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**INTERFERONS IN IMMUNITY TO
*CHLAMYDIA PNEUMONIAE***

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Cover photo: *Chlamydia pneumoniae*-infected F4/80⁺ macrophage.

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Ao meu avô, Antonio Carlos Gigliotti

“Uma busca começa sempre com a sorte de principiante, e termina sempre com a prova do conquistador” (A quest always starts with beginner’s luck and always ends with the trial of the conqueror)- Paulo Coelho, “O Alquimista”

“Do or do not. There is no try”- Master Yoda
in “The Empire Strikes Back”

ABSTRACT

The cytokine IFN- γ is the architect behind an amazing immunological program of host resistance to intracellular bacterial and protozoal infections. IFN- γ activates macrophages, making them into inhospitable habitats for parasites attempting to grow inside them. The family of obligate intracellular Gram-negative bacteria Chlamydia is an example of such pathogens. The overall aim of this thesis was to unravel resistance to infection with the human respiratory pathogen *C. pneumoniae*. Specific focus was placed on innate immune responses to *C. pneumoniae* and the regulation and role of IFN- γ in the outcome of infection. An experimental mouse model of lung infection and a macrophage model of *in vitro* infection were used for this purpose.

A protective role for infection-induced IFN- γ in restricting *C. pneumoniae* growth *in vivo* was observed, though IFN- γ was not required for resolution of infection. IL-12 and/or IL-23 was a necessary but not an absolute requirement for expression of IFN- γ . IFN- γ -dependent protection was in part mediated by iNOS expression. TNF- α , known to be synergistic with IFN- γ , was not required for restricting Chlamydial growth. Innate immune cells in the lung constituted an important source of IFN- γ and were essential for restricting *C. pneumoniae* growth and for containment of bacteria in the lungs. However, NK cells were not implicated in such protective IFN- γ release. On the other hand, lung macrophages isolated from *C. pneumoniae*-infected mice expressed IFN- γ . Moreover, bone marrow-derived macrophages (BMM ϕ) conferred upon transfer to RAG-1^{-/-}/IFN- γ ^{-/-} mice, enhanced resistance to *C. pneumoniae* infection via their ability to release IFN- γ . Innate IFN- γ was however not required for protection conferred by CD4⁺ or CD8⁺ T cells. Innate and T cell-derived IFN- γ are also non-redundant (complementary) in protecting mice against *C. pneumoniae*.

C. pneumoniae-infected BMM ϕ also expressed IFN- γ *in vitro*. Such IFN- γ release was IL-12-independent but required instead IFN- α/β and restricted chlamydial growth. IFN- α/β , and not IFN- γ , was required for iNOS-mediated protection in BMM ϕ . The molecular details of BMM ϕ -derived IFN- γ expression revealed a TLR4-MyD88-dependent pathway of IFN- α and IFN- γ induction. Also surprising was the presence of a TLR4- and MyD88-independent, infection-induced NF- κ B activation and pro-inflammatory cytokine expression. Phosphorylation of STAT1 during infection was IFN- α/β -dependent, and necessary for increased IFN- γ expression and for restricting Chlamydial growth. Expression of IFN- γ and restriction of *C. pneumoniae* growth also required NF- κ B activation, but such activation was independent of IFN- α/β , revealing a dual pathway of *C. pneumoniae*-induced IFN- γ expression in BMM ϕ : a TLR4-MyD88-IFN- α/β -STAT1-dependent pathway, and a TLR4-independent pathway leading to NF- κ B activation.

IFN- α/β was also protective *in vivo* by cooperating with IFN- γ for activation of STAT1, which was required for restricting Chlamydial growth. Different from the *in vitro* situation, IFN- γ was sufficient on its own for this effect and did not require IFN- α/β for its expression.

In summary, IFN- γ is important for restricting *C. pneumoniae* growth. Innate IFN- γ is protective both in lungs and in BMM ϕ . IFN- α/β are pivotal in regulating protective responses in BMM ϕ , including IFN- γ release, but are dispensable for IFN- γ expression and protection *in vivo*. This discrepancy may be a qualitative feature in *C. pneumoniae* pattern recognition by different cell types; lung cells convey the generation of protective, IL-12-driven responses, while IFN- α/β -driven protection in BMM ϕ is essential.

LIST OF ORIGINAL ARTICLES

This thesis is based on the following papers which will be referred to by their Roman numerals.

- I. Rottenberg ME, **Gigliotti Rothfuchs A**, Gigliotti D, Svanholm C, Bandholtz L and Wigzell H. Role of innate and adaptive immunity in the outcome of primary infection with *Chlamydia pneumoniae*, as analyzed in genetically modified mice. *J. Immunol.* 1999, 162, 2829-2836.
- II. Rottenberg ME, **Gigliotti Rothfuchs A**, Gigliotti D, Ceausu M, Une C, Levitsky V and Wigzell H. Regulation and role of IFN- γ in the innate resistance to infection with *Chlamydia pneumoniae*. *J. Immunol.* 2000, 164, 4812-4818.
- III. **Gigliotti Rothfuchs A***, Kreuger MR*, Wigzell H and Rottenberg ME. Macrophages, CD4⁺ or CD8⁺ cells are each sufficient for protection against *Chlamydia pneumoniae* infection through their ability to secrete IFN- γ . *J. Immunol.* 2004, 172: 2407-2415.
- IV. **Gigliotti Rothfuchs A**, Gigliotti D, Palmblad K, Andersson U, Wigzell H and Rottenberg ME. IFN- α/β -dependent, IFN- γ secretion by bone marrow-derived macrophages controls an intracellular bacterial infection. *J. Immunol.* 2001, 167, 6453-6461.
- V. **Gigliotti Rothfuchs A**, Trumstedt C, Wigzell H and Rottenberg ME. Intracellular bacterial infection-induced IFN- γ is critically but not solely dependent on TLR4-MyD88-IFN- α/β -STAT1 signaling. 2004. *Submitted for publication.*
- VI. **Gigliotti Rothfuchs A**, Trumstedt C, Wigzell H and Rottenberg ME. Protective role of IFN- α/β against murine infection with *Chlamydia pneumoniae*. *Manuscript.*

* Contributed equally to this work.

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ABBREVIATIONS

APC	Antigen-presenting cell
BMM ϕ	Bone marrow-derived macrophage
CAP	Community-acquired pneumonia
CIITA	Class II trans-activator
DC	Dendritic cell
ds	double-stranded
EB	Elementary body
GAS	γ -activated site
GPI	Glycosylphosphatidylinositol
γ_c R	Common cytokine receptor γ chain
HSP	Heat-shock protein
ICE	IL-1 β -converting enzyme
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IFNAR1	α -chain of the IFN- α / β R
IFNAR2	β -chain of the IFN- α / β R
IFNGR1	α -chain of the IFN- γ R
IFNGR2	β -chain of the IFN- γ R
I κ B	Inhibitor of κ B
IKK	I κ B kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPC	IFN-producing cell
IRAK	IL-1R-associated kinase
IRF	IFN regulatory factor
ISRE	IFN stimulatory response element
JAK	Janus kinase
LBP	LPS-binding protein
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
Mal	MyD88 adaptor-like

MAPK	Mitogen-activated protein kinase
MCMV	Mouse Cytomegalovirus
MHC	Major histocompatibility complex
MoPn	<i>C. trachomatis</i> mouse pneumonitis agent
MPO	Myeloperoxidase
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor κ B
NK	Natural killer
NO	nitric oxide
NOD	Nucleotide-binding oligomerization domain
NRAMP	Natural resistance-associated macrophage protein
OAS	2'-5' Oligoadenylate synthetase
PAMP	Pathogen-associated molecular pattern
Phox	NADPH Oxidase
PKR	dsRNA-dependent protein kinase
Poly(I:C)	Polyinosinic-polycytidylic acid
PRR	Pattern-recognition receptor
R	Receptor
RAG	Recombination-activation gene
RB	Reticulate body
SARM	Sterile α and Armadillo motif
STAT	Signal transducer and activator of transcription
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TICAM	TIR-containing adaptor molecule
TIR	Toll/IL-1R domain
TIRAP	TIR-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor-necrosis factor
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor inducing IFN-β
USF	Upstream stimulatory factor

INTRODUCTION

Living at the expense of someone else is a successful way of life, for a bug at least, since infection is still the predominant cause of death in the world. Most infectious diseases are caused by pathogens smaller than a single mammalian cell, so an entire host encompasses a vast resource-abundant milieu for parasitism. Thus, a mammalian host must invest heavily in generating cells dedicated to defense; cells of the immune system. In maintaining a disease-free state, the immune system must first recognize an invader and consequently deploy appropriate, multifaceted defense-mechanisms which in an integrated fashion, will eliminate or curb pathogen growth.

The innate immune system is an evolutionarily conserved, ancient component of host defense, present in both plants and animals (1). During a primary infection, the pre-determined, fixed responses of innate immunity are the first to come into play. This does not necessarily imply innate cell-mediated responses and could simply be the action of epithelial barriers providing mechanical, chemical and microbiological protection as well as the antibody-independent pathways of complement activation. In mammals the cellular component of innate immunity consists of both myeloid and lymphoid cells bearing germline-encoded receptors, i.e., of pre-determined specificities; phagocytic cells (granulocytes, monocytes/macrophages and dendritic cells [DCs]); lymphocytes lacking genetically-rearranged receptor specificities; Natural killer (NK) cells, and lymphocytes lacking or displaying only poor genetically-rearranged receptor specificities; T cells bearing $\gamma:\delta$ T-cell receptors (TCRs) and CD5⁺ (B-1) B cells. These cells have a swift, hard-wired response to infecting microbes, serving as a first line of defense to prevent pathogens from establishing themselves in the host. Sometimes, infecting microorganisms manage to subvert and overwhelm innate immunity. The delayed but pathogen-specific clonal responses of the adaptive immune system are then required for curbing pathogen growth, which often leads to clearance of infection and memory to re-infection. The cells of the adaptive immune system are lymphocytes bearing somatic rearranged receptors with randomly-generated specificities, that are clonally distributed on individual cells. These are the thymus-educated T cells expressing $\alpha:\beta$ TCRs together with either of the co-receptors CD4 or CD8, and the CD5⁻ (B-2) B cells expressing antibodies.

Signals are required for both alerting the host of infecting pathogens and for the specific activation of lymphocytes. The innate immune system, which mediates recognition of invading pathogens through their germline-encoded receptors, provides both signals and is therefore central in the initiation of immune responses (reviewed in (2)). This is mediated on the one hand through induction of pro-inflammatory cytokines and chemokines (and thus inflammation) and on the other hand through antigen presentation and induction of co-stimulatory molecules (and thus activation of adaptive immunity). Cytokines are important not only in the initiation of the immune response, but also for mediating the actions of T cells. In this respect, the Interferons (IFN)- α , IFN- β and IFN- γ are important components of the immunological arsenal deployed during an immune response. IFN- α/β act primarily during the innate and IFN- γ during both the innate and adaptive phase of the immune response.

The core of this thesis lies on the expression, regulation and role of particularly IFN- γ in resistance to intracellular infection. The original articles reported herein focus on the immune response against the most recently described member of the Chlamydia family; *Chlamydia pneumoniae*, a human respiratory pathogen. Understanding immunity to infection is the first step in vaccine design, and a thorough understanding of the immune response against *C. pneumoniae* may unravel several aspects regarding Chlamydial biology which may be useful in the eradication of not only *C. pneumoniae* but of other Chlamydia too.

PATTERN RECOGNITION BY THE IMMUNE SYSTEM

Innate immune recognition is special in that it mediates interactions between products of different genomes where the host genome is constantly selecting against the survival of the targets that it has evolved to recognize (2). Evolutionary pressure has driven the effective recognition of structures that are essential for microbial survival, i.e., attempting to vary these structures to avoid immune recognition could be lethal or strongly disadvantageous for a microbe's survival and growth. These structures are termed pathogen-associated molecular patterns (PAMPs) and are shared by large groups of pathogens. They are recognized by a relatively limited number of germline-encoded receptors present on innate immune cells such as macrophages and DCs (but also on surface epithelia and endothelial cells) termed pattern-recognition receptors (PRRs). There are several families of PRRs and these can be cell-surface expressed, present in intracellular compartments or humoral. The different family of PRRs include C-type lectins, proteins containing a Leucine-rich repeat (LRR), scavenger receptors, pentraxins, lipid transferases and integrins (reviewed in (3)). PAMP recognition often leads to direct induction of effector functions such as opsonization, complement activation, phagocytosis, apoptosis (reviewed in (3, 4)), but also to the activation of pro-inflammatory signaling pathways, discussed below.

TLRs and Their Ligands

Toll-like receptors (TLRs) constitute a family of mammalian, closely related transmembrane PRRs that respond to an array of microbial products (reviewed in (4-6)). They are characterized structurally by an extracellular LRR domain and a cytoplasmic Toll/IL-1R (TIR) domain (reviewed in (6, 7)). As derived from the name, TIR domains are also involved in signaling downstream of the IL-1R and IL-18R, which share many similarities with TLR signaling (reviewed in (7)). TIR domains also play a role in plant host-defense, suggesting that they may be one of the first evolved host-defense domains (8). It is unclear whether TLRs directly bind their particular PAMPs, but a special feature of these PRRs is their function as signaling receptors. TLRs mediate signal transduction events leading to induction of inflammatory responses, such as production of pro-inflammatory cytokines, chemokines and expression of co-stimulatory molecules. Thus, unlike other PRRs which seem mainly to

mediate effector functions, the initiation of also adaptive immune responses can be driven by TLR-dependent PAMP recognition.

The TLR family contains to date 10 members identified in mammals and those TLRs with identified ligands are involved in the recognition of several important PAMPs (reviewed in (9) and Figure 1). TLR2 recognizes the broadest range of PAMPs. These include bacterial peptidoglycans (10, 11), the yeast cell-wall component zymosan (12), glycosylphosphatidylinositol (GPI) anchors from *Trypanosoma cruzi* (13) and mycobacterial lipoarabinomannan (14, 15). The broad specificity of TLR2 probably stems from the fact that it is able to pair with other TLRs, so far, TLR1 and TLR6. Though not formally demonstrated, dimerization with either TLR1 or TLR6 seems to allow TLR2 to discriminate between di- and triacylated lipoproteins (discussed in (5, 16)).

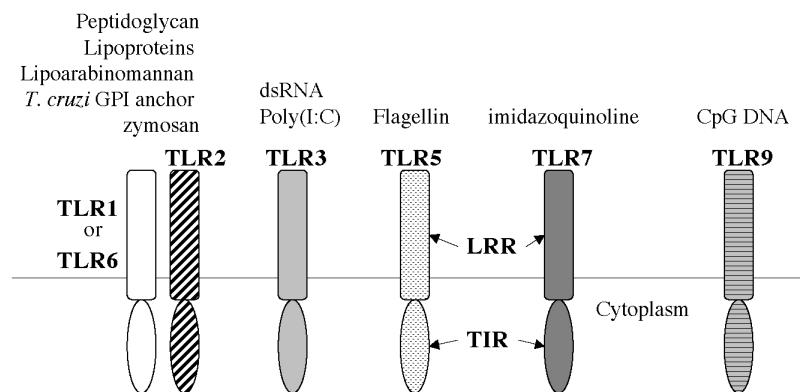


Figure 1: TLRs and Their Ligands.

TLR3 recognizes double-stranded (ds) RNA and synthetic dsRNA, such as polyinosinic-polycytidylic acid [poly(I:C)] (17). dsRNA is a specific viral product and can be generated during the replication of both RNA and DNA viruses. Though not a TLR, the cytosolic dsRNA-dependent protein kinase (PKR) also recognizes dsRNA and is considered a PRR (discussed later).

