaptive immune response in the absence of MyD88. *J Clin Invest* 114:1790-1799.
94. Plant, L., H. Wan, and A. B. Jonsson. 2006. MyD88-dependent signaling affects the development of meningococcal sepsis by nonlipooligosaccharide ligands. *Infect Immun* 74:3538-3546.
95. Ku, C. L., H. von Bernuth, C. Picard, S. Y. Zhang, H. H. Chang, K. Yang, M. Chrabieh, A. C. Issekutz, C. K. Cunningham, J. Gallin, S. M. Holland, C. Roifman, S. Ehl, J. Smart, M. Tang, F. J. Barrat, O. Levy, D. McDonald, N. K. Day-Good, R. Miller, H. Takada, T. Hara, S. Al-Hajjar, A. Al-Ghoniaim, D.

- Speert, D. Sanlaville, X. Li, F. Geissmann, E. Vivier, L. Marodi, B. Z. Garty, H. Chapel, C. Rodriguez-Gallego, X. Bossuyt, L. Abel, A. Puel, and J. L. Casanova. 2007. Selective predisposition to bacterial infections in IRAK-4-deficient children: IRAK-4-dependent TLRs are otherwise redundant in protective immunity. *J Exp Med* 204:2407-2422.
96. von Bernuth, H., C. Picard, Z. Jin, R. Pankla, H. Xiao, C. L. Ku, M. Chrabieh, I. B. Mustapha, P. Ghandil, Y. Camcioglu, J. Vasconcelos, N. Sirvent, M. Guedes, A. B. Vitor, M. J. Herrero-Mata, J. I. Arostegui, C. Rodrigo, L. Alsina, E. Ruiz-Ortiz, M. Juan, C. Fortuny, J. Yague, J. Anton, M. Pascal, H. H. Chang, L. Janniere, Y. Rose, B. Z. Garty, H. Chapel, A. Issekutz, L. Marodi, C. Rodriguez-Gallego, J. Banchereau, L. Abel, X. Li, D. Chaussabel, A. Puel, and J. L. Casanova. 2008. Pyogenic bacterial infections in humans with MyD88 deficiency. *Science* 321:691-696.
97. Naiki, Y., K. S. Michelsen, N. W. Schroder, R. Alsabeh, A. Slepkin, W. Zhang, S. Chen, B. Wei, Y. Bulut, E. M. Peterson, and A. Moshe. 2005. MyD88 is pivotal for the early inflammatory response and subsequent bacterial clearance and survival in a mouse model of Chlamydia pneumoniae pneumonia. *J Biol Chem*.
98. Netea, M. G., B. J. Kullberg, L. E. Jacobs, T. J. Verver-Jansen, J. van der Ven-Jongekrijg, J. M. Galama, A. F. Stalenhoef, C. A. Dinarello, and J. W. Van der Meer. 2004. Chlamydia pneumoniae stimulates IFN-gamma synthesis through MyD88-dependent, TLR2- and TLR4-independent induction of IL-18 release. *J Immunol* 173:1477-1482.
99. Netea, M. G., B. J. Kullberg, J. M. Galama, A. F. Stalenhoef, C. A. Dinarello, and J. W. Van der Meer. 2002. Non-LPS components of Chlamydia pneumoniae stimulate cytokine production through Toll-like receptor 2-dependent pathways. *Eur J Immunol* 32:1188-1195.
100. Bulut, Y., E. Faure, L. Thomas, H. Karahashi, K. S. Michelsen, O. Equils, S. G. Morrison, R. P. Morrison, and M. Arditi. 2002. Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway. *J Immunol* 168:1435-1440.
101. Sasu, S., D. LaVerda, N. Qureshi, D. T. Golenbock, and D. Beasley. 2001. Chlamydia pneumoniae and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. *Circ Res* 89:244-250.
102. Vabulas, R. M., P. Ahmad-Nejad, C. da Costa, T. Miethke, C. J. Kirschning, H. Hacker, and H. Wagner. 2001. Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. *J Biol Chem* 276:31332-31339.
103. Prebeck, S., C. Kirschning, S. Durr, C. da Costa, B. Donath, K. Brand, V. Redecke, H. Wagner, and T. Miethke. 2001. Predominant role of toll-like receptor 2 versus 4 in Chlamydia pneumoniae-induced activation of dendritic cells. *J Immunol* 167:3316-3323.
104. Da Costa, C. U., N. Wantia, C. J. Kirschning, D. H. Busch, N. Rodriguez, H. Wagner, and T. Miethke. 2004. Heat shock protein 60 from Chlamydia pneumoniae elicits an unusual set of inflammatory responses via Toll-like receptor 2 and 4 in vivo. *Eur J Immunol* 34:2874-2884.
105. Mueller, M., S. Postius, J. G. Thimm, K. Gueinzius, I. Muehldorfer, and C. Hermann. 2004. Toll-like receptors 2 and 4 do not contribute to clearance of

- Chlamydomonas pneumoniae* in mice, but are necessary for the release of monokines. *Immunobiology* 209:599-608.
106. Rodriguez, N., N. Wantia, F. Fend, S. Durr, H. Wagner, and T. Miethke. 2006. Differential involvement of TLR2 and TLR4 in host survival during pulmonary infection with *Chlamydia pneumoniae*. *Eur J Immunol* 36:1145-1155.
  107. Rodriguez, N., F. Fend, L. Jennen, M. Schiemann, N. Wantia, C. U. Prazeres da Costa, S. Durr, U. Heinzmann, H. Wagner, and T. Miethke. 2005. Polymorphonuclear neutrophils improve replication of *Chlamydia pneumoniae* in vivo upon MyD88-dependent attraction. *J Immunol* 174:4836-4844.
  108. Ingalls, R. R., P. A. Rice, N. Qureshi, K. Takayama, J. S. Lin, and D. T. Golenbock. 1995. The inflammatory cytokine response to *Chlamydia trachomatis* infection is endotoxin mediated. *Infect Immun* 63:3125-3130.
  109. Costa, C. P., C. J. Kirschning, D. Busch, S. Durr, L. Jennen, U. Heinzmann, S. Prebeck, H. Wagner, and T. Miethke. 2002. Role of chlamydial heat shock protein 60 in the stimulation of innate immune cells by *Chlamydia pneumoniae*. *Eur J Immunol* 32:2460-2470.
