universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

The innate immune response to *Listeria monocytogenes*:
Interaction with host cells and signal transduction

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat.)

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Wien, am 25. Mai 2010

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1 Abstract

Listeria monocytogenes, a gram-positive, facultative intracellular pathogen, is the causative agent of many severe foodborne diseases in humans. Once in the intestinal tract of humans, the *Listeria* surface protein Internalin A (Inl A) interacts with its receptor E-cadherin and the bacterium is taken up by the intestinal epithelial cell. The mouse E-cadherin differs from its human homologue by a single amino acid exchange that strongly reduces the affinity to Inl A. To enable oral infection studies with moderate *Listeria* doses in the mouse model, our aim was to create a murinized *L. monocytogenes* LO28 strain. Therefore we tried to replace the truncated LO28 Internalin A gene with a mutated version of the Internalin A gene from the *L. monocytogenes* EGDe strain.

In addition to the uptake into intestinal epithelial cells, *Listeria* can be taken up by macrophages. Once recognized, many signaling pathways are triggered in the cell, leading amongst others to type I IFN gene induction. We investigated the role of the signal transducers TBK1, p38 MAPK, IKKβ and JNK in the induction of IFN-β. Since IFN-β production increases host cell death, the impact of these signal transducers on the death of *Listeria* infected macrophages was studied as well. Our data suggest that all proteins are needed for full IFN-β induction, but IKKβ inhibition showed the slightest effect. Concerning the cell death, only p38 MAPK and JNK seem to have an impact.

Interferons subsequently induce STAT-JAK signaling. We examined the role of Stat1 F77A macrophages, which are unable to form Stat1 oligomers due to the aminoacid substitution in the N-terminus, on the intracellular replication of *Listeria.* Further, we analyzed gene induction and cell death in Stat1 F77A macrophages.

Our results show that *Listeria* replication is altered in Stat1 F77A macrophages compared to wild type cells. In the mutant cells, the induction of Stat dependent genes is strongly decreased. We could not observe a difference concerning host cell death in the Stat1 F77A macrophages.

2 Zusammenfassung

Listeria monocytogenes ist ein gram-positives, fakultativ intrazelluläres Humanpathogen, das für viele schwerwiegende Lebensmittelinfektionen im Menschen verantwortlich ist.
Wenn sich *Listerien* im Verdauungstrakt des Menschen befinden, interagiert deren Oberflächenprotein Internalin A (Inl A) mit dem Rezeptor E-Cadherin, wodurch die Bakterien von den Darmepithelzellen aufgenommen werden.
Der E-Cadherin Rezeptor in der Maus unterscheidet sich vom menschlichen durch einen Aminosäureaustausch, der die Affinität zu Inl A reduziert. Um orale Infektionen mit einer angemessenen Dosis an *L. monocytogenes* LO28 im Maus Model zu ermöglichen, muss die Interaktion zwischen Internalin A und E-cadherin funktionieren.
Deshalb sollte hier das trunkierte Inl A Gen des Stammes LO28 durch ein vollständiges Gen des *L. monocytogenes* EGDe Stammes erstezt werden, das zusätzlich Mutationen zur Erhöhung der Affinität zum Maus E-Cadherin trägt.
Zusätzlich zur Aufnahme in Darmepithelzellen können *Listerien* auch von Makrophagen aufgenommen werden. Einmal vom Immunsystem erkannt werden viele Signaltransduktionswege angeschaltet, die unter anderem zur Produktion von Typ I Interferonen führen.
Wir untersuchten die Rolle der Signaltransduktionsmoleküle TBK1, p38 MAPK, IKKβ und JNK bezüglich IFN-β Induktion und auch den Tod infizierter Makrophagen, da IFN-β nekrotischen Zelltod verstärkt. Unsere Daten zeigen, dass alle Proteine für eine vollständige IFN-β Induktion notwendig sind, wobei IKKβ Inhibition den geringsten Effekt aufweist. Bezüglich des Zelltods spielen nur p38 MAPK und JNK eine Rolle.

Interferone sind weiters verantwortlich für die Aktivierung des JAK-STAT Signaltransduktionsweges. Wir analysierten die Auswirkung einer Stat1 F77A Mutation auf die intrazelluläre Vermehrung von *Listerien.* Aufgrund der Mutation im N-Terminus ist eine Oligomerisierung von Stat1 F77A Molekülen nicht mehr möglich. Weiters untersuchten wir die Geninduktion und den Zelltod von *Listerien* infizierten Stat1 F77A Makrophagen.

Unsere Ergebnisse zeigten einen deutlichen Effekt der Stat1 F77A Mutation auf die intrazelluläre Vermehrung von *L. monocytogenes*. Weiters konnten wir zeigen, dass die Induktion der untersuchten, durch Interferon induzierten, Gene stark vermindert ist. Bezüglich des Zelltodes konnten wir keinen Unterschied zwischen mutierten und Wildtyp Makrophagen feststellen.

3 Introduction

3.1 *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive facultative intracellular bacterium. It is the etiological agent of listeriosis, a severe human foodborne infection, characterized by diseases like febrile gastroenteritis, meningitis, encephalitis, sepsis, abortions and perinatal infections (Dussurget et al., 2004).
In addition, *L. monocytogenes* causes infections in a variety of other vertebrates, including domesticated and wild birds and other mammals.
Listeria can be found throughout the environment in soil, vegetation and animals, but contaminated food is the major source of infection in epidemic, as well as in sporadic cases. Therefore the gastrointestinal tract is thought to be the primary site of entry into the host. Usually, the clinical course of infection starts about 20 hours after the ingestion of heavily contaminated food in cases of gastroenteritis, whereas the incubation period for the invasive illness is generally around 20 to 30 days (Allerberger and Wagner, 2010; Vázquez-Boland et al., 2001).

3.1.1 Life cycle of *Listeria monocytogenes*

Listeria monocytogenes can invade and replicate in a variety of cell types including epithelial cells and macrophages. After entry the bacterium lyses the internalization vacuole and escapes into the

cytoplasm of the host cell, where the bacteria start to replicate. When the bacterium is in the cytosol, it uses an actin based motility process to move through the cytosol and to invade neighbouring cells through the formation of a double-membrane protrusion, which results in the formation of a so-called secondary vacuole. Afterwards this compartment is lysed, making a new intracellular infection cycle in adjacent cells possible (Dussurget et al., 2004).

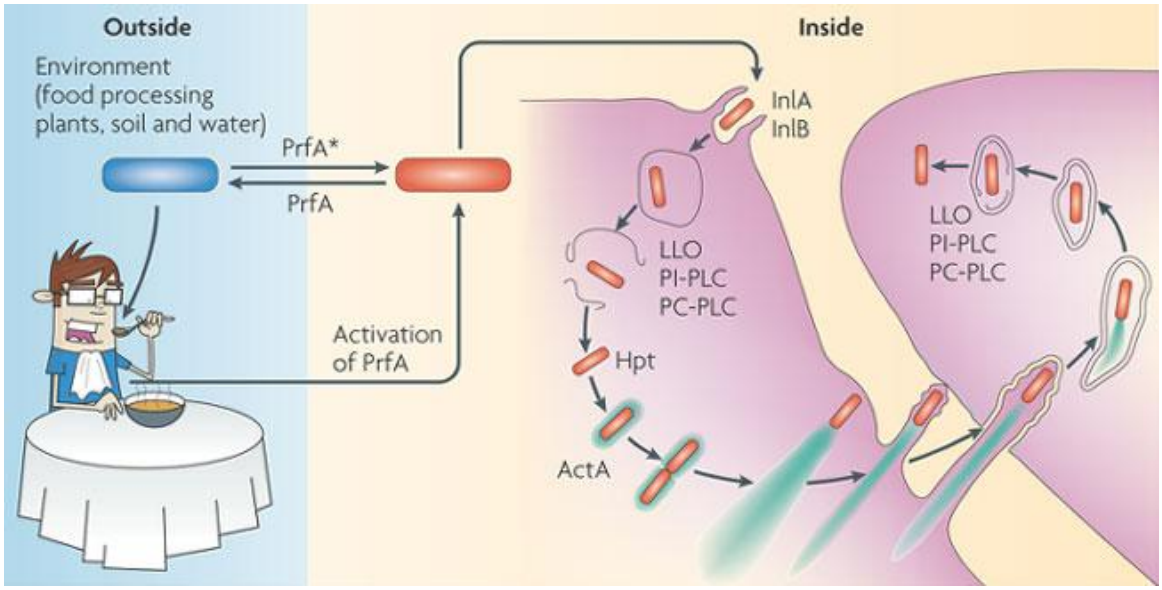


Figure 1: *Life cycle of L. monocytogenes. The pathogen* can survive in different environments. The transcriptional activator PfrA plays an important role in the switch between life outside and inside of host cells. On the right hand side the life cyle of *L. monocytogenes* is shown (Freitag et al., 2009).

Two molecules, Internalin A (InlA) and Internalin B (InlB), mediate the bacterial invasion into target cells. These proteins belong to the internalin family, characterized by the presence of leucine-rich repeats (LRRs), which are involved in protein-protein interactions. Two LRR regions are separated by an inter-repeat region (IR). These regions are necessary and sufficient to promote *Listeria* entry into human epithelial cells.

The receptor for the 800-amino acid protein InlA is called E-cadherin and belongs to the cadherin superfamily of cell adhesion molecules.
The 882 amino acid transmembrane protein consists of a long N-terminal extracellular domain, a transmembrane domain and a relatively short cytoplasmic domain.
The ectodomain of E-cadherin is required for bacterial adherence but also the intracytoplasmic domain is absolutely necessary for entry of microbes into the host cell, because its phosphorylation regulates the binding of E-cadherin to its interactors (Bonazzi et al., 2009; Dussurget et al., 2004).
The interaction of InlA and E-cadherin is species specific and will be discussed in chapter 3.1.3.

InlB (630 amino acids) is located in the same operon as InlA. The two proteins can be transcribed bicistronically or alternatively, InlB expression can be regulated independently via σB. InlB is essential for entry of *Listeria monocytogenes* into many cell types, for example hepatocytes and other nonepithelial cells.
The hepatocyte growth factor/scatter factor, also called Met, has been identified as the main receptor for InlB. Met belongs to the family of tyrosin kinase receptors. InlB binds Met through the concave surface of the LRR region, which leads to transient phosphorylation of Met (Bonazzi et al., 2009a; Dussurget et al., 2004).

After invasion of host cells, *Listeria* resides in a vacuole that is lysed by Listeriolysin O (LLO) in concert with two phospholipases C (PLCs). LLO is a member of the pore-forming, cholesterol-dependent cytolysin family. When *Listeria* is located in the cytoplasm, it starts to multiply and moves by polymerizing the host cell actin through the activity of the virulence factor ActA. Actin based motility allows *Listeria* to spread from an infected cell into adjacent cells after the

transient formation of a double-membrane vacuole, which is then lysed by the activities of LLO/PLC (Dussurget et al., 2004).
Mutants lacking LLO fail to reach the cytoplasm and are nonvirulent (Portnoy et al., 2002).

Cytosolic LLO is degraded by the N-end rule pathway, restricting its function to the vacuolar environment and inhibiting the lysis of host cells (Dussurget et al., 2004; Schnupf et al., 2007).
The pH for optimal pore formation by LLO is between 5,5 and 6,0 (the pH of the early phagosome).
Listeria secretes two PLCs, which also play a role in the lysis of intracellular vacuoles. The PI-PLC is specific for phosphatidylinositol (PI) and glycosyl-PI-anchored proteins, whereas the phosphatidylcholin (PC)-PLC is a broad range PLC. The PC-PLC can also promote lysis of primary vacuoles in human epithelial cell lines, when LLO is absent. Together with LLO, the PI-PLC induces hydrolysis of PI and production of diacylglycerol in macrophages (Dussurget et al., 2004).
Mutants lacking both PLCs show a defect in vacuolar escape (Portnoy et al., 2002).

Cytosolic *Listeria* move through the cytoplasm by polymerization of actin filaments. This "motor" makes it possible to spread from one cell to another adjacent cell. The surface protein ActA is the only bacterial factor required for this activitiy. The *Listeria* actin-based motility is stimulated by a central repeat domain containing four proline-rich repeats. This domain binds members of the enabled/basodilator-stimulated phosphoprotein family of proteins. These molecules modulate bacterial speed and directionality.
The amino-terminal region of ActA is the only important region for inducing bacterial movement. It binds and activates Arp2/3, a seven

protein host complex, which induces actin polymerization and the generation of a dendritic array of actin filaments (Dussurget et al., 2004).
ActA minus mutants are not able to spread from cell to cell or to form plaques in tissue culture monolayers (Portnoy et al., 2002).
Listeria moving at the tip of actin-comet tails can form protrusions that are engulfed by neighboring cells.

The most important *Listeria* virulence genes are regulated by the transcriptional activator PrfA, which belongs to the Crp/Fnr family. At temperatures of about 37°C, the temperature of the host, PrfA regulated virulence factors are produced at higher levels. At temperatures lower than 30°C, the low levels of expression of virulence genes are correlated to undetectable levels of PfrA, although its transcript is synthesized. At low temperatures, the untranslated leader region of the *prfA* messenger RNA forms a stable secondary structure (called a thermosensor) masking the Shine-Dalgarno sequence, which averts the translation of PfrA. This thermosensor melts at higher temperatures, resulting in the translation of PrfA (Dussurget et al., 2004).

3.1.2 Listeria monocytogenes strains

The species *L. monocytogenes comprises 13 serovars*, but only serovars 1/2a, 1/2b and 4b are responsible for 98% of listeriosis reported in humans (Dussurget et al., 2004; Sleator et al., 2009).
In our lab, we are mainly using *L. monocytogenes* EGDe (serovar 1/2a) and LO28 (serovar 1/2c), which are both fully virulent in the mouse model.

One crucial difference between these two strains is a nonsense mutation in the *inlA* gene of LO28. In *Listeria* LO28, the adenine at position 1637 is deleted, resulting in a frameshift, creating a stop codon (TAA) at position 1729. This leads to a truncated version of InlA, lacking a cell wall anchor.
Western blot analysis showed that the amount of InlA found in whole cell extracts of EGDe and LO28 was quite similar, but for LO28 cultures a larger quantity of InlA could be found in the supernatant than in EGDe supernatants, due to the absence of a cell wall anchoring motif (Jonquières et al., 1998).
These findings would suggest that in vivo infection with *Listeria monocytogenes* LO28 is not as effective as infection with EGDe, due to the lower amount of InlA found on the *Listeria* LO28 cell wall, that is important for InlA/E-cadherin interaction, leading to uptake into intestinal cells.

Also important for our studies is the fact, that *Listeria monocytogenes* LO28 induces an about 10 times stronger type I interferon response in infected bone marrow derived macrophages (BMDMs) and bone marrow derived dendritic cells (BMDCs) as well as in RAW 264.7 macrophages compared to the strain EGDe (Reutterer et al., 2008).

3.1.3 Species specificity of InlA E-cadherin interaction

InlA specifically interacts with the N-terminal EC repeat of E-cadherin, termed EC1. This interaction is species specific, for example in rat and mouse it is completely impaired, whereas the interaction between InlA and E-cadherin is intact in humans, guinea pigs and gerbils.

This species specificity is due to a single aminoacid within the EC1 domain of E-cadherin. Species allowing an interaction harbor a prolin at position 16 of the EC-1 domain, whereas in mice and rats there is a glutamic acid (Bonazzi et al., 2009b; Lecuit et al., 1999).

Prolin at position 16 makes the loop hydrophobic and uncharged, whereas the glutamic acid in the E-cadherin loop results in a charged and hydrophilic region. Therefore it is obvious that InlA E-cadherin interaction is dependent on hydrophobic interactions at this position. However this is not sufficient for InlA binding to the EC1 domain of cadherins, as InlA does not interact with N-cadherin, where prolin is also located at position 16. In 2002 the crystal structure of InlA alone and in complex with E-cadherin was solved, showing a hydrophobic binding pocket in the InlA molecule, which creates the binding site for E-cadherin.

The affinity of InlA to E-cadherin is not very high, which could be an advantage for the bacterium, as InlA has to dissociate from the receptor once the bacterium has been internalized.

To solve the interaction problem of *Listeria* InlA and the mouse E-cadherin, either the bacterium or the mouse have to be modified. Both scenarios have already been put into practice. On the one hand, a murinized *Listeria* strain has been created and on the other hand there is a humanized mouse (see Figure 2) (Bonazzi et al., 2009b).

The transgenic mouse expressing human E-cadherin at the level of the intestine, allowed InlA-mediated intestinal *Listeria* infections in an animal model for the first time, providing evidence that InlA, but not InlB, is the molecule necessary for crossing the intestinal barrier after oral infection (Bonazzi et al., 2009b; Lecuit et al., 2001).

Some years later the murinized *Listeria monocytogenes* strain EGDe was created (Wollert et al., 2007). Serine at position 192 was replaced by asparagine, bridging the gap between InlA and E-cadherin and tyrosin 369 substituted by serine allows the formation of a hydrogen bond. Changing these two aminoacids increases the affinity of InlA to the murine E-cadherin by four orders of magnitude, which is comparable to the affinity of the unmodified InlA with human E-cadherin.

These two substitutions extend the binding specificity of InlA to include formerly incompatible murine E-cadherin, by creating additional, stabilizing contacts (Bonazzi et al., 2009b; Wollert et al., 2007).

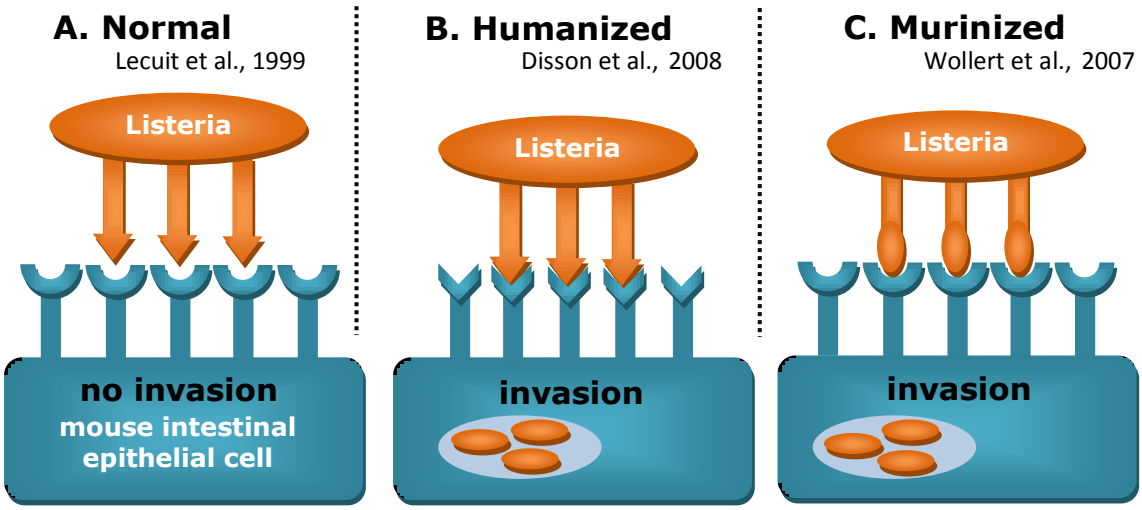


Figure 2: *Model systems for analysis of L. monocytogenes infection in mice.* **A.** InlA is unable to bind to the E-cadherin of the mouse. **B.** Human E-cadherin is expressed in transgenic mouse – interaction InlA/E-catherin is possible. **C.** InlA is modified via rational protein engineering – interaction InlA/E-cadherin is possible (Sleator et al., 2009; modified figure).

3.1.4 Innate immune response to *Listeria monocytogenes* infection

After infection with *Listeria monocytogenes*, innate immune responses are rapidly activated and are essential for controlling bacterial growth and dissemination and preventing spreading of the pathogen, which could result in systemic lethal infection.

Early resistence is due to the action of phagocytes. Neutrophils can kill bacteria through engulfment and resulting production of reactive nitrogen and oxygen intermediates and extracellular bacteria through release of extracellular traps.

Neutrophils secrete chemokines, like MCP-1, and cytokines, like CSF-1, which are necessary for recruitment of macrophages to the site of infection.

In response to infection, macrophages secrete cytokines like tumor necrosis factor α (TNFα) and interleukin 12 (IL-12) which induce the secretion of interferon γ (IFN-γ) by natural killer cells (NK-cells), resulting in the enhanced activation and bactericidal activity of macrophages.

Also in macrophages the production of reactive oxygen and nitrogen intermediates plays an important role in the clearing of the bacteria (Zenewicz and Shen, 2007).

Innate immune responses are activated stepwise. *Listeria* are recognized by specific receptors and downstream signaling cascades are activated. TLR2 and TLR5 seem to play a role in recognition of extracellular *Listeria* (Pamer, 2004). Nucleotide-binding oligomerization domain 2 (NOD2) and NACHT/LRR/PYD-containing protein 3 (NALP3) are important for recognizing intracellular Listeria, but the receptor, triggering tank binding kinase 1 (TBK1) and IκB kinase ε (IKKε or IKKi) activation and subsequent IFN-β induction is still unknow (Zenewicz et al., 2007).