TLR5 is the only TLR recognizing a protein ligand; flagellin (18). Flagellin is a component of the flagellum found in both Gram-positive and -negative bacteria. While flagellin is known to be a potent immunogen on which much of the clonal selection theory was based, several factors explain why it is also a PAMP (reviewed in (19)). These include the fact that flagellin is expressed in a highly repetitive pattern, elicits a potent immune response even in plants and most importantly, the advantage of motility for bacterial survival has presumably imposed structural constraints on flagellin allowing it to be targeted by the innate immune system.

The natural ligands for TLR7, TLR8 and TLR10 have not been identified (reviewed in (9)). Functional speculations have been made based on structural homology to other TLRs with known ligands. TLR7 and TLR8 are highly homologous to TLR9. TLR9 recognizes unmethylated CpG motifs in DNA (20), infrequent in vertebrate genomes but very common in bacterial DNA. The ability of TLR7 to recognize imidazoquinolines (21) as well as other synthetic compounds related to nucleic acids, suggests that TLR7 (and possibly TLR8) may be involved in recognition of microbial nucleic acid-like structures (reviewed in (6)). Similarly, TLR10 is related to TLR1 and TLR6 and may thus be likely to pair with TLR2.

TLR4 Signaling

Lipopolysaccharide (LPS) is probably the most studied bacterial immunogen to date, but it was not until recently that TLR4 was characterized as the signal-transducing receptor for LPS. TLR signaling is described using LPS-mediated activation of TLR4 as an example (Figure 2). Accessory proteins are required for LPS signaling through TLR4. LPS-binding protein (LBP), produced by the liver, is known to bind LPS via the lipid-A moiety and this complex interacts with the GPI-anchored receptor CD14 (reviewed in (22)). Because CD14 lacks a cytoplasmic signaling domain, it serves mainly to “recruit” LPS to TLR4. Studies indicate that TLR4 makes direct contact with LPS (23-25). The small protein MD2, which associates with the extracellular part of TLR4, has also been shown to be necessary for LPS-mediated TLR4 signaling (26). The number of accessory proteins involved in TLR4 signaling suggest that the full complement of molecules for a particular TLR to mediate recognition may be yet uncharacterized for most TLRs, but it seems that TLRs most probably constitute the signaling core of such complexes, as is the case for TLR4. The essential role of LBP, CD14, MD2 and TLR4 in LPS-mediated signaling is confirmed by the hyporesponsiveness of LBP^{-/-}, CD14^{-/-}, MD2^{-/-} and TLR4^{-/-} mice to LPS-induced lethality (26-29).

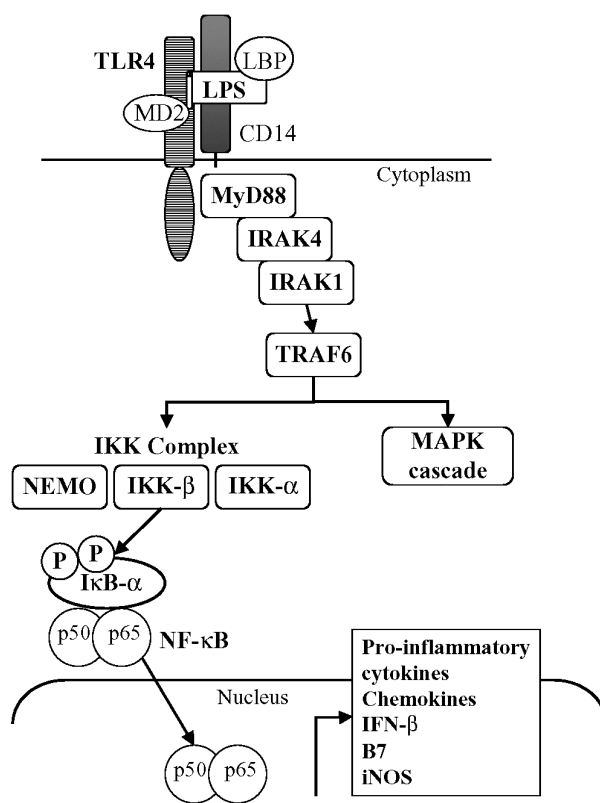


Figure 2: Schematics of TLR4 Signaling. Details of MAPK signaling have been omitted for the sake of simplicity but are known to promote pro-inflammatory cytokine release. Adapted from (5).

Activation of NF- κ B

The NF- κ B family of transcription factors include the proteins p50, p52, p65/RelA, RelB and c-Rel and are key regulators of immune responses (reviewed in (30)). NF- κ B transcription factors have a Rel homology domain which contains a nuclear-localization sequence and is involved in DNA-binding, dimerization with other Rel proteins and interaction with the family of inhibitors of NF- κ B (I κ B). The NF- κ B family is evolutionarily conserved with several homologs found in *Drosophila* (reviewed in (31)). In unstimulated cells, they are found as cytosolic homo- or heterodimers (primarily the combination of p50/p65). The I κ B family includes I κ B- α (the main actor) as well as I κ B- β , I κ B- ϵ and Bcl-3. They associate with NF- κ B dimers and keep them sequestered in the cytosol. Upon stimulation, I κ B become phosphorylated, (on serine residues 32 and 36 for I κ B- α) which target them for ubiquitination and consequently degradation (reviewed in (32)). This allows the otherwise I κ B-sequestered NF- κ B dimers to translocate from the cytosol into the nucleus (33), where they bind to κ B sites on target genes and mediate their transcriptional induction. Several proteins have been shown to phosphorylate I κ B and thereby activate NF- κ B, but the classical I κ B-phosphorylating unit is the I κ B kinase (IKK) complex, constituted of IKK- α , IKK- β and a third regulatory subunit IKK- γ /NEMO.

The generation of mice with targeted disruptions in members of the NF- κ B and I κ B family have revealed many lethal phenotypes, but also enhanced susceptibility in the non-lethal phenotypes of p50^{-/-}, p52^{-/-}, RelB^{-/-}, c-Rel^{-/-} and Bcl-3^{-/-} mice to a variety of viral, bacterial and parasitic infections (reviewed in (30)). Most deficiencies revealed susceptibility to *Listeria monocytogenes* and *Toxoplasma gondii* infections. Due to the multiple defects often found in these animals though, understanding how different populations specifically contributed to susceptibility is difficult.

Upon PAMP recognition, the TIR domain of all known TLRs interacts with the TIR domain present in the molecule myeloid differentiation factor (MyD) 88. This cytosolic adaptor initiates a common signaling cascade that subsequently leads to nuclear translocation of NF- κ B. The same signaling steps are also known to occur upon IL-1R or IL-18R engagement and in fact all proteins described for IL-1R signaling have also been implicated in LPS signaling (reviewed in (7)). Via its death-domain, MyD88 recruits IL-1R-associated kinase (IRAK)-1 and IRAK-2 (or in myeloid cells IRAK-M) to the cytoplasmic tail of the receptor. The importance of MyD88 in recruiting IRAK1 is confirmed in MyD88^{-/-} mice, which also fail to activate NF- κ B following IL-1 or IL-18 stimulation (34). IRAK-1^{-/-} mice also show severe ablation in response to IL-1, IL-18 (35) and LPS stimulation (36). This ablation though, is not complete for TLR responses and a newly described IRAK family member, IRAK4, has been shown to be necessary for IL-1-, TLR2-, TLR3-, TLR4- and TLR9-driven responses (37) and for activation of IRAK-1 itself (38), making it so far the IRAK member most proximal to the receptor TIR domain.

Following IRAK recruitment, Tumor necrosis factor (TNF) receptor-associated factor (TRAF) 6 becomes involved in the signaling cascade and consequently leads to IKK- β activation, degradation of I κ B, nuclear translocation of NF- κ B and induction of target genes involved in host defense. These include expression of pro-inflammatory

cytokines (IL-1, IL-6 and TNF- α), chemokines, IFN- β , inducible nitric oxide synthase (iNOS) and co-stimulatory molecules (reviewed in (31)).

Activation of MAP Kinase Cascade

Signaling downstream of TRAF6 also leads to activation of the mitogen-activated protein kinase (MAPK) cascade (39). This is a complicated, multi-step cytosolic cascade (reviewed in (40)) leading to activation of MAPKs that can translocate to the nucleus and regulate gene expression. Two important MAPKs are p38 and c-Jun N-terminal kinase. These are involved primarily in cell proliferation and differentiation, but also in inflammation. The overall importance of this pathway in host defense is not fully understood.

MyD88-Independent Signaling

The expression of pro-inflammatory cytokines are absent in LPS-stimulated MyD88^{-/-} mice, but LPS-stimulated macrophages from the same display only an early delay in the activation of NF- κ B and MAPKs (41). This has been similarly shown for LPS-stimulated MyD88^{-/-} but not TLR4^{-/-} DCs, which also retain the ability to functionally mature, i.e., to up-regulate expression of co-stimulatory molecules (42). Identical observations have been made for TLR3 signaling (17). Thus, except for pro-inflammatory cytokine release, signaling through TLR3 and TLR4 proceed in the absence of MyD88, suggesting that presence of other cytoplasmic adaptors in mediating signaling by these TLRs (Figure 3). Today, four other TIR adaptor proteins are recognized (reviewed in (43)). The TIR-containing adaptor protein (TIRAP), also known as MyD88 adaptor-like (Mal), was initially thought to be the adaptor responsible for TLR4-dependent, MyD88-independent signaling (44, 45). That fact that LPS-induced DC maturation was intact in TIRAP^{-/-} (46) and TIRAP^{-/-}/MyD88^{-/-} mice (47) strongly suggests against a role for TIRAP/Mal in the MyD88-independent pathway (47). LPS-stimulated MyD88^{-/-} but not TLR4^{-/-} macrophages have been shown to express a set of IFN- α / β -inducible genes (48). Furthermore, LPS- and poly(I:C)-dependent expression of IFN- β requires Interferon regulatory factor (IRF)3 (49, 50), activation of which was also intact in TIRAP^{-/-}/MyD88^{-/-} mice (47). Thus IRF3, IFN- β and IFN- β -induced genes form part of the MyD88-independent response during TLR3 and TLR4 signaling.

A third adaptor called TIR-domain-containing adaptor inducing IFN- β (TRIF) (51), also known as TIR-containing adaptor molecule (TICAM)-1 (52) has recently been reported. TRIF^{-/-} mice (53) or mice with a chemically-induced mutation occurring in the TRIF protein (54) showed severe impairment of TLR3- and TLR4-mediated (late) NF- κ B activation, including impairment of MyD88-independent responses, such as expression of co-stimulatory molecules (55) and IRF3-induced genes (53, 54). Thus, TRIF seems to mediate activation of IRF3 and the induction of IFN- α / β -driven responses and mice with the chemically-induced mutation in TRIF were also highly susceptible to mouse Cytomegalovirus (MCMV) infection (54). It is unclear why TRIF^{-/-} mice also had reduced expression of pro-inflammatory cytokines following TLR4 stimulation (53), since this is considered an MyD88-dependent effect. A fourth adaptor called TRIF-related adaptor molecule (TRAM) has recently been described to

work with TRIF in TLR4-specific activation of IRF3 and NF- κ B (45). TRAM^{-/-} cells also display impaired pro-inflammatory cytokine production following LPS stimulation as well as impaired NF- κ B and IRF3 activation (56). It seems therefore that TRIF/TRAM work together with MyD88 for expression of pro-inflammatory cytokines. The fifth TIR-containing adaptor is Sterile α and Armadillo motif (SARM) (57), and though it has the characteristics of a signaling molecule, its function remains unknown.

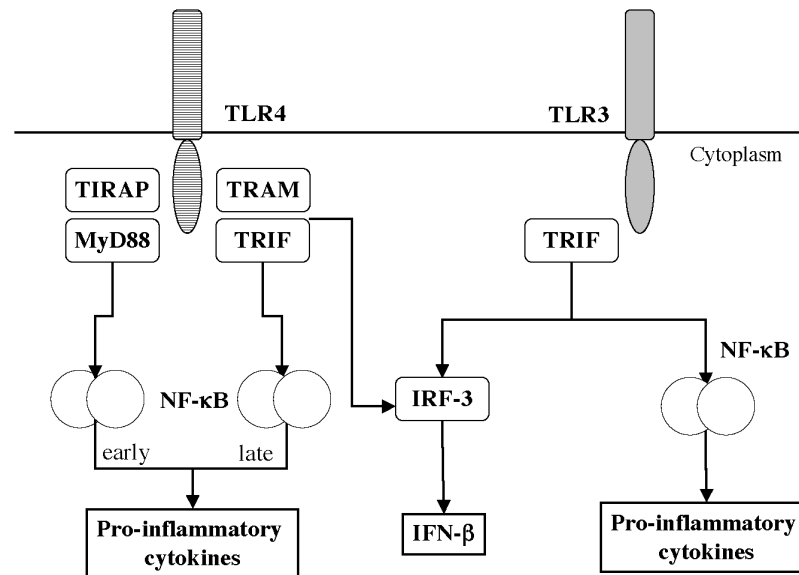


Figure 3: Schematics of Adaptor Molecules Involved in Signaling Downstream of TLR3 and TLR4. TRIF mediates MyD88-independent activation of IRF3, leading to release of IFN- β . Adapted from (56).

The fact that the host employs several accessory molecules for bringing LPS and TLR4 together suggests that responding to LPS is a fine-tuned, complicated process, but obviously important since so much effort is put into this. Similarly, it is unclear why so many different cytosolic adaptors are involved in LPS (and TLR3) signaling. The dual nature of LPS may be part of the answer. Facing the (lethal) risk of LPS-induced toxicity, the host still opts to respond to LPS and thus be able to recognize and mount an immune response against invading, Gram-negative bacteria. In this regard, possessing a signaling cascade with multiple adaptors also opens the possibility for the host in fine-tuning its response to such an important but potentially lethal immunogen. Because the recruitment of multiple adaptors allows response specificity it is possible that the other TLRs strictly signaling through MyD88 may also turn out to have other, as-yet undiscovered adaptors involved in fine-tuning their responses.

TLRs in Host Defense

The question whether or not responding to LPS was advantageous for host resistance was addressed already some time ago with LPS-hyporesponsive C3H/HeJ mice (58), which today are known to have a mutation in TLR4 (59). These animals were reported to be more susceptible to infection with several Gram-negative bacteria, (60-63). More recently, enhanced mortality was observed in TLR2^{-/-} and MyD88^{-/-} mice

to *Staphylococcus aureus* infection in comparison to WT controls (64). TLR2^{-/-} mice displayed enhanced mortality to *Streptococcus pneumoniae* due to reduced brain bacterial clearance (65) and reduced control of *Borrelia burgdorferi* growth in tissues (66).

The role of TLR signaling in parasitic infections has also been studied. *T. gondii*-infected MyD88^{-/-} mice displayed enhanced mortality associated to impaired Interleukin (IL)-12 production by DCs (67), suggesting an important role for TLR signaling in control of *T. gondii*, though the particular TLR(s) associated to IL-12 expression *in vivo* was not reported. A protective role for TLR2-dependent signaling was recently demonstrated during *Mycobacterium bovis* (68) infection and a role for TLR2 but particularly MyD88 in *M. avium* infection of mice (69). A delay in clearance of two other lung pathogens, namely the Gram-negative bacterium *Haemophilus influenzae* (70) and Respiratory syncytial virus (71) have also been shown. The latter observation is particularly interesting, suggesting that TLR4 can recognize PAMPs other than LPS, in this case the Respiratory syncytial virus fusion protein (71).