  110. Chopra, I., C. Storey, T. J. Falla, and J. H. Pearce. 1998. Antibiotics, peptidoglycan synthesis and genomics: the chlamydial anomaly revisited. *Microbiology* 144 ( Pt 10):2673-2678.
  111. Nagarajan, U. M., D. M. Ojcius, L. Stahl, R. G. Rank, and T. Darville. 2005. *Chlamydia trachomatis* Induces Expression of IFN- $\gamma$ -Inducible Protein 10 and IFN- $\beta$  Independent of TLR2 and TLR4, but Largely Dependent on MyD88. *J Immunol* 175:450-460.
  112. Derbigny, W. A., S. C. Hong, M. S. Kerr, M. Temkit, and R. M. Johnson. 2007. *Chlamydia muridarum* infection elicits a beta interferon response in murine oviduct epithelial cells dependent on interferon regulatory factor 3 and TRIF. *Infect Immun* 75:1280-1290.
  113. Seki, E., H. Tsutsui, N. M. Tsuji, N. Hayashi, K. Adachi, H. Nakano, S. Futatsugi-Yumikura, O. Takeuchi, K. Hoshino, S. Akira, J. Fujimoto, and K. Nakanishi. 2002. Critical roles of myeloid differentiation factor 88-dependent proinflammatory cytokine release in early phase clearance of *Listeria monocytogenes* in mice. *J Immunol* 169:3863-3868.
  114. Edelson, B. T., and E. R. Unanue. 2002. MyD88-dependent but Toll-like receptor 2-independent innate immunity to *Listeria*: no role for either in macrophage listericidal activity. *J Immunol* 169:3869-3875.
  115. Ozoren, N., J. Masumoto, L. Franchi, T. D. Kanneganti, M. Body-Malapel, I. Erturk, R. Jagirdar, L. Zhu, N. Inohara, J. Bertin, A. Coyle, E. P. Grant, and G. Nunez. 2006. Distinct roles of TLR2 and the adaptor ASC in IL-1 $\beta$ /IL-18 secretion in response to *Listeria monocytogenes*. *J Immunol* 176:4337-4342.
  116. Hauf, N., W. Goebel, F. Fiedler, Z. Sokolovic, and M. Kuhn. 1997. *Listeria monocytogenes* infection of P388D1 macrophages results in a biphasic NF- $\kappa$ B (RelA/p50) activation induced by lipoteichoic acid and bacterial phospholipases and mediated by IkappaB $\alpha$  and IkappaB $\beta$  degradation. *Proc Natl Acad Sci U S A* 94:9394-9399.
  117. Stockinger, S., T. Materna, D. Stoiber, L. Bayr, R. Steinborn, T. Kolbe, H. Unger, T. Chakraborty, D. E. Levy, M. Muller, and T. Decker. 2002. Production of type I IFN sensitizes macrophages to cell death induced by *Listeria monocytogenes*. *J Immunol* 169:6522-6529.

118. Stockinger, S., B. Reutterer, B. Schaljo, C. Schellack, S. Brunner, T. Materna, M. Yamamoto, S. Akira, T. Taniguchi, P. J. Murray, M. Muller, and T. Decker. 2004. IFN regulatory factor 3-dependent induction of type I IFNs by intracellular bacteria is mediated by a TLR- and Nod2-independent mechanism. *J Immunol* 173:7416-7425.
119. O'Riordan, M., C. H. Yi, R. Gonzales, K. D. Lee, and D. A. Portnoy. 2002. Innate recognition of bacteria by a macrophage cytosolic surveillance pathway. *Proc Natl Acad Sci U S A* 99:13861-13866.
120. McCaffrey, R. L., P. Fawcett, M. O'Riordan, K. D. Lee, E. A. Havell, P. O. Brown, and D. A. Portnoy. 2004. A specific gene expression program triggered by Gram-positive bacteria in the cytosol. *Proc Natl Acad Sci U S A* 101:11386-11391.
121. Leber, J. H., G. T. Crimmins, S. Raghavan, N. P. Meyer-Morse, J. S. Cox, and D. A. Portnoy. 2008. Distinct TLR- and NLR-mediated transcriptional responses to an intracellular pathogen. *PLoS Pathog* 4:e6.
122. Carneiro, L. A., L. H. Travassos, and S. E. Girardin. 2007. Nod-like receptors in innate immunity and inflammatory diseases. *Ann Med* 39:581-593.
123. Mariathasan, S., D. S. Weiss, K. Newton, J. McBride, K. O'Rourke, M. Roose-Girma, W. P. Lee, Y. Weinrauch, D. M. Monack, and V. M. Dixit. 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440:228-232.
124. Suzuki, T., L. Franchi, C. Toma, H. Ashida, M. Ogawa, Y. Yoshikawa, H. Mimuro, N. Inohara, C. Sasakawa, and G. Nunez. 2007. Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigella-infected macrophages. *PLoS Pathog* 3:e111.
125. Girardin, S. E., I. G. Boneca, L. A. Carneiro, A. Antignac, M. Jehanno, J. Viala, K. Tedin, M. K. Taha, A. Labigne, U. Zathringer, A. J. Coyle, P. S. DiStefano, J. Bertin, P. J. Sansonetti, and D. J. Philpott. 2003. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 300:1584-1587.
126. Chamaillard, M., M. Hashimoto, Y. Horie, J. Masumoto, S. Qiu, L. Saab, Y. Ogura, A. Kawasaki, K. Fukase, S. Kusumoto, M. A. Valvano, S. J. Foster, T. W. Mak, G. Nunez, and N. Inohara. 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol* 4:702-707.
127. Park, J. H., Y. G. Kim, C. McDonald, T. D. Kanneganti, M. Hasegawa, M. Body-Malapel, N. Inohara, and G. Nunez. 2007. RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs. *J Immunol* 178:2380-2386.
128. Inohara, N., T. Koseki, J. Lin, L. del Peso, P. C. Lucas, F. F. Chen, Y. Ogura, and G. Nunez. 2000. An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways. *J Biol Chem* 275:27823-27831.