Recently, the protein LRRFIP1 was identified as cytoplasmic nucleic acid sensor, mediating IFN-β induction in response to *L.monocytogenes* infection. LRRFIP1 signals via β-catenin and subsequent activation of IRF3. Nevertheless, it does not affect PRR-triggered activation of IRF3 or NFκB (also see chapter 3.3.2) (Yang et al., 2010).

Type I interferons, usually associated with antiviral immune responses, are important for clearing of many viral pathogens. Concerning *Listeria monocytogenes* infection, type I interferons are detrimental for the host.Previous studies have shown that the induction of type I interferons decreases the viability of *Listeria* infected host cells. Mice that lack the receptor for type I interferons have increased resistance to *Listeria* infection (Pamer, 2004; Reutterer et al., 2008; Stockinger et al., 2002, 2009).

3.1.5 Cell death of eukaryotic cells after *L.monocytogenes* infection

Many pathogenic microorganisms are able to cause eukaryotic cell death, either as a consequence of infection or via production of toxic products.

The diversity of processes mediating cell death necessitated the introduction of several terms for the different ways a cell can die. Three of the best characterized cell death types are probably apoptosis, necrosis and pyroptosis.

Apoptosis is described as an active, programmed cell death. Apoptotic cells show autonomous cellular dismantling that avoids inflammation.

Necrosis is a passive, accidental cell death resulting from environmental perturbations. Necrosis leads to release of inflammatory cellular content.
Pyropotosis is characterized by caspase-1 activation, which processes the pro-forms of the inflammatory cytokines, IL-1β and IL-18. Pyroptosis features cell lysis and also releases inflammatory cellular contents (Fink and Cookson, 2005).

Earlier studies in our lab showed that *Listeria monocytogenes* induced cell death of macrophages is best classified as necrosis. This conclusion could be drawn due to the cellular morphology and the lack of requirement for typical events necessary for other cell death types, like caspase activation and loss of the mitochondrial membrane potential (Zwaferink et al., 2008).
One important aspect of *Listeria* induced cell death is the ability of type I IFNs to increase the death of infected macrophages via an NO-dependent enhancement of necrosis (Stockinger et al., 2002; Zwaferink et al., 2008).

3.2 Macrophages

Macrophages are cells of the innate immune system. They originate from stem cells in the bone marrow, circulate in the blood as monocytes and differentiate into macrophages when they enter organs such as the liver (Kupffer cells), the central nervous system (microglia) or the lung (alveolar macrophages). According to the requirements of the tissue, their morphology and functions differ (Abbas, 2010; Takahashi, 2001).

Their main function is to recognize invading microorganisms, phagocytose and kill the microbes. For this purpose macrophages express pattern recognition receptors (PRRs), which sense general structures of microbes called pathogen associated molecular patterns (PAMPs) (see chapter 3.3). Activation by IFN-γ is of vital importance to enhance macrophage effector functions. This cytokine is secreted by NK cells in response to activating ligands on the surface of infected host cells or in response to IL-12. Activated macrophages produce cytokines, which are necessary for inducing an inflammatory reaction, stimulate and recruite other cells, like T-cells and NK-cells and produce growth factors for fibroblasts and endothelial cells that participate in the remodelling of tissue after infections and other tissue injuries. Macrophages typically respond very early to microbes and because of their long survival at sites of infection, they are the dominant effector cells at later stages of the innate immune response (Abbas, 2010).

3.3 Pattern Recognition Receptors

The innate immune system possesses a limited number of germline-encoded PRRs, which are important for the recognition of invading microorganisms. Concerning their location it can be distinguished between three main classes of PRRs: PRRs located on the cell surface, (TLRs), receptors in the endosomal membrane (TLRs) and cytoplasmic PRRs.
All receptors have in common that they recognize microbial components, so called PAMPs, which leads to the activation of signaling pathways and further induces the expression of a variety of genes (Akira et al., 2006).

3.3.1 Toll like receptors

TLRs are type I integral membrane glycoproteins and defined by the presence of a Toll/interleukin-1 (IL-1) receptor (TIR) domain in their cytosolic regions, which is necessary for the signal transduction, and an extracellular leucine-rich repeat region that plays an important role in the recognition of microbial by-products (Dunne and O'Neill, 2005; O'Neill, 2003).
Up to date 13 members of the TLR familiy have been identified in mammals. 11 could be found in humans and 13 in the mouse genome. TLRs1-9 are conserved in both species (Dunne et al., 2005).
TLRs are expressed on many immune cells, including macrophages, dendritic cells, B cells, specific types of T cells and also on non immune cells such as fibroblasts and epithelial cells (Akira et al., 2006).

Cell surface stainings with antibodies to specific TLRs revealed that TLR1, TLR2, TLR4, TLR5 and TLR6 are localized on the plasma membrane whereas TLR3, TLR7, TLR8 and TLR9 are preferentially expressed in intracellular compartments such as the endosome.
The latter group of TLRs recognizes nucleic acid structures whereas TLR1, TLR2, TLR4, TLR5 and TLR6 generally recognize cell wall components of microorganisms (Dunne et al., 2005).

3.3.2 Cytoplasmic receptors

When pathogens bypass the membrane-associated PRRs and invade the cytoplasm, they are detected by cytosolic receptors (Akira et al., 2006; Lee and Kim, 2007).
Cytoplasmic PRRs can be classified into three different families: interferon (IFN)-inducible proteins, caspase-recruiting domain (CARD) helicases and NOD-like receptors (NLRs). IFN-inducible proteins such as double-stranded RNA (dsRNA)-dependent protein kinase (PKR) or 2',5'-oligoadenylate synthetase 1 (OAS-1) and CARD helicases are mainly important in antiviral defense, whereas NLRs primarily mediate antibacterial defense (Lee et al., 2007; Behera et al., 2002).

PKR is a ubiquitously expressed serine-threonine kinase that is dramatically induced by IFN-γ. At the N-terminus two dsRNA-binding domains can be found and a conserved kinase domain at the C terminus. Once activated by dsRNA, PKR dimerizes, autophosphorylates and in turn phosphorylates the α-subunit of eukaryotic initiation factor 2 (eIF-2), leading to decreased protein synthesis in mammalian cells (Hu et al., 2009; Kumar et al., 1994).

Retinoic acid-inducible protein I (RIG-I) and melanoma differentiation associated gene 5 (MDA5) are cytoplasmic helicases containing a CARD domain.
They play an important role in detecting viruses that enter the cytosol of cells, or viral RNA synthesized in the cytosol (Andrejeva et al., 2004). Both receptors are able to recognize dsRNA via their RNA helicase domain. Furthermore they have two CARDs, which are critical for initiating downstream signaling pathways (Lee et al., 2007). LGP2 (laboratory of genetics and physiology 2) is also a RIG-I like helicase that can recognize RNA. Unlike RIG-I and MDA5, LGP2 lacks the CARDs and thus has no signaling capability (Li et al., 2009; Murali et al., 2008).

NLRs are a growing family of regulatory proteins having a conserved tripartite domain structure: the C-terminal leucine-rich repeat (LRR) domain for recognizing ligands directly or indirectly, the NACHT domain and the N-terminal effector domain that generates downstream signaling. NLRs contain two major subfamilies called nucleotide-binding oligomerization domain (NOD) and NACHT/LRR/PYD-containing proteins (NAPLS). The larger subgroup of NLR proteins are the NALPs with 14 members identified in humans (Le Bourhis et al., 2007; Pétrilli et al., 2007). In general the NLR family is composed of 22 members in mammals (Martinon and Tschopp, 2005).
NLRs are believed to remain in an inactive form until binding of a ligand, resulting in conformational changes, making downstream signaling possible. The activated NLR may then serve as a molecular platform for protein complexes such as inflammasomes (for NALPs) or nodosomes (for Nods) by triggering the activation of downstream effector molecules initiated via self-association and induced proximity of binding partners. The activation regulates intracellular signaling

pathways such as nuclear factor κB (NF-κB), caspase 1 inflammasome and mitogen-activated protein kinases (MAPKs), which finally leads to altered gene expression of proteins, which play an essential role in inflammatory responses, cell fate or antimicrobial activity (Le Bourhis et al., 2007).
For example, NOD1 specifically recognizes meso-diaminopimelic acid, a peptidoglycan product mainly present in gram-negative bacteria, whereas NALP3 and NOD2 sense muramyl dipeptide (MDP), a pepdidoglycan constituent, which can be found in gram-positive as well as in gram-negative bacteria (Abbas, 2010; Martinon et al., 2005).

Recognition of cytosolic DNA via cytoplasmic receptors

The induction of type I IFN genes by *Listeria monocytogenes* requires the S/T protein kinase TBK1 and transcription factor interferon regulatory factor 3 (IRF3), but not TLRs or NOD proteins (Stockinger et al., 2004). Removal of DNA from *Listeria* extracts abolishes their ability to activate type I IFNs, suggesting that DNA is the ligand. On the one hand DNA may be released during *Listeria* infection as a consequence of LLO-mediated dirsruption of the vacuole (see chapter 3.1.1) and on the other hand live *Listeria* may actively secrete DNA (Stetson and Medzhitov, 2006).

Chemically synthesized dsDNA, like poly(dA-dT), triggers antiviral response through IFN-β promoter stimulator 1 (IPS-1) and TBK1/IKKε, including type I IFNs independently of TLRs and NLRs (Ishii et al., 2006). Whereas in case of Listeria infection IPS-1 is not needed for IFN-β induction (Soulat et al., 2006). Recently it has

been shown that cytosolic poly(dA-dT) is converted into 5'-ppp RNA by DNA-dependent RNA polymerase III to induce IFN-β via the RIG-I pathway, indicating RNA polymerase III as a DNA sensor that links DNA release by pathogenic bacteria and viruses in the host cell cytosol to IFN-β production and innate immunity (Chiu et al., 2009).

LRRFIP1 was recently identified as cytoplasmic interferon-inducing sensor of nucleic acids in macrophages. This protein is able to detect dsRNA as well as dsDNA, which leads to activation of β-catenin and binding of IRF-3. Subsequently more p300 acetyltransferase is recruited to the IFN-β enhanceosome, leading to increased IFN-β induction. LRRFIP1 seems to play a crucial role in recognizing pathogens, like L.monocytogenes and vesicular stomatitis virus, resulting in innate immune responses, such as IFN-β expression (Yang et al., 2010).

In 2007 a protein named DLM-1 or Z-DNA binding protein 1 (ZBP1) was identified as cytoplasmic DNA sensor, termed DNA dependent activator of IFN-regulatory factors (DAI).
In some cell types, but not in macrophages, DNA binding to DAI leads to type I IFN induction through the activation of IRF3 and, probably, IRF7. Notably, this receptor may itself recruit IRF3 and TBK1/IKKi in a DNA-dependent manner (Takaoka et al., 2007; Takaoka and Shinohara, 2008).

AIM2 (absent in melanoma 2), a HIN-200 family member, also recognizes cytoplasmic dsDNA. AIM2 contains a carboxy-terminal oligonucleotide/oligosaccharide-binding domain and a pyrin domain at the N-terminus, which is important for interaction with ASC to activate an inflammosome. This further leads to the activation of caspase-1, a cystein protease that processes the inactive pro-IL1β

and proIL18 to their active proinflammatory cytokines IL1β and IL18, respectively. Additionally AIM2/ASC interaction leads to the formation of the ASC pyroptosome, which induces pyroptotic cell death in cells containing caspase1 (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009).

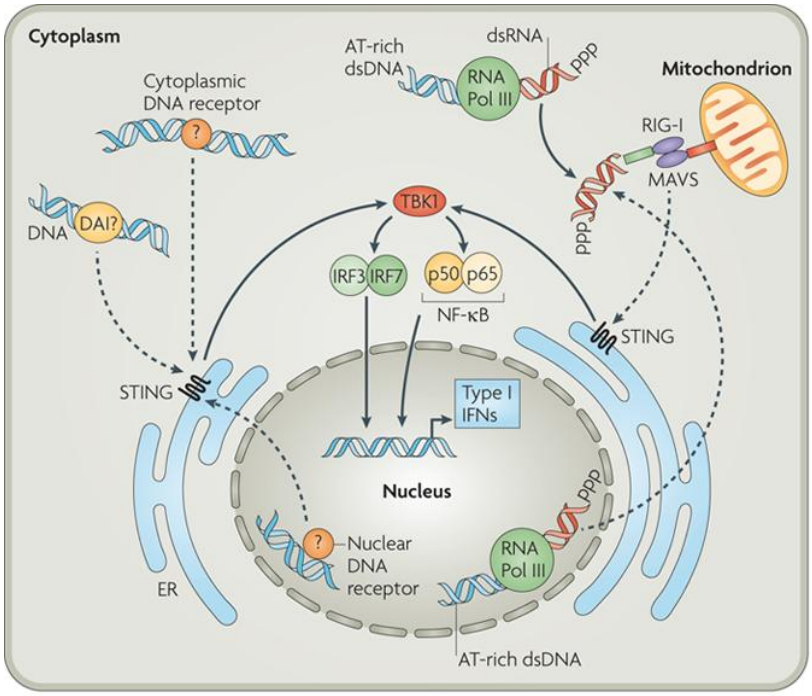


Figure 3: *Innate immune responses activated by cytosolic DNA*. DAI, as well as an unknown receptor for cytosolic DNA trigger type I IFN expression via TBK1 activation. Polymerase III converts cytosolic DNA into 5'triphosphate dsRNA, which is sensed by RIG-I (Hornung and Latz, 2010).

3.4 Signaling pathways triggerd by PRR stimulation

3.4.1 TLR signaling

Ligand binding to TLRs facilitates their dimerization and initiates downstream signaling pathways by using adaptor molecules such as Myeloid differentiation factor 88 (MyD88), TIR-domain containing adapter protein (TIRAP or MAL), TIR domain containing adapter protein inducing IFN-β (TRIF), and TRIF related adapter molecule (TRAM). All these proteins contain a Toll/interleukin 1 receptor (TIR) domain (Takeda and Akira, 2005).
In general a MyD88 dependent and a MyD88 independent pathway can be distinguished. All TLRs use MyD88 except TLR3, which functions independently of MyD88. TLR 4 can utilize both pathways (Dunne et al., 2005).

MyD88 dependent signaling

MyD88 has a C-terminal TIR domain and an N-terminal death domain and associates with the TIR domain of TLRs. The pathway will be further explained in the context of TLR4. After ligand binding to the TLR4, TIRAP recruits MyD88 to the receptor by binding to Phosphatidylinositol 4,5-bisphosphate (PIP2) concentrated on the plasma membrane, interacting with MyD88 via their TIR domains, and thus promoting the interaction between TLR4 and MyD88 through TIR-TIR interactions. Then MyD88 recruits IL-1R associated kinase 4 (IRAK-4) via the interaction of the death domains, present in both molecules. IRAK-4 mediates the phosphorylation of IRAK-1, making

the association with TNFR associated factor (TRAF6) possible. This interaction initiates two distinct signaling pathways.
One pathway leads to the activation of activator protein 1 (AP-1) transcription factor, composed of c-Jun and c-Fos, through phosphorylation of Mitogen-activated protein kinase kinase 6 (MKK6) and 7, which in turn activate the p38 MAPK and c-Jun kinase (JNK) pathways, respectively and the other pathway activates the IKK complex (Dunne et al., 2005; Takeda et al., 2005). Once activated, the IKK complex induces phosphorylation, leading to ubiquitination and subsequent proteasomal degradation of the inhibitor IκB, that is then followed by release and nuclear translocation of NFκB (Schmid and Birbach, 2008). Both pathways make use of the Transforming growth factor β activated kinase 1 (TAK1)/TAK1 binding protein (TAB) complex (Thiefes et al., 2005).

MyD88 independent signaling

The Myd88-independent pathway is initiated by the recruitment of TRIF to the cytoplasmic TIR domain of TLRs (Lee et al., 2007). In case of TLR4, TRIF is recruited to the receptor on the plasma membrane through myristoylated TRAM, which is localized on the membrane and exclusively used in TLR4 signaling (Rowe et al., 2006). Via the interaction of TRIF with Receptor interacting protein 1 (RIP1), TRAF6 and TRAF3, downstream signaling pathways are activated.
RIP1 plays a role in NF-κB activation but not in IRF3 activation. In certain cells TRAF6 may activate NF-κB and AP-1 through TAK1 activation to induce the expression of inflammatory cytokine genes. TRAF3 is essential for IRF3 activation and subsequent IFN-β production. The interaction of TRAF3 with TRIF activates the redundant S/T kinases TBK1 and IKKi, which further phosphorylate

interferon regulatory factor 3 (IRF3). The activated IRF3 translocates into the nucleus and induces the expression of IFN-β and subsequent IFN-β inducible genes (Dunne et al., 2005; Lee et al., 2007; Takeda and Akira, 2007).

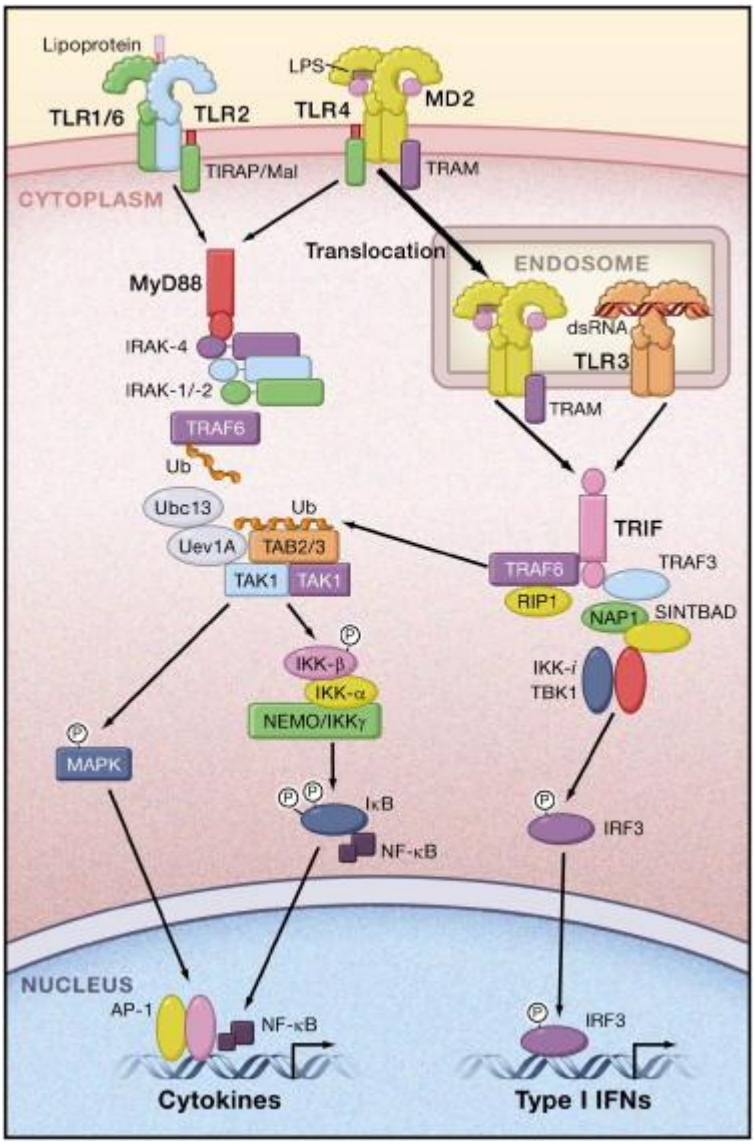


Figure 4: *TLR2, TLR3 and TLR4 signaling pathways* (Takeuchi and Akira, 2010).

3.4.2 RIG-I/MDA5 signaling

When ligand binding activates RIG-I or MDA5, an adaptor protein is recruited. This protein was simultaneously identified by four different groups and is therefore known as mitochondrial antiviral signaling (MAVS), IFN-β promoter stimulator 1 (IPS-1), virus induced signaling adapter (VISA) or CARD adapter inducing IFN-β (CARDIF) (Gack et al., 2010). This adaptor protein contains a CARD domain on the N-Terminus, which is important for CARD-CARD interactions with the adaptor protein IPS-1.
IPS-1 can directly interact with TRAF3, which activates TBK1/IKKi, leading to the sequential phosphorylation of IRF3 and subsequently to IFN-β induction. IPS-1 can also associate with TRAF6 and RIP1, inducing the expression of proinflammatory cytokine genes by activating the NFκB-pathway and the MAPK-pathway (Lee et al., 2007).