It is not always clear from the above studies whether the reported TLR deficiencies are affecting the innate and/or the adaptive immune response. A role for TLRs specifically in dictating the quality of adaptive immune responses has been suggested (reviewed in (72)), and it is supported primarily by two studies. In the first study, MyD88^{-/-} mice immunized with ovalbumin in Freund's complete adjuvant (a strong inducer of IFN- γ) failed to induce antigen-specific IFN- γ and IgG2a but showed unaffected IgE and IgG1 responses (73). On the other hand, immunization of ovalbumin with alum (a strong inducer of IL-4) resulted in comparable levels of antigen-specific IgG1 in MyD88^{-/-} and WT mice (73). Thus, IL-4-driven responses may either be dependent on other as-yet uncharacterized TLRs or other signaling PRRs, or occur by default in the absence of TLR stimulation. The latter suggestion is supported by the fact that MyD88^{-/-} mice stimulated with a potent IFN- γ -inducing stimulant, namely soluble tachyzoite extract from *T. gondii*, display a complete inability to mount IFN- γ responses and instead display an IL-4 response (74). IL-12p40^{-/-} mice stimulated with the same extract did not produce IL-4 but displayed instead residual IFN- γ production (74). This supports a more critical role for MyD88 signaling in promoting IFN- γ responses rather than, as previously believed, IL-12 stimulation.

IFN- α/β

Sources of IFN- α/β

IFN- α/β were described almost 50 years ago (75), based on their ability to “interfere” with viral replication, hence their name “interferon”. In both humans and mice, there is only a single IFN- β , but 15 different IFN- α genes in humans and 10 in mice. They are encoded by intronless genes and all bind to the same IFN- α/β R. Though other related IFNs exist, such as IFN- δ in pigs, IFN- τ in cattle and sheep and IFN- ω in cattle and humans, only IFN- α/β have been studied in detail (reviewed in (76, 77)). Most cell types, including fibroblasts, macrophages and DCs, are capable of producing IFN- α/β (reviewed in (77)). But the main IFN- α/β -producing cell (IPC) has been

identified in humans (78) and in mice (79) as a rare type of DC with plasmacytoid morphology. In the human setting, such IPCs have been shown to induce *in vitro* IFN- γ production from naïve CD4⁺ T cells upon stimulation with virus (80). Though the latter has not been demonstrated in mice, it is clear that mouse IPCs are a major *in vivo* source of protective IFN- α production during MCMV infection (79) where endogenous IFN- α/β are known to be protective (81). IFN- α is readily used in humans for the treatment of various cancers (reviewed in (82-85)) and chronic hepatitis (reviewed in (86)).

Biological Functions of IFN- α/β

Anti-Viral Effects

Though several immunoregulatory effects have been ascribed to IFN- α/β (described below) the direct anti-viral effects that lead to their discovery are beyond any doubt their hallmark. Several pathways of innate immunity have been described in inhibition of viral growth, the best characterized are those involving Mx proteins, PKR, and 2'-5' Oligoadenylate synthetase (OAS) (reviewed in (76)).

Mx proteins are cytoplasmic GTPases specifically induced by IFN- α/β . Exactly how they inhibit viral replication is not fully understood. Mx1 was discovered in the A2G mouse strain shown to have an inherent resistance to influenza virus (87). Though Mx1 is functional in wild mice (88), it seems to be inactivated in inbred laboratory strains (except for the A2G mouse). Thus, Mx proteins are not involved in resistance to infection in experimental models, though cells transfected with functional Mx or transgenic mice expressing functional Mx show enhanced resistance to several viruses (reviewed in (89)).

PKR is a serine/threonine kinase mediating not only anti-viral activity but also signal transduction such as activation of NF- κ B through activation of the IKK complex or phosphorylation of I κ B- α (reviewed in (90)). PKR is transcriptionally induced preferentially by IFN- α/β and enzymatic activation of the protein requires dsRNA, making PKR a cytosolic PRR involved in recognition of dsRNA. PKR-dependent viral growth inhibition entails the phosphorylation of the translation initiation factor eIF2, resulting in its inactivation and consequently block of host protein synthesis, necessary for viral replication (reviewed in (76, 91)).

As for PKR, OAS is transcriptionally induced by IFN- α/β and likewise activated by dsRNA. Activated OAS uses ATP to produce short strings of 2'-5'-linked (oligo) adenyates at the 3' end of mRNA molecules. These are necessary in turn for activation of RNaseL, which cleaves single-stranded RNA and consequently blocks host transcription (reviewed in (76, 91)). The combined actions of PKR and OAS thus result in the generalized inhibition of host translation and transcription which prevents the assembly of new virus particles, inducing an anti-viral state in the IFN- α/β -stimulated cell. Though PKR and OAS are classical IFN- α/β -regulated genes, they may also be induced by IFN- γ . The importance of the PKR and OAS pathways in resistance against viral infection is demonstrated by PKR^{-/-}/RNaseL^{-/-} mice which display enhanced susceptibility to encephalomyocarditis virus (92). The large number of viral-evasion proteins specifically targeting the PKR and OAS pathways (reviewed

in (93)) further supports the protective role of IFN- α/β , PKR and OAS against viral infections.

Immunoregulatory Effects

Enhancement of NK-cell cytotoxicity was one of the first described immunoregulatory functions of IFN- α/β (94) as well as IL-2-independent NK-cell proliferation (95). IFN- α/β have also been shown to promote bystander (i.e. TCR-independent) proliferation of memory CD8⁺ T cells (96) and to directly prevent death of activated T cells (97). Activation of macrophages to kill *Leishmania major* has also been shown through exogenous administration of IFN- α/β (98). In an experimental system using a model antigen, maturation/activation of DCs by IFN- α/β has been shown to promote T-cell proliferation and IFN- γ secretion, as well as antibody production and isotype switching (99). Also involving DC stimulation, virus-induced IFN- α/β was recently shown to mediate cross priming of CD8⁺ T cells (100). The use of IFN- α/β as natural adjuvants in vaccine design has also been suggested from findings made in an influenza vaccine model (101). This vast array of immunoregulatory functions ascribed to IFN- α/β should imply a protective role for it during most intracellular infections. It is therefore interesting to note that at least during experimental infections, this does not seem to be the case. The use of IFN- α/β R^{-/-} mice has clearly confirmed the protective role of IFN- α/β during viral, but not bacterial or protozoan infections (reviewed in (102)). It is possible however that in such animals, one is only observing the effects of IFN- α/β on the innate response and not its immunoregulatory role.

IFN- γ

Sources of IFN- γ

IFN- γ was first identified as a phytohemagglutinin (PHA)-induced, IFN- α/β -like factor produced by lymphocytes able to block the cytopathic effects of Sindbis virus (103). It has since then become a key player in immunity to infection (reviewed in (102)) and a mediator of tumor immuno-surveillance (104). Unlike IFN- α , mouse and human IFN- γ are each encoded by a single gene, unrelated to the genes encoding IFN- α and IFN- β . IFN- γ signals through a heterodimeric IFN- γ R present on most nucleated cells.

IFN- γ is the architect behind an amazing immunological program governing defense against most (if not all) intracellular infections (discussed below). Yet the phenotype of mice with targeted deletions in IFN- γ , its receptor or components of the receptor signaling machinery (reviewed in (91)), confirm that the effects of this pleiotropic cytokine are confined to the immune system. This is also supported by the phenotype of humans having deficiencies in IFN- γ (reviewed in (105, 106)). Thus, IFN- γ is produced *by* cells of the immune system *for* the sake of an immune response.

The main cellular sources of IFN- γ are NK cells (107) of the innate and activated CD4⁺ (108) and CD8⁺ (109) T cells of the adaptive immune system. The

original monopoly of the above-mentioned lymphocytes on IFN- γ production has recently been challenged. A series of independent studies (including those presented in this thesis) show that different myeloid populations also express IFN- γ , discussed in detail later.

Biological Functions of IFN- γ

Cellular response to IFN- γ can be viewed as an integrated “molecular program of resistance” to intracellular pathogens (91). The fact that IFN- γ mediates resistance primarily against intracellular bacteria and protozoa is confirmed by the enhanced susceptibility of IFN- γ ^{-/-} or IFN- γ R^{-/-} mice to several experimental infections (reviewed in (102), (110-115)). This is also demonstrated in Signal transducer and activator of transcription (STAT)1^{-/-} mice (116-118), which are also susceptible to viruses (119, 120). STAT1 is a molecule central to both IFN- α/β R and IFN- γ R signaling (discussed later). The importance of IFN- γ -mediated immunity is also supported by a rare human immunodeficiency known as Mendelian susceptibility to mycobacterial infection. This condition is characterized by genetic deficiencies in IL-12, IL-12R, IFN- γ R and STAT1, which often lead to fatal infections with otherwise atypical, non-pathogenic mycobacteria and also enhanced susceptibility to salmonella (reviewed in (105, 106)). These clinical findings clearly confirm the essential role of the IL-12-IFN- γ -STAT1 pathway in resistance against intracellular infections.

Some recent studies have employed the use of oligonucleotide microarrays to analyze gene expression following IFN- γ stimulation (120-123). Differences in the number of IFN- γ -responsive genes reported by these studies may reflect differences in the oligonucleotide arrays, cut-off for fold induction/repression, cell type, concentration of IFN- γ and time-point for analysis. One study using bone marrow-derived macrophages (BMM ϕ) revealed a striking number of more than a 1000 IFN- γ -responsive genes (123). In this study, treatment with IFN- γ suppressed almost as many genes as it induced, but a closer look revealed that genes directly related to immunity and inflammation were more likely to be induced than repressed by IFN- γ . This is in line with the general view that IFN- γ is a macrophage-activating factor (124, 125). In fact, the activation state of macrophages is an important determinant in the outcome of intracellular infection. The biological consequences of IFN- γ are thus best described in macrophages. These include antigen presentation by MHC class I and II, induction of the respiratory burst enzyme NADPH Oxidase (Phox), induction of iNOS, induction of tryptophan catabolism via Indoleamine 2,3-dioxygenase (IDO) and induction of natural resistance-associated macrophage protein (NRAMP). Many of these aspects are also applicable to other phagocytic and non-phagocytic cells and overlap with actions of other cytokines.

Antigen Presentation

The expression of MHC class I and class II molecules following IFN- γ stimulation was one of the first effects described for IFN- γ and involves a series of molecules (reviewed in (91)). Though MHC class I is expressed constitutively on all nucleated cells (except desidual trophoblasts), IFN- γ and to a lesser extent IFN- α/β can

further enhance its expression. IFN- γ not only regulates actual expression of MHC class I, but also of other components required for MHC class I antigen processing and presentation. These include expression of β_2 microglobulin, the transporter associated with antigen processing (TAP)-1 and -2 and the proteasome regulatory subunits LMP2, LMP7 and MECL1, which together generate the “immune” proteasome, better equipped at generating peptides suitable for presentation on MHC class I. Expression of MHC class II is restricted to professional antigen-presenting cells (APCs) macrophages, DCs and B cells but is also strongly induced by IFN- γ . Unlike class I induction, the class II pathway is regulated by a single IFN- γ -inducible transcription factor, the class II trans-activator (CIITA). CIITA controls expression of both chains of the MHC class II molecule as well as the accessory invariant chain and MHC H-2M/HLA-DM.

Antimicrobial effector molecules Phox, iNOS, IDO and NRAMP

The respiratory burst is a well-characterized component of phagocyte resistance that includes IFN- γ -regulated molecules. It involves a series of components in this case those of the multi-enzyme Phox. Phox catalyzes the reduction of molecular oxygen to superoxide anion, a highly reactive, antimicrobial oxygen species. Superoxide anion can be converted by superoxide dismutase into hydrogen peroxide. Through another respiratory-burst enzyme, myeloperoxidase (MPO), hydrogen peroxide can together with chlorine ions form hypochlorous acid (bleach). There are (at least) seven characterized subunits of the Phox, the cytosolic p40-phox, p47-phox p67-phox and the GTPase Rac2, and the membrane-bound cytochrome C-forming components gp91-phox, p22-phox and Rap1A (reviewed in (126)). Expression of p47-phox, p67-phox and definitely gp91-phox have been reported to be regulated by IFN- γ (127-130), sometimes in synergism with TNF- α (127, 129). Loss of function in Phox leads to Chronic Granulomatous Disease, where patients suffer from bacterial infections and IFN- γ therapy is often applied. The generation of p47-phox^{-/-}, gp91-phox^{-/-}, Rac2^{-/-} and MPO^{-/-} mice confirm a protective role for the respiratory burst against primarily bacterial infections but also against fungi (reviewed in (131)).

iNOS, an inducible isoform of NOS, catalyzes high-output, calcium-independent production of nitric oxide (NO) gas (and L-citrulline) from L-arginine and molecular oxygen. Like Phox, iNOS is also expressed in neutrophils and macrophages but is induced by IFN- γ in many other cell types. NO is a highly reactive and diffusible nitrogen radical with both anti-tumor and anti-microbial activities (reviewed in (131-133)). NO interacts with superoxide anion to form the highly toxic, antimicrobial molecule peroxynitrite. Cytokines, primarily IFN- γ and TNF- α are potent inducers of iNOS, as are microbial products such as LPS (reviewed in (132)). Since L-arginine is the only physiological substrate for iNOS, regulation of its availability is rate-limiting for NO production as are co-factors for iNOS enzymatic activity (reviewed in (134)). In various cell types including macrophages, IFN- γ together with either TNF- α or LPS often leads to the co-induction of iNOS and such rate-limiting enzymes (135-141). Thus IFN- γ seems not only involved in iNOS expression, but also in orchestrating an overall cellular commitment for NO production. The use of NO inhibitors or NO-generating compounds (reviewed in (132)) and iNOS^{-/-}

mice (reviewed in (131, 133)) have revealed an important role for iNOS-derived NO in resistance to several intracellular bacteria, intracellular protozoa and viruses.

IDO catalyzes the conversion of L-tryptophan into L-kynurenine and is induced by IFN- γ in various cell types including macrophages. By restricting the cellular pools of L-tryptophan, intracellular parasites requiring this essential amino acid for replication are “starved”. Several studies show synergism for TNF- α in enhancing IFN- γ -mediated IDO induction. IDO is clearly mediating *in vitro* growth control of *T. gondii* (142-146) and Chlamydia species (147-152) though more recent findings also suggest an *in vitro* growth-restricting role for IDO in *Rickettsia conorii* (153), CMV (154), *S. aureus* (155) and *Trypanosoma brucei* (156) infections.

NRAMP was identified as the candidate gene for the Ity/Lsh/Bcg locus, which was known to confer resistance against intracellular parasites such as *Salmonella typhimurium*, *M. bovis* and *L. donovani* (157). Though constitutively expressed in macrophages, NRAMP is enhanced by IFN- γ stimulation. NRAMP might be a transporter in the phagolysosomal membrane of macrophages (158) but its exact function is unclear.

Regulation of IFN- γ Production

Cross-linking of the TCR and co-stimulation are strong inducers of IFN- γ expression in T cells (reviewed in (159)). APC-derived cytokines are the key inducers for IFN- γ release from NK cells, but their role in enhancing IFN- γ production from T cells is also appreciated.