129. Park, J. H., Y. G. Kim, M. Shaw, T. D. Kanneganti, Y. Fujimoto, K. Fukase, N. Inohara, and G. Nunez. 2007. Nod1/RICK and TLR signaling regulate chemokine and antimicrobial innate immune responses in mesothelial cells. *J Immunol* 179:514-521.
130. Werts, C., L. le Bourhis, J. Liu, J. G. Magalhaes, L. A. Carneiro, J. H. Fritz, S. Stockinger, V. Balloy, M. Chignard, T. Decker, D. J. Philpott, X. Ma, and S.

- E. Girardin. 2007. Nod1 and Nod2 induce CCL5/RANTES through the NF-kappaB pathway. *Eur J Immunol* 37:2499-2508.
131. Masumoto, J., K. Yang, S. Varambally, M. Hasegawa, S. A. Tomlins, S. Qiu, Y. Fujimoto, A. Kawasaki, S. J. Foster, Y. Horie, T. W. Mak, G. Nunez, A. M. Chinnaiyan, K. Fukase, and N. Inohara. 2006. Nod1 acts as an intracellular receptor to stimulate chemokine production and neutrophil recruitment in vivo. *J Exp Med* 203:203-213.
  132. Uehara, A., S. Yang, Y. Fujimoto, K. Fukase, S. Kusumoto, K. Shibata, S. Sugawara, and H. Takada. 2005. Muramyl dipeptide and diaminopimelic acid-containing desmuramyl peptides in combination with chemically synthesized Toll-like receptor agonists synergistically induced production of interleukin-8 in a NOD2- and NOD1-dependent manner, respectively, in human monocytic cells in culture. *Cell Microbiol* 7:53-61.
  133. Netea, M. G., G. Ferwerda, D. J. de Jong, T. Jansen, L. Jacobs, M. Kramer, T. H. Naber, J. P. Drenth, S. E. Girardin, B. Jan Kullberg, G. J. Adema, and J. W. Van der Meer. 2005. Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release. *J Immunol* 174:6518-6523.
  134. van Heel, D. A., S. Ghosh, M. Butler, K. Hunt, B. M. Foxwell, D. Mengin-Lecreulx, and R. J. Playford. 2005. Synergistic enhancement of Toll-like receptor responses by NOD1 activation. *Eur J Immunol*.
  135. Kobayashi, K., N. Inohara, L. D. Hernandez, J. E. Galan, G. Nunez, C. A. Janeway, R. Medzhitov, and R. A. Flavell. 2002. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 416:194-199.
  136. Abreu, M. T., M. Fukata, and M. Arditi. 2005. TLR signaling in the gut in health and disease. *J Immunol* 174:4453-4460.
  137. Inohara, Chamaillard, C. McDonald, and G. Nunez. 2005. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu Rev Biochem* 74:355-383.
  138. Gutierrez, O., C. Pipaon, N. Inohara, A. Fontalba, Y. Ogura, F. Prosper, G. Nunez, and J. L. Fernandez-Luna. 2002. Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. *J Biol Chem* 277:41701-41705.
  139. Kim, J. G., S. J. Lee, and M. F. Kagnoff. 2004. Nod1 is an essential signal transducer in intestinal epithelial cells infected with bacteria that avoid recognition by toll-like receptors. *Infect Immun* 72:1487-1495.
  140. Girardin, S. E., R. Tournibize, M. Mavris, A. L. Page, X. Li, G. R. Stark, J. Bertin, P. S. DiStefano, M. Yaniv, P. J. Sansonetti, and D. J. Philpott. 2001. CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive *Shigella flexneri*. *EMBO Rep* 2:736-742.
  141. Opitz, B., S. Forster, A. C. Hocke, M. Maass, B. Schmeck, S. Hippenstiel, N. Suttorp, and M. Krull. 2005. Nod1-mediated endothelial cell activation by *Chlamydomydia pneumoniae*. *Circ Res* 96:319-326.
  142. Travassos, L. H., L. A. M. Carneiro, S. E. Girardin, I. G. Boneca, R. Lemos, M. T. Bozza, R. C. P. Domingues, A. J. Coyle, J. Bertin, D. J. Philpott, and M. C. Plotkowski. 2005. Nod1 Participates in the Innate Immune Response to *Pseudomonas aeruginosa*. *J. Biol. Chem.* %R 10.1074/jbc.M501649200 280:36714-36718.

143. Opitz, B., A. Puschel, W. Beermann, A. C. Hocke, S. Forster, B. Schmeck, V. van Laak, T. Chakraborty, N. Suttorp, and S. Hippenstiel. 2006. *Listeria monocytogenes* activated p38 MAPK and induced IL-8 secretion in a nucleotide-binding oligomerization domain 1-dependent manner in endothelial cells. *J Immunol* 176:484-490.
144. Viala, J., C. Chaput, I. G. Boneca, A. Cardona, S. E. Girardin, A. P. Moran, R. Athman, S. Memet, M. R. Huerre, A. J. Coyle, P. S. DiStefano, P. J. Sansonetti, A. Labigne, J. Bertin, D. J. Philpott, and R. L. Ferrero. 2004. Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. *Nat Immunol* 5:1166-1174.
145. Kobayashi, K. S., M. Chamaillard, Y. Ogura, O. Henegariu, N. Inohara, G. Nunez, and R. A. Flavell. 2005. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 307:731-734.
146. Stetson, D. B., and R. Medzhitov. 2006. Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity* 24:93-103.
147. Chin, A. I., P. W. Dempsey, K. Bruhn, J. F. Miller, Y. Xu, and G. Cheng. 2002. Involvement of receptor-interacting protein 2 in innate and adaptive immune responses. *Nature* 416:190-194.
148. Kim, Y.-G., J.-H. Park, M. H. Shaw, L. Franchi, N. Inohara, and G. N' Oez. 2008. The Cytosolic Sensors Nod1 and Nod2 Are Critical for Bacterial Recognition and Host Defense after Exposure to Toll-like Receptor Ligands. *Immunity* 28:246-257.