3.4.3 NLR signaling

Upon ligand binding to NODs, the CARD-domain containing serine/threonine kinase RIP2, also known as RIPK2, Rip-like interacting caspase-like apoptosis-regulatory protein kinase (RICK), CARD-containing interleukin (IL)-1 beta converting enzyme (ICE) associated kinase (CARDIAK) and cholecystokinin (CCK), is recruited to the receptor via CARD-CARD interaction (Kobayashi et al., 2002). Oligomerization of NODs induces physical proximity of RIP2 proteins and the IKK subunits, leading to NF-κB activation. Also MAP kinases such as JNK, p38 and ERK can be activated (Lee et al., 2007; Martinon et al., 2005).

NALPs have the ability to recruit the adaptor Apoptosis-associated speck-like protein with CARD domain (ASC) through a homotypic PYD–PYD interaction, which in turn recruits caspase-1 via CARD–CARD interaction, leading to the cross-activation of Caspase-1 which subsequently processes immature pro-IL-1β and pro-IL-18 into the mature forms IL-1β and IL-18.

IL-1β and IL-18 bind to the IL-1 receptor (IL-1R) and IL-18 receptor (IL-18R) complexes, respectively. Similar to TLRs, these receptors have a cytoplasmic TIR domain that can recruit adaptors such as MyD88, which is responsible for activating NF-kB and other signaling cascades as mentioned in chapter 3.4.1 (Lee et al., 2007; Martinon et al., 2005).

3.5 Interferons

Interferons (IFNs) are cytokines, which are produced in response to microbes and play an important role in the immune response. They can be differentiated into type I, type II and type III interferons. Classically, type I interferons have been identified as mediators of the early innate immune response to viral infections and type II interferons as antibacterial immunregulators (Decker et al., 2005). Type III interferons also induce a type I interferon like response (Zhou et al., 2007).

There are several members of the type I IFN family, which share a structural homology and are encoded by genes in a single cluster on chromosome 9 (Abbas, 2010). This group includes IFN-α, which can be subdivided into 13 different subtypes, IFN-β, IFN-ε, IFN-κ, IFN-ω and additionally three others, which can be only found in pigs and mice. Type I IFNs interact with the type I IFN receptor, which consist of the IFNAR I and the IFNAR II chain (Decker et al., 2005).

The only type II interferon is IFN-γ, which has already been mentioned in chapter 3.2. It is produced by T lymphocytes and NK cells and plays an important role in macrophage activation, where it induces a cell-autonomous microbial state against ingested intracellular pathogens. Type II IFNs are recognized by the type II IFN or IFN-γ receptor (IFNGR), which is built of the IFNGR I and IFNGR II chains (Abbas, 2010; Decker et al., 2005).

The type III interferons comprise IFN-λ1 (IL-29), IFN-λ2 and IFN-λ3 (IL-28A/B) in humans. Although they exhibit antiviral activity similar to type I interferons, they have evolved independently of type I interferons (Onoguchi et al., 2006). This group of interferons

interacts with cell surface receptors composed of interleukin-10 receptor β and interleukin-28 receptor (Zhou et al., 2007).

All IFN receptors regulate gene expression by activating the Janus kinase (JAK) – signal transducer and activator of transcription (STAT) pathway to induce several hundred IFN-stimulated genes (ISGs) that can be type-specific or common to two or all receptor types (Decker et al., 2005).

IFN-α and IFN-β are the main type I IFNs that are synthesised during viral and bacterial infections. IFNs cause cells to produce enzymes that interfere with transcription of viral RNA or DNA and viral replication. They increase expression of class I major histocompatibility complex (MHC) molecules and the cytotoxic activity of NK cells. Type I IFNs promote the sequestration of lymphoctes in lymph nodes and in humans, type I IFNs also stimulate the development of T_H1 cells (Abbas, 2010). In addition, the IFN-α/β system also modulates the oncogenic process and bone metabolism (Takaoka and Taniguchi, 2003).
IFN-α and IFN-β are massively produced in most cell types in response to viral and bacterial infections. Type I IFNs are currently recognized as pivotal cytokines bridging innate and adaptive immune systems (Takaoka and Yanai, 2006).

3.6 The IFN-β enhanceosome

Transcriptional enhancers are cis-regulated elements, which have an essential role in eukaryotic gene expression.
Enhancers consist of multiple binding sites on the DNA, creating a combinatorial code, which directs transcription factors and polymerases to their specific promoter in response to stimulation.
There is a wide range concerning length and complexity of enhancers. Binding of proteins on enhancers with binding sites tightly clustered in a short DNA fragment, leads to the formation of an enhanceosome.

The best characterized compact human enhanceosome forms on the virus inducible enhancer of the IFN-β gene. In 2007 the crystal structure of the transcription factors bound to the enhancer was described.
It showed that assembly of a set of transcription factors, each of which can also bind, together with different partner proteins, to other enhancers, is necessary to fully activate the promoter.
The IFN-β enhanceosome consists of the heterodimer ATF-2/c-Jun, IFR3 and IRF7, and NFκB (p50/RelA). These proteins bind to a 55bp stretch on the DNA. The IFN-β enhancer has been subdivided into 4 positive regulatory domains (PRDs). The IRFs bind to PRDI and PRDIII, NFκB binds to PRDII and ATF-2/c-Jun bind to PRDIV. In case of virus infection, all these transcriptionfactors bind to their designated PRD and activate the transcription of the IFN-β gene. An IFN-β response should only occur, when the right set of transcription factors binds to the enhancer and only when all of them are present (Panne et al., 2007).

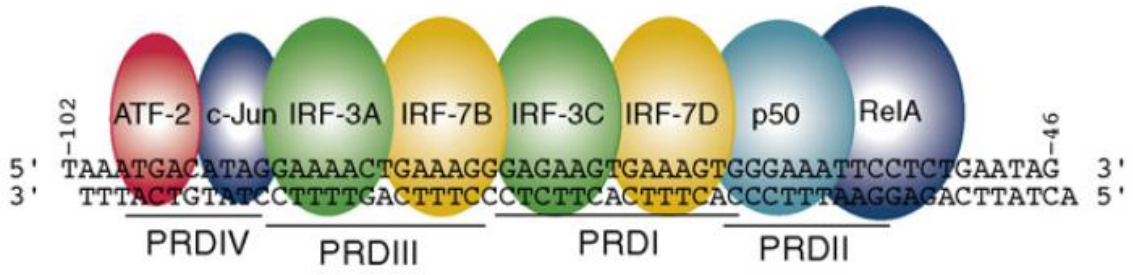


Figure 5: *The IFN-β enhanceosome*. Transcriptionfactors bound to the IFN-β enhancer.

3.7 Interferons and the JAK/STAT pathway

Type I and type II interferons can both activate the JAK/STAT signaling pathway.

Type I interferons interact with the type I IFN receptor, composed of the IFNAR I and the IFNAR II chain, which are permanently associated with the JAKs Tyrosine Kinase 2 (TYK2) or JAK1, respectively (Decker et al., 2002).
JAKs have tandem kinase-homologous domains at the C-terminus. The first is a non-catalytic regulatory domain, whereas the second has tyrosine kinase activity (Rawlings et al., 2004).
IFN binding to the receptor induces the tyrosine phosphorylation of cytokine-receptor cytoplasmic domains via JAKs, which then provides binding sites for the Src-homology-2 (SH2) domain of the STAT proteins (Levy and Darnell, 2002).
The STAT proteins are then recruited to the JAKs, whereupon they are phosphorylated on a single tyrosine residue 701 (in case of STAT1) or 692 (in case of STAT2). For full Stat1 activity, an additional phosphorylation at serine 727 is necessary. Phosporylated STATs are able to interact through SH2 domain – phosphotyrosine interaction, which results in the formation of homo- or heterodimers, which further translocate to the nucleus, where they act as transcription factors.
STAT1 homodimers bind to gamma IFN-activated site (GAS) elements, palindromic DNA recognition sites located in the promoter or enhancer region of IFN regulated genes. In case of STAT1/STAT2 heterodimer formation, another protein, called IRF9 or p48, joins the complex. This transcription factor complex, termed ISGF3, binds to interferon stimulated response elements (ISRE) on the DNA and

activates the transcription of several type I IFN stimulated genes (ISGs) (Decker et al., 2002; Levy et al., 2002).

Type II interferons (IFN-γ) bind to the IFNGR, consisting of IFNGR I and IFNGR II chains associated with JAK1 and JAK2. IFN-γ stimulation leads to the tyrosine phosphorylation of the receptor chains and subsequent recruiting, binding and phosphorylation of STAT1 on tyrosin 701.
STAT1 homodimers are build up, tansclocate to the nucleus and bind to promoters containing GAS sites to regulate gene activation (Decker et al., 2002).

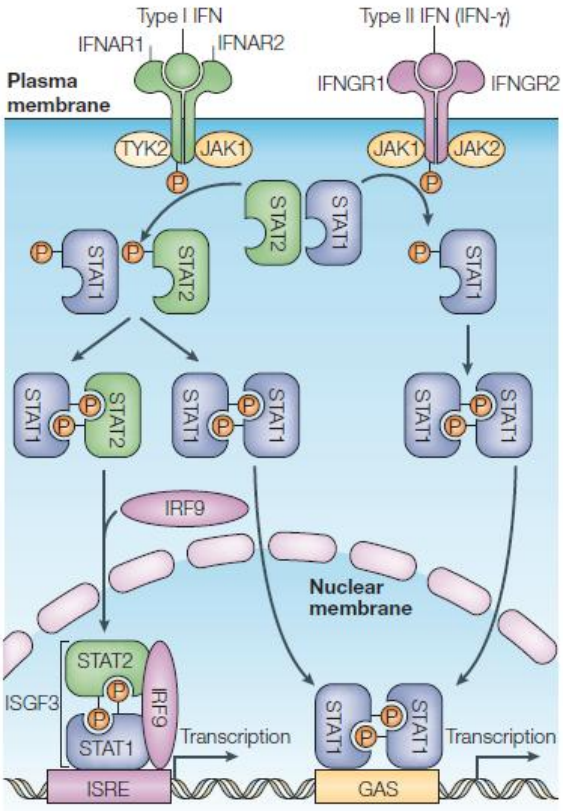


Figure 6: *JAK-STAT pathway.* Binding of interferons to their receptor activates JAKs, resulting in the phosphorylation of the receptor. Stat molecules are recruited to the phosphorylated receptor, are also phosphorylated and Stat1 homodimers or Stat1-Stat2 heterodimers are formed. The complexes translocate to the nucleus, bind to their respective site and activate gene transcription (Decker et al., 2005).

Interferon production is regulated via a positive feedback loop. Activation of TBK1 or IKKε leads to serine phosphorylation of IRF3, which triggers the induction of IFN-β and in some cells of IFN-α4. These "early" IFNs lead to the formation of ISGF3 and one of its target genes *Irf7*. IRF7, which in turn is, like IRF3, also phosporylated by TBK1/IKKi induces the expression of a second stronger wave of IFNs (Decker et al., 2002, 2005).

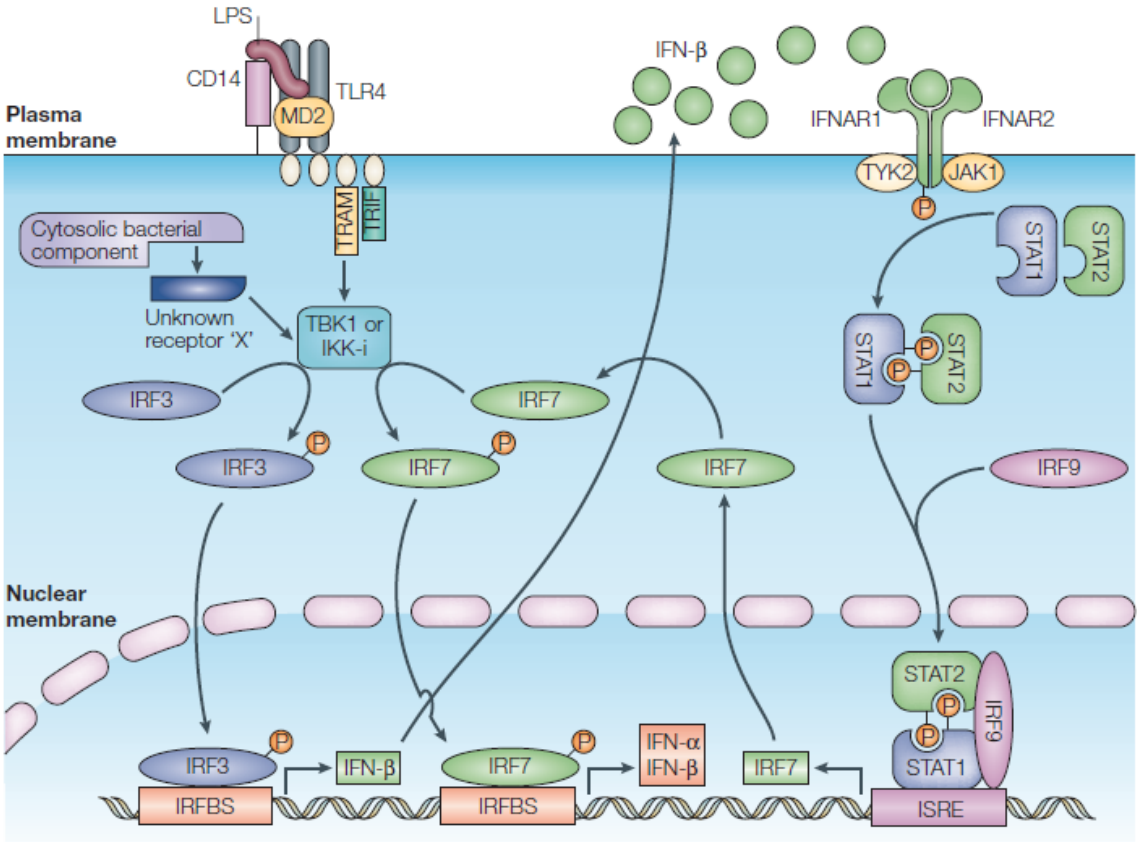


Figure 7: *Activation of IRF3 and IRF7 and resulting type I IFN production.* TLR4, or an unknown receptor in case of cytosolic bacteria, activate TBK1 or IKKε, which in terms phosphorylate IRF3. Phosphorylated IRF3 translocates to the nucleus and induces the transcription of IFN-β. IFN-β binds to its receptor, activates the Jak-Stat pathway, resulting in the induction of IRF7. IRF7 is again phosphorylated by TBK1 or IKKε, translocates to the nucleus and activates the transcription of a second wave of type I interferons (Decker et al., 2005).

3.7.1 STATs

So far seven STAT proteins were identified, denoted STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6, in mammalian cells. These proteins consist of several conserved functional domains. The domain on the N-terminus is responsible for tetramerization of all STATs (with the probable exception of STAT2), and is also important for receptor recognition and phosphatase recruitment for some STATs. The N-domain is followed by a coiled-coil domain that participates in protein–protein interactions, a DNA binding domain, a linker domain implicated in DNA binding, an SH2 domain that mediates STAT dimerization and receptor binding, and a carboxy-terminal transactivation domain (see Figure 8) (Meyer and Vinkemeier, 2004).

For the translocation of STATs into the nucleus components of the Ran-dependent nuclear import machinery are used. Furthermore the karyopherin importin α5 plays an essential role (Levy et al., 2002; Meyer et al., 2004). For the nuclear export, the STAT molecules have to be in an unphosphorylated mode. For STAT1 and STAT3, there is evidence that TC45 (a nuclear tyrosine phosphatase) is a relevant STAT nuclear phosphatase (Levy et al., 2002).Unphosphorylated STATs can bind to the exportin CRM1 via leucine-rich nuclear export signals and move back to the cytoplasm (Meyer et al., 2004).

3.7.2 STAT1 F77A mutant

The ~130 residues N-terminal domain of STAT1 plays an important role in many protein-protein interactions. Mutation of a single residue (Phe^{77} to Ala) located on the surface of the N-domain of STAT1 precludes polymerization of STAT1 homodimers and dephosphorylation of tyrosin 701 (Meyer et al., 2004).

The formation of STAT tetramers seems to be conserved throughout the STAT family and for some genes, especially those with multiple GAS in their promoters, the STAT tetramer binding seems to be important to fully activate gene expression. Activation of endogenouse STAT1 target genes is impaired in the STAT1 F77A mutant.

Nuclear import is not affected in the STAT1 F77A mutant but due to the defect in dephosphorylation, persistent DNA binding and accumulation of STAT1 F77A molecules in the nucleus was observed (Meyer et al., 2004; Vinkemeier and Meyer, 2005; Zhong et al., 2005).

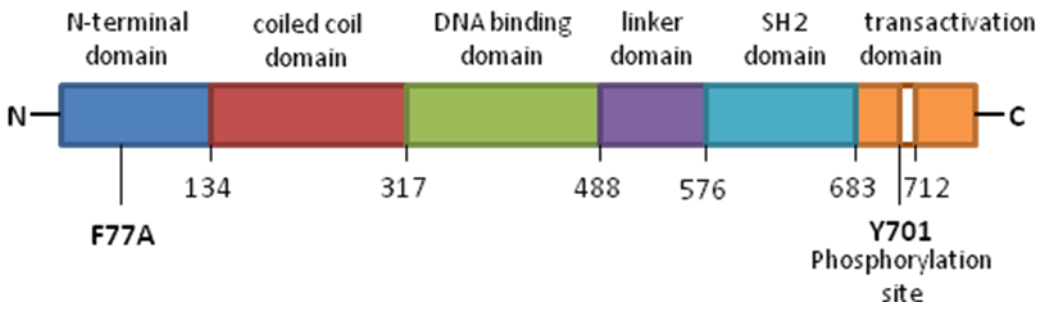


Figure 8: *Domain organisation of Stat1*. The site of the F77A mutation and the tyrosin phosphorylation site are indicated.

4 Aims

The main project of my diploma thesis was to replace the *inlA* gene of *Listeria monocytogenes* LO28 by the mutated version (S192N, Y369S) of EGDe *inlA*. This should enable oral infection experiments with a moderate dose of *Listeria* LO28.
The mutated *inlA* (S192N, Y369S) can interact with the mouse E-cadherin on intestinal epithelial cells. The exchange of the LO28 *inlA* by the EGDe *inlA* ensures that *inlA* is anchored in the cell wall of the bacterium and not secreted because of a nonsense mutation, which is present in the LO28 strain.

Further I investigated the role of p38 MAPK, JNK, TBK1/IKKε or IKKβ on IFN-β induction and cell death in bone marrow derived macrophages (BMDMs). For this purpose we used pharmacological inhibition of the kinases.
We wanted to clarify, if in case of *Listeria monocytogenes* LO28 infection all transcription factors activated by these proteins are necessary for full IFN-β induction. Additionally, we examined cell death after inhibiton of the above-mentioned kinases.

The third project investigated the Stat1 F77A mutant. We focused on the intracellular replication of *L. monocytogenes* LO28 in Stat1 F77A mutated BMDM. Additionally, we investigated the impact of the mutation on the induction of IFN-β and IFN-induced genes as well as on host cell death.

5 Results

The modification of the *inlA* locus in *Listeria monocytogenes* LO28 is carried out in a two step process. The first step is to knock-out *inlA* in *Listeria* LO28 and the second step is the insertion of the mutated version of EGDe *inlA* in the *Listeria* LO28 *inlA* knock-out. For the insertion of *inlA*, five different strategies have been developed.

5.1 Strategy for creating the *inlA* knock-out in *L. monocytogenes*

The genome of *Listeria monocytogenes* LO28 has not been completely sequenced so far, therefore we had to sequence the gene locus and the flanking regions of *inlA* of *Listeria* LO28 (for primer sequences see materials).

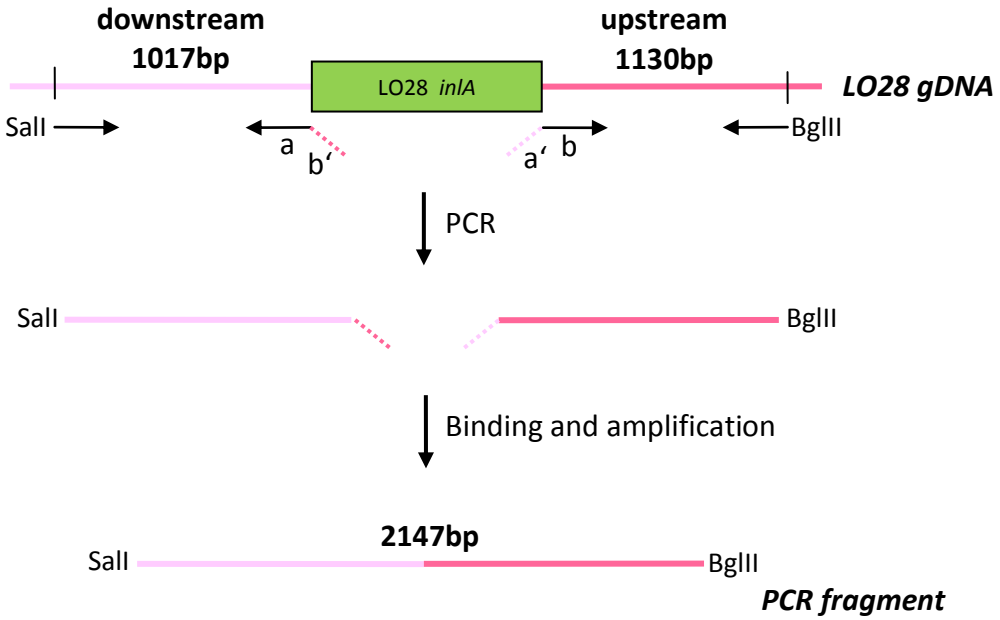


Figure 9: *Overlap extension PCR*. Flanking regions of *L. monocytogenes* LO28 *inlA* were amplified with specific primers and with an additional PCR the 2147bp product was generated from both fragments.