Role of IL-12

IL-12 is the best characterized APC-derived cytokine involved in regulation of IFN- γ production (reviewed in (160, 161)). This heterodimeric cytokine is composed of an inducible p40 subunit and a primarily constitutive p35 subunit, generating bioactive IL-12p70. IL-12 was originally described as “NK-cell stimulatory factor” or “cytotoxic lymphocyte maturation factor”, both products of Epstein-Barr virus-transformed human B-cell lines that could activate NK-cell cytotoxicity (162, 163) and IFN- γ production (162), as well as augment PHA-driven T-cell proliferation (162, 163) and IFN- γ production (162). The ability of IL-12 to excel in IFN- γ production lies not only in its efficiency at low concentrations, but also in its synergism with several other factors for IFN- γ release. IL-12 is for example synergistic with cytokines such as IL-1, TNF- α and IL-2 for IFN- γ expression from NK cells (162, 164, 165). Still, the best-described synergism is that between IL-12 and IL-18, a cytokine related to IL-1 in terms of structure, receptor family and function (reviewed in (166)). IL-18 is also produced by APCs and like IL-1, must be cleaved by IL-1 β -converting enzyme (ICE), also known as Caspase-1, to be functional (167, 168).

IL-18 was described as an “IFN- γ -inducing factor” with the ability to induce IFN- γ production from TCR-stimulated CD4⁺ T cells better than IL-12 itself, though it also displayed synergism with IL-12 for IFN- γ production (169). This synergism between IL-18 and IL-12 also applied to the generation of IFN- γ -producing CD4⁺ T cells *in vitro*, but a role for IL-18 alone in this process was excluded (170). The

inability of IL-18 to stimulate IFN- γ production in the absence of IL-12 was confirmed by the fact that naïve T cells from IL-18^{-/-} mice stimulated *in vitro* with IL-12 produced IFN- γ similar to WT controls, while stimulation with IL-18 could not produce the same result in T cells from IL-12p40^{-/-} mice (171). Still, while TCR-stimulated CD4⁺ T cells isolated from *M. bovis*-infected IL-18^{-/-} mice produced IFN- γ in almost equal amounts to WT controls, cells from IL-12^{-/-}/IL-18^{-/-} mice stimulated under similar conditions failed to induce IFN- γ altogether, confirming also the co-operative action of IL-12 and IL-18 on CD4⁺ T cells (171). Thus, IL-18 is alone a poor enhancer of IFN- γ but in the presence of IL-12, stimulates synergistic IFN- γ release. This has been ascribed to the fact that IL-12 up-regulates both chains of the IL-18R (172). Similarly, IL-18 up-regulates expression of IL-12R β 2 in resting T cells (173), so that IL-12 and IL-18 work in concert to augment the responsiveness of cells for each other and consequently, for the expression of IFN- γ .

It is possible that under certain circumstances, IL-18 may suffice for IFN- γ production in the absence of IL-12. This is supported by a model using mice primed with heat-killed *Propionibacterium acnes*. IL-18^{-/-} mice primed with *P. acnes* and consequently challenged with LPS display reduced levels of IFN- γ in the serum despite normal levels of IL-12, as do T cells isolated from *P. acnes*-primed IL-18^{-/-} mice stimulated *in vitro* with anti-CD3 (171). Reduced serum levels of IFN- γ is also observed in *P. acnes*-primed, LPS-stimulated ICE^{-/-} mice (167, 168). Further support for a distinct role for IL-12 and IL-18 in IFN- γ expression is a report analyzing the transcriptional activity of the IFN- γ promoter in human CD4⁺ T cells. In transfected CD4⁺ T cells, IL-18 was found to directly stimulate a luciferase reporter plasmid coupled to the IFN- γ promoter while IL-12 could only do so in the presence of TCR-stimulation (174), though it is unclear whether reporter activity truly reflects IFN- γ production.

IL-12 also augments IFN- γ production following both TCR activation (175) and co-stimulation via B7:CD28 interactions (176, 177). Thus for T cells, the fact that APCs can deliver simultaneous cytokine and TCR stimulatory signals immensely facilitates IFN- γ induction, though it is clear that IL-12 (nor IL-18) alone is enough for substantial IFN- γ expression in T cells.

The overall importance of IL-12 in IFN- γ production is seen in IL-12p40^{-/-} mice which display reduced IFN- γ production by NK cells following LPS stimulation and reduced IFN- γ but enhanced IL-4 production by T cells (primarily CD4⁺) following immunization with a model antigen (178). STAT4 is the main transcription factor mediating IL-12-driven transcription of IFN- γ (reviewed in (160)). In line with IL-12p40^{-/-} mice, STAT4^{-/-} mice also displayed impaired IL-12-dependent IFN- γ production and enhanced IL-4 responses (179), though interestingly, both IL-12p40^{-/-} and STAT4^{-/-} mice have residual (IL-12-independent) IFN- γ production. In fact, recent data suggests that IL-12 is not an absolute requirement for IFN- γ production nor is its absence responsible for enhanced IL-4 responses (74). The importance of IL-12 in generating IFN- γ -dominated responses during different intracellular infections is nevertheless well characterized (reviewed in (160, 161, 180)).

Two other IL-12-related cytokines have recently been described that add to the complexity of IL-12-driven responses. IL-23 is composed of the IL-12p40 subunit and a novel p19 subunit (181). It signals via a similar pathway to IL-12p70, using both the IL-12R β 1 chain and STAT4 (182), but is not as efficient in inducing IFN- γ expression.

The other cytokine, IL-27 is composed of an IL-12p40-related molecule and a novel p28 subunit (183). In comparison to IL-23, IL-27 is a potent inducer of IFN- γ in naïve T cells but the role of either cytokine in promoting IFN- γ production in different settings awaits further analysis.

Role of IFN- α/β

While IL-12 is required for induction of IFN- γ during intracellular bacterial and intracellular protozoal infections, the same is not consistently true for viral infections where IL-12p70 is not always detected (reviewed in (180)). During Lymphocytic choriomeningitis virus (LCMV) and Vesicular stomatitis virus infections, IL-12 is actually not required for induction of T cell-derived IFN- γ (184). Such observations may be explained by the fact that IFN- α/β actually inhibit the production of IL-12 and consequently IFN- γ production, both *in vitro* as well as during LCMV and MCMV infections (185). Levels of IFN- α/β induced during MCMV infection are lower than during LCMV, probably explaining why (early) IL-12-dependent, NK cell-derived IFN- γ release is observed during MCMV (186, 187). In fact, inhibition of endogenous IFN- α/β augments IL-12-driven responses during MCMV and allows the appearance of early IL-12 and IFN- γ production during LCMV (185). The association between high titers of IFN- α/β and reduced IFN- γ expression has also been suggested in mice infected with hyper-virulent strains of *M. tuberculosis* (188).

While IFN- α/β are known to promote IFN- γ production from human T cells (189), fewer studies report the same in mice. Direct support for this comes from LCMV infection, where IFN- α/β R^{-/-} mice cannot mount a protective CD8⁺ T-cell-derived IFN- γ response even though an IL-12-dependent IFN- γ production attempts to compensate for the absence of IFN- α/β -driven IFN- γ (190). Also, exogenous IFN- α/β have been shown to be potent stimulators of IFN- γ release from bone marrow-derived NK cells when used in combination with IL-12 or IL-2, but not alone (191). Interestingly, splenocytes from LPS-hyporesponsive C57Bl/10ScCr mice stimulated with different heat-inactivated Gram-negative bacteria showed impaired IFN- γ production (192). This effect could be reverted by addition of exogenous IFN- α or IFN- β or by supplementing splenocyte cultures with WT C57Bl/10ScSn macrophages. Though C57Bl/10ScCr mice do not express TLR4 (59) they are also defective in IL-12R signaling (193). Thus, this study clearly supports an IL-12-independent pathway for IFN- γ production in the experimental setting.

IL-18 has been shown to work together with IFN- α/β in the release of IFN- γ from human NK and T cells (194, 195), from mouse splenocytes stimulated with heat-killed, Gram-negative bacteria (196) and from CD8⁺ T cells during LCMV infection (197). In fact, a previous discrepancy between human and murine cells in the ability of IFN- α/β to stimulate IFN- γ through STAT4 activation has been challenged. It was previously suggested that IFN- α/β were unable to synergize with IL-12 for STAT4-dependent IFN- γ production in mice because of a minisatellite insertion in the mouse STAT2 gene that disrupted the ability of IFN- α/β to activate STAT4 (198). Now, not only has IFN- β been shown to activate STAT4 in Con A-activated mouse splenocytes (196) but direct binding of STAT4 to the IFN- γ promoter (just like IL-12) has been shown in IFN- α -stimulated splenocytes or in splenocytes from LCMV-infected mice (199).

Myeloid IFN- γ

Expression of IFN- γ by other populations other than NK or T cells has been an issue of some controversy but is starting to gain acceptance. Actually the first reports on macrophage-derived IFN- γ are not recent (200, 201). Few studies on myeloid IFN- γ have been made using human material. Except for an isolated study showing that human neutrophils secrete IFN- γ when stimulated *in vitro* with either LPS, IL-12 or TNF- α (202), other studies essentially involve IFN- γ expression by alveolar macrophages infected *in vitro* with *M. tuberculosis* (203), obtained from sarcoidosis patients (204) or from bronchoalveolar lavage of patients with tuberculosis (205). The overwhelming majority of studies were thus made with mouse cells and show that IFN- γ can be produced *in vitro* by macrophages stimulated with LPS (206, 207), IL-12 (208), IL-12 and *M. bovis* (209), *Legionella pneumophila* (210), IL-12 and IL-18 (211-215) or IFN- γ itself (216). These studies have used a variety of macrophage populations, including resting or thioglycollate-elicited peritoneal macrophages, BMM ϕ and alveolar macrophages. A role for IL-12 in myeloid-derived IFN- γ expression is also supported by studies with DCs. Splenic DCs infected *in vitro* with *L. monocytogenes* produced IFN- γ in an IL-12-dependent manner (217). A role for STAT4 in such IL-12-dependent DC and macrophage IFN- γ has also been reported (213-215). Overall, these reports clearly suggest that IL-12 is an important factor for myeloid-derived IFN- γ even though it was not until later that the IL-12R was formally demonstrated on macrophages (212) or DCs (218). Furthermore, IL-12- and IL-18-stimulated murine (219, 220) and human (221) B cells have also been reported to express IFN- γ . It is clear that the sole combination of IL-12 and IL-18 suffices for production of substantial amounts of IFN- γ from non-T cells.

The relevance of myeloid IFN- γ is nevertheless unclear. Few reports have addressed its actual role during intracellular infection, even *in vitro*. Except for *T. gondii* (214) and *L. monocytogenes* (217), the other pathogens used in studies of myeloid IFN- γ (including *C. pneumoniae* in this thesis) have been respiratory pathogens; *Nocardia asteroides* (222), mycobacteria species (205, 209) and *L. pneumophila* (210). Only during *T. gondii* and *L. pneumophila* infection was the involvement of IFN- γ in restricting pathogen growth studied. Though the biological significance was not addressed, expression of myeloid IFN- γ was shown *ex vivo* during *N. asteroides*, *M. tuberculosis* and *L. monocytogenes*. A protective role for IFN- γ *in vivo* is well known in the latter two infections (110-112) as well as during *L. pneumophila* (223). The role of IFN- γ in protection against *Nocardia* is unknown though a patient with inherited IL-12 deficiency had clinical *N. asteroides* infection (224) and IFN- γ treatment of Chronic Granulomatous Disease patients suffering from *Nocardia* infections protected patients against *Nocardia* dissemination (225).

Together with NK cell-derived IFN- γ , myeloid-derived IFN- γ might play an important role in the innate response against infection (discussed in (226)). Further, even small amounts of IFN- γ could suffice for induction of macrophage responses in the absence of NK cells, and contribute to early pathogen containment. It seems logical that IFN- γ , the prototypical macrophage-activating cytokine, should be produced in an autocrine/paracrine fashion by macrophages themselves. As for DC-derived IFN- γ ,

since DCs may be important in governing the differentiation of T cells during an immune response (227), DC-derived IFN- γ could possibly influence this process.

IFN- α/β R and IFN- γ R Signaling

Since IFN- α/β and IFN- γ signal through related pathways that lead to some overlap in gene expression, such signaling is addressed together. The details of IFN- α/β R and (particularly) IFN- γ R signaling have been extensively characterized (reviewed in (76, 91, 228)). The IFN- γ R is a heterodimeric receptor consisting of a high-affinity, ligand-binding α -chain (IFNGR1) and a β -chain (IFNGR2) required for signaling (Figure 4). Associated to the different receptor chains are Janus kinases (JAKs), JAK1 binding to IFNGR1 and the β -chain of the IFN- α/β R (IFNAR2), JAK2 to IFNGR2 and Tyk2 binding to the α -chain of the IFN- α/β R (IFNAR1). JAKs are associated to these receptor subunits even in the absence of ligand binding and are essential for IFN-dependent responses. Bioactive IFN- γ works as a homodimer, binding two IFNGR1 subunits. Less is known how IFN- α and IFN- β bind to IFNAR1, but for both IFN- α/β and IFN- γ , ligand binding mediates aggregation of the different receptor subunits, leading to the assembly of an active receptor complex. This in turn leads to the activation of receptor-associated JAKs through auto- and trans-phosphorylation on tyrosine residues and thus the initiation of intracellular signal transduction. Activated JAKs phosphorylate in turn tyrosine residues on the cytoplasmic tails of the IFN- α/β R and IFN- γ R, creating docking sites for STATs. STATs are both signaling molecules and transcription factors. In unstimulated cells, STATs remain in the cytosol but when activated, transduce to the nucleus and activate transcription of target genes. STATs contain SH2 domains and are therefore capable of recognizing phosphorylated tyrosine residues. Upon receptor-tyrosine phosphorylation by JAKs, STATs, via their SH2

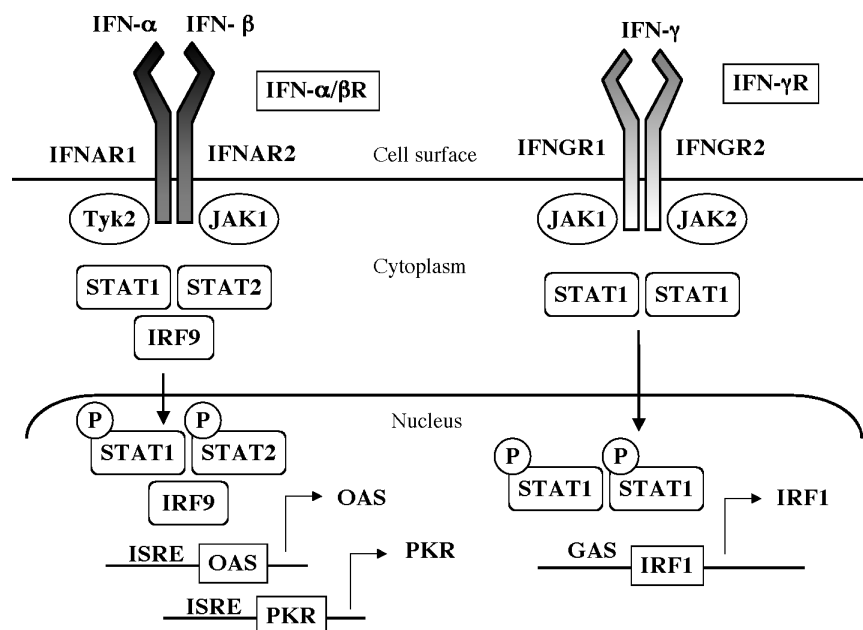


Figure 4: Schematic Diagram of IFN- α/β R and IFN- γ R Signaling. See text and (76, 91, 228) for details.

domains, dock onto the receptor and become activated by JAKs that phosphorylate STATs at single tyrosine residues. This enable STATs to form complexes with each other and adaptor proteins that together will dictate the DNA-binding ability of the transcription factor.