149. Dobrovolskaia, M. A., and S. N. Vogel. 2002. Toll receptors, CD14, and macrophage activation and deactivation by LPS. *Microbes Infect* 4:903-914.
150. Honda, K., and T. Taniguchi. 2006. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol* 6:644-658.
151. Takaoka, A., Z. Wang, M. K. Choi, H. Yanai, H. Negishi, T. Ban, Y. Lu, M. Miyagishi, T. Kodama, K. Honda, Y. Ohba, and T. Taniguchi. 2007. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* 448:501-505.
152. O'Connell, R. M., S. A. Vaidya, A. K. Perry, S. K. Saha, P. W. Dempsey, and G. Cheng. 2005. Immune activation of type I IFNs by *Listeria monocytogenes* occurs independently of TLR4, TLR2, and receptor interacting protein 2 but involves TNFR-associated NF kappa B kinase-binding kinase 1. *J Immunol* 174:1602-1607.
153. Soulat, D., A. Bauch, S. Stockinger, G. Superti-Furga, and T. Decker. 2006. Cytoplasmic *Listeria monocytogenes* stimulates IFN-beta synthesis without requiring the adapter protein MAVS. *FEBS Lett* 580:2341-2346.
154. Isaacs, A., and J. Lindenmann. 1957. Virus interference. I. The interferon. *Proc R Soc Lond B Biol Sci* 147:258-267.
155. Hardy, M. P., C. M. Owczarek, L. S. Jermin, M. Ejdeback, and P. J. Hertzog. 2004. Characterization of the type I interferon locus and identification of novel genes. *Genomics* 84:331-345.
156. Ank, N., H. West, C. Bartholdy, K. Eriksson, A. R. Thomsen, and S. R. Paludan. 2006. Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. *J Virol* 80:4501-4509.

157. Onoguchi, K., M. Yoneyama, A. Takemura, S. Akira, T. Taniguchi, H. Namiki, and T. Fujita. 2007. Viral infections activate types I and III interferon genes through a common mechanism. *J Biol Chem* 282:7576-7581.
158. Pestka, S., C. D. Krause, and M. R. Walter. 2004. Interferons, interferon-like cytokines, and their receptors. *Immunol Rev* 202:8-32.
159. Decker, T., M. Muller, and S. Stockinger. 2005. The yin and yang of type I interferon activity in bacterial infection. *Nat Rev Immunol* 5:675-687.
160. Baig, E., and E. N. Fish. 2008. Distinct signature type I interferon responses are determined by the infecting virus and the target cell. *Antivir Ther* 13:409-422.
161. Le Bon, A., and D. F. Tough. 2002. Links between innate and adaptive immunity via type I interferon. *Curr Opin Immunol* 14:432-436.
162. Bracci, L., V. La Sorsa, F. Belardelli, and E. Proietti. 2008. Type I interferons as vaccine adjuvants against infectious diseases and cancer. *Expert Rev Vaccines* 7:373-381.
163. Bogdan, C. 2000. The function of type I interferons in antimicrobial immunity. *Current Opinion in Immunology* 12:419-424.
164. Kadowaki, N., and Y. J. Liu. 2002. Natural type I interferon-producing cells as a link between innate and adaptive immunity. *Hum Immunol* 63:1126-1132.
165. Bogdan, C., J. Mattner, and U. Schleicher. 2004. The role of type I interferons in non-viral infections. *Immunological Reviews* 202:33-48.
166. Mancuso, G., A. Midiri, C. Biondo, C. Beninati, S. Zummo, R. Galbo, F. Tomasello, M. Gambuzza, G. Macri, A. Ruggeri, T. Leanderson, and G. Teti. 2007. Type I IFN signaling is crucial for host resistance against different species of pathogenic bacteria. *J Immunol* 178:3126-3133.
167. Bukholm, G., B. P. Berdal, C. Haug, and M. Degre. 1984. Mouse fibroblast interferon modifies *Salmonella typhimurium* infection in infant mice. *Infect Immun* 45:62-66.
168. Auerbuch, V., D. G. Brockstedt, N. Meyer-Morse, M. O'Riordan, and D. A. Portnoy. 2004. Mice lacking the type I interferon receptor are resistant to *Listeria monocytogenes*. *J Exp Med* 200:527-533.
169. Qiu, H., Y. Fan, A. G. Joyee, S. Wang, X. Han, H. Bai, L. Jiao, N. Van Rooijen, and X. Yang. 2008. Type I IFNs enhance susceptibility to *Chlamydia muridarum* lung infection by enhancing apoptosis of local macrophages. *J Immunol* 181:2092-2102.
170. Nagarajan, U. M., D. Prantner, J. D. Sikes, C. W. Andrews, Jr., A. M. Goodwin, S. Nagarajan, and T. Darville. 2008. Type I IFN signaling exacerbates *Chlamydia muridarum* genital infection in a murine model. *Infect Immun*.
171. Reutterer, B., S. Stockinger, A. Pilz, D. Soulat, R. Kastner, S. Westermayer, T. Rulicke, M. Muller, and T. Decker. 2008. Type I IFN are host modulators of strain-specific *Listeria monocytogenes* virulence. *Cell Microbiol* 10:1116-1129.
172. Carrero, J. A., B. Calderon, and E. R. Unanue. 2004. Type I interferon sensitizes lymphocytes to apoptosis and reduces resistance to *Listeria* infection. *J Exp Med* 200:535-540.
173. O'Connell, R. M., S. K. Saha, S. A. Vaidya, K. W. Bruhn, G. A. Miranda, B. Zarnegar, A. K. Perry, B. O. Nguyen, T. F. Lane, T. Taniguchi, J. F. Miller, and G. Cheng. 2004. Type I interferon production enhances susceptibility to *Listeria monocytogenes* infection. *J Exp Med* 200:437-445.

174. Carrero, J. A., B. Calderon, and E. R. Unanue. 2004. Listeriolysin O from *Listeria monocytogenes* is a lymphocyte apoptogenic molecule. *J Immunol* 172:4866-4874.
175. Carrero, J. A., B. Calderon, and E. R. Unanue. 2006. Lymphocytes are detrimental during the early innate immune response against *Listeria monocytogenes*. *J Exp Med* 203:933-940.