The first step to create the *inlA* knock-out (k/o) was an overlap extension polymerase chain reaction (PCR) with a proof reading polymerase. As shown in figure 9, primer for the flanking regions of *inlA* of *L. monocytogenes* LO28 were designed.

The outer primers (InlAdown_for, InlAup_rev) contain restriction enzyme sites for SalI or BglII, whereas the inner primers (InlAdown_rev, InlAup_for) have an attached sequence, which is complementary to the 5’ end of the upstream fragment in case of a and to the 3’ end of the downstream fragment in case of b.

The downstream fragment (1017bp) and the upstream fragment (1130bp) were amplified and an additional PCR (using primer pair InlAdown_for and InlAup_rev) was accomplished for binding and amplification of the two DNA fragments, resulting in a 2147bp PCR product.

This DNA fragment was ligated into the temperature sensitive shuttle vector pMAD, using the restriction enzymes SalI and BglII. The ligation was accomplished at 16°C over night.

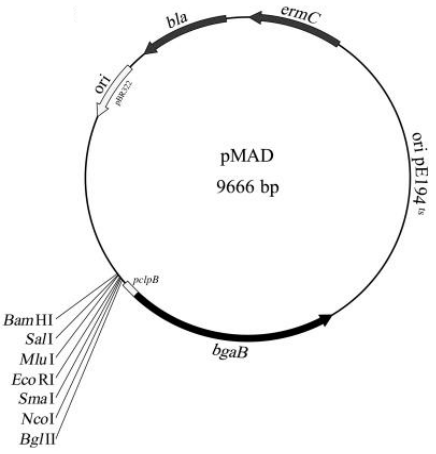


Figure 10: *Physical map of pMAD.* Unique restriction enzyme sites in the multiple cloning site are indicated. *pclpB* is a DNA fragment from *S. aureus* containing the *clpB* promoter region. *bgaB* is a DNA fragment containing the promoterless *bgaB* gene encoding a thermostable β-galactosidase. *ermC* is the gene for the erythromycin resistance in gram-positive bacteria. *bla* is the gene for β-lactamase, conferring resistance to β-lactam antibiotics. The ori pE194 is a temperature sensitive origin of replication for gram-positive bacteria, not working at temperatures above 37°C (Arnaud et al., 2004).

The vector containing the insert was transformed into *E.coli* DH5α cells. Positive transformants were selected by growth on LB-ampicillin plates at 37°C. From overnight cultures of the grown colonies the plasmid was purified via Mini Prep, digested with the restriction enzymes SalI and BglII and loaded on a 1% agarose gel to check for the presence of the 2147bp insert. The insert was sequenced, using the primers seqInlALO28_1, seqInlALO28_2, seqInlALO28_3 and seqInlALO28_4, to ensure that there were no point mutations.

The plasmid containing the correct insert was transformed into *L. monocytogenes* LO28 via electroporation. The integration of the plasmid into the genome took place at 42°C, the non-permissive temperature, which prevents the replication of the plasmid.
10ml Brain-Heart-Infusion Medium (BHI) containing erythromycin were inoculated with the bacteria and grown at 42°C over night. On the next day 10ml fresh BHI with erythromycin were again inoculated with 100µl of the overnight (O/N) culture and incubated under the same conditions. This procedure was repeated for three days.
On the third day 100µl of an appropriate dilution (1:1.000.000) were plated on BHI plates containing erythromycin and incubated at 42°C O/N.
Several clones where selected and used to inoculate 10ml BHI with erythromycin for growth over night at 42°C. On the next day

bacterial glycerol stocks were prepared and stored at -80°C. From 5ml of the rest of the O/N-cultures, genomic DNA from the *Listeria* was purified via Phenol/Chloroform extraction.

Site directed integration of pMAD into the genome of *Listeria monocytogenes* LO28 requires homologous genomic sequences. Therefore the vector could either integrate via the downstream region or the upstream region of *inlA*. The integration was analyzed via PCR with one primer (bgal) binding close to the multiple cloning site of pMAD and the other primer (EGD_for1) binding outside of the knock-out region. The length of the PCR product indicates if the plasmid integrated down- or upstream. Both scenarios are shown in figure 11.

For the release of the plasmid, *Listeria* from the glycerol stock with the integrated plasmid were used to inoculate 10ml BHI and incubated without selection pressure at 28°C (the permissive temperature) for four days (passage of O/N culture as described for integration of the plasmid). 100µl of an appropriate dilution were plated on BHI plates containing no antibiotics. A colony-lift was performed to transfer clones to a BHI plate with erythromycin.
Comparison with the original plate identified clones, which lost their antibiotic resistance, due to the homologous recombination event. Theoretically, two different types of reversion in either case of integration were possible. On the one hand excision of the vector could represent an exact reversion of the integration process, resulting in unchanged gDNA of *L. monocytogenes* LO28, on the other hand excision could result in the knock-out of InlA (Figure 11).
After reversion of the plasmid the genomic DNA was analyzed by PCR (for primer sequences see materials).

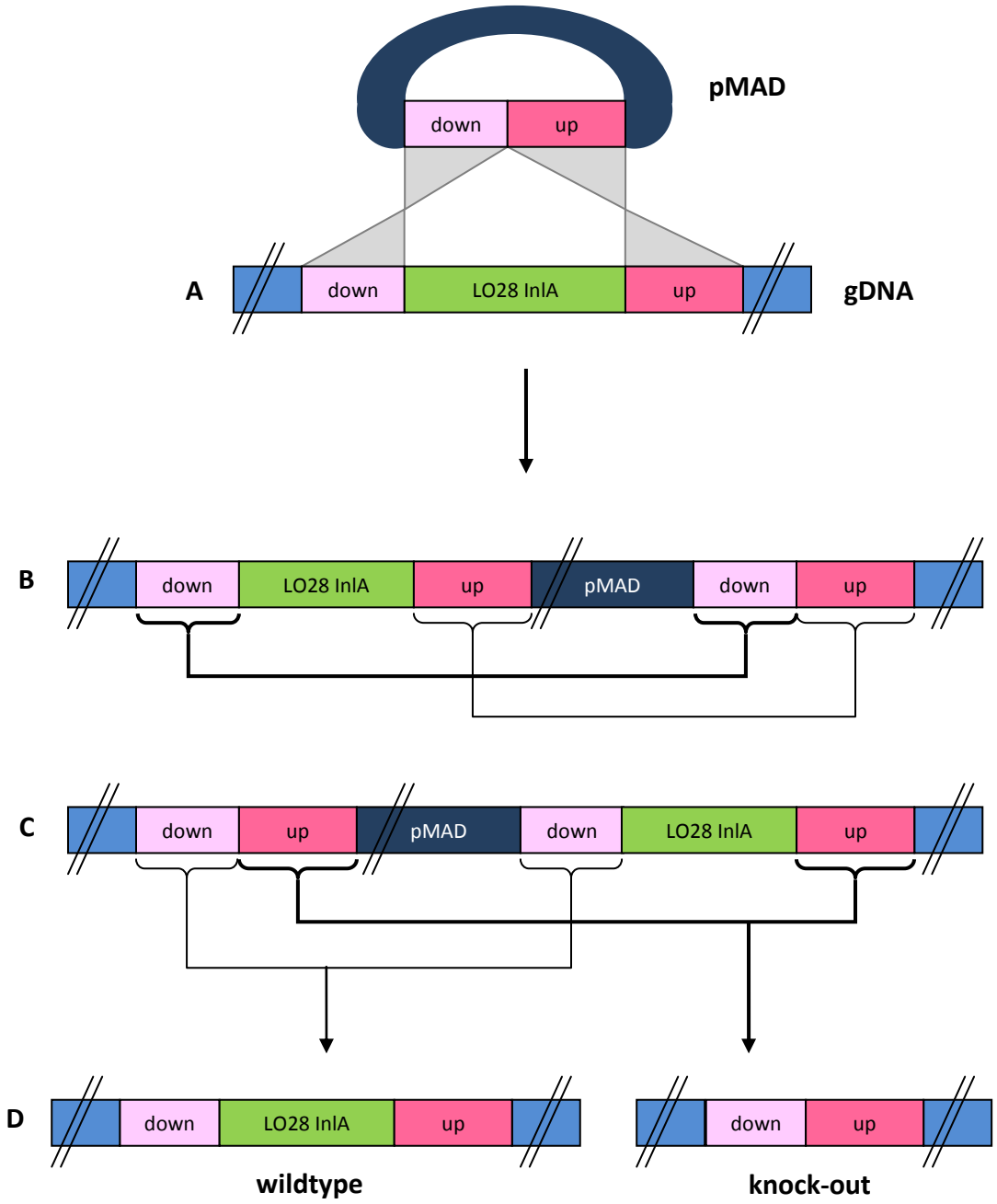


Figure 11: *Integration and reversion of pMAD*. **A.** Homologous recombination of pMAD with insert and gDNA of *L. monocytogenes* LO28. **B.** Integration of pMAD via the upstream region. **C.** Integration of pMAD via the downstream region. **D**. Reversion of pMAD integration. The thinner lines indicate the reversion to the wildtype situation and the thicker lines indicate the reversion to the *inlA* knock-out.

5.2 Strategies for the insertion of EGD *inlA*^{mut}

5.2.1 Strategy 1

Overview: The flanking regions of LO28 inlA and the mutated EGD inlA (S192N, Y369S) are amplified and and a fragment containing all three parts can be generated with an overlap extension PCR. This DNA fragment is cloned into pMAD and via homolgous recombination the EGD inlA is inserted into the LO28 inlA knock-out genome.

The first step for the insertion of *L. monocytogenes* EGD *inlA* with the point mutations S192N and Y369S into the LO28 *inlA* knock-out are three different PCRs (Figure 12).
One PCR to amplify the downstream region of InlA. Therefore the genomic LO28 DNA has to be used as template. The forward primer (InlAdownfor_Nco or down190_NcoI) includes an NcoI restriction enzyme site and the reverse primer (a, down_rev_lig) has an appendix (b') complementary to the 5' end of EGD *inlA* (b).
The same has to be done for the upstream region with a forward primer (d, up_for_lig) containing a complementary sequence (c') to the 3' end of EGD InlA (c). The reverse primer (InlAuprev_SalI or up224_SalI) includes a SalI restriction enzyme site for cloning into the shuttle vector pMAD.
The third PCR amplifies the EGD *inlA* containing the point mutations S192N, Y369S. The plasmid pAUL-inlA2 with the above mentioned EGD *inlA* was kindly provided by Wolf-Dieter Schubert (Helmholtz Center for infection research Braunschweig). To accomplish this PCR, primers (b, InlA_for_lig and d, InlA_rev_lig) with complementary regions to the 3' end of the downstream fragment (a') and to the 5' end to the upstream fragment (d') of LO28 *inlA* have to be used.

When the three fragments are amplified, they can be used for an additional overlap extension PCR to get one fragment with a length of 4570 or 2837bp, depending on the length of the downstream and upstream fragment. The primers for the overlap extension PCR have a restriction enzyme site for NcoI or SalI, respectively.

When the whole fragment is amplified it has to be cloned into the temperature-sensitive shuttle vector pMAD via NcoI and SalI. For the rest of the procedure see chapter 5.1. Figure 13 shows the possibilities of integration and reversion of pMAD containing EGD *inlA* S192N, Y369S into the *L. monocytogenes inlA* knock-out genome.

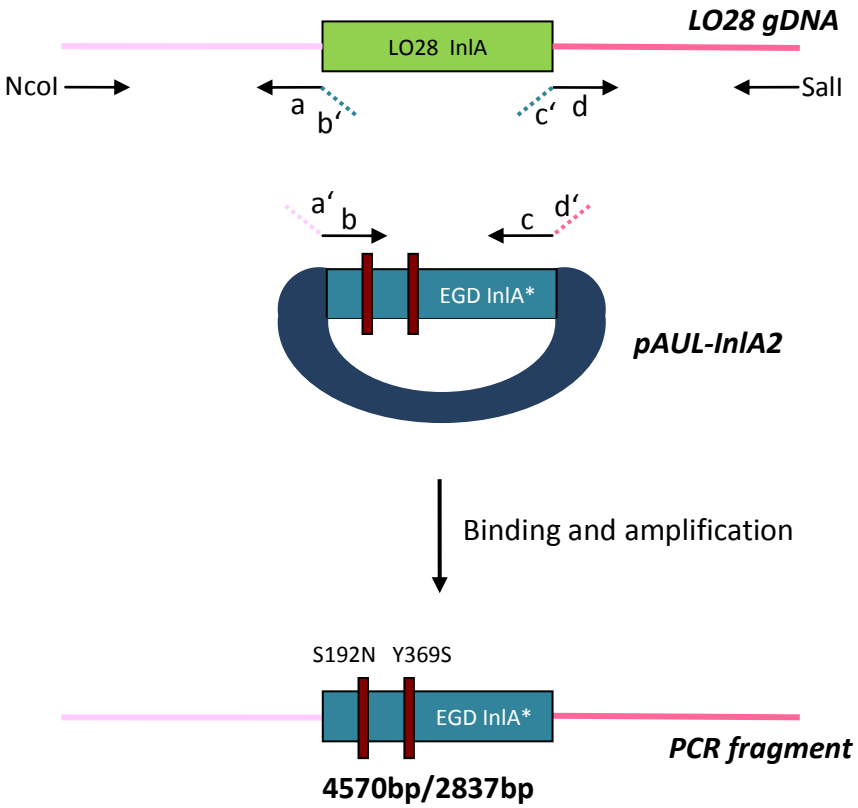


Figure 12: *Overlap extension PCR* to amplify the fragment needed for the insertion of the mutated EDG InlA into the *L. monocytogenes* LO28 *inlA* knock-out.

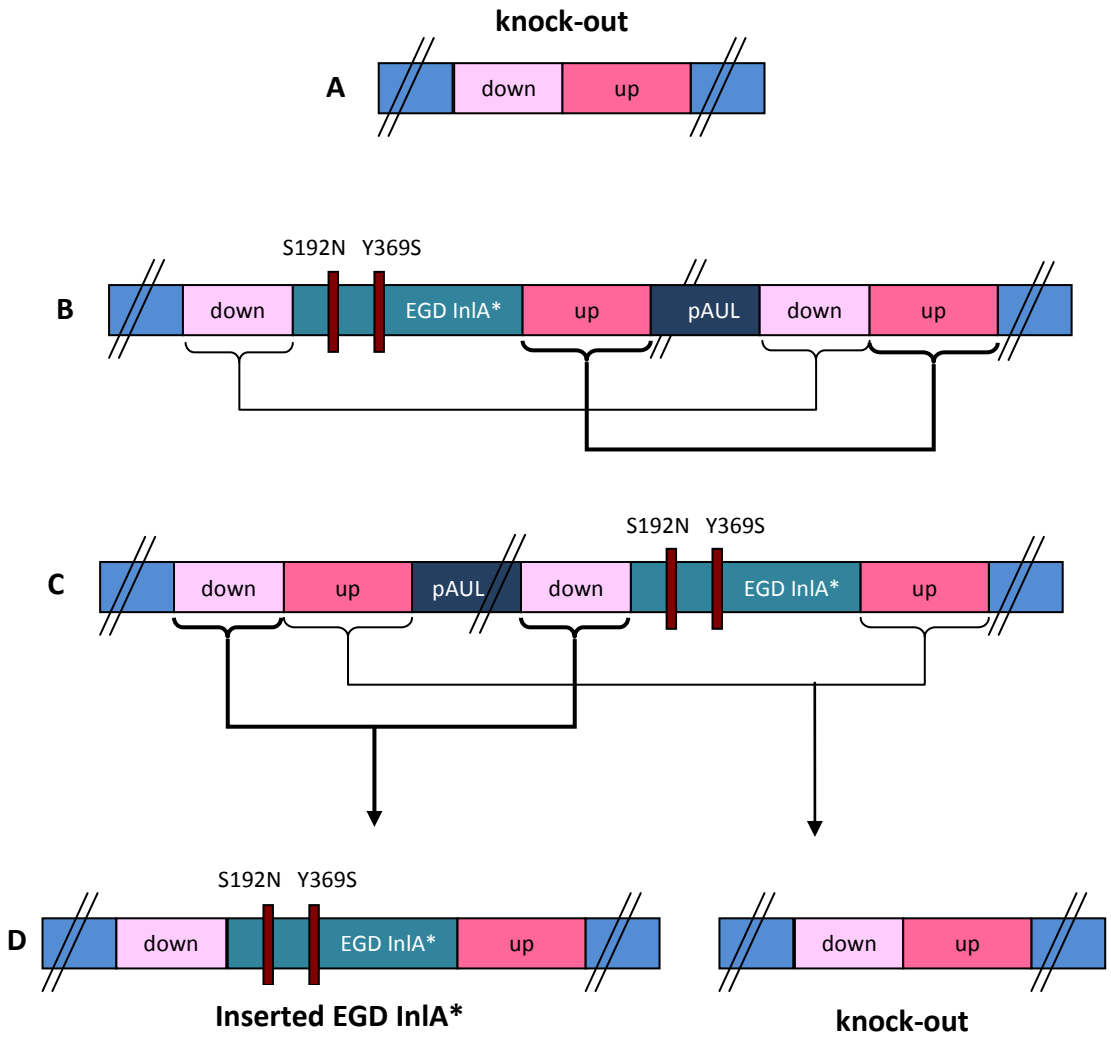


Figure 13: *Integration and reversion of pAUL-inlA2 containing EGD inlA* (S192N, Y369S).* **A**. *inlA* knock-out in LO28 gDNA. **B**. Integration of pAUL-inlA2 with EGD InlA* via the downstream region. **C**. Integration of pAUL-inlA2 with EGD *inlA** via the upstream region. **D**. Reversion of pAUL-inlA2 indicated with thicker lines resulting in inserted EGD *inlA** in the LO28 genome and with thinner lines resulting in the *inlA* knock-out situation.

5.2.2 Strategy 2

Overview: The flanking regions of LO28 inlA and the 5' and the 3' end of the mutated EGD inlA are amplified. With two overlap extension PCRs two fragments containing the downstream region and the 5' end of inlA or the upstream region and the 3' end of inlA can be generated. The fragments are Topo-cloned to get one large fragment, which is further cloned into pMAD. Via homolgous recombination the EGD inlA (S192N, Y369S) is inserted into the LO28 inlA knock-out genome.

Four independent PCRs have to be accomplished. PCR1 and PCR2 amplify the downstream and upstream region of LO28 *inlA* (see strategy 1). PCR3 and PCR4 amplify the 5' end and the 3' end of EGD *inlA* containing the point mutations. For PCR3 primer b (InlA_for_lig) with a complementary region to the 3' end of the downstream fragment (a') was used as forward primer. The reverse primer (InlA_AflII_rev) has an NcoI restriction enzyme site and binds to a region in EGD *inlA* containing an AflII restriction enzyme site.
For PCR4 the complementary version without an NcoI restriction enzyme site of this primer has to be used as forward primer (InlA_AflII_for) and a primer with a complementary region to the 5' end of the upstream fragment (d') has to be used as reverse primer (InlA_rev_lig).
Having the four fragments, two overlap extension PCRs can be accomplished. One to get the downstream region/InlA 5' fragment (#1) and another one to get the InlA 3'/upstream region fragment (#2).
Then fragment #1 has to be Topo-cloned into the vector pGEM and can be sequenced to ensure that there are no other point mutations, except S192N and Y369S. The vector containing the correct insert

and fragment #2 have to be digested with the restriction enzymes AflII and NcoI and fragment #2 can be cloned into pGEM and again sequenced.

When the whole fragment, consisting of the downstream region of LO28 *inlA*, EGD *inlA* S192N and Y369S and the upstream region of LO28 *inlA*, is generated, pGEM and pMAD have to be digested with the restriction enzymes SalI and NcoI and the fragment can be cloned into pMAD.

The rest of the procedure is described in chapter 5.1. Figure 14 shows the possibilities of integration and reversion of pMAD containing EGD *inlA* S192N, Y369S into the *L. monocytogenes inlA* knock-out genome.