Stimulation with IFN- γ leads to formation of primarily STAT1:STAT1 homodimers and stimulation with IFN- α/β leads to formation of STAT1:STAT2:IRF9 heterotrimers (reviewed in (76)). These transcription-factor complexes bind to distinct elements in the promoters of IFN-responsive genes. STAT1 homodimers bind to so-called IFN- γ -activated sites (GAS) while STAT1:STAT2:IRF9 heterotrimers bind to IFN-stimulated response elements (ISREs). Overlap exists, and it is known that IFN- α stimulation also leads to the formation of STAT1 homodimers and under certain conditions, IFN- γ -activated STAT1 homodimers can interact with IRF9 that redirect the STAT1 homodimers to ISRE sequences (229). However, duration and degree of stimulation with a particular IFN and cell-type specificity affect the formation of the particular type of STAT1-containing transcription factor, thus adding diversity to the (genes induced and) responses elicited by IFN- α/β and IFN- γ .

STAT1-Independent Signaling

Confirming the central role of STAT1 in mediating IFN- α/β - and IFN- γ -dependent responses, STAT1^{-/-} mice were unable to manifest any biologic responses to either IFN- α or IFN- γ (116, 119). In line, these animals also display marked susceptibility to viral (116, 119), intracellular bacterial (116) and intracellular protozoan infections (117, 118). It was surprising though, that IFN- γ was shown to regulate gene expression even in the absence of STAT1 (230), revealing the presence of IFN- γ -dependent but STAT1-independent signaling. Using oligonucleotide microarrays, this alternative signaling pathway has been further studied in IFN- γ -stimulated mouse embryonic fibroblasts (MEFs) (122) and IFN- α - or IFN- γ -stimulated BMM ϕ (120). IFN- γ -dependent, STAT1-independent genes expressed in MEFs required JAK1 and many are also known to be induced by Platelet-derived growth factor, a potent inducer of cell proliferation (122). The fact that some genes, like *c-myc*, were induced in STAT1^{-/-} MEFs but repressed in the presence of STAT1 further add to the regulatory diversity elicited by IFN signaling though a biological relevance for such signaling remains to be clarified. Also, IFN- γ repressed M-CSF-dependent proliferation of bone marrow cells in the presence but induced it in the absence of STAT1 (120). A physiological relevance for IFN-dependent, STAT1-independent signaling was demonstrated by the enhanced susceptibility of IFN- α/β R^{-/-}/IFN- γ R^{-/-} mice to both Sindbis and MCMV infections in comparison to STAT1^{-/-} mice (120). A relevant situation for STAT1-independent but IFN-dependent, anti-viral responses may arise if STAT1 activation is blocked by viruses, as reported during measles (231) and human papillomavirus (232) infections. A final intriguing finding on IFN-dependent, STAT1-independent signaling comes from LCMV-infected mice where early production of IFN- γ is absent. An explanation for this is given by the fact that in STAT1^{-/-} but not WT mice, IFN- α/β stimulate LCMV-induced IFN- γ release (233). These findings have however not been confirmed by others.

CHLAMYDIA

Microbiology

The word “Chlamydia” comes from the Greek for “cloak-like mantle”, based on the erroneous belief that the Chlamydial inclusion “cloaked” the nucleus of infected cells (234). The genus Chlamydia consists of the four species, *C. pneumoniae*, *C. trachomatis*, *C. psittaci*, and *C. pecorum*. Despite intra-species differences, Chlamydia share some primary biological features (reviewed in (235)); I) Chlamydia are obligate intracellular gram-negative bacteria; II) Chlamydia share a common, developmental cycle; and III) Chlamydia share a similar genome organization. These features consequently point towards a common immunobiology, addressed later.

Life Cycle

In being obligate intracellular parasites, host-cell entry is not an option but rather a pre-requisite for survival (and disease). In meeting such requirements, Chlamydia possess an unique, biphasic developmental cycle (reviewed in (235-237)) (Figure 5). It involves a small (100 to 200 nm) metabolically inactive, infectious form called the Elementary body (EB) and a non-infectious, metabolically active, larger (500 to 1000 nm) form called the Reticulate body (RB). Surprisingly, many details pertaining to this life cycle are unclear.

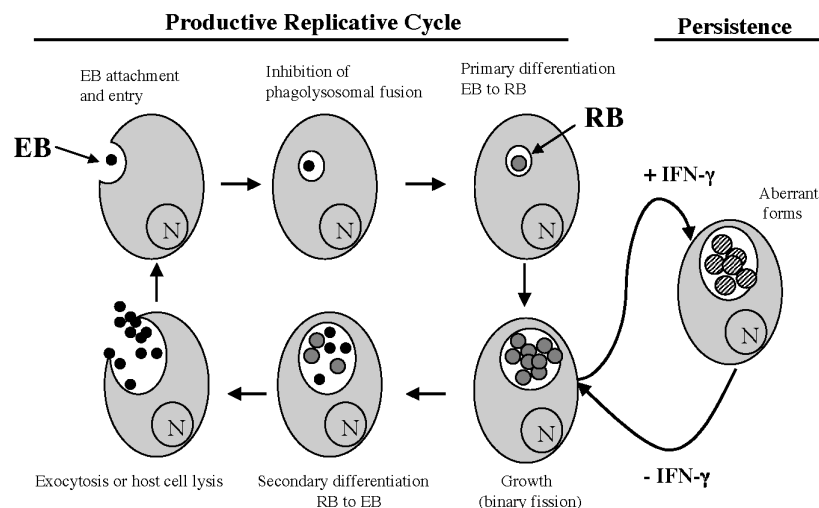


Figure 5: Chlamydia Life Cycle. See text and (235-237) for details.

It is known that Chlamydia enter cells through endocytosis, pinocytosis and phagocytosis. This process involves several Chlamydial molecules (reviewed in (236)), including the most abundant Chlamydial surface protein, Major outer membrane protein. It is also clear that membrane-bound EBs avoid (via uncharacterized

mechanisms) fusion with lysosomes (238-240) and then somehow differentiate into RBs. RBs reside within an intracytoplasmic, parasitophorous vacuole, termed the Chlamydial “inclusion”, which is the only environment known to support Chlamydial replication. Sphingomyelin from the host-cell exocytic pathway seems to accumulate on the inclusion surface (241), making the inclusion into a “modified” Golgi-derived vesicle which is retained intracellularly (reviewed in (242)). It has long been known that Chlamydia contain needle-like projections, but it was not until sequencing of Chlamydia genomes that the presence of a type III secretion machinery was confirmed (243-246). This type III secretion system seems to be present in both EBs and RBs (247). It supports the notion that Chlamydia are able to acquire nutrients from the cytoplasm although the inclusion is not freely permeable (248) and are able to modify the inclusion membrane so as to camouflage it into an exocytic vesicle. A recent analysis of Chlamydial gene expression during the developmental cycle through the use of oligonucleotide microarrays has suggested a role for a novel Chlamydial gene homologous to human early endosomal antigen 1 (involved in endosomal trafficking and fusion in mammalian cells) in avoiding phagolysosomal fusion (249).

After multiple rounds of replication, probably through binary fission, RBs re-differentiate back into EBs that are released either through exocytosis or host-cell lysis to initiate new rounds of infection. Only infectious EBs perpetuate the life cycle. The length of the developmental cycle has been studied *in vitro* and may take 48h to 72h depending on the Chlamydial strain, host cell and environmental conditions.

Chlamydial Persistence

Persistence is defined as a long-term association between Chlamydia and the host in which the organism remains viable, but in a culture-negative state (reviewed in (250, 251)). It should not be confused with chronic infections where organisms are actively replicating. All strains of Chlamydia may become persistent. Persistence has not been specifically addressed in works presented in this thesis but the subject merits discussion. Persistent infection *in vitro* seems to involve incomplete chlamydial growth, if any. It has been suggested to be a developmental stage triggered as a consequence of stress, such as during nutrient deprivation, elevated temperature or presence of IFN- γ . Morphological criteria have been described for persistent forms of Chlamydia. Stress-induced atypical inclusions are generally smaller in diameter and contain RB-like aberrant bodies, larger in diameter than typical RBs. Such aberrant forms are refractory to antibiotic treatment, since they are essentially non-replicative. Resumption of growth and the eventual release of EBs is observed upon stress removal, leading to a reorganization of the aberrant form into a replicative organism (Figure 5). The mechanisms behind this shift are not known.

Aberrant Chlamydial inclusions express a special gene and protein profile including lower levels of LPS, outer membrane proteins, genes involved in cytokinesis and DNA replication and the up-regulation of stress-induced heat-shock protein (HSP)-60 (252-254). HSP-60 colocalizes with macrophages in atherosclerotic plaques (255), causes the production of pro-inflammatory cytokines by macrophages *in vitro* (255-257) and the oxidation of low-density lipoprotein (258), which is involved in atherosclerosis. Also, anti-HSP-60 immune reactivity correlates with the pathological consequence of Chlamydial infections (reviewed in (259, 260)). Recently, the use of

oligonucleotide microarrays has revealed an even more detailed picture of the Chlamydial gene expression profile during IFN- γ -mediated persistence *in vitro* (261). It gives firm support for the idea that the Chlamydia “persistence stimulon” is more than a just a simple stress response, but rather a global response permitting bacterial survival via an alternative developmental stage.

Evidence for Chlamydial persistence has also been documented *in vivo* during *C. pneumoniae* infection (262), during genital infection with the *C. trachomatis* mouse pneumonitis agent (MoPn) (263) and lung infection with a *C. trachomatis* trachoma strain (264). Mice which have been previously infected intranasally with *C. pneumoniae* or genitally with MoPn, were allowed to resolve primary infection, i.e., to become culture-negative for Chlamydia, and were then treated with an immunosuppressant such as cortisone which caused the re-activation of infection, suggesting that the organisms had become “culture-negative” but were still present and viable *in vivo*. It is therefore unclear during Chlamydia infections, if the reduction in culturable Chlamydia observed as the infection starts to subside is actually a sign of clearance of the organism by the host or simply the induction of persistence.

Though persistence has long been recognized as a major factor in the pathogenesis of Chlamydial disease (reviewed in (250)), atypical aberrant forms have only been associated with persistence *in vitro*. Re-activation of Chlamydia infection in mice, though supportive of persistence, does not imply that aberrant forms are actually present *in vivo* during the persistent stage of infection. Recently, atypical inclusions could be cultured from *C. trachomatis*-infected patients refractory to antibiotic treatment and in two individuals, atypical *C. trachomatis* inclusions were also detected *in vivo* by electron microscopy (265). Although one cannot draw too many conclusions on this small sample size, it is the first line of evidence that atypical, aberrant forms of Chlamydia also exist *in vivo*.

Chlamydia Infections

Chlamydia are among the most widespread bacterial pathogens in the world, causing different afflictions characterized by asymptomatic infection to late-stage disease in humans and animals (reviewed in (236)). *C. trachomatis* and *C. pneumoniae* are human pathogens while *C. pecorum* infects cattle and *C. psittaci* infects birds (occasionally causing zoonosis in humans). Below the waist, *C. trachomatis* serovars (strains) D to K are major causes of sexually-transmitted disease worldwide, with pathological complications such as pelvic inflammatory disease and its sequelae, ectopic pregnancy or tubal infertility. Above the waist, *C. trachomatis* serovars A to C are agents of ocular disease, causing conjunctivitis and trachoma, the most common cause of preventable blindness worldwide. Systemic disease caused by *C. trachomatis* Lymphogranuloma venereum strains occur, but are not as common. While primary *C. trachomatis* infections are often asymptomatic, tubal and ocular scarring are the long-term consequences of persistent (or recurrent) infections.

C. pneumoniae Respiratory Tract Infections

C. pneumoniae was first isolated in 1965 from the conjunctiva of a Taiwanese child, identified as a human respiratory pathogen in 1986 and finally established as a separate species of Chlamydia in 1989 (reviewed in (266)). Sero-prevalence of over 50%, observed worldwide, is reached by age 20 and continues to rise with age, reaching approximately 75% in the elderly (reviewed in (266)). *C. pneumoniae* is today recognized as an “atypical” agent of community-acquired pneumonia (CAP), associated in 6 to more than 20% of cases, ranking often as the third most common etiological agent (reviewed in (267)). The incidence of *C. pneumoniae*-related bronchitis is lower, approximately 5% (reviewed in (237)) while incidences as high as 25% have also been reported (268). Less well characterized are the associations of *C. pneumoniae* to pharyngitis (269), otitis (270) and sinusitis (271).

There are no obvious clinical manifestations specific to *C. pneumoniae*-induced respiratory tract infections (reviewed in (237, 266, 272, 273)), except for possibly chronic cough. Shortness of breath has also been reported (274). Transmission is probably from person-to-person, via respiratory secretions (275). Sero-epidemiological studies in family settings suggest that transmission is relatively inefficient (276). It is estimated that approximately 70% of *C. pneumoniae* respiratory infections are mild or asymptomatic, with 30% causing upper and lower respiratory disease, including CAP (reviewed in (237)). Infection may present itself as biphasic, starting often with upper respiratory involvement, usually pharyngitis, which may regress and be followed weeks later by a cough and lower respiratory affliction. Though *C. pneumoniae* causes disease only in a minority of cases, pneumonia *per se* may be fatal and may require hospitalization. Pneumonia (together with influenza) is the sixth leading cause of death in the USA and *C. pneumoniae* is probably the second most common agent of CAP requiring hospitalization (reviewed in (277)). Long-lasting, i.e., chronic, respiratory disease due to *C. pneumoniae* has also been reported (278, 279) as well as epidemics in schools (280, 281) and among military conscripts (282-284). In fact, the first epidemic described was identified solely because of a routine radiographic tuberculosis survey (285), supporting the often mild nature of the infection. Like *C. trachomatis*, severe disease due to *C. pneumoniae* is rare but case reports reveal severe systemic disease (273, 286), respiratory distress syndrome (287), multiorgan dysfunction (288, 289) and fulminant pneumonia (290). Altogether, observations clearly highlight the importance of *C. pneumoniae* as a respiratory pathogen.

Association Between *C. pneumoniae* and Atherosclerosis

The association between *C. pneumoniae* and atherosclerosis is undoubtedly one of the most fascinating findings in the field. A Finnish sero-epidemiological study was the first to coin an association between acute myocardial infarction and *C. pneumoniae* serology (291). These findings were followed by direct demonstration (292) and even isolation (293) of *C. pneumoniae* in atherosclerotic lesions. The significance of these findings are however inconclusive. Several studies in experimental models and antibiotic clinical trials have attempted to address this etiological issue. Infection of rabbits with *C. pneumoniae* induced inflammatory changes in the aorta while treatment

of the same with antibiotics prevented pathology (294). Similarly, in two different mouse models of atherosclerosis, *C. pneumoniae* infection accelerated disease progression (295, 296).

The first clinical study analyzing the effect of antibiotic treatment on the outcome of cardiovascular disease reported a decrease in adverse cardiovascular events and mortality (297). A total of 13 other studies have since been performed (reviewed in (298)). Despite many of these studies yielding positive results, all were limited by size, short observation periods and short antibiotic therapy regimens. The WIZARD trial is the only large, adequately powered, clinical trial that has been concluded. Even though antibiotic-treated subjects had a statistically significant, 33% reduction in mortality and myocardial infarction during the first 6 months of the study, the overall results were negative. Treatment fall-off after 3 months of antibiotic therapy questioned whether the duration of treatment was adequate or not. This has been taken into account in two other large trials, ACES and PROVE IT, which include 1 to 2 years of antibiotic treatment. These trials are estimated to be concluded by March 2004. Until then, a role for anti-*C. pneumoniae* therapy in atherosclerosis remains unsettled.