176. Zwaferink, H., S. Stockinger, S. Reipert, and T. Decker. 2008. Stimulation of inducible nitric oxide synthase expression by beta interferon increases necrotic death of macrophages upon *Listeria monocytogenes* infection. *Infect Immun* 76:1649-1656.
177. Marriott, H. M., and D. H. Dockrell. 2007. The role of the macrophage in lung disease mediated by bacteria. *Exp Lung Res* 33:493-505.
178. Boehm, U., T. Klamp, M. Groot, and J. C. Howard. 1997. Cellular responses to interferon-gamma. *Annu Rev Immunol* 15:749-795.
179. Frucht, D. M., T. Fukao, C. Bogdan, H. Schindler, J. J. O'Shea, and S. Koyasu. 2001. IFN-gamma production by antigen-presenting cells: mechanisms emerge. *Trends Immunol* 22:556-560.
180. van den Broek, M. F., U. Muller, S. Huang, R. M. Zinkernagel, and M. Aguet. 1995. Immune defence in mice lacking type I and/or type II interferon receptors. *Immunol Rev* 148:5-18.
181. Newport, M. J., C. M. Huxley, S. Huston, C. M. Hawrylowicz, B. A. Oostra, R. Williamson, and M. Levin. 1996. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. *N Engl J Med* 335:1941-1949.
182. Dorman, S. E., and S. M. Holland. 1998. Mutation in the signal-transducing chain of the interferon-gamma receptor and susceptibility to mycobacterial infection. *J Clin Invest* 101:2364-2369.
183. Cooter, T., K. Ramsey, G. Mirampuri, C. Poulsen, and G. Byrne. 1997. Dissemination of *Chlamydia trachomatis* chronic genital tract infection in gamma interferon gene knock out mice. *Infection and Immunity* 65:2145-2152.
184. Perry, L. L., K. Feilzer, and H. D. Caldwell. 1997. Immunity to *Chlamydia trachomatis* is mediated by T helper 1 cells through IFN-gamma-dependent and -independent pathways. *J Immunol* 158:3344-3352.
185. Johansson, M., K. Schon, M. Ward, and N. Lycke. 1997. Genital tract infection with *Chlamydia trachomatis* fails to induce protective immunity in gamma interferon receptor-deficient mice despite a strong local immunoglobulin A response. *Infect Immun* 65:1032-1044.
186. Ito, J. I., and J. M. Lyons. 1999. Role of gamma interferon in controlling murine chlamydial genital tract infection. *Infect Immun* 67:5518-5521.
187. Rothfuchs, A. G., D. Gigliotti, K. Palmblad, U. Andersson, H. Wigzell, and M. E. Rottenberg. 2001. IFN-alpha beta-dependent, IFN-gamma secretion by bone marrow-derived macrophages controls an intracellular bacterial infection. *J Immunol* 167:6453-6461.
188. Harty, J. T., and M. J. Bevan. 1995. Specific immunity to *Listeria monocytogenes* in the absence of IFN gamma. *Immunity* 3:109-117.
189. Portnoy, D. A., R. D. Schreiber, P. Connelly, and L. G. Tilney. 1989. Gamma interferon limits access of *Listeria monocytogenes* to the macrophage cytoplasm. *J Exp Med* 170:2141-2146.



190. Harty, J. T., R. D. Schreiber, and M. J. Bevan. 1992. CD8 T cells can protect against an intracellular bacterium in an interferon gamma-independent fashion. *Proc Natl Acad Sci U S A* 89:11612-11616.
191. Decker, T., S. Stockinger, M. Karaghiosoff, M. Muller, and P. Kovarik. 2002. IFNs and STATs in innate immunity to microorganisms. *J. Clin. Invest.* 109:1271-1277.
192. Schindler, C., D. E. Levy, and T. Decker. 2007. JAK-STAT signaling: from interferons to cytokines. *J Biol Chem* 282:20059-20063.
193. Varinou, L., K. Ramsauer, M. Karaghiosoff, T. Kolbe, K. Pfeffer, M. Muller, and T. Decker. 2003. Phosphorylation of the Stat1 transactivation domain is required for full-fledged IFN-gamma-dependent innate immunity. *Immunity* 19:793-802.
194. Ramana, C. V., M. Chatterjee-Kishore, H. Nguyen, and G. R. Stark. 2000. Complex roles of Stat1 in regulating gene expression. *Oncogene* 19:2619-2627.
195. van Boxel-Dezaire, A. H., M. R. Rani, and G. R. Stark. 2006. Complex modulation of cell type-specific signaling in response to type I interferons. *Immunity* 25:361-372.
196. van Boxel-Dezaire, A. H., and G. R. Stark. 2007. Cell type-specific signaling in response to interferon-gamma. *Curr Top Microbiol Immunol* 316:119-154.
197. Meraz, M. A., J. M. White, K. C. Sheehan, E. A. Bach, S. J. Rodig, A. S. Dighe, D. H. Kaplan, J. K. Riley, A. C. Greenlund, D. Campbell, K. Carver-Moore, R. N. DuBois, R. Clark, M. Aguet, and R. D. Schreiber. 1996. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* 84:431-442.
198. Dupuis, S., E. Jouanguy, S. Al-Hajjar, C. Fieschi, I. Z. Al-Mohsen, S. Al-Jumaah, K. Yang, A. Chapgier, C. Eidenschenk, P. Eid, A. Al Ghonaium, H. Tufenkeji, H. Frayha, S. Al-Gazlan, H. Al-Rayes, R. D. Schreiber, I. Gresser, and J. L. Casanova. 2003. Impaired response to interferon-alpha/beta and lethal viral disease in human STAT1 deficiency. *Nat Genet* 33:388-391.
199. Dupuis, S., C. Dargemont, C. Fieschi, N. Thomassin, S. Rosenzweig, J. Harris, S. M. Holland, R. D. Schreiber, and J. L. Casanova. 2001. Impairment of mycobacterial but not viral immunity by a germline human STAT1 mutation. *Science* 293:300-303.
200. Lad, S. P., E. Y. Fukuda, J. Li, L. M. de la Maza, and E. Li. 2005. Up-regulation of the JAK/STAT1 signal pathway during Chlamydia trachomatis infection. *J Immunol* 174:7186-7193.