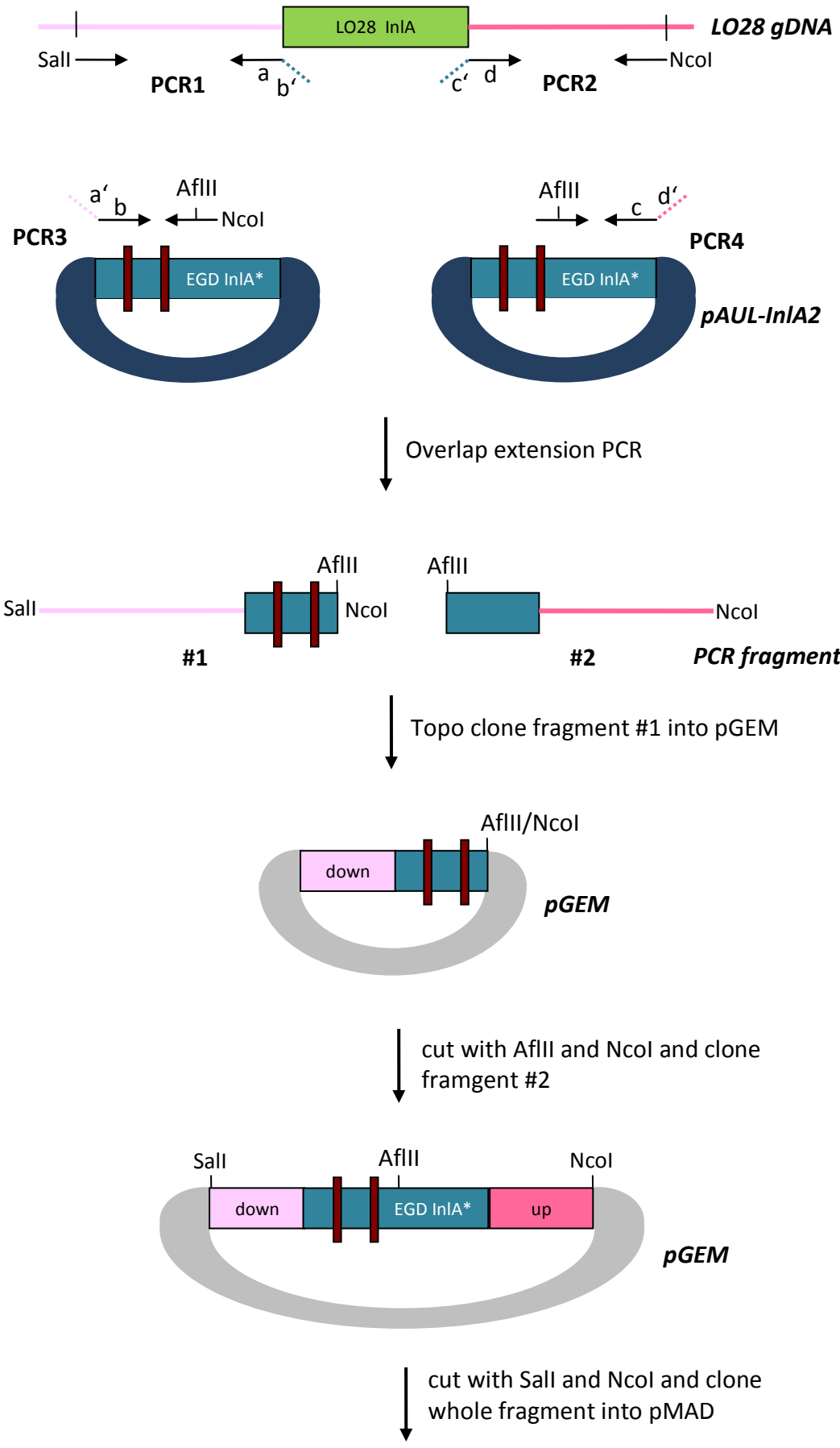

LO28 InlA
LO28 gDNA
SalI
NcoI
PCR1
PCR2
a
b'
c'
d
a'
b
AflII
NcoI
PCR3
EGD InlA*
AflII
c
d'
PCR4
EGD InlA*
pAUL-InlA2
Overlap extension PCR
AflII
AflII
SalI
NcoI
NcoI
#1
#2
PCR fragments
Topo clone fragment #1 into pGEM
AflII/NcoI
down
pGEM
cut with AflII and NcoI and clone framgent #2
SalI
AflII
NcoI
down
EGD InlA*
up
pGEM
cut with SalI and NcoI and clone whole fragment into pMAD

Figure 14: *Overlap extension PCR and pGEM/Topo cloning.* Overlap extension PCR creating two PCR fragments (#1 and #2). Fragment #1 is Topo-cloned into the vector pGEM and then digested with AflII and NcoI restriction enzymes. The second fragment is additionally cloned into the vector resulting in the full length EGD *inlA* S192N, Y369S with the flanking downstream and upstream region. After digestion with SalI and NcoI it can be cloned into pMAD and integrated into the *Listeria* LO28 genome.

5.2.3 Strategy 3

Overview: Two PCRs, amplifying the downstream region and the 5' end of LO28 inlA or the upstream region and the 3' end of LO28 inlA, are carried out. The two fragments are ligated, cloned into pMAD and a partial inlA knock-out is generated in the LO28 genome via homologous recombination. Through a second homologous recombination the mutated EGD inlA, located in the pAUL-InlA2 vector, is inserted in the partial inlA k/o Listeria LO28 genome.

PCR1 amplifies the downstream fragment and the 5' end of LO28. The forward primer (InlAdownfor_NcoI or down190NcoI) contains an NcoI restriction enzyme site and the reverse primer (InlA_2_rev), binding to LO28 *inlA*, contains a BamHI restriction enzyme site.
PCR2 amplifies the 3' end of LO28 *inlA* and the upstream fragment. The primer (InlA_3_for) binding to LO28 *inlA* has a BamHI restriction enzyme site and the reverse primer (InlAuprev_SalI or up224_SalI), binding to the upstream fragment, contains a SalI restriction enzyme site.
After digestion with NcoI/BamHI or BamHI/SalI the two fragments can be ligated resulting in a fragment containing only the 5' and the 3' end of LO28 *inlA* and the flanking regions (Figure 15).

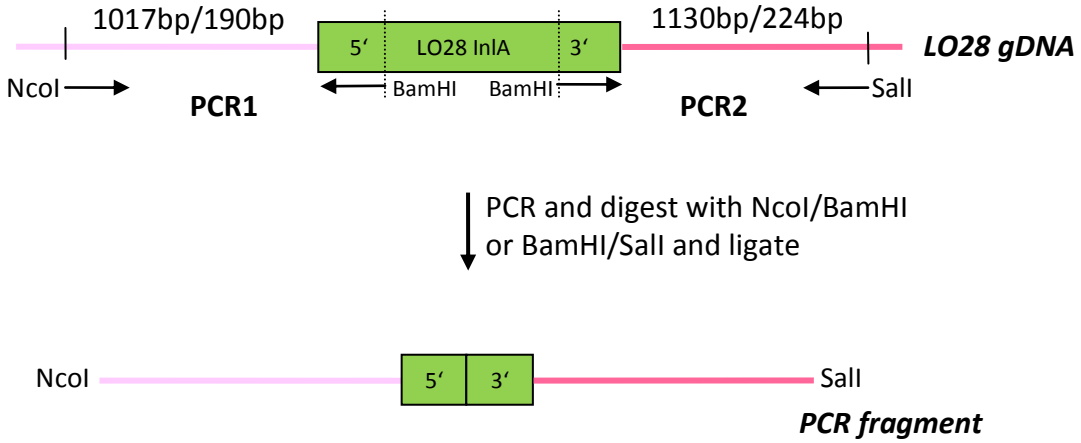


Figure 15: *PCR and ligation*. Forward and reverse primers, binding to LO28 *inlA*, contain a BamHI restriction enzyme site. The PCR fragments are digested and ligated.

The PCR fragment can be cloned into the temperature-sensitive shuttle vector pMAD via NcoI and SalI. pMAD is transformed into *L. monocytogenes* LO28 and can integrate into the genome through homologous recombination. This event can take place via the downstream region/5’ end of *inlA* or via the 3’ end of *inlA*/upstream region. After reversion of the plasmid integration, the partial *inlA* knock-out is generated. The middle part of *inlA* containing the amino acids S192 and Y369 and the nonsense mutation, which is responsible for the truncated version of *inlA* are deleted(Figure 16).

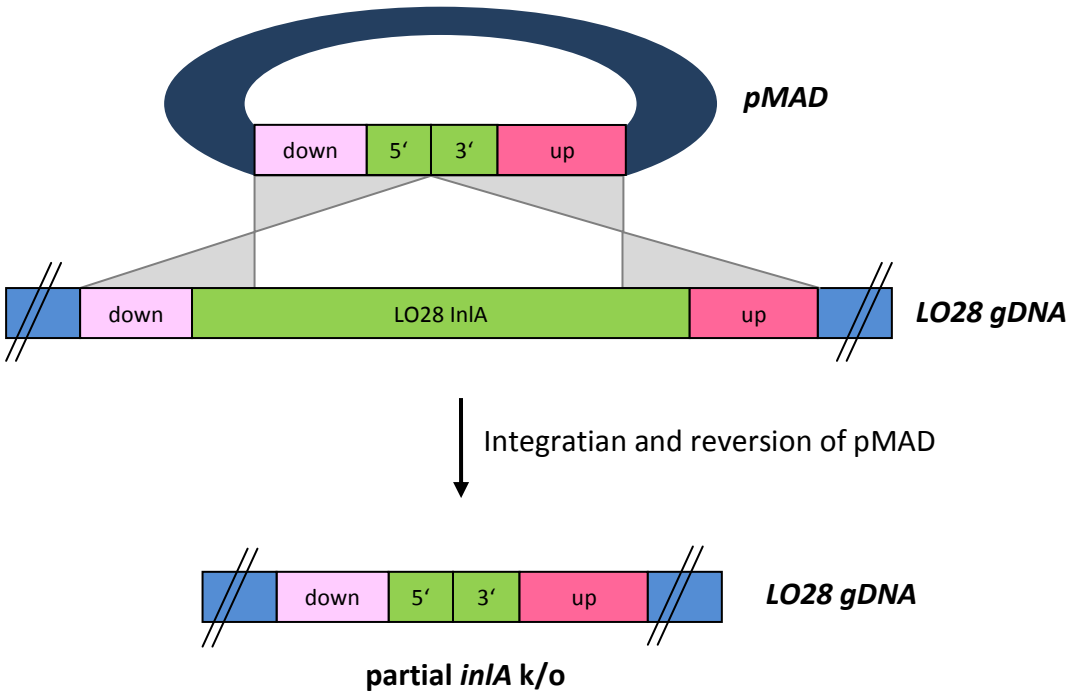


Figure 16: *Homologous recombination creating a partial inlA k/o in the Listeria LO28 genome.* Homologous recombination can take place via downstream/5’ region or via 3’/upstream region. After reversion of pMAD the partial *inlA* k/o in the *Listeria* LO28 genome is created.

Listeria LO28 with the partial *inlA* k/o are used for a second transformation. pAUL-InlA2, containing the EGD *inlA* S192N, Y369S, is introduced and through homolgous recombination the plasmid can integrate into the *Listeria* LO28 genome. The homologous recombination can take place via the 5’ or the 3’ end of *inlA*. Both strategies are shown in figure 17. The same is true for the reversion of plasmid integration, which can result in the initial situation (partial *inlA* k/o) or in the insertion of EGD *inlA* S192N, Y369S in the LO28 genome (Figure 17).

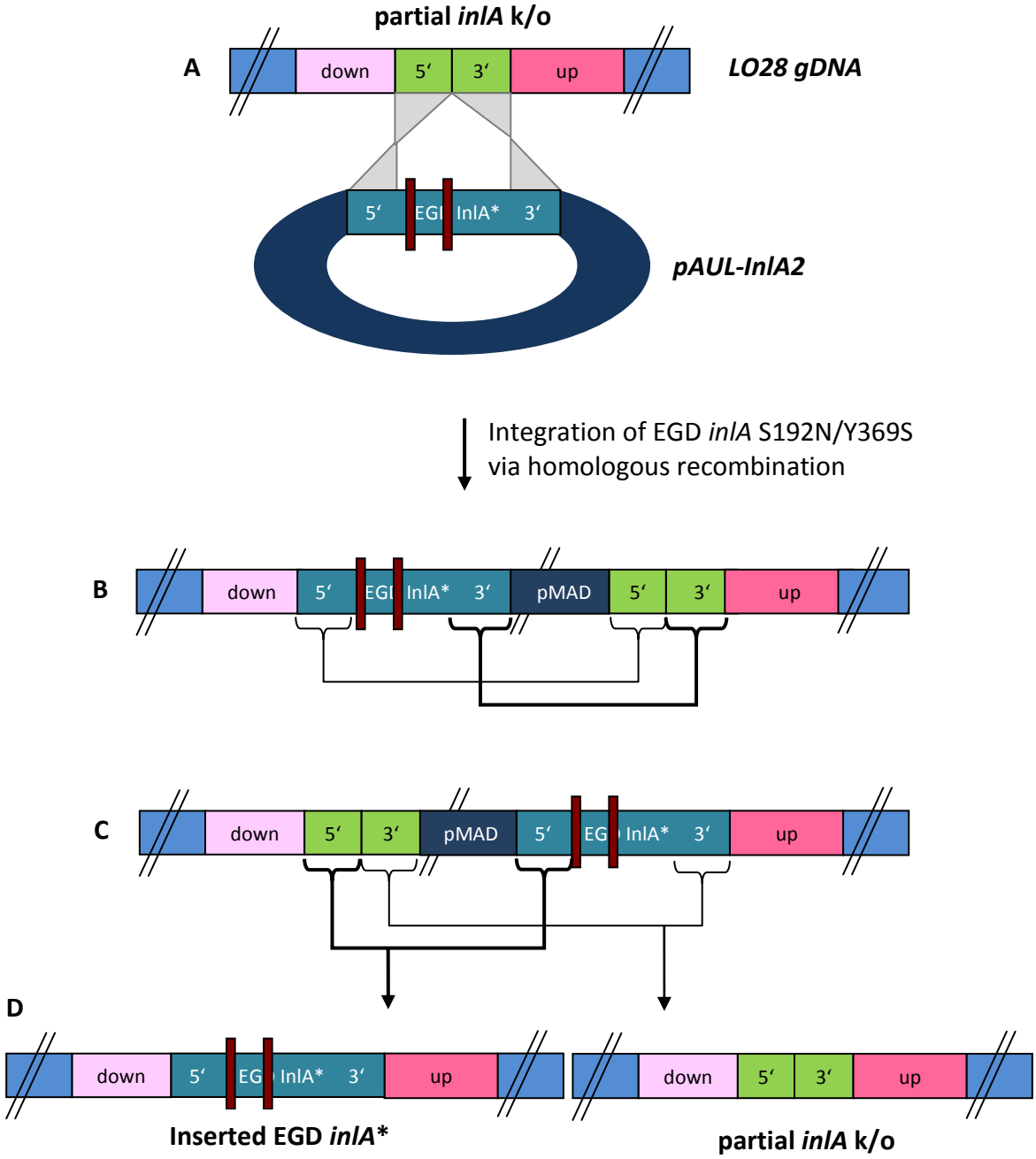


Figure 17: *Integration and reversion of pAUL-inlA2 containing EGD inlA* (S192N, Y369S).* **A**. Partial *inlA* knock-out in LO28 gDNA. **B**. Integration of pAUL-inlA2 with EGD *inlA** via 5’ end. **C**. Integration of pAUL-inlA2 with EGD *inlA** via 3’ end. **D**. Reversion of pAUL-inlA2 integration indicated with thicker lines resulting in inserted EGD *inlA** in the LO28 genome and with thinner lines resulting in the partial *inlA* knock-out situation.

5.2.4 Strategy 4

Overview: Two PCRs, amplifying the downstream region and the 5' end of LO28 inlA or the upstream region and the 3' end of LO28 inlA, are carried out. These two fragments are used for an overlap extension PCR, cloned into pMAD and a partial inlA knock-out is generated in the LO28 genome via homologous recombination. Through a second homologous recombination the mutated EGD inlA, located in the pAUL-InlA2 vector, is inserted in the partial inlA k/o Listeria LO28 genome.

Strategy 4 is the same as strategy 3 except the initial step. Instead of primers containing a BamHI restriction enzyme site, primers with an appendix have to be designed. Primer a (Start_lig_rev) has a complementary region to the beginning of the 3' end of *inlA* (b'), whereas primer b (Stop_lig_for) has a complementary region to the end of the 5' end of *inlA* (a').With the amplified fragments, an overlap extension PCR can be accomplished, creating a partial *inlA* k/o, which consists of the downstream region, the 5' and the 3' end of LO28 *inlA* and the upstream region (Figure 18). For the rest of the procedure see strategy 3.

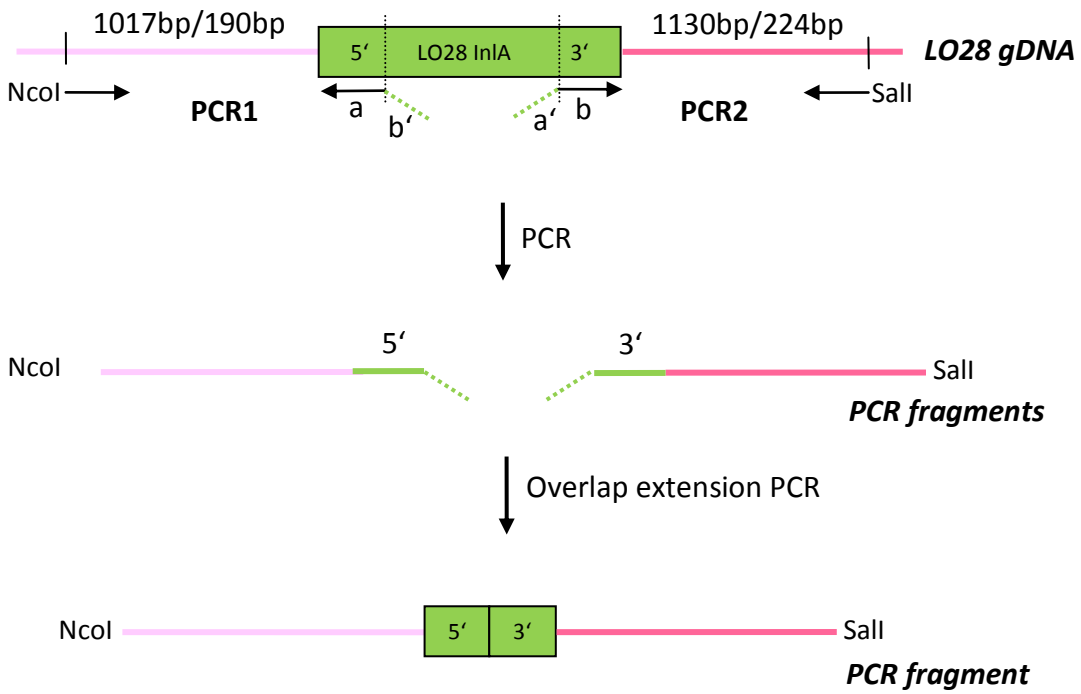


Figure 18: *Overlap extension PCR* to create the partial *inlA* knock-out, which can be used for the integration of EGD *inlA* S192N, Y369S via homologous recombination.

5.2.5 Strategy 5

Overview: The flanking regions of LO28 inlA and the mutated EGD inlA (S192N, Y369S) are amplified. The three fragments are ligated, cloned into pMAD and via homolgous recombination the EGD inlA is inserted into the LO28 inlA knock-out genome

3 PCRs have to be accomplished. PCR1 amplifies the downstream region. The forward primer (InlAdownfor_NcoI or down190_NcoI) contains an NcoI restriction enzyme site and the reverse primer (a, down_rev) binds exactly before the start codon of LO28 *inlA*. PCR2 amplifies the upstream region. The forward primer (d, up_for) binds directly after the stop codon of LO28 *inlA* and the reverse primer

(InlAuprev_SalI or up224_SalI) contains a SalI restriction enzyme site.

PCR3 is for the amplification of EGD *inlA* S192N, Y369S (primer pair: InlA_for and InlA_rev). With these three fragments T4 ligations can be accomplished. There are several options of ligation but after NcoI and SalI digestion and agarose gel electrophoresis only the variant with the correct arrangement of the fragments also has the correct size (Figure 19).

For the rest of the procedure see strategy 1.