Experimental Chlamydia Infections

Infection with *C. trachomatis*

In comparison to other experimental mouse models of infection, relatively few studies have addressed immunity to Chlamydia. Such studies have focused mainly on infection with different serovars of *C. trachomatis*, particularly MoPn. This is a mouse strain not naturally occurring as a human pathogen. Nevertheless, MoPn has been extensively used as a model for both lung and genital-tract infection. Genital-tract infection with MoPn closely resembles the human disease (reviewed in (299)). The use of genetically-modified mice has helped dissect immunity to *C. trachomatis* infection (reviewed in (299, 300)), and will serve later as a discussion basis for the results presented in this thesis where *C. pneumoniae* infection has been used. At this point it suffices to say that a protective role for CD4⁺ and CD8⁺ T cells and IFN- γ has been reported during *C. trachomatis* infection (reviewed in (301)).

Infection with *C. pneumoniae*

A mouse model for *C. pneumoniae* using the first described respiratory isolate AR-39, has been described (302). Intranasal infection induced pneumonia in animals and no differences in susceptibility were observed between the inbred mouse strains (C57Bl/6, BALB/c and C3H/HeN) tested. The work presented in this thesis is based on a similar *C. pneumoniae* lung model which uses the respiratory isolate Kajaani 6 (303). This isolate was obtained during an epidemic outbreak in military garrisons in northern Finland (304). Intranasal inoculation with 10⁶ inclusion-forming units of Kajaani 6 (a dose 3 times lower than for AR-39) induces similar pathological features as that described for the original model, possibly suggesting that this Finnish isolate is slightly more virulent. Still, whole genome sequence comparisons between different *C. pneumoniae* isolates reveal little variation (245, 305). Also, when an atherosclerotic isolate was compared to a respiratory one, no differences were found (293). Even

though these comparisons were not between AR-39 and Kajaani 6, it still seems plausible that different *C. pneumoniae* isolates should induce similar features in experimental settings based on their high genomic conservation. The main discrepancy between the two models is that systemic dissemination has been reported in WT mice infected with AR-39 (306). Infectious dose may account for this, as it has only been observed in the Kajaani 6 model following high-dose infection (303).

Characteristic observations (reviewed in (307)) made in both models include the establishment of a patchy interstitial pneumonia, i.e., affected regions interspersed among areas of normal tissue. Inflammatory infiltrates are first neutrophilic and later mononuclear. Chlamydia is often detected in the inflamed areas (308). This response is very similar to that observed during *C. trachomatis* lung infection (309). Bacterial lung levels also correlate with the degree of lung pathology and active infection is eventually resolved (with sequential ablation of lung pathology) in immunocompetent mice, or bacteria are at least not culture-positive from lungs 60 days after infection (302). Ultrastructural studies revealed the presence of Chlamydial inclusions (containing EBs, intermediate forms and RBs) in both epithelial cells and macrophages, as well as dividing RBs (308). The persistent form was not described. Such findings are also recorded in the lungs of *C. trachomatis*-infected mice (310). The *C. pneumoniae* mouse model seems consistent with human disease to the point that WT mice infected via the respiratory route acquire a mild, non-lethal lung infection with ensuing pneumonia. Protective immunity in this model has not been studied in any greater detail.

AIMS OF THIS THESIS

The general aim of this thesis was to characterize the immune response during experimental *C. pneumoniae* infection. Specific aims were to:

- I) Study the relative role of innate and adaptive immunity during Chlamydia lung infection, with emphasis on the regulation and role of particularly IFN- γ and IFN- α/β in protection.
- II) Study the regulation and expression of cytokines in Chlamydia-infected macrophages and their role in restricting Chlamydial growth.
- III) Study pattern recognition and signal transduction pathways activated in Chlamydia-infected macrophages.

DISCUSSION OF OBTAINED RESULTS

The importance of IFN- γ in protective immunity to intracellular infections has been addressed, as has the function of macrophages as important effectors (and even producers) of IFN- γ -mediated responses. PRR-bearing macrophages in the lungs might recognize invading *C. pneumoniae* and through these (and other) innate receptors contribute to activation of effector mechanisms. Understanding the innate immune interplay between macrophages and *C. pneumoniae* may be central for unraveling immunity to this pathogen. Specific interest regarding IFN- γ and innate immunity to *C. pneumoniae* is therefore emphasized in the discussion. It should be stressed that the content of this section requires of the reader prior knowledge of Papers I to VI, since for the sake of space, a summary of each work has been omitted.

IFN- γ in Resistance to *C. pneumoniae* Lung Infection

Role of IFN- γ in the Outcome of *C. pneumoniae* Infection (Papers I to III)

The single most concise observation made throughout the different studies, was the protective role of endogenous IFN- γ during infection with *C. pneumoniae*. Lung levels of *C. pneumoniae* were dramatically enhanced in IFN- γ R^{-/-} (Papers I, II & VI) and IFN- γ ^{-/-} (Paper VI) mice compared to WT controls. Enhanced *C. pneumoniae* levels and *C. pneumoniae* DNA were also observed in IFN- γ R^{-/-} BMM ϕ compared to WT BMM ϕ (Paper IV). This seems obvious in light of the described massive anti-microbial program regulated by IFN- γ . The susceptibility of IFN- γ ^{-/-} or IFN- γ R^{-/-} mice to various intracellular bacteria, intracellular protozoa and some viruses also anticipates this result (both discussed under Biological Functions of IFN- γ). Though IFN- γ ^{-/-}, IFN- γ R^{-/-} or anti-IFN- γ -depleted mice also display enhanced susceptibility following MoPn (311) or *C. trachomatis* genital strain (312, 313) infections, variations in susceptibility to IFN- γ have however been reported. Whereas IFN- γ ^{-/-} mice developed more severe genital-tract infections when *C. trachomatis* genital strain was used, minimal effects were noted on resolution of genital infection with MoPn (314, 315). These differences were not related to cytokine production *in vivo*, but rather to the differential susceptibility of the strains to IFN- γ -mediated effector mechanisms *in vitro* (315, 316). Thus the mouse virulent MoPn seems to have adapted to the inhibitory, species-specific effects of IFN- γ . But reports do show a protective role for IFN- γ during MoPn lung infection (317) and during MoPn genital infection in SCID mice (318).

Host-cell polarization state has also been shown to affect the degree of IFN- γ -mediated inhibition. Larger quantities of IFN- γ are required for *C. trachomatis* growth inhibition in polarized cells (319) and is suggested to be due to larger intracellular pools of L-tryptophan, which cannot be produced by some Chlamydia strains, in these cells (320).

Discrepancies have also been reported on the role of IFN- γ during primary contra re-infection. Protection may for instance depend on genetic background, with IFN- γ being protective during primary infection of C57Bl/6 but not BALB/c WT mice,

but involved in protection of both strains during re-infection (321). Such differences may however be due to the degree of success of the anti-IFN- γ depletion and have not been confirmed in IFN- γ ^{-/-} mice. Using the same *C. pneumoniae* isolate, the results in this thesis do not reveal differences between IFN- γ R^{-/-} mice on the 129Sv/Ev or C57Bl/6 background although both carry the H-2^b haplotype (Paper VI). In all, genetic background does not seem to affect the study of *C. pneumoniae* infection in mice.

IFN- γ -Independent Resolution of Infection

While IFN- γ was clearly important for restricting bacterial growth in the lungs, it was not required for resolving infection (Paper I, II & VI). This suggests the presence of IFN- γ -independent mechanisms of protection involved in resolution of infection or alternatively in inducing persistence. The 100% mortality observed in Recombination-activation gene (RAG)-1^{-/-}/IFN- γ R^{-/-} or RAG-1^{-/-}/IFN- γ ^{-/-} mice, which is not seen in IFN- γ R^{-/-} or IFN- γ ^{-/-} mice (Papers II & III) also clearly supports the existence of T- and B-cell-dependent, IFN- γ -independent protection.

Unpublished observations from our laboratory reveal enhanced *C. pneumoniae* levels in the lungs of B-cell-deficient μ MT^{-/-} (Igh6) mice at 24 but not 7 or 14 days after infection in comparison to WT controls, suggesting a protective role for B cells later during *C. pneumoniae* infection. These findings are further supported by μ MT^{-/-}/CD8^{-/-} mice which display higher susceptibility than μ MT^{-/-} or CD8^{-/-} mice at 14 and 24 days, suggesting that CD8⁺ T cells and B cells synergize in the resolution of infection (M. E. Rottenberg, unpublished observations). Lung infection of μ MT^{-/-} mice with MoPn also reveals enhanced susceptibility (322) though μ MT^{-/-} mice infected genitally with a *C. trachomatis* genital strain do not (323, 324). While re-infection of μ MT^{-/-} mice has not been published for *C. pneumoniae*, μ MT^{-/-} mice after genital infection with MoPn are more susceptible to re-infection (325). When μ MT^{-/-} animals are also depleted of CD4⁺ cells, they are unable to resolve secondary infection (326). In summary, IFN- γ -independent mechanisms of protection exist during Chlamydia infection, are probably important later during primary infection or during re-infection, and probably involve antibody production.

Regulation of IFN- γ Expression During *C. pneumoniae* Infection (Papers II & VI)

It is already appreciated that IL-12 can regulate IFN- γ expression on several immune cells (discussed under Regulation of IFN- γ Production: Role of IL-12). In light of this, it seems obvious that genital infection of IL-12p40^{-/-} mice with a *C. trachomatis* genital strain, and genital or lung infection of IL-12p40^{-/-} or anti-IL-12-depleted mice with MoPn result in slight to dramatically increased susceptibility to infection, which are associated to reduction in IFN- γ production (315, 327, 328). Similarly, *C. pneumoniae*-infected IL-12p40^{-/-} mice displayed enhanced susceptibility to infection and reduced expression of IFN- γ in the lungs (Paper II). Similar observations have been made with *C. pneumoniae*-infected, anti-IL-12-depleted mice (329). In all, IL-12-dependent IFN- γ seems to be an important protective pathway elicited during Chlamydia infections. The cellular source of this IL-12 *in vivo* is however not fully studied during *C. pneumoniae* infection and includes at least macrophages (Paper III).

Administration of exogenous IL-12 also confers protection to naïve mice against challenge with *C. trachomatis* and is paralleled by IFN- γ secretion *in vivo* (330, 331). Still, observations made in *C. psittaci*-infected mice suggest that despite the necessity for IL-12 in inducing early IFN- γ production, IFN- γ but not IL-12 was required for resolving infection (332). Similarly, while IL-12p40^{-/-} and IFN- γ R^{-/-} mice are both susceptible to *C. pneumoniae* compared to WT controls, IFN- γ R^{-/-} mice are more susceptible than IL-12p40^{-/-} (lacking both IL-12 and IL-23) animals (Paper II). These findings suggest the presence of alternative, IL-12-independent pathways for IFN- γ release during Chlamydia infection. IL-18 could be involved in such a response and has been implicated in IL-12-independent IFN- γ release from CD4⁺ T cells in *T. cruzi*-infected mice (333). STAT4-independent IFN- γ -mediated protection was also observed in *T. gondii*-infected mice receiving exogenous IL-2 + IL-18 (334). Still, during MoPn lung infection, IL-18^{-/-} or ICE^{-/-} mice did not display enhanced susceptibility to infection though a slight reduction in IFN- γ production was observed (328). During *C. pneumoniae* lung infection, ICE^{-/-} mice seem to have a delayed resolution of infection (A. Gigliotti Rothfuchs & M. E. Rottenberg, unpublished observations), which is not observed in IL-12p40^{-/-} mice (Paper II). This suggests a dissociation between IL-12- and IL-18-driven responses *in vivo* with only a marginal role for IL-18 in protection against Chlamydia. It also suggests an IL-12- and IL-18-independent control of infection.

In line, a role for both IL-12- and IL-18-independent, IFN- γ -mediated protection has also been observed during *L. monocytogenes* infection, so other factors may govern IFN- γ expression even during bacterial infections (335). In such a situation, it is possible that cytokines such as IFN- α/β , shown to regulate IFN- γ expression mostly but not exclusively during viral infections, (discussed under Regulation of IFN- γ Production: Role of IFN- α/β) may be involved. This however, does not seem to be the case in *C. pneumoniae* infection since lungs from IFN- α/β R^{-/-} mice did not display diminished expression of IFN- γ or increased levels of *C. pneumoniae* (Paper VI). Thus, the IL-12-independent stimulus of IFN- γ production in the lungs of *C. pneumoniae*-infected mice is unclear. Still, infection of IL-12p40^{-/-} and IL-12p40^{-/-}/IL-18^{-/-} mice has not been compared and a role for other IL-12-family members cannot be excluded.

IFN- γ -Mediated Effector Mechanisms in the Control of *C. pneumoniae* (Papers I to III)

As previously described, IDO is clearly implicated in IFN- γ -mediated *in vitro* inhibition of Chlamydial replication, at least in human cells. Differences in susceptibility of various Chlamydia strains to IFN- γ exist, and are ascribed to that strain's ability to acquire or synthesize L-tryptophan. The IFN- γ -sensitive *C. pneumoniae* (150) lacks L-tryptophan biosynthesis genes (245, 246) as probably do IFN- γ -sensitive *C. trachomatis* trachoma strains (315, 316) where partial sequencing has revealed a truncation in a L-tryptophan biosynthesis gene (336). On the other hand, L-tryptophan biosynthesis genes are present in MoPn and *C. trachomatis* genital strains (244-246) which are more resistant to the actions of IFN- γ (315, 316). Thus, L-tryptophan biosynthesis seems to be an adaptive virulence determinant in Chlamydia, corroborating the importance of IFN- γ -dependent IDO in restricting Chlamydial growth. Such a role for IDO in generating a persistent form of Chlamydia has been

shown by the re-activation of infection upon addition of L-tryptophan to *C. trachomatis*-infected, IFN- γ -treated cells (337).

During *C. pneumoniae* infection, IDO mRNA expression was only observed in the lungs of RAG-1^{-/-} mice, where levels of IL-12p40 and IFN- γ were much higher than in WT controls. A clear requirement for IFN- γ in induction of IDO was shown by the complete ablation of IDO transcripts in lungs of RAG-1^{-/-}/IFN- γ R^{-/-} mice, which were also extremely susceptible to infection. Findings from other experimental infections where IFN- γ is important for resistance, have also correlated IDO expression to protection. For example, IDO (but not iNOS) expression and L-tryptophan depletion in the lungs of *T. gondii*-infected mice was dependent on IFN- γ (338). Similar data from *T. gondii* infection showed that IDO expression, L-tryptophan degradation and increased L-kynurenine formation occurred in the presence of IFN- γ and IRF-1 but not TNFRp55 (339). Also, enhanced IDO expression is observed in the placenta of *L. monocytogenes*-infected mice, expression of which is completely abrogated in the absence of IFN- γ (340). Still, these findings do not strictly show a causative role for IDO-mediated protection in experimental infections.