201. Gil, M. P., E. Bohn, A. K. O'Guin, C. V. Ramana, B. Levine, G. R. Stark, H. W. Virgin, and R. D. Schreiber. 2001. Biologic consequences of Stat1-independent IFN signaling. *Proc Natl Acad Sci U S A* 98:6680-6685.
202. Ramana, C. V., M. P. Gil, Y. Han, R. M. Ransohoff, R. D. Schreiber, and G. R. Stark. 2001. Stat1-independent regulation of gene expression in response to IFN-gamma. *Proc Natl Acad Sci U S A* 98:6674-6679.
203. Zimmerer, J. M., G. B. Lesinski, M. D. Radmacher, A. Ruppert, and W. E. Carson, 3rd. 2007. STAT1-dependent and STAT1-independent gene expression in murine immune cells following stimulation with interferon-alpha. *Cancer Immunol Immunother* 56:1845-1852.
204. Endo, T. A., M. Masuhara, M. Yokouchi, R. Suzuki, H. Sakamoto, K. Mitsui, A. Matsumoto, S. Tanimura, M. Ohtsubo, H. Misawa, T. Miyazaki, N. Leonor, T. Taniguchi, T. Fujita, Y. Kanakura, S. Komiya, and A. Yoshimura.

1997. A new protein containing an SH2 domain that inhibits JAK kinases. *Nature* 387:921-924.
205. Young, H. A., and J. H. Bream. 2007. IFN-gamma: recent advances in understanding regulation of expression, biological functions, and clinical applications. *Curr Top Microbiol Immunol* 316:97-117.
206. Schoenborn, J. R., and C. B. Wilson. 2007. Regulation of interferon-gamma during innate and adaptive immune responses. *Adv Immunol* 96:41-101.
207. Nguyen, K. B., W. T. Watford, R. Salomon, S. R. Hofmann, G. C. Pien, A. Morinobu, M. Gadina, J. J. O'Shea, and C. A. Biron. 2002. Critical Role for STAT4 Activation by Type 1 Interferons in the Interferon-gamma Response to Viral Infection. *Science* 297:2063-2066.
208. Freudenberg, M. A., T. Merlin, C. Kalis, Y. Chvatchko, H. Stubig, and C. Galanos. 2002. Cutting Edge: A Murine, IL-12-Independent Pathway of IFN- $\gamma$  Induction by Gram-Negative Bacteria Based on STAT4 Activation by Type I IFN and IL-18 Signaling. *J Immunol* 169:1665-1668.
209. Muller, U., U. Steinhoff, L. F. Reis, S. Hemmi, J. Pavlovic, R. M. Zinkernagel, and M. Aguet. 1994. Functional role of type I and type II interferons in antiviral defense. *Science* 264:1918-1921.
210. Takaoka, A., Y. Mitani, H. Suemori, M. Sato, T. Yokochi, S. Noguchi, N. Tanaka, and T. Taniguchi. 2000. Cross talk between interferon-gamma and -alpha/beta signaling components in caveolar membrane domains. *Science* 288:2357-2360.
211. Mattei, F., G. Schiavoni, F. Belardelli, and D. F. Tough. 2001. IL-15 is expressed by dendritic cells in response to type I IFN, double-stranded RNA, or lipopolysaccharide and promotes dendritic cell activation. *J Immunol* 167:1179-1187.
212. Ohteki, T., K. Suzue, C. Maki, T. Ota, and S. Koyasu. 2001. Critical role of IL-15-IL-15R for antigen-presenting cell functions in the innate immune response. *Nat Immunol* 2:1138-1143.
213. Nguyen, K., L. P. Cousens, L. A. Doughty, G. C. Pien, J. E. Durbin, and C. A. Biron. 2000. Interferon alpha/beta-mediated inhibition and promotion of interferon gamma: STAT1 resolves a paradox. *Nature Immunology* 1:70-76.
214. Sica, A., L. Dorman, V. Viggiano, M. Cippitelli, P. Ghosh, N. Rice, and H. A. Young. 1997. Interaction of NF-kappaB and NFAT with the interferon-gamma promoter. *J Biol Chem* 272:30412-30420.
215. Kojima, H., Y. Aizawa, Y. Yanai, K. Nagaoka, M. Takeuchi, T. Ohta, H. Ikegami, M. Ikeda, and M. Kurimoto. 1999. An essential role for NF-kappa B in IL-18-induced IFN-gamma expression in KG-1 cells. *J Immunol* 162:5063-5069.
216. Shirey, K. A., J. Y. Jung, G. S. Maeder, and J. M. Carlin. 2006. Upregulation of IFN-gamma receptor expression by proinflammatory cytokines influences IDO activation in epithelial cells. *J Interferon Cytokine Res* 26:53-62.
217. Maniatis, T., J. V. Falvo, T. H. Kim, T. K. Kim, C. H. Lin, B. S. Parekh, and M. G. Wathlet. 1998. Structure and function of the interferon-beta enhanceosome. *Cold Spring Harb Symp Quant Biol* 63:609-620.
218. Doyle, S., S. Vaidya, R. O'Connell, H. Dadgostar, P. Dempsey, T. Wu, G. Rao, R. Sun, M. Haberland, R. Modlin, and G. Cheng. 2002. IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* 17:251-263.

219. Tamura, T., H. Yanai, D. Savitsky, and T. Taniguchi. 2008. The IRF family transcription factors in immunity and oncogenesis. *Annu Rev Immunol* 26:535-584.
220. Sato, M., H. Suemori, N. Hata, M. Asagiri, K. Ogasawara, K. Nakao, T. Nakaya, M. Katsuki, S. Noguchi, N. Tanaka, and T. Taniguchi. 2000. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. *Immunity* 13:539-548.
221. Sakaguchi, S., H. Negishi, M. Asagiri, C. Nakajima, T. Mizutani, A. Takaoka, K. Honda, and T. Taniguchi. 2003. Essential role of IRF-3 in lipopolysaccharide-induced interferon-beta gene expression and endotoxin shock. *Biochem Biophys Res Commun* 306:860-866.