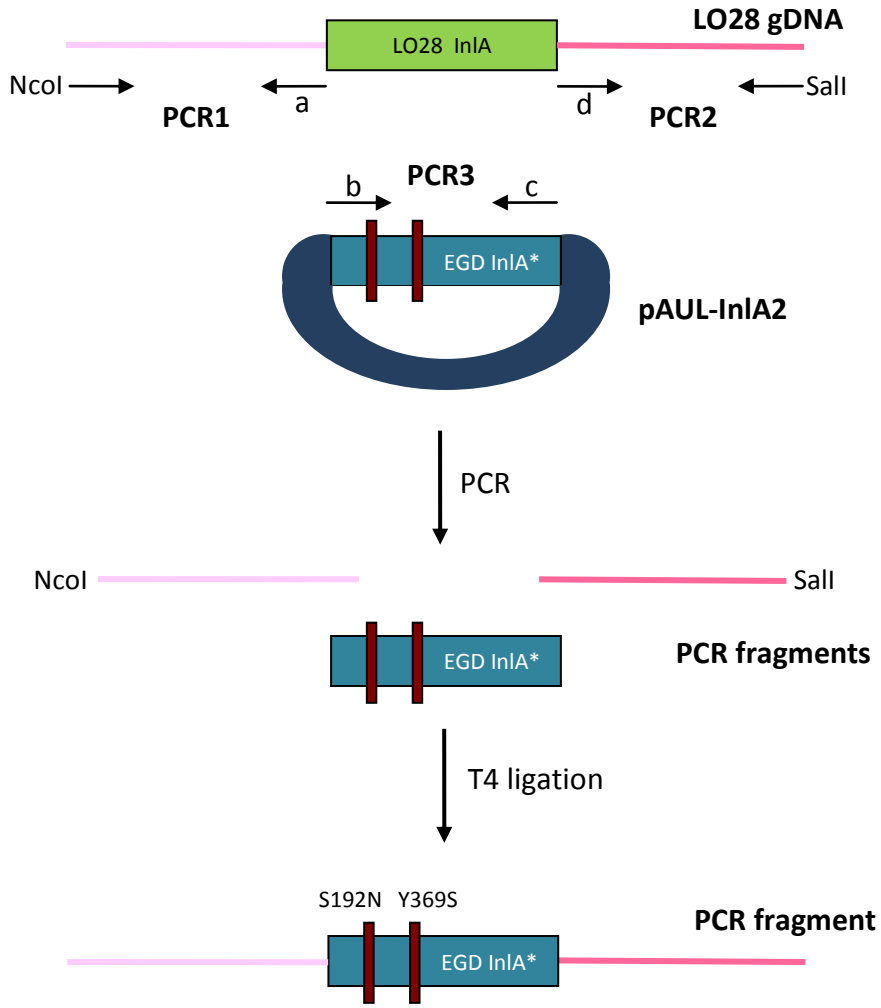
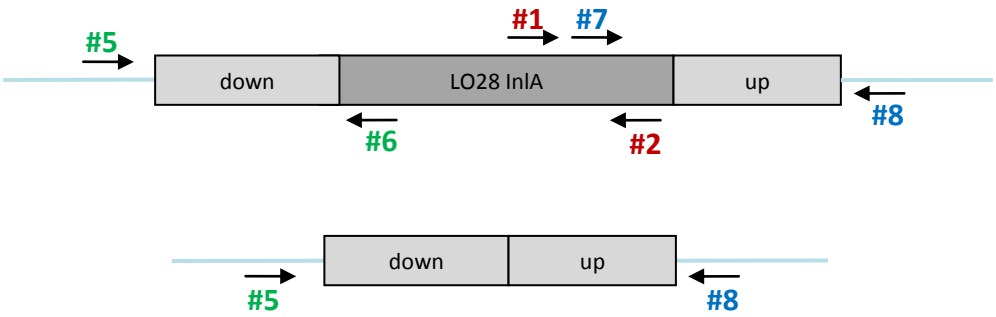


Figure 19: *PCR and T4 ligation*. The downstream and upstream fragment of LO28 *inlA* and the EGD *inlA* S192N, Y369S are amplified and used for T4 ligation.

5.3 *InlA* knock-out in *L. monocytogenes* LO28

For analyzing the *inlA* knock-out in *L. monocytogenes* LO28, several PCRs were accomplished. Figure 20 shows the agarose gelelektrophoreses of the PCRs with primer pairs, where at least one primer binds to the LO28 locus if present. For the *inlA* k/o no band could be observed, using the primer pairs #1/#2, #5/#6 and #7/#8. The wildtype sample shows the bands with the correct size (see table 1). The water control shows no DNA. In the scheme above the binding sites of the primers are indicated.



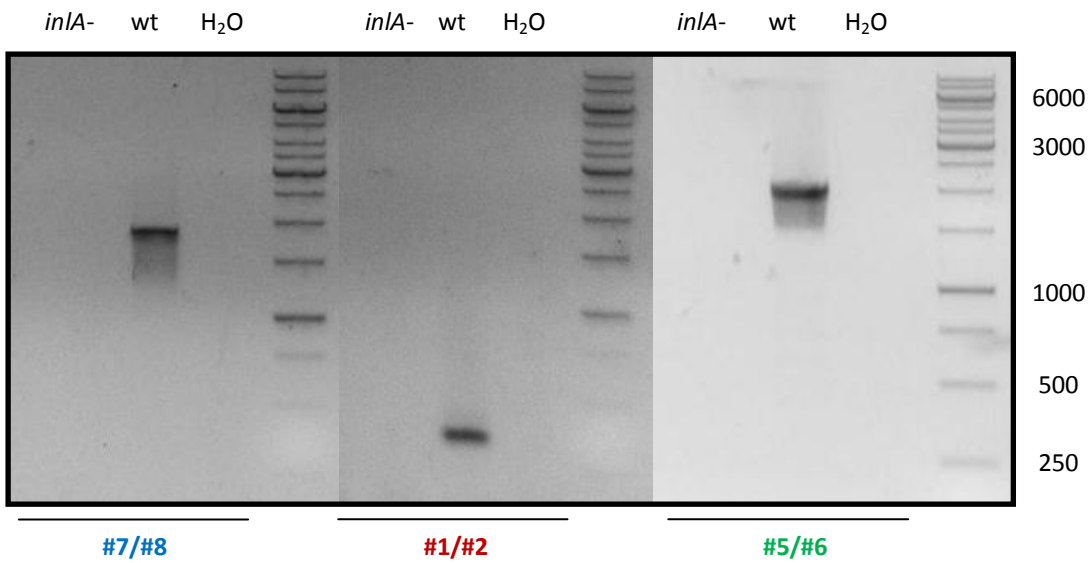


Figure 20: *Control of inlA k/o.* Agarose gel electrophoresis of PCRs for controlling the *inlA* k/o is shown. The scheme above shows the binding sites for the primer. The following primer pairs have been used #7/#8, #1/#2 and #5/#6. *inlA-*, internalin A knock-out; wt, wildtype.

Figure 21 shows the primer pairs binding outside the *inlA* locus of *Listeria* LO28 and one primer pair binding to the shuttle vector pMAD, to anlayze the reversion of the plasmid.

Using the primer pairs #3/#4 and #5/#6 results in a DNA fragment in the *inlA* k/o, as well as in the wildtype situation. The amplified fragments of the *inlA* k/o are 2403bp (length of LO28 InlA) smaller than the fragments of the wildtype.

Primer pair #9/#10 binds to the vector pMAD, which has been used for the transformation of *L. monocytogenes* LO28. In the *inlA* k/o no DNA could be observed, asserting that the reversion of the plasmid has happened. The PCR conditions and the length of the PCR fragments are shown in table 1.

For the water controls no DNA could be observed.

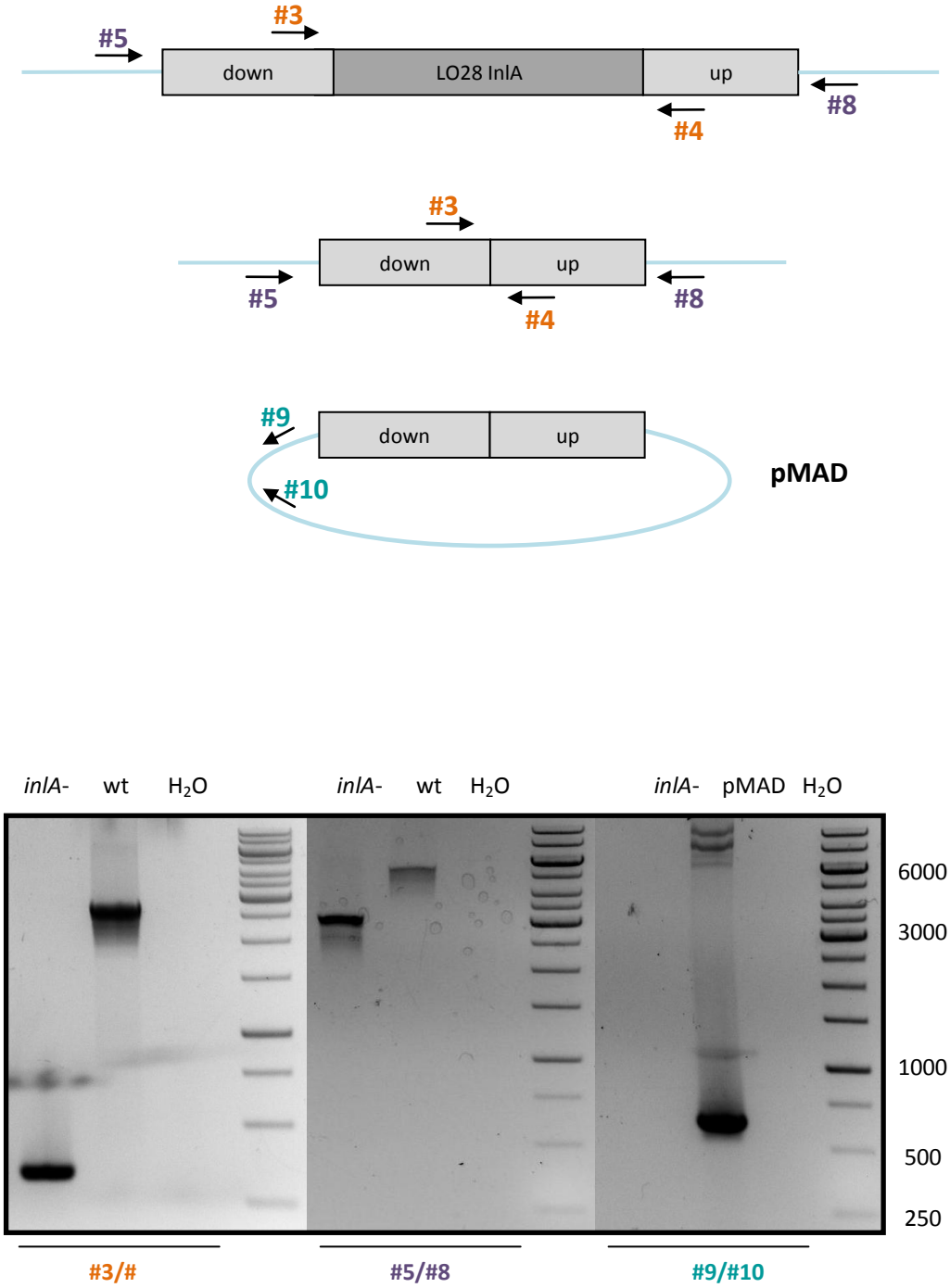


Figure 21: *Control of inlA k/o.* Agarose gel electrophoresis of PCRs for controlling the *inlA* k/o are shown. The scheme above shows the binding sites for the primer. The following primer pairs have been used #3/#4, #5/#8 and #9/#10. *inlA*-, internalin A knock-out; wt, wildtype.

Oligos	gene	Ta (°C)	extension time (sec)	LO28 wt	LO28 *ΔinlA*	pMAD
#1/#2	InlA	57	30	326	-	-
#3/#4	InlA	54	120	2736	337	-
#5/#6	down	54	120	2043	-	-
#7/#8	up	54	60	1880	-	-
#9/#10	pMAD	57	60	-	-	630
#5/#8	locus	54	330	5457	3053	-

Table 1: Primerpairs with binding sites, annealing temperature (Ta), extension time and length of PCR fragment are listed.

5.4 Insertion of EGDe *inlA* into *L. monocytogenes* LO28

The insertion of the *Listeria monocytogenes* EGD *inlA* S192N, Y369S could not be accomplished so far.
Strategy 3, 4 and 5 failed, for strategy 1 and 2 the initial PCRs were done.

5.5 The role of TBK, p38 MAPK, IKKβ and JNK in IFN-β induction and death of macrophages upon infection with *Listeria monocytogenes*

The effect of pharmacological inhibition of TBK1/IKKε, p38 MAPK, IKKβ and JNK on IFN-β induction was analyzed by real-time PCR. Bone marrow–derived macrophages (BMDM) were treated with the respective inhibitors 1 hour before infection and during the infection. The cells were infected with *L. monocytogenes* LO28 with a multiplicity of infection (MOI) of 10 and cell extracts were taken 4, 6 and 8 hours after infection.

After TBK1/IKKε inhibition no IFN-β could be observed. When using the p38 inhibitor we monitored an about 4-fold decrease of IFN-β induction at the 4h timepoint (see Figure 22), but looking at later timepoints (see Figure 24), the IFN-β induction increased and reached a higher level than non-treated BMDM at 8 hours.

The IKKβ inhibitor slightly decreased IFN-β induction and after JNK inhibiton the IFN-β induction was strongly decreased, comparable to TBK1/IKKε inhibition (see Figure 22).

Combination of two of the inhibitors always resulted in the reduction of IFN-β to the level achieved by the inhibitor with the stronger effect (see Figure 23). This leads us to the conclusion that combination of inhibitors do not show a syngergistic effect.

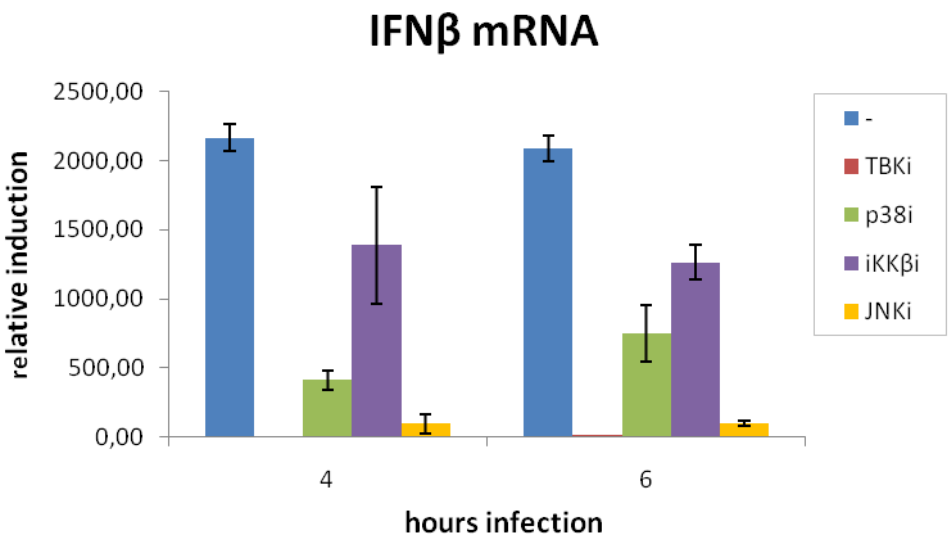


Figure 22: *Relative IFN-β induction* by infection of BMDM with *Listeria monocytogenes* LO28 following treatment with TBK1/IKKε inhibitor, p38 MAPK inhibitor, IKKβ inhibitor and JNK inhibitor.

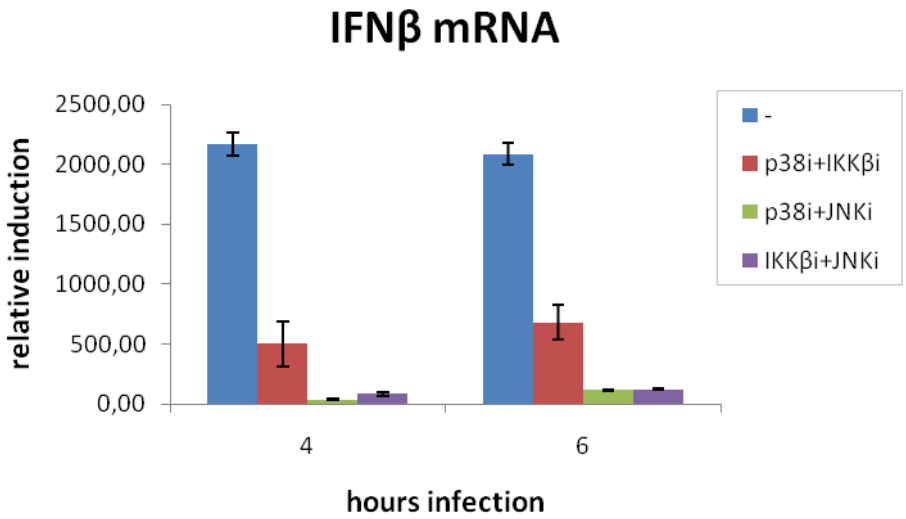


Figure 23: *Relative IFN-β induction* by infection of BMDM with *Listeria monocytogenes* LO28 following treatment with p38 MAPK+IKKβ inhibitor, p38 MAPK+JNK inhibitor and IKKβ+JNK inhibitor.

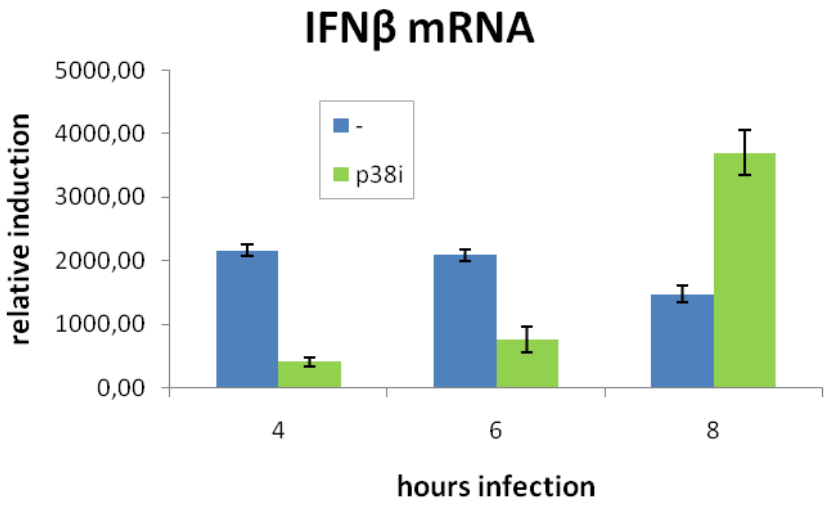


Figure 24: *Relative IFN-β induction* by infection of BMDM with *Listeria monocytogenes* LO28 following treatment with p38 MAPK inhibitor.

The cell viability of BMDM, treated with inhibitors 1 hour before and during infection, was evaluated by Flow Cytometry. The cells were infected with *L. monocytogenes* LO28 and after 24 hours the cells were stained with propidium iodide (PI), a fluorescent molecule able to intercalate into the DNA of dead cells.

Figure 25 shows the percent of cell death after the different treatments and *Listeria* LO28 infection for 24 hours. It can be seen that after TBK1/IKKε inhibition the rate of dead cells is slightly increased. After p38 MAPK and JNK inhibition cell death is reduced by half compared to control cells.

The IKKβ inhibitor could not be used for this experiment because after incubation longer than 10 hours all BMDM died, when the inhibitor was used in the recommended concentration of 6μM. Sublethal concentrations of 1,3-1,5μM failed to block IκBα degradation (see Figure 26).

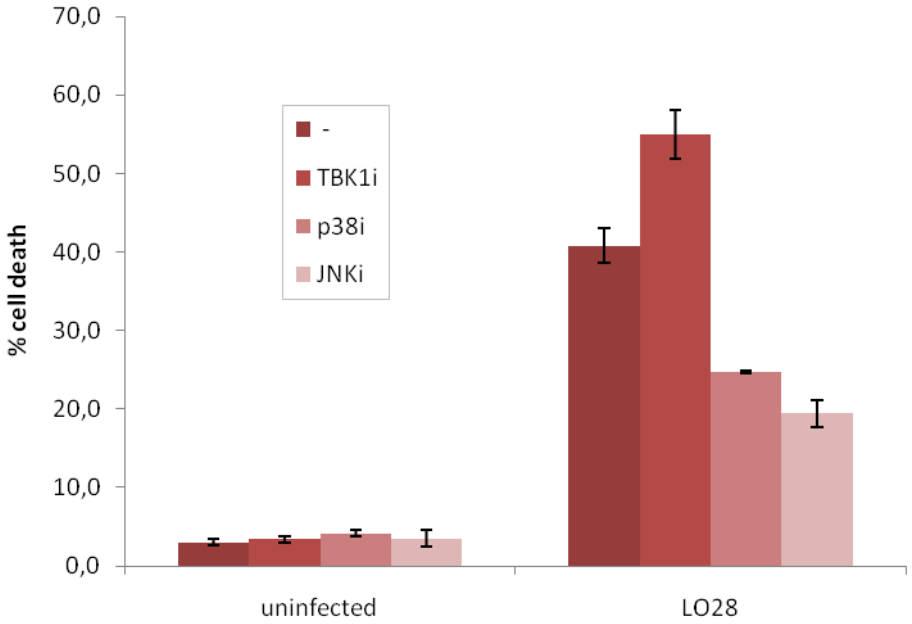


Figure 25: *Cell death analysis* of *L. monocytogenes* LO28-infected BMDM after treatment with TBK1/IKKε inhibitor, p38 MAPK inhibitor and JNK inhibitor. PI staining was performed 24h after infection.