In mouse fibroblasts, epithelial cells and macrophages, iNOS was credited for mediating IFN- γ -dependent inhibition of chlamydial growth (341-345) and iNOS^{-/-} mice showed a more severe pathology and increased dissemination following MoPn infection (346). These observations are in agreement with the dramatically reduced expression of iNOS in the lungs of *C. pneumoniae*-infected IFN- γ R^{-/-} mice and the enhanced lung levels of *C. pneumoniae* recorded in iNOS^{-/-} mice (Paper I). IFN- γ -mediated, iNOS-dependent expression was also observed in the absence of T and B cells (Paper II), suggesting that innate IFN- γ is important for inducing iNOS. However, IFN- γ -mediated, iNOS-independent protection does occur since iNOS^{-/-} mice are more resistant than IFN- γ R^{-/-} mice. Moreover, treatment of MoPn-infected cells with IFN- γ also resulted in bacterial control in the absence of NO (347).

Similar to IDO, gp91-phox seems only to be induced as a compensation in the lungs of *C. pneumoniae*-infected RAG-1^{-/-} mice (Paper II). While presence of gp91-phox was associated with IFN- γ -mediated protection in such animals, the outcome of *C. pneumoniae* infection in gp91-phox^{-/-} mice has not been evaluated. Genital infection with MoPn in p47-phox^{-/-} mice had no effect on susceptibility or resolution of infection (348). Though superoxide-dependent inhibition of *C. psittaci* in lymphokine-activated macrophages has been reported (349), other studies did not show a reactive oxygen-dependent restriction of Chlamydial growth in IFN- γ -activated epithelial cells (350) or macrophages (351). Thus, Phox is probably not involved in the control of Chlamydia infection. Likewise, IFN- γ -mediated iron deprivation has been suggested to control Chlamydial infections *in vitro* in some studies (352-354) but not others (355).

Exogenous administration of certain antibacterial peptides to epithelial cells or fibroblasts lead to the restriction of Chlamydial growth (356, 357), but it is unclear to what degree IFN- γ regulates such processes. Another effector molecule probably not involved in IFN- γ -mediated protection in *C. pneumoniae* is NRAMP. It was not necessary for control of either *C. pneumoniae*, MoPn lung infection, or during *in vitro* infection of macrophages with MoPn or a Lymphogranuloma venereum strain of *C. trachomatis* (358).

An immunoregulatory role for IFN- γ during MoPn lung infection has been suggested where IFN- $\gamma^{-/-}$ mice exhibited a partial IL-4-dependent immunopathology together with disseminated infection (317). This role does not seem to apply to *C. pneumoniae* infection where expression of IL-4 and IL-10 in the lungs of infected IFN- $\gamma R^{-/-}$ mice are similar to that of WT mice (Paper I). However, IL-13 expression and *C. pneumoniae* levels were increased upon transfer of IFN- $\gamma^{-/-}$ CD4⁺ or IFN- $\gamma^{-/-}$ CD8⁺ T cells into RAG-1^{-/-}/Common cytokine receptor γ chain ($\gamma_c R$)^{-/-} mice, so a role for IFN- γ in regulating the cytokine balance cannot be fully excluded (M. E. Rottenberg, unpublished observations). IFN- γ might also modify the outcome of infection by regulating chemokine expression (359). It is therefore possible that altered levels of IFN- γ -regulated adhesion molecules or chemokines may account for quantitative changes in the inflammatory infiltrate (reviewed in (91)), including the presence of granulocytes mainly in the bronchial lumen (Paper II, data not shown). The relative role of these mechanisms in Chlamydia infection is unknown.

The purigermic P2X₇ nucleotide receptor expressed on macrophages and DCs is known to mediate ATP-dependent killing of *M. bovis* (360) in a process that triggers phagolysosomal fusion and is independent of Phox, iNOS and NRAMP (361). Expression of P2X₇R on monocytes has been shown to be synergistically up-regulated by IFN- γ and TNF- α (362). Similar to Mycobacteria, ATP-dependent P2X₇R signaling has recently been shown to promote growth control of MoPn by triggering phagolysosomal fusion in macrophages (363). This molecule is therefore a novel candidate for IFN- γ -mediated, iNOS-independent control of *C. pneumoniae*.

Another novel IFN- γ -regulated mechanism is that of the growing family of small GTPases, including IGTP, LRG-47, IRG-47 and TGTP (364). They localize to the endoplasmic reticulum but their functions are unclear. Interestingly, IGTP^{-/-} (365) and LRG-47^{-/-} and IRG-47^{-/-} (366) mice displayed enhanced mortality to *T. gondii*, and IRG-47^{-/-} also to *L. monocytogenes* (366), while expressing intact levels of IL-12p40, IFN- γ and iNOS. Further, LRG-47^{-/-} but not IGTP^{-/-} or IRG-47^{-/-} mice have recently been shown to be susceptible to *M. tuberculosis* in an iNOS-independent way and susceptibility was associated to impaired phagosome maturation (367). Such GTPases may be potential IFN- γ -inducible effectors in resistance to Chlamydia.

Cellular Sources of IFN- γ During *C. pneumoniae* Infection (Papers II & III)

IFN- γ secreted as a result of innate-immune responses is important for restricting Chlamydial growth. This is demonstrated by the increased susceptibility of MoPn lung-infected nude mice treated with anti-IFN- γ neutralizing antibodies compared to undepleted nude controls (318) and the severe susceptibility of *C. pneumoniae*-infected RAG-1^{-/-}/IFN- $\gamma R^{-/-}$ (Paper II) or RAG-1^{-/-}/IFN- $\gamma^{-/-}$ (Paper III) mice compared to RAG-1^{-/-} controls. Such severe susceptibility in RAG-1^{-/-}/IFN- $\gamma^{-/-}$ was associated with the dissemination of *C. pneumoniae* infection into hearts, spleens and livers (Paper III and data not shown), suggesting that innate IFN- γ is important for restricting the spread of *C. pneumoniae* *in vivo*.

NK cells are known to participate in the resistance against a number of bacterial and protozoan infections through their ability to secrete IFN- γ (reviewed in (368, 369)). However, the involvement of NK cells in the control of Chlamydia infection is unclear. During MoPn genital infection, anti-asialo-GM1-mediated NK-cell depletion lead to a

reduction in early IFN- γ expression in the genital tract and though depletion had no effect on bacterial levels early during infection, the course of infection was prolonged in the treated group (370). Differences in bacterial levels early after infection in anti-asialo-GM1-treated, MoPn lung-infected mice were also not observed in other studies (371), but reduced lung levels of IFN- γ following anti-asialo-GM1 treatment in SCID mice are recorded (318). Thus, NK cells seem to be a source of IFN- γ during MoPn infection though a protective role early during infection, when NK cells would be expected to act, is not reported.

On the other hand, anti-asialo-GM1-mediated depletion of NK cells in *C. pneumoniae*-infected RAG-1^{-/-} mice did not alter susceptibility to infection and only a partial decrease in lung IFN- γ expression was observed (Paper II). This was in contradiction with the general belief that NK cells are important sources of innate IFN- γ during intracellular infections, an issue first described for *L. monocytogenes* infection where a similar approach of anti-asialo-GM1 depletion was used (372). In fact, this very same result has been challenged by findings showing that anti-asialo-GM1-treated, *L. monocytogenes*-infected RAG-2^{-/-} mice have intact IFN- γ levels in the sera and that macrophages and DCs are important sources of innate IFN- γ during infection (217). In line, NK1.1 depletion in RAG-1^{-/-} mice resulted only in a slight increase in susceptibility to *L. monocytogenes* (E. Eriksson, unpublished observations). The role of NK cells as the main innate IFN- γ source *in vivo* has also been challenged during *S. typhimurium* infection where granulocytes and macrophages were shown to be the main source of IFN- γ during primary infection (373).

Despite that ablation of NK-cell cytotoxicity in spleens confirmed efficacy of depletion in *C. pneumoniae*-infected RAG-1^{-/-} mice, it could not be excluded that small number of lung NK cells surviving treatment were responsible for the partially reduced IFN- γ levels detected. To exclude this, γ_cR ^{-/-} mice which are deficient in IL-2R, IL-4R, IL-7R, IL-9R, IL-15R and IL-21R signaling, were crossed with RAG-1^{-/-} mice (Paper III). γ_cR ^{-/-} mice have severe cellular abnormalities such as the lack of peripheral lymph nodes, greatly diminished B- and T-cell numbers and a complete absence of NK cells (374). There are many unanswered questions regarding NK-cell development, but *in vitro* models suggest some degree of involvement for IL-2, IL-7, IL-15 and possibly IL-21 (reviewed in (375)) in generation of NK cells. Because *C. pneumoniae*-infected RAG-1^{-/-}/ γ_cR ^{-/-} and RAG-1^{-/-} mice showed similar levels of IFN- γ and bacteria in the lungs, it was possible to conclude NK cells were not required for IFN- γ expression in these mice, and that innate γ_cR signaling was also not involved in protection *in vivo* (Paper III). This finding confirms the previous study in NK cell-depleted RAG-1^{-/-} mice, where it was also shown that RAG-1^{-/-}/Perforin^{-/-} mice were as susceptible as RAG-1^{-/-} controls (Paper II). The combined data thus excludes the participation of NK cells in protective innate immune (IFN- γ - and perforin-mediated cytotoxic) responses against *C. pneumoniae*.

Further, bronchoalveolar lavage cells, F4/80⁺ and CD14⁺ macrophages isolated from the lungs of *C. pneumoniae*-infected RAG-1^{-/-} mice expressed IFN- γ (Paper III). The protective role of this expression is suggested by the enhanced susceptibility of either RAG-1^{-/-}/IFN- γ R^{-/-} or RAG-1^{-/-}/IFN- γ ^{-/-} mice to *C. pneumoniae* (Papers II & III). Transfer of WT but not IFN- γ ^{-/-} BMM ϕ into *C. pneumoniae*-infected RAG-1^{-/-}/IFN- γ ^{-/-} mice protected them from infection (Paper III), clearly supporting a protective role for macrophage-derived IFN- γ production in innate immune control of *C. pneumoniae* *in*

vivo. IFN- γ secretion by lung macrophages has also been shown *in vivo* during *M. tuberculosis* infection in mice as well as in *M. tuberculosis*-infected patients (discussed under Regulation of IFN- γ Production: Myeloid IFN- γ). *In vivo* expression of IFN- γ by granulocytes and macrophages has also been reported in *S. typhimurium*-infected mice (373). Even though at this point the participation of other non-lymphoid, innate cells in IFN- γ -mediated protection cannot be excluded, a protective role for macrophage-derived IFN- γ against intracellular bacterial infections has not been previously described *in vivo*.

Both CD4⁺ and CD8⁺ T cells produce IFN- γ in response to *C. trachomatis* and are probably complementary in warranting protective levels of IFN- γ during infection (reviewed in (301)). But although adaptive immune protection against *C. trachomatis* in mice can be demonstrated, for example, by transfer of Chlamydia-specific CD4⁺ or CD8⁺ T cells, there is some degree of controversy regarding the level of protection conferred by each subset (reviewed in (299, 300)) with some studies suggesting that CD4⁺ are more protective than CD8⁺ T cells. During *C. pneumoniae* infection both CD4⁺ cells and CD8⁺ cells mediate protection, albeit with a different kinetics where CD8⁺ are clearly protective earlier during infection but CD4⁺ seem to act later (Paper I). Antibody-depletion studies also support a protective role for CD8⁺ cells during primary and particularly during re-infection (376). In line, both CD4⁺ T cells and CD8⁺ T cells are required for (partial) protection against *C. pneumoniae* by DNA vaccination, an effect which requires IFN- γ (377). IFN- γ release by T cells has also been associated to the protective ability of DNA vaccines against *C. trachomatis* (378, 379). Further, reconstitution of RAG-1^{-/-}/ γ_c R^{-/-} mice with WT but not IFN- γ ^{-/-} CD4⁺ or CD8⁺ T cells protected them against *C. pneumoniae* infection (Paper III), confirming a protective role for IFN- γ -producing T cells during *C. pneumoniae* infection. Of interest, RAG-1^{-/-}/ γ_c R^{-/-} mice do possess the ability to secrete normal levels of innate IFN- γ , indicating that T cell-derived and non-lymphoid-derived IFN- γ mediate non-redundant effects (Paper III). Although IFN- γ has been shown to be involved in triggering T cells (380), it was also shown that in absence of innate IFN- γ , CD4⁺ and CD8⁺ T cells could still mediate protection through their ability to release IFN- γ (Paper III).

Lysis of cells containing intracellular RBs would be an attractive way to interfere with infection since this form is not infectious. However, Perforin^{-/-}, Fas^{-/-} and Fas-ligand^{-/-} mice do not display enhanced susceptibility to *C. trachomatis* infection (381). The same is observed in Perforin^{-/-} mice infected with *C. pneumoniae* (Paper I). On the other hand, Chlamydia-specific or naïve CD8⁺ T cells derived from IFN- γ ^{-/-} mice, failed to provide protection under conditions where the WT CD8⁺ T cells did (382), further confirming a role for IFN- γ release in CD8⁺ T-cell-mediated resistance to Chlamydia.

T-cell recognition of infected cells may lead to secretion of IFN- γ that mediates restriction of intracellular Chlamydial growth. In this respect, *C. trachomatis* has been shown to suppress IFN- γ -inducible MHC class II expression via degradation of Upstream stimulatory factor (USF)-1, a constitutively and ubiquitously expressed transcription factor required for IFN- γ -dependent induction of CIITA (383). Similarly, *C. trachomatis*-induced IFN- β has been suggested to mediate the inhibition of IFN- γ -dependent expression of MHC class II (384). The ability to degrade USF-1 was mediated by secretion into the cell cytosol of a Chlamydia-derived protease which turned out to degrade RFX5 as well, a transcription factor required for both constitutive

and IFN- γ -inducible MHC class I expression (385). A homologue of the *C. trachomatis* protease has been found in *C. pneumoniae* and it too, degrades RFX5 (386). These evasion strategies clearly confirm the protective role of T cells and IFN- γ against Chlamydia infections.

In summary, IFN- γ production by both T cells and innate immune cells seems to be not only important but also complementary for protection against *C. pneumoniae*. The combined data suggests that innate release of IFN- γ is required earlier (approximately 1 to 2 weeks) during infection, while T-cell-derived IFN- γ production is required much later (approximately 3 weeks) for protection. This is also supported by the fact that innate IFN- γ was not required for the protective, IFN- γ -dependent actions of T cells (Paper III) and that differences in *C. pneumoniae* levels between WT and RAG-1^{-/-} mice were also only observed 3 weeks after infection. The innate, probably macrophage-derived IFN- γ , restricts initial bacterial growth and prevents systemic dissemination while CD4⁺ or CD8⁺ T-cell-derived IFN- γ is needed later for bringing down bacterial levels and thus for resolution of infection.