222. Marie, I., J. E. Durbin, and D. E. Levy. 1998. Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. *EMBO J* 17:6660-6669.
223. Karaghiosoff, M., R. Steinborn, P. Kovarik, G. Kriegshauser, M. Baccarini, B. Donabauer, U. Reichart, T. Kolbe, C. Bogdan, T. Leanderson, D. Levy, T. Decker, and M. Muller. 2003. Central role for type I interferons and Tyk2 in lipopolysaccharide-induced endotoxin shock. *Nat Immunol* 4:471-477.
224. Nakaya, T., M. Sato, N. Hata, M. Asagiri, H. Suemori, S. Noguchi, N. Tanaka, and T. Taniguchi. 2001. Gene induction pathways mediated by distinct IRFs during viral infection. *Biochem Biophys Res Commun* 283:1150-1156.
225. Taniguchi, T., and A. Takaoka. 2001. A weak signal for strong responses: interferon-alpha/beta revisited. *Nat Rev Mol Cell Biol* 2:378-386.
226. Honda, K., H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N. Shimada, Y. Ohba, A. Takaoka, N. Yoshida, and T. Taniguchi. 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* 434:772-777.
227. Honda, K., H. Yanai, T. Mizutani, H. Negishi, N. Shimada, N. Suzuki, Y. Ohba, A. Takaoka, W. C. Yeh, and T. Taniguchi. 2004. Role of a transductional-transcriptional processor complex involving MyD88 and IRF-7 in Toll-like receptor signaling. *Proc Natl Acad Sci U S A* 101:15416-15421.
228. Kawai, T., S. Sato, K. J. Ishii, C. Coban, H. Hemmi, M. Yamamoto, K. Terai, M. Matsuda, J. Inoue, S. Uematsu, O. Takeuchi, and S. Akira. 2004. Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nat Immunol* 5:1061-1068.
229. Shenoy, A. R., B. H. Kim, H. P. Choi, T. Matsuzawa, S. Tiwari, and J. D. MacMicking. 2007. Emerging themes in IFN-gamma-induced macrophage immunity by the p47 and p65 GTPase families. *Immunobiology* 212:771-784.
230. MacMicking, J. D., G. A. Taylor, and J. D. McKinney. 2003. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. *Science* 302:654-659.
231. Singh, S. B., A. S. Davis, G. A. Taylor, and V. Deretic. 2006. Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science* 313:1438-1441.
232. Nelson, D. E., D. P. Virok, H. Wood, C. Roshick, R. M. Johnson, W. M. Whitmire, D. D. Crane, O. Steele-Mortimer, L. Kari, G. McClarty, and H. D. Caldwell. 2005. Chlamydial IFN-gamma immune evasion is linked to host infection tropism. *Proc Natl Acad Sci U S A* 102:10658-10663.
233. MacMicking, J. D., C. Nathan, G. Hom, N. Chartrain, D. S. Fletcher, M. Trumbauer, K. Stevens, Q. W. Xie, K. Sokol, N. Hutchinson, and et al. 1995.

- Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* 81:641-650.
234. Endres, R., A. Luz, H. Schulze, H. Neubauer, A. Futterer, S. M. Holland, H. Wagner, and K. Pfeffer. 1997. Listeriosis in p47(phox<sup>-/-</sup>) and TRp55<sup>-/-</sup> mice: protection despite absence of ROI and susceptibility despite presence of RNI. *Immunity* 7:419-432.
  235. Collazo, C. M., G. S. Yap, G. D. Sempowski, K. C. Lusby, L. Tessarollo, G. F. Woude, A. Sher, and G. A. Taylor. 2001. Inactivation of LRG-47 and IRG-47 reveals a family of interferon gamma- inducible genes with essential, pathogen-specific roles in resistance to infection. *J Exp Med* 194:181-188.
  236. Bernstein-Hanley, I., J. Coers, Z. R. Balsara, G. A. Taylor, M. N. Starnbach, and W. F. Dietrich. 2006. The p47 GTPases Igtp and Irgb10 map to the Chlamydia trachomatis susceptibility locus Ctrq-3 and mediate cellular resistance in mice. *Proc Natl Acad Sci U S A* 103:14092-14097.
  237. Segal, B. H., T. L. Leto, J. I. Gallin, H. L. Malech, and S. M. Holland. 2000. Genetic, biochemical, and clinical features of chronic granulomatous disease. *Medicine (Baltimore)* 79:170-200.
  238. Nicholson, S., G. Bonecini-Almeida Mda, J. R. Lapa e Silva, C. Nathan, Q. W. Xie, R. Mumford, J. R. Weidner, J. Calaycay, J. Geng, N. Boechat, C. Linhares, W. Rom, and J. L. Ho. 1996. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J Exp Med* 183:2293-2302.
  239. Denis, M. 1991. Tumor necrosis factor and granulocyte macrophage-colony stimulating factor stimulate human macrophages to restrict growth of virulent Mycobacterium avium and to kill avirulent M. avium: killing effector mechanism depends on the generation of reactive nitrogen intermediates. *J Leukoc Biol* 49:380-387.
  240. Roshick, C., H. Wood, H. D. Caldwell, and G. McClarty. 2006. Comparison of gamma interferon-mediated antichlamydial defense mechanisms in human and mouse cells. *Infect Immun* 74:225-238.
  241. Williams, B. R. 2001. Signal integration via PKR. *Sci STKE* 2001:RE2.
  242. Sadler, A. J., and B. R. Williams. 2008. Interferon-inducible antiviral effectors. *Nat Rev Immunol* 8:559-568.
  243. Hemmi, H., T. Kaisho, K. Takeda, and S. Akira. 2003. The roles of Toll-like receptor 9, MyD88, and DNA-dependent protein kinase catalytic subunit in the effects of two distinct CpG DNAs on dendritic cell subsets. *J Immunol* 170:3059-3064.
  244. Toshchakov, V., B. W. Jones, P. Y. Perera, K. Thomas, M. J. Cody, S. Zhang, B. R. Williams, J. Major, T. A. Hamilton, M. J. Fenton, and S. N. Vogel. 2002. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. *Nat Immunol* 3:392-398.