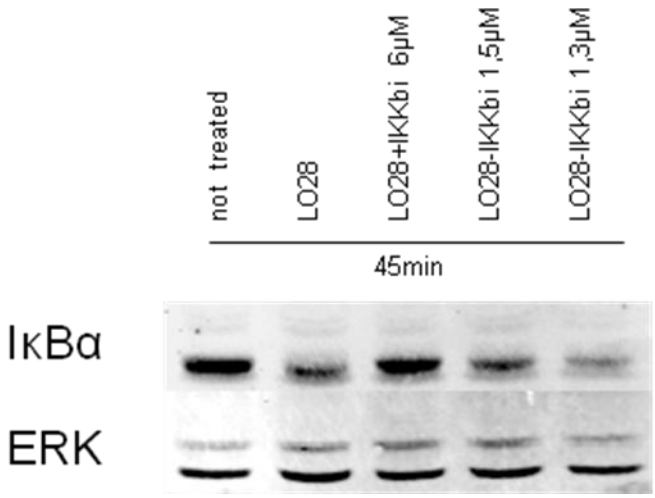


Figure 26: *Western blot analysis*. The IKKβ inhibitor was used at the recommended concentration of 6µM and at lower concentrations of 1,5µM and 1,3µM in *Listeria* infected BMDM. IκBα degradation could be observed at the lower concentrations of the inhibitor. The protein extracts were taken 45min after infection and ERK was used as loading control.

To ascertain that the slightly increased cell death after TBK1/IKKε inhibition and *Listeria* LO28 infection did not result from residual endogenous IFN-β and type I IFN signaling, we determined the tyrosin phosphorylation of Stat1. TBK1/IKKε inhibition completely abrogated Stat1 tyrosine phosphorylation following *Listeria* LO28 infection, indicating that there is no residual IFN-β induction (see Figure 27).

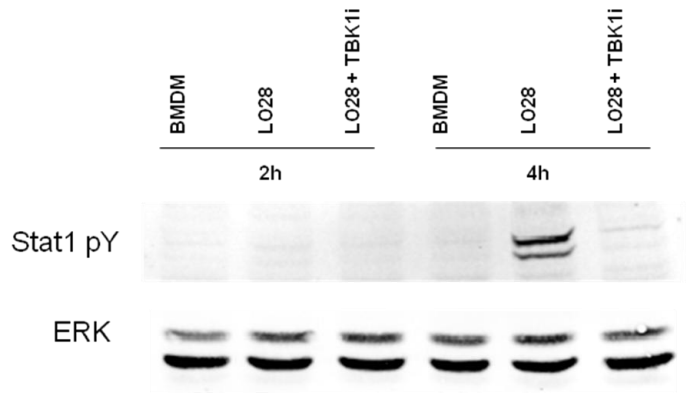


Figure 27: *Western Blot analysis*. Stat1 tyrosine phosphorylation after TBK1/IKKε inhibitor treatment and *Listeria* LO28 infection was examined. The cell extracts were taken 2h and 4h after infection and ERK was used as loading control.

The TBK1/IKKε inhibitor was shown to block p38 MAPK activation (Clark et al., 2009). To examine the role of TBK1 without affecting other pathways, knock-down experiments were performed. Compared to wildtype controls, TBK1 siRNA transfected into BMDM did not cause a difference in cell death after *L. monocytogenes* LO28 infection for 24 hours. The macrophages were infected with an MOI of 30 (Figure 28).

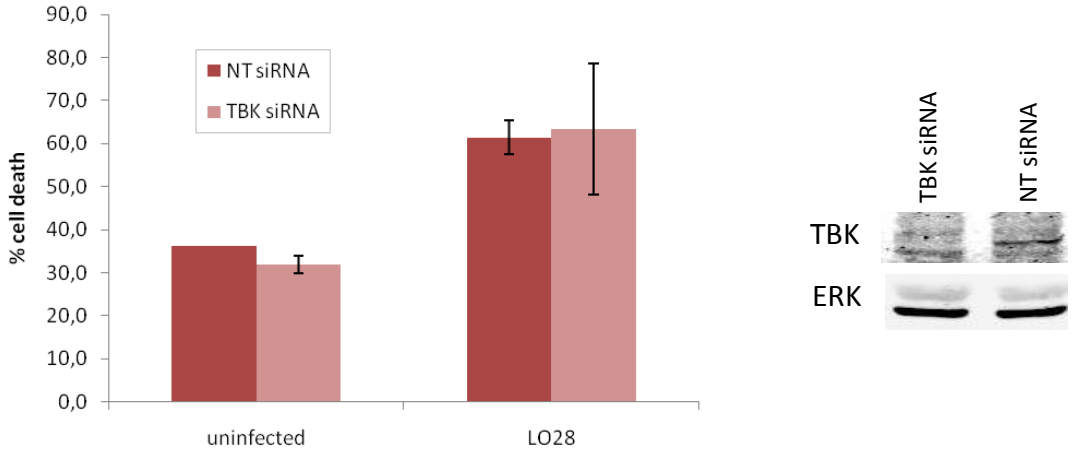


Figure 28: *Cell death analysis* of *L. monocytogenes* LO28 infected BMDM after TBK1 siRNA knock-down. PI staining was performed 24h after infection. The TBK1 knock-down efficiency was controlled by western blot analysis. ERK was used as loading control. NT siRNA, non target siRNA.

To obtain further genetic confirmation of the results with inhibitors, IKKβ deficient (IKKβ-/-) BMDM were used to investigate the viability of macrophages after *Listeria* LO28 infection. The spontaneous cell death was reduced to more than a half in IKKβ-/- BMDM, compared to wildtype cells, whereas the cell death rate after infection for 24h was only slightly decreased (see Figure 29).

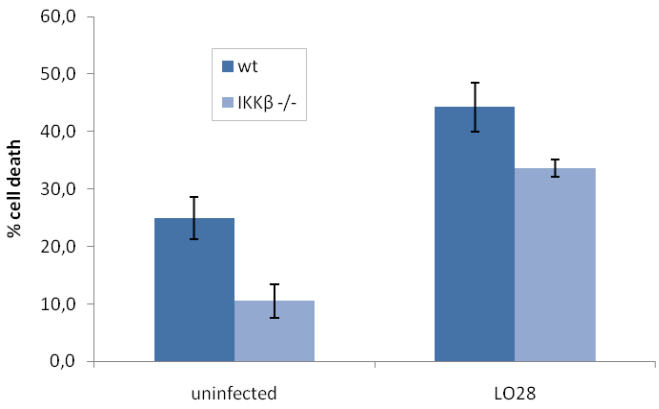


Figure 29: *Cell death* of *L. monocytogenes* LO28 infected IKKβ-/- and wildtype BMDM. PI staining was performed 24h after infection.

We also determined the induction of the IFN-β gene in IKKβ-/- macrophages. A difference to wt macrophages was observed at the later timepoint. 6 hours after Listeria infection, with an MOI of 10, the IFN-β induction in IKKβ-/- cells was slightly decreased (see Figure 30).

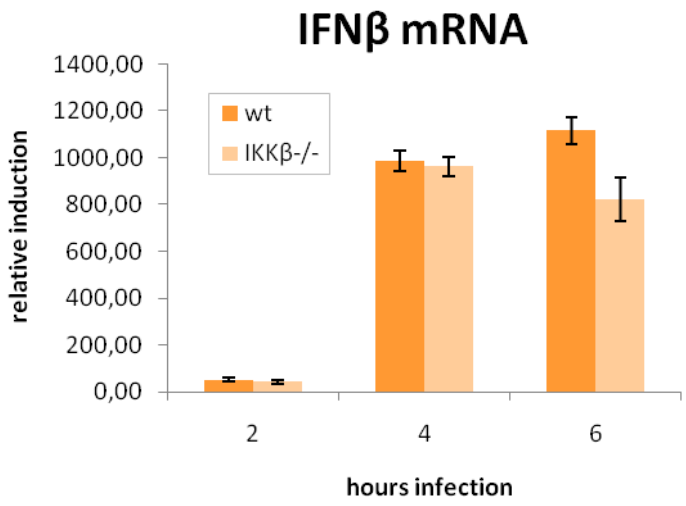


Figure 30: *Relative IFN-β induction* of wt and IKKβ-/- BMDM after *Listeria monocytogenes* LO28 infection.

To explain the different results obtained with the IKKβ inhibitor and the IKKβ-/- macrophages we examined whether the IKKβ inhibitor influences the phosphorylation of p38 MAPK, AKT, JNK and the tyrosin phosphorylation of Stat1. Western blot analysis revealed that the IKKβ inhibitor has no inhibitory function on either of these kinases. However, the tyrosin phosphorylation of Stat1 was decreased compared to the non treated LO28-infected cells, which could be due to the decreased IFN-β induction after IKKβ inhibitor treatment (Figure 31).

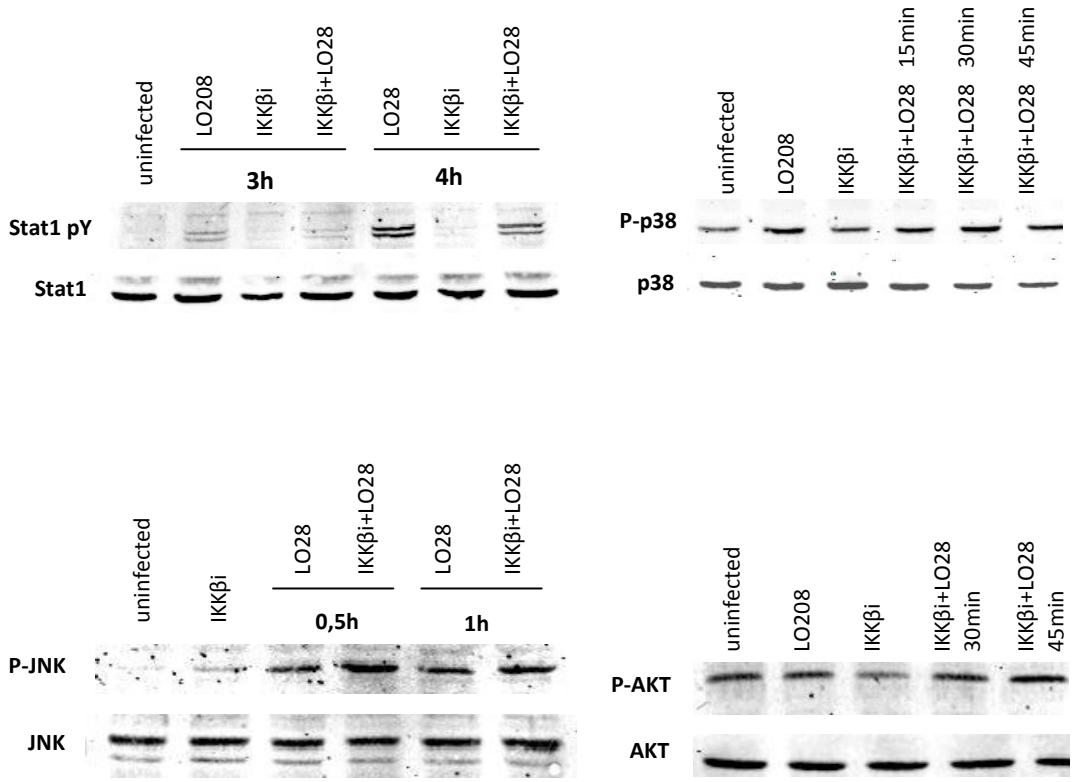


Figure 31: *Western blot analysis*. The influence of the IKKβ inhibitor on the phosphorylation of Stat1 at tyrosine 701, or the phosphorylation of p38 MAPK, JNK or AKT was investigated. The unphosphorylated proteins are shown as loading controls.

Since Listeria DNA was suggested to stimulate IFN-β gene transcription we investigated the IFN-β induction in RAW 264.7 macrophages and in BMDM after inhibitor treatment and poly(dA-dT) transfection. The cells were treated with the inhibitors 1 hour before and during the transfection and the cell extracts were taken 2 and 4 hours after transfection.

The effect of the inhibitors after poly(dA-dT) transfection in RAW 264.7 macrophages was comparable to the effect after *Listeria* LO28 infection in BMDM, with the exception of p38 MAPK inhibition

(compare Figure 22 and 32). 4 hours after poly(dA-dT) transfection, IFN-β induction was completely reduced.

Treatment with the TBK1/IKKε inhibitor and the JNK inhibitor showed a strong decrease in the IFN-β induction. At the 4 hour timepoint the p38 MAPK inhibitor had the strongest effect (see Figure 32).

Using two inhibitors in combination always showed a strong decrease in IFN-β induction in RAW 264.7 cells (see Figure 33).

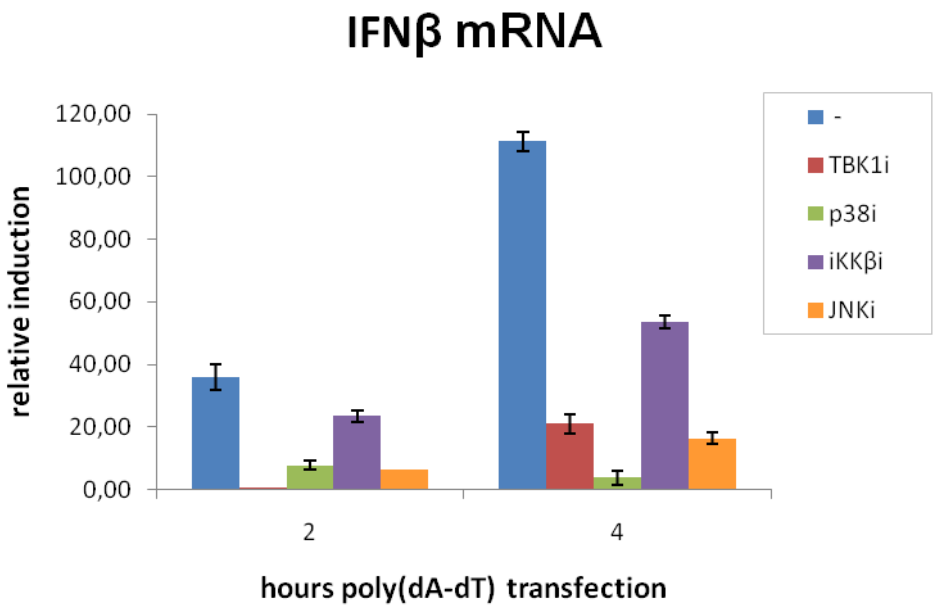


Figure 32: *Relative IFN-β induction* in RAW 264.7 macrophages after inhibitor treatment and poly(dA-dT) transfection. A TBK1/IKKε inhibitor, a p38 MAPK inhibitor, an IKKβ inhibitor and a JNK inhibitor were used. Cell extracts were taken 2 and 4 hours after transfection.

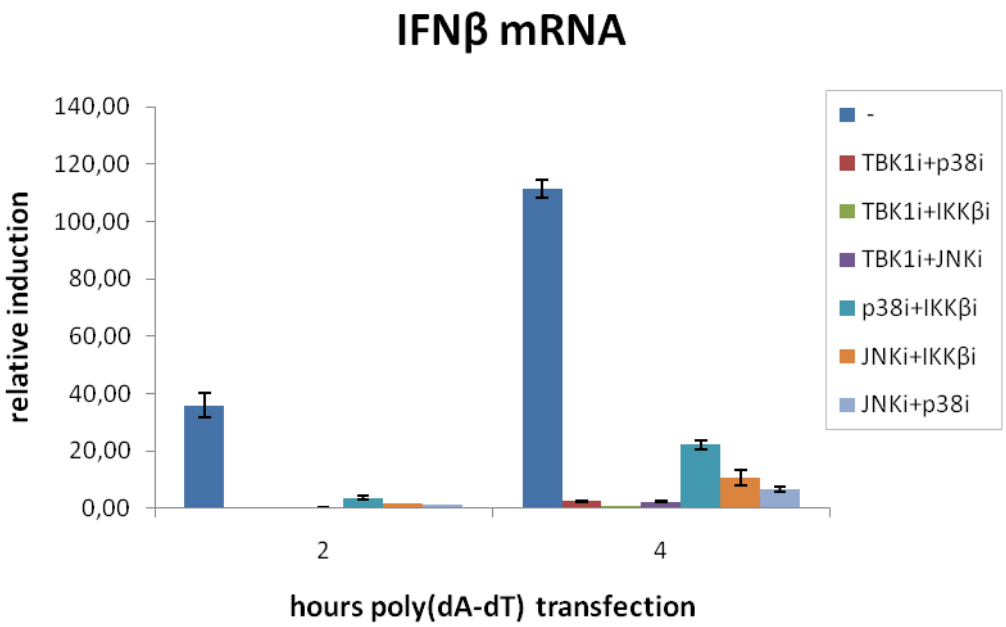


Figure 33: *Relative IFN-β induction* in RAW 264.7 macrophages after inhibitor treatment and poly(dA-dT) transfection. The inhibitors were used in combinations. Cell extracts were taken 2 and 4 hours after transfection.

In poly(dA-dT) transfected BMDM only the TBK1/IKKε inhibitor decreased IFN-β induction by about 50% . The p38 inhibitor and the IKKβ inhibitor had no effect on the IFN-β induction. Treatment with the JNK inhibitor resulted in an increase of IFN-β induction after poly(dA-dT) transfection, which is stronger at the 2 hour timepoint compared to the 4 hour timepoint (see Figure 34).

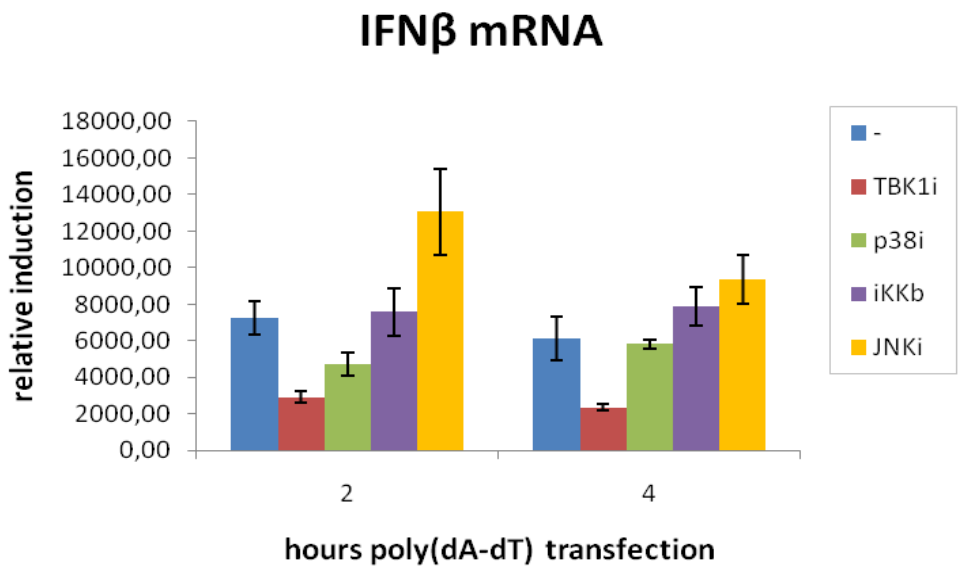


Figure 34: *Relative IFN-β induction* in BMDM after inhibitor treatment and poly(dA-dT) transfection. A TBK1/IKKε inhibitor, a p38 MAPK inhibitor, an IKKβ inhibitor and a JNK inhibitor were used. Cell extracts were taken 2 and 4 hours after transfection.

Overview of inhibitors and their effects

inhibitor	kinase	Inhibition of IFN-β induction			Inhibition of cell death
		BMDM LO28	BMDM poly(dA/dT)	RAW 264.7 poly(dA/dT)	BMDM LO28
BX795	TBK1/IKKε	+++	++	++	-
SB203580	p38 MAPK	++	+	+++	+
BIX02550	IKKβ	+	no effect	+	toxic
SP600125	JNK	+++	-	++	+

Table 2: *Overview of inhibitors and their effect on IFN-β induction and cell death.* LO28, infection with *L. monocytogenes* LO28; poly(dA-dT), transfection with poly(dA-dT); +++, completely inhibited; ++, strongly inhibited; +, slightly inhibited; -, enhanced

5.6 Intracellular *Listeria* growth and gene expression in Stat1 F77A macrophages

We investigated the intracellular growth of *Listeria monocytogenes* LO28 in wildtype and Stat1 F77A BMDM. To this end colony forming unit assays were performed. The cells were infected at an MOI of 7,5 and samples were taken at the indicated timepoints. For each genotype we also included samples, which were after pretreatment with IFN-γ (5ng/µl) for about 12 hours. IFN-γ remained present in the medium for the whole experiment.Comparing the two genotypes, we observed that the reduced growth of *Listeria* caused by IFN-γ treatment of wt BMDM was not observed in the Stat1 F77A BMDM. (see Figure 35).

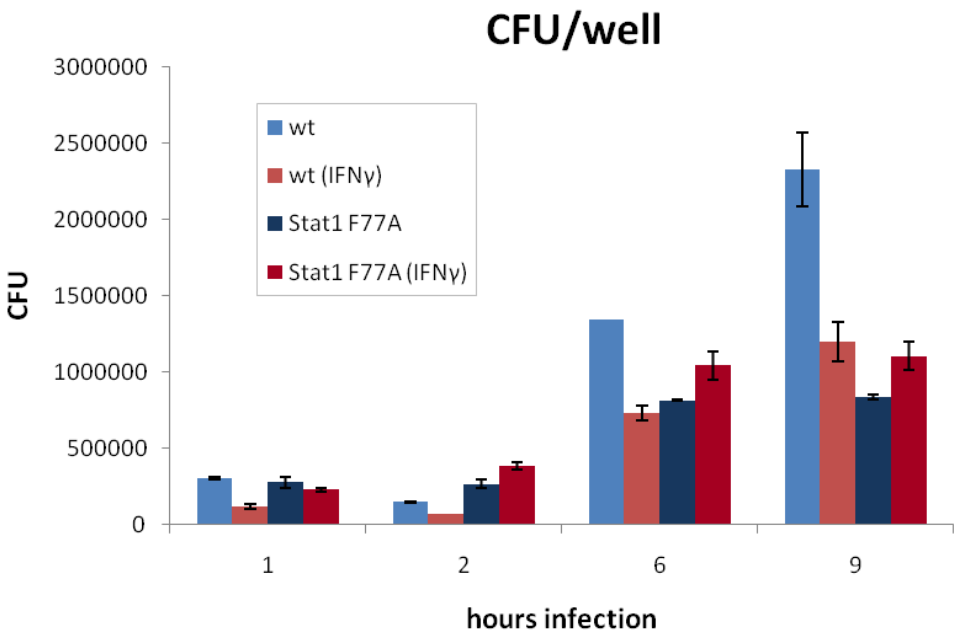


Figure 35: *CFU assay*. CFU of *Listeria monocytogenes* LO28 per well at the stated timepoints with- or without IFN-γ pretreatment..

We further investigated the expression of four different genes after *Listeria* LO28 infection or IFN-γ treatment in Stat1 F77A BMDM. The cells were infected at an MOI of 10 and cell extracts were taken 2, 4, 6 and 8 hours after infection. IRF1, Gbp2 and Tap1 induction was strongly reduced in Stat1 F77A BMDM compared to wildtype macrophages in either infected or IFN-γ treated cells (see Figure 36, 37).

The Mx2 expression was only analyzed after Listeria LO28 infection. Stat1 F77A BMDM showed a decreased induction of Mx2, especially 4 and 6 hours after infection (see Figure 36).

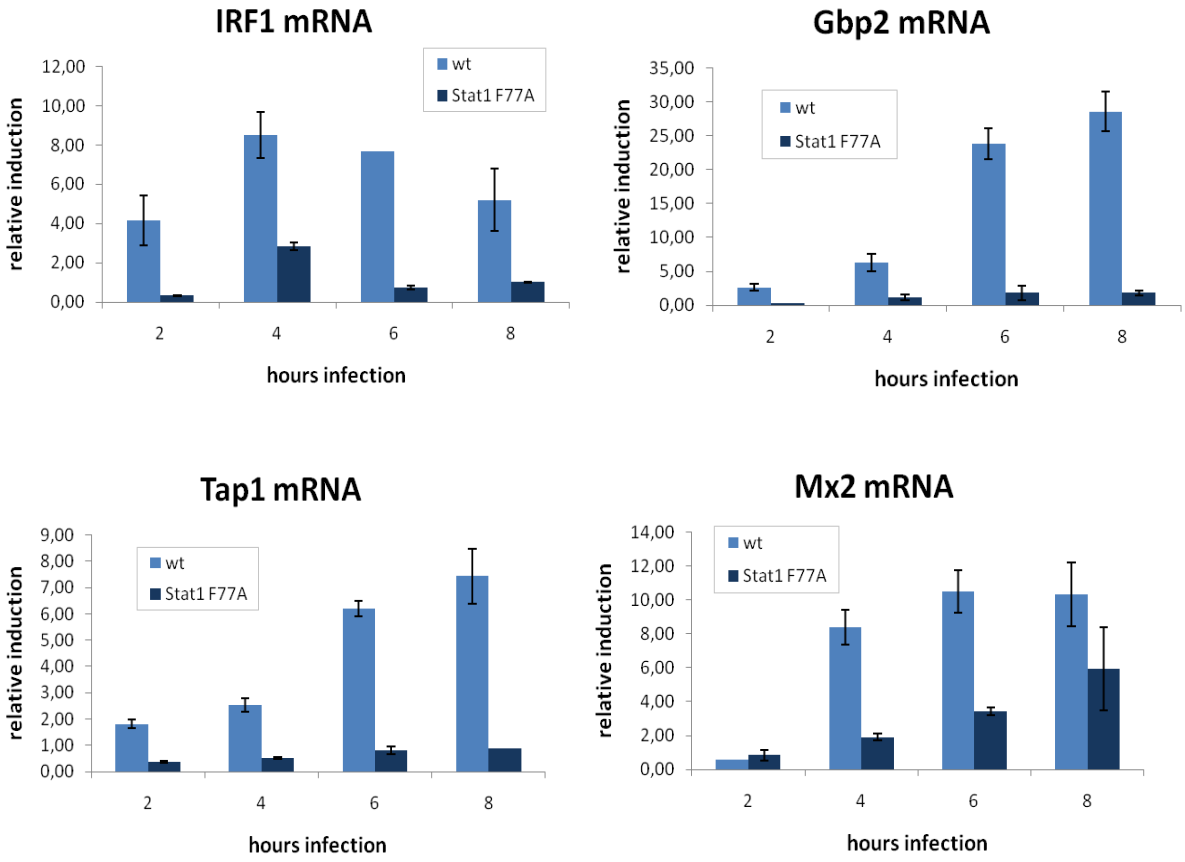


Figure 36: *Gene induction after L. monocytogenes LO28 infection.* Expression of IRF1, Gbp2, Tap1 and Mx2 genes is shown for wildtype and Stat1 F77A BMDM.

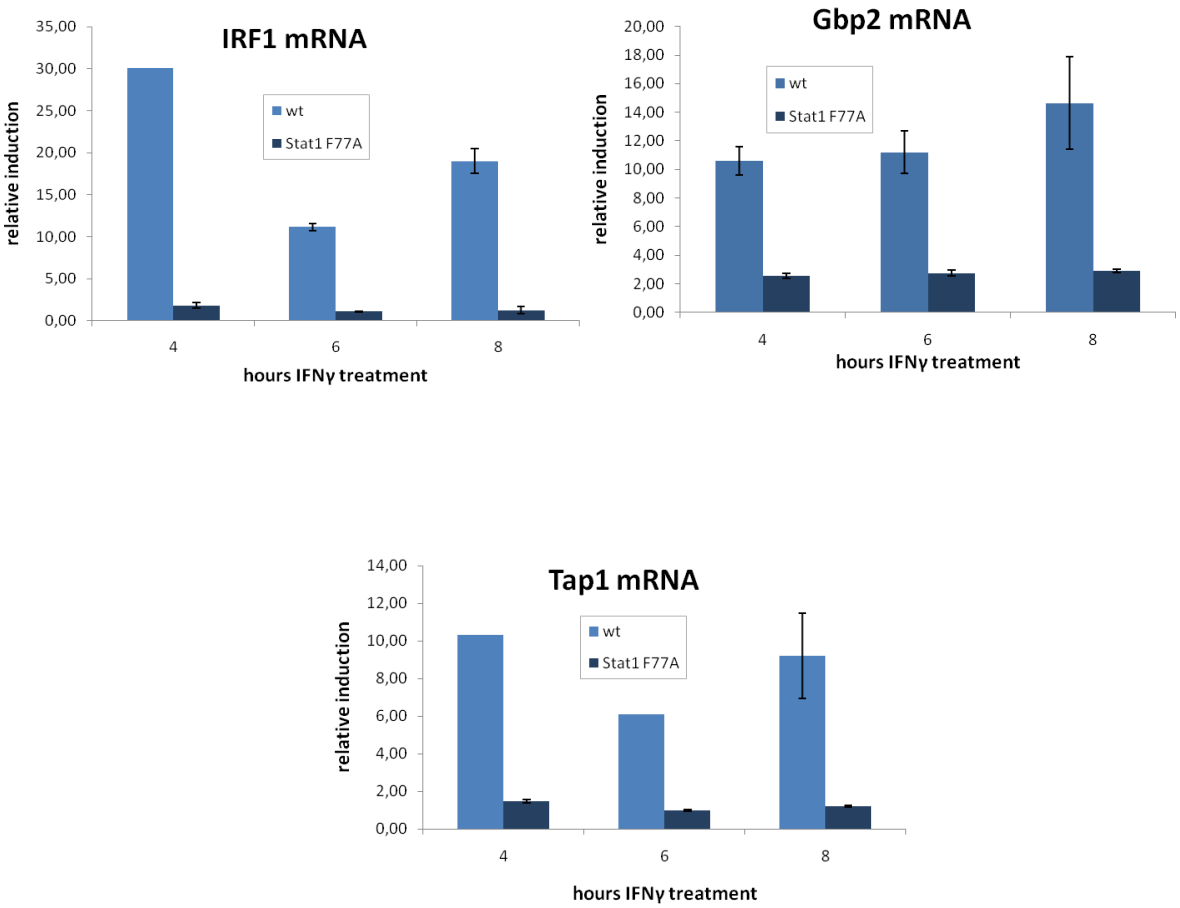


Figure 37: *Gene induction after IFN-γ treatment.* Expression of IRF1, Gbp2 and Tap1 genes is shown for wildtype and Stat1 F77A BMDM.

For cell death analysis, PI staining was performed. The cells were infected at an MOI of 30 and stained after 24 hours. We could not observe any significant difference in Stat1 F77A BMDM compared to wildtype cells (see Figure 38).

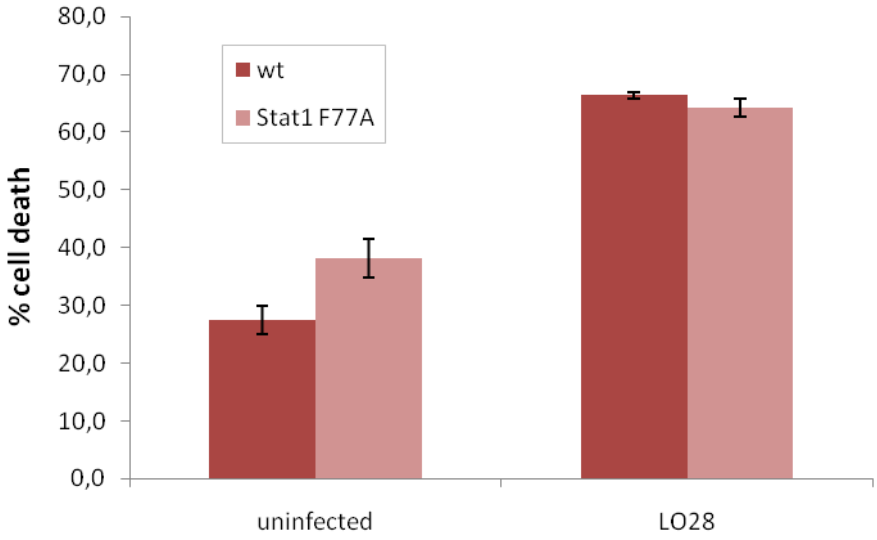


Figure 38: *Cell death* of *L. monocytogenes* LO28-infected wildtype and Stat1 F77A BMDM. PI staining was performed 24h after infection.

6 Discussion

Listeria monocytogenes, a gram-positive food-borne pathogen, causes severe diseases with high mortility rates in immunocompromised humans and infections of the fetus in pregnant women (Sleator et al., 2009). Therefore it is important to study the immune responses, caused by this microbe. The model organism used for infections with *Listeria* is the mouse. Due to the failure in the interaction of InternalinA, the protein important for the gastrointestinal uptake of *Listeria,* and its receptor E-cadherin in mice, it is difficult to investigate oral infections with moderate doses of the pathogen.
There are two strategies to avoid this problem. On the one hand a humanized mouse line was generated and on the other hand a murinized pathogen was engineered (Sleator et al., 2009; Wollert et al., 2007).

Our lab is mainly interested in the innate immune response to *Listeria monocytogenes.* Compared to other strains *Listeria* LO28 causes robust induction of the type I IFN genes (Reutterer et al., 2008). Therefore this strain is used for our experiments. The aim was to create murinized *Listeria* LO28, where two amino acids of InlA are substituted, making a productive interaction between the InlA and the E-cadherin of mice possible. Furthermore we aimed at exchanging the LO28 InlA with the EGDe version, which has a cell wall anchor motif in contrast to LO28 (Jonquières et al., 1998).

The InlA of *Listeria monocytogenes* LO28 could be knocked out via homologous recombination. The insertion of the mutated EGDe InlA was not successful so far.

We have developed five different strategies, but three of them failed. Strategy 3 and 5, as described in the results, failed due to the output of DNA after T4 ligation. Concerning strategy 4, the overlap extension PCR did not work, which could probably be resolved by using another, more potent proof-reading polymerase.
Recently, the overlap extension PCR of strategy 1 and strategy 2 was carried out succesfully, therefore the next step of cloning the fragment into the temperature-sensitive shuttle vector and the sequencing of the amplified PCR fragment can be performed.
The insertion of the mutated EGDe *inlA* into the *inlA* knock-out *Listeria* will progress further and numerous applications of the recombinant stains are on our list of future experiments.

Type I IFN plays a major role in the innate immune response to *Listeria monocytogenes*. Earlier studies revealed the structure of the virus-induced IFN-β enhancer, showing the assembly of several transcription factors on the DNA. The IFN-β enhanceosome is built of ATF-2/c-Jun, IRF3/IRF7 and NFκB (Panne et al., 2007). We wanted to investigate, *L. monocytogenes* LO28 infection employs all of these transcription factors to fully induce IFN-β in BMDM.
Using inhibitors against kinases, which are involved in the signaling after *Listeria* infection allowed us to suppress the activation of the above mentioned transcription factors. Our data show that TBK1/IKKε and JNK, leading to the activation of IRF3/IRF7 or c-Jun, respectively, are essential for IFN-β induction. Inhibition of these proteins resulted in total loss of IFN-β mRNA.
Inhibition of the p38 MAPK or IKKβ, playing a role in phosphorylation of ATF-2 or NFκB, respectively, did not show these dramatic effects. In case of IKKβ, the IFN-β induction after *Listeria* infection was only reduced by about 50 percent.

Additionally we examined the IFN-β induction of IKKβ-/- BMDM after *Listeria* infection. There was no difference in IFN-β induction compared to wildtype cells, except for the 6 hour timpoint, where a slight decrease in IFN-β induction was noted in IKKβ-/- cells.
The strongest effect on IFN-β induction after Listeria infection of IKKβ-/- BMDM, or in IKKβ inhibitor treated cells was always observed at the latest analysed timepoint.
Interestingly, the p38 MAPK inhibitor delayed IFN-β induction. For the early timepoints IFN-β is reduced by approximately 70%, but IFN-β induction after 8 hours of *Listeria* infection increased to more than 200% compared to the non treated macrophages.
To define the maximum of the IFN-β induction after p38 MAPK inhibition, experiments with later time points would be useful.
Our findings reveal that all transcription factors (c-Jun, ATF-2, IRF3, IRF7 and NFκB) are necessary for full IFN-β induction after *L. monocytogenes* LO28 infection, but that compared to IRF3/7 and ATF2 NFkB appears to be of minor importance.
Although IKKα does not seem to be critical for cytokine induced IκB degradation and subsequent NFκB activation, it functions in the nucleus to activate the expression of NFκB responsive genes through phosphorylation and subsequent acetylation of specific residues in histone H3 (Anest et al., 2003; Yamamoto et al., 2003). This activity may explain the difference between treating macrophages with an IKK inhibitor and the use of IKKb-/- macrophages.

To compare Listeria infection with the effects of its proposed PAMP for IFN-β stimulation, we transfected inhibitor treated RAW 264.7 macrophages and BMDM with poly(dA-dT), activating signaling exclusively via intracellular receptors. The cytosolic receptor *for L. monocytogenes*, which leads to IFN-β gene transcription has not been identified, but it is without controversy that IFN-β is induced via TBK1

and IRF3 (Perry et al., 2004; Stockinger et al., 2004). Concerning poly(dA-dT), it is possible that the DNA dependent RNA polymeraseIII converts poly(dA-dT) into 5'-ppp RNA leading to IFN-β induction via RIG-I (Chiu et al., 2009).

After inhibitor treatment and poly(dA-dT) transfection in RAW 264.7 macrophages, we saw that the TBK1/IKKε, p38 MAPK and the JNK inhibitor had a stronger effect compared to the IKKβ inhibitor treated cells, which is in agreement with our results after *Listeria* infection. Regarding the inhibitor treated, poly(dA-dT) transfected BMDM, the IFN-β induction was only reduced after TBK1/IKKε inhibitor treatment and after p38 MAPK inhibition at early timepoints. The IKKβ inhibitor had no effect on the IFN-β gene expression and the JNK inhibitor enhanced IFN-β induction. We do not have an explanation for the increased IFN-β induction after JNK inhibition, but the generally very weak effects could be due to a poor transfection rate. To draw any conclusions of the poly(dA-dT) transfection in BMDM, further investigations would be necessary, but in case of inhibitor treated, poly(dA-dT) transfected RAW 264.7 macrophages, the results are in line with the data of the inhibitor treated, *Listeria* infected BMDM.

To this date there are no published data about the IKKβ inhibitor, BIX02550 (kindly provided by Boehringer-Ingelheim) and its side effects. Therefore we investigated the effect of the inhibitor on the phosphorylation of several proteins, including p38 MAPK, JNK, AKT and Stat1. We could not observe an inhibition of the phosphorylation of any of these proteins, except for Stat1, where the phosphorylation was slightly decreased, which could be due to the reduced IFN-β induction after IKKβ inhibition.

We also investigated the cell death of *L. monocytogenes* LO28-infected BMDM after inhibitor treatment. NFκB and p38 MAPK play an important role in maintaining cell survival in macrophages. Published data showed that inhibition of one of these proteins leads to compromised survival of macrophages (Park et al., 2005).

Furthermore, a correlation between IFN-β induction and cell death after *L. monocytogenes* infection in macrophages has been demonstrated (Stockinger et al., 2002).

Surprisingly, we observed that after TBK1/IKKε inhibition, the percentage of dead macrophages after *Listeria* LO28 infection is slightly increased. We would have expected a higher viability of *Listeria* infected macrophages after TBK1/IKKε inhibition, due to the fact that IFN-β induction is recduced to a minimum.

In recent publications (Clark et al., 2009), a number of side effects elicited by the TBK1/IKKε inhibitor were investigated. To be able to study the contribution of TBK1 on the cell death of macrophages, we generated an siRNA knock-down of TBK1 in BMDM. This did not result in a significant effect on macrophage death, suggesting that the slightly increased cell death caused by the TBK1/IKKε inhibitor may be due to side effects of the chemical component (Clark et al., 2009).

Inhibition of p38 MAPK, reduced cell death of *Listeria* infected BMDM after 24 hours. This is in contrast to recently published data, suggesting that the p38 MAPK and IKKα/β, synergize to induce transcription of two major anti-apoptotic genes, *Bfl-1/AI* and *Pai-2* (Park et al., 2005). Our results would suggest that in case of *Listeria* infected macrophages p38 MAPK has a proapoptotic effect.

Cell survival assays in presence of the IKKβ inhibitor could not be performed, due to the fact that all macrophages, either infected or not, died within 12 hours.

Using the inhibitor in a lower concentration led to the survival of the cells, but drastically reduced the inhibitory function. Therefore we

used IKKβ-/- BMDM to investigate the cell death after *L. monocytogenes* infection. In infected IKKβ-/- macrophages, slightly more cells survived.
Cell death in uninfected IKKβ-/- cells was reduced compared to wildtype cells. Earlier studies showed that IKKβ-/- mice have a severe defect in NFκB activation (Li et al., 1999). This would suggest that NFκB plays a role in the activation of pro-apoptotic genes. To confirm these data, the experiment would need to be repeated. Due to the lack of cells, it was performed only once.
JNK is known to have a dual role in both apoptotic and anti-apoptotic signaling pathways, indicating that the function of JNK is