C. pneumoniae Pattern Recognition in Macrophages

Regulation of IFN- γ in *C. pneumoniae*-Infected Macrophages (Paper IV, Figure 6)

With support for macrophage-derived IFN- γ during *C. pneumoniae* lung infection (Paper III), it may not be surprising that BMM ϕ were found to express IFN- γ at the mRNA and protein level, early and transiently after *in vitro* infection with *C. pneumoniae* as well. While the vast majority of findings regarding myeloid IFN- γ production have implied an IL-12-dependent pathway (discussed under Regulation of IFN- γ Production: Myeloid IFN- γ), this was one of the first reports suggesting instead an IFN- α/β -dependent production of IFN- γ . A previous report suggested that endogenous IFN- α/β primed macrophages for IFN- γ expression following LPS

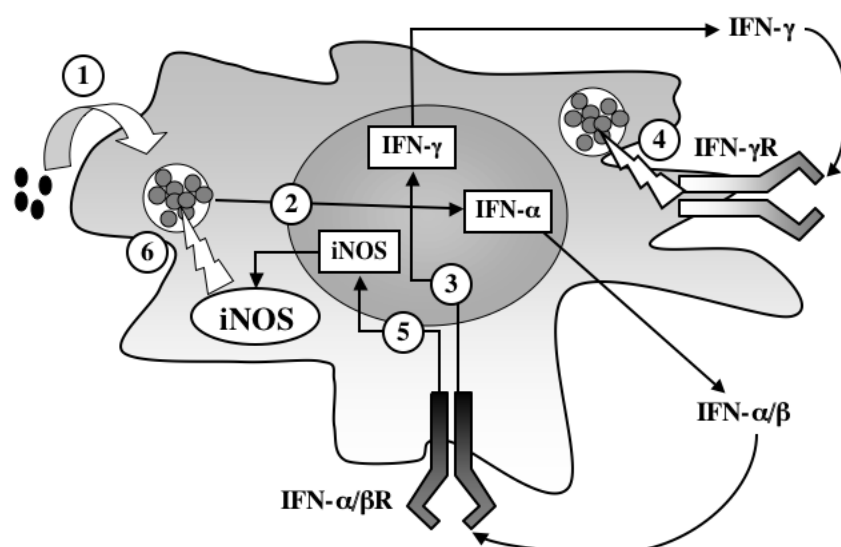


Figure 6: Role of IFN- α/β in *C. pneumoniae* Growth Restriction. Infection of macrophages with *C. pneumoniae* (1) leads to expression of IFN- α (2). IFN- α/β signaling regulates expression of IFN- γ (3) and iNOS (5) both which mediate restriction of bacterial growth (4 and 6).

stimulation (387). More recent findings also support a role for IFN- α/β in inducing IFN- γ in DCs (388), probably involving IL-15 as well (389). The fact that IFN- α/β can inhibit IL-12 expression (185) may account for the low levels of IL-12 recorded in *C. pneumoniae*-infected BMM ϕ , possibly inadequate for induction of IFN- γ . Alternatively, infection-induced IL-10 was shown to inhibit IL-12 but not IFN- α/β or iNOS expression, and to reduce the duration of IFN- γ expression, suggesting that IL-10 may prevent an IL-12-dependent pathway of IFN- γ expression, as the *in vivo* situation (Paper II & A. Gigliotti Rothfuchs, unpublished observations). The inhibitory role of IL-10 in IL-12-dependent IFN- γ release by macrophages has been reported by others (207, 213, 214).

Increased iNOS and NO production in *C. pneumoniae*-infected BMM ϕ was IFN- α/β -dependent but IFN- γ -independent, and important for restricting Chlamydial growth. It is unclear why IFN- γ was not required for iNOS induction in BMM ϕ as is the case in *C. pneumoniae*-infected mice (Paper I), though studies in *C. trachomatis*-infected fibroblasts also show a role for infection-induced IFN- α/β in inducing NO (390). IFN- α/β -dependent iNOS induction has also been recorded in *L. major*-infected BMM ϕ (98). Such IFN- α/β -dependent NO production has been shown to mediate early containment of *L. major in vivo* (391).

While IFN- γ was shown to mediate protective effect(s) in an iNOS-independent way, the mechanism is unknown.IDO has not clearly been shown to be protective in mouse cells, and its expression was not detected in *C. pneumoniae*-infected BMM ϕ . Still, a role for IDO in IFN- γ -dependent Chlamydial growth control in BMM ϕ cannot be excluded. As discussed for *C. pneumoniae* lung infection, GTPases and P2X₇R signaling are two potential candidates for IFN- γ -dependent protective effects.

TLRs in Regulation of IFN- α/β -Dependent IFN- γ Expression in *C. pneumoniae*-Infected Macrophages (Paper IV)

TLRs can trigger IFN- α/β expression in the presence of MyD88 (via TLR7 (21) and TLR9 (392)) or in the absence of MyD88 using TRIF (via TLR3 and TLR4 (53)) and TRAM (TLR4 (56)). It was therefore surprising that expression of IFN- α in *C. pneumoniae*-infected BMM ϕ was dependent on TLR4 and MyD88 signaling (Paper V). Though TLR9 signaling induces IFN- α in a MyD88-dependent fashion, TLR9^{-/-} BMM ϕ were not more susceptible to infection, suggesting that expression of IFN- α in such BMM ϕ is probably intact (Paper V, data not shown). TLR7 also signals via MyD88 but because it lacks a natural ligand(s) and has not yet been implicated in pattern recognition, it is difficult to speculate its involvement in *C. pneumoniae*-induced IFN- α expression. Although the presence of a TLR4-mediated, MyD88-independent pathway cannot be excluded, it clearly is not sufficient to induce IFN- α responsive-genes in these cells. Only TLR3 and TLR4 have been described to date as having MyD88-independent signaling. Because no report has been made concerning the presence of dsRNA in Chlamydia, TLR3 is an unlikely candidate for mediating Chlamydia pattern recognition. Similarly, whether TLRs other than TLR2, TLR4, TLR6 or TLR9 also participate in *C. pneumoniae*-induced IFN- α expression remains to be explored.

Another finding at variance with other reports was the requirement of STAT1 signaling in IFN- γ expression by BMM ϕ (Paper V). STAT1 is clearly implicated in

signaling downstream of both the IFN- α/β R and IFN- γ R (discussed under IFN- α/β R and IFN- γ R Signaling) but its requirement for actual IFN- γ expression is not clear. One report suggests that STAT1 inhibits rather than stimulates IFN- α/β -dependent IFN- γ production in NK and T cells (233). Signaling by the γ_c R was also implicated in IFN- α/β -dependent IFN- γ expression in *C. pneumoniae*-infected BMM ϕ and IFN- α/β R signaling was required for IL-15 expression, a cytokine that signals through the γ_c R (Paper V). In line, it has recently been shown that IL-15 and IL-21 (another cytokine that signals through the γ_c R) activated the binding of STATs, including STAT1, STAT3 and STAT4 to regulatory sites on the IFN- γ gene in NK and T cells (393). It seems possible then that IFN- α/β -induced IL-15 activates via the γ_c R, direct STAT1-dependent activation of IFN- γ transcription.

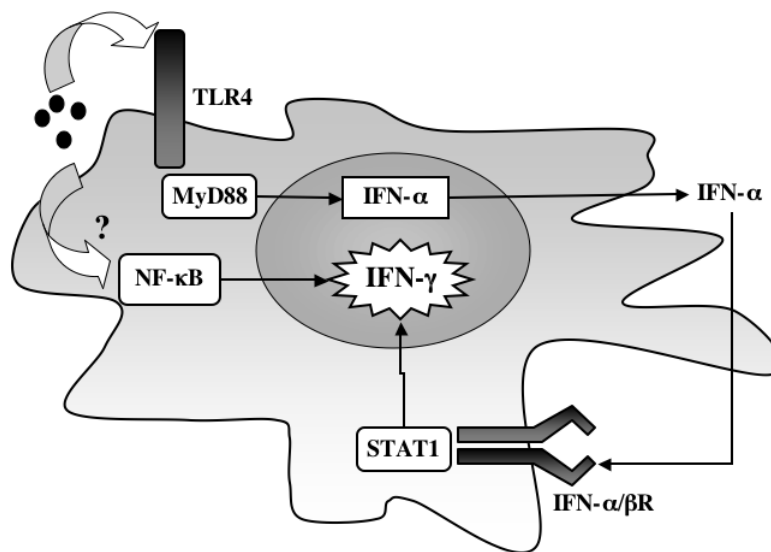


Figure 7: Molecular Pathways Controlling Macrophage Secretion of IFN- γ after Infection with *C. pneumoniae*. IFN- γ expression is regulated by a TLR4-MyD88-STAT1 pathway and by a TLR4-independent pathway leading to NF- κ B activation.

Activation of NF- κ B and Expression of Pro-inflammatory Cytokines (Paper V)

Activation of NF- κ B seems to occur independently of TLR4 and MyD88 in *C. pneumoniae*-infected BMM ϕ , suggesting the involvement of a TLR-independent pathway of NF- κ B activation in these cells. Interestingly, NF- κ B-dependent expression of pro-inflammatory cytokines IL-1 α , IL-6 and TNF- α did not require TLR4 or MyD88 in *C. pneumoniae*-infected BMM ϕ . The involvement of other TLRs in MyD88-independent, pro-inflammatory cytokine expression seems also unlikely since expression of pro-inflammatory cytokines is abolished in MyD88^{-/-} cells following stimulation with specific TLR ligands (41, 394). However, this is not formally excluded as other TLRs not requiring MyD88 may yet be characterized. Though TRIF^{-/-} and TRAM^{-/-} mice reveal a role for these adaptors in LPS-driven induction of pro-inflammatory cytokines (53, 56), this is irrelevant in *C. pneumoniae*-infected BMM ϕ since TLR4 itself is not required for pro-inflammatory cytokine expression.

An alternative, TLR-independent pathway for PAMP recognition with ensuing NF- κ B activation and production of pro-inflammatory cytokines has recently been

suggested to be mediated by the Nucleotide-binding oligomerization domain (NOD) family of proteins (reviewed in (395)). Peptidoglycans are present in all bacteria and NOD1 has been shown to recognize peptidoglycans from Gram-negative bacteria while NOD2 can recognize peptidoglycans from all bacteria (reviewed in (396)). The role of NOD1 and NOD2 in *Chlamydia* pattern recognition is unknown. Preliminary data from our laboratory suggests however that treatment of *C. pneumoniae*-infected BMM ϕ with cytochalasin D, an inhibitor of phagocytosis, does not affect expression of pro-inflammatory cytokines or activation of NF- κ B but blocks IFN- α and IFN- γ expression. This is in line with results where antibiotic-treatment of BMM ϕ reduced IFN- α and IFN- γ but not the pro-inflammatory response (Paper IV & data not shown). This suggests that intracellular recognition is not required for NF- κ B activation and consequent induction of pro-inflammatory cytokines. However, phagocytosis and viable bacteria are required for *C. pneumoniae*-induced, TLR-dependent IFN- α expression. The inflammatory response to *C. pneumoniae* remains therefore enigmatic (Figure 7).

IFN- α/β in Resistance to *C. pneumoniae* Lung Infection (Paper VI)

The unaltered (or possibly even reduced) susceptibility of IFN- α/β R^{-/-} mice to *C. pneumoniae* infection does not relate to the *in vitro* data showing enhanced Chlamydial growth in IFN- α/β R^{-/-} BMM ϕ . Although administration of exogenous IFN- α/β or inducers thereof, have protected or ameliorated disease in mice infected with intracellular bacteria (397, 398), intracellular protozoa (399-401), or fungi (402), a protective role for endogenous IFN- α/β during non-viral infections has only been documented early during *L. major* infection (391) and during a low-dose infection with *M. tuberculosis* in IFN- α/β R^{-/-} mice (403). However, endogenous IFN- α/β does participate together with IFN- γ in protection against *C. pneumoniae*, as demonstrated by the enhanced susceptibility of IFN- α/β R^{-/-}/IFN- γ R^{-/-} mice compared with IFN- γ R^{-/-} mice. This observation, together with the previous findings in *L. major* and *M. tuberculosis*, constitute the only supportive data on a protective role for endogenous IFN- α/β during non-viral, experimental infections. The cooperative, protective effect of IFN- α/β and IFN- γ are probably mediated through activation of STAT1. Such a positive cross-talk between IFN- α/β and IFN- γ at the level of STAT1 is well described and feasible since STAT1 is important for signaling downstream of both the IFN- γ R and the IFN- α/β R (discussed under IFN- α/β R and IFN- γ R Signaling, and reviewed in (404)). The protective effect mediated by IFN- α/β is however, only evident in the absence of IFN- γ .

The reason why IFN- α/β are pivotal in regulating protective responses in BMM ϕ , including the release of IFN- γ , but *in vivo* are not required for IFN- γ expression (which is largely IL-12-dependent) and seem only secondary to IFN- γ -mediated protection, is currently unknown. This discrepancy may be a qualitative feature in *C. pneumoniae* pattern recognition by different cell types. *In vivo*, the different cell types in the lung convey the generation of protective, IL-12-driven responses, while *in vitro*, IFN- α/β -driven protection in BMM ϕ are essential. In line, bone marrow-derived DCs infected *in vitro* with *C. pneumoniae* express IFN- γ in the

absence of IFN- α / β R signaling and could therefore fit in with the IL-12-biased pathway of IFN- γ release which seems to dominate *in vivo* (Paper II).

CONCLUDING REMARKS

Implications for Vaccine Design

Asymptomatic or mild, clinical disease ensues in most cases of *C. pneumoniae* infection, as is the case for *C. trachomatis*. For *C. trachomatis* though, serious pathological complications to otherwise mild/asymptomatic infection occur in the form of blindness from trachoma or infertility and ectopic pregnancy from salpingitis (reviewed in (236, 307)). The association of *C. pneumoniae* infection to chronic obstructive lung disease, asthma and atherosclerosis become therefore even more impending since more than 50% of the population worldwide are sero-positive for *C. pneumoniae*. A vaccine against either *C. trachomatis* or *C. pneumoniae* would therefore not only reduce the incidence of clinical disease (trachoma, salpingitis, CAP and upper respiratory disease) but also, and equally important, reduce the incidence of the severe pathological complications of Chlamydia infection. This is particularly important considering the link between *C. pneumoniae* and atherosclerosis.

The sensitization to a more severe disease in individuals vaccinated against *C. trachomatis* (reviewed in (236)) suggests that failing to first understand the immune response to Chlamydia may consequently lead to failure in vaccine design. Since TLR ligands essentially mediate adjuvant effects such as co-stimulation and cytokine release, understanding the fine molecular details of pattern recognition might facilitate at some point, the selective mustering of a host response, through the use of a particular PAMP, natural or modified for the purpose of inducing that wanted response. The only partial protection obtained in experimental vaccines with Chlamydial antigens clearly suggest that appropriate antigens must first be identified for a vaccine to be optimal. Vaccination would also need to avoid generating persistence. It is not clear how this will be achieved but vaccination strategies will in any case require the generation of ample, adaptive IFN- γ responses for protection.

General Conclusions

A set of basic conclusions can be drawn from the papers presented in this thesis and are summarized below:

- 1) IFN- γ restricts *C. pneumoniae* growth.
- 2) Innate-immune cells are an important source of IFN- γ and macrophages (but not NK cells) are involved in IFN- γ release.
- 3) CD4⁺ and CD8⁺ cells have a protective role that depends on their capacity to secrete IFN- γ . T-cell-derived IFN- γ plays a protective role at late times during primary infection, synergizes in protection with non-lymphoid IFN- γ , but is not dependent on the latter for protection.

- 4) IFN- γ -independent mechanisms also contribute in the control of infection.
- 5) IL-12/IL-23 regulates expression of IFN- γ *in vivo*.
- 6) IFN- α/β is not required for IFN- γ expression *in vivo* but restricts *C. pneumoniae* growth in a STAT1-dependent way in the absence of IFN- γ .
- 7) IFN- α/β is important for restricting *C. pneumoniae* growth in macrophages by regulating expression of IFN- γ and iNOS, in a STAT1-dependent manner.
- 8) TLR4 and MyD88 are required for IFN- α expression in *C. pneumoniae*-infected macrophages but not for activation of NF- κ B and expression of pro-inflammatory cytokines.
- 9) NF- κ B is required for expression of IFN- γ in *C. pneumoniae*-infected macrophages.
- 10) Working with Chlamydia is fun!

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