  245. Xu, X., Y. L. Sun, and T. Hoey. 1996. Cooperative DNA binding and sequence-selective recognition conferred by the STAT amino-terminal domain. *Science* 273:794-797.
  246. Adhikari, A., M. Xu, and Z. J. Chen. 2007. Ubiquitin-mediated activation of TAK1 and IKK. *Oncogene* 26:3214-3226.
  247. Kim, T. W., K. Staschke, K. Bulek, J. Yao, K. Peters, K. H. Oh, Y. Vandenburg, H. Xiao, W. Qian, T. Hamilton, B. Min, G. Sen, R. Gilmour, and X. Li. 2007. A critical role for IRAK4 kinase activity in Toll-like receptor-mediated innate immunity. *J Exp Med* 204:1025-1036.

248. Gencay, M. M., M. Tamm, A. Glanville, A. P. Perruchoud, and M. Roth. 2003. Chlamydia pneumoniae activates epithelial cell proliferation via NF-kappaB and the glucocorticoid receptor. *Infect Immun* 71:5814-5822.
249. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 162:3749-3752.
250. Cusson-Hermance, N., S. Khurana, T. H. Lee, K. A. Fitzgerald, and M. A. Kelliher. 2005. Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF- $\kappa$ B activation but does not contribute to interferon regulatory factor 3 activation. *J Biol Chem* 280:36560-36566.
251. Opitz, B., A. Puschel, B. Schmeck, A. C. Hocke, S. Rosseau, S. Hammerschmidt, R. R. Schumann, N. Suttrop, and S. Hippenstiel. 2004. Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized Streptococcus pneumoniae. *J Biol Chem* 279:36426-36432.
252. Lu, C., A. Wang, M. Dorsch, J. Tian, K. Nagashima, A. J. Coyle, B. Jaffee, T. D. Ocain, and Y. Xu. 2005. Participation of Rip2 in lipopolysaccharide signaling is independent of its kinase activity. *J Biol Chem* 280:16278-16283.
253. Hata, N., M. Sato, A. Takaoka, M. Asagiri, N. Tanaka, and T. Taniguchi. 2001. Constitutive IFN-alpha/beta signal for efficient IFN-alpha/beta gene induction by virus. *Biochem Biophys Res Commun* 285:518-525.
254. Levy, D. E., and J. E. Darnell, Jr. 2002. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 3:651-662.
255. Yang, T., P. Stark, K. Janik, H. Wigzell, and M. E. Rottenberg. 2008. SOCS-1 protects against Chlamydia pneumoniae-induced lethal inflammation but hampers effective bacterial clearance. *J Immunol* 180:4040-4049.
256. Cousens, L. P., J. S. Orange, H. C. Su, and C. A. Biron. 1997. Interferon-alpha/beta inhibition of interleukin 12 and interferon-gamma production in vitro and endogenously during viral infection. *Proceedings of the National Academy of Sciences of the United States of America* 94:634-639.
257. Lee, C. K., R. Gimeno, and D. E. Levy. 1999. Differential regulation of constitutive major histocompatibility complex class I expression in T and B lymphocytes. *Journal of Experimental Medicine* 190:1451-1464.
258. Perfettini, J. L., V. Hospital, L. Stahl, T. Jungas, P. Verbeke, and D. M. Ojcius. 2003. Cell death and inflammation during infection with the obligate intracellular pathogen, Chlamydia. *Biochimie* 85:763-769.
259. Trinchieri, G. 1998. Interleukin-12: A cytokine at the interface of inflammation and immunity. *Advances in Immunology* 70:83-240.
260. Hibbert, L., S. Pflanz, R. De Waal Malefyt, and R. A. Kastelein. 2003. IL-27 and IFN-alpha signal via Stat1 and Stat3 and induce T-Bet and IL-12Rbeta2 in naive T cells. *J Interferon Cytokine Res* 23:513-522.
261. Nishibori, T., Y. Tanabe, L. Su, and M. David. 2004. Impaired development of CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells in the absence of STAT1: increased susceptibility to autoimmune disease. *J Exp Med* 199:25-34.
262. Lieberman, L. A., M. Banica, S. L. Reiner, and C. A. Hunter. 2004. STAT1 plays a critical role in the regulation of antimicrobial effector mechanisms, but not in the development of Th1-type responses during toxoplasmosis. *J Immunol* 172:457-463.

- 263. Carlin, J. M., and J. B. Weller. 1995. Potentiation of interferon-mediated inhibition of Chlamydia infection by interleukin-1 in human macrophage cultures. *Infect Immun* 63:1870-1875.
- 264. Summersgill, J. T., N. N. Sahney, C. A. Gaydos, T. C. Quinn, and J. A. Ramirez. 1995. Inhibition of Chlamydia pneumoniae growth in HEp-2 cells pretreated with gamma interferon and tumor necrosis factor alpha. *Infect Immun* 63:2801-2803.
- 265. Mannonen, L., E. Kamping, T. Penttila, and M. Puolakkainen. 2004. IFN-gamma induced persistent Chlamydia pneumoniae infection in HL and Mono Mac 6 cells: characterization by real-time quantitative PCR and culture. *Microb Pathog* 36:41-50.
- 266. Durbin, J. E., R. Hackenmiller, M. C. Simon, and D. E. Levy. 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* 84:443-450.
- 267. Fritz, J. H., L. Le Bourhis, G. Sellge, J. G. Magalhaes, H. Fsihi, T. A. Kufer, C. Collins, J. Viala, R. L. Ferrero, S. E. Girardin, and D. J. Philpott. 2007. Nod1-mediated innate immune recognition of peptidoglycan contributes to the onset of adaptive immunity. *Immunity* 26:445-459.
- 268. Herskovits, A. A., V. Auerbuch, and D. A. Portnoy. 2007. Bacterial ligands generated in a phagosome are targets of the cytosolic innate immune system. *PLoS Pathog* 3:e51.
- 269. Serbina, N. V., W. Kuziel, R. Flavell, S. Akira, B. Rollins, and E. G. Pamer. 2003. Sequential MyD88-independent and -dependent activation of innate immune responses to intracellular bacterial infection. *Immunity* 19:891-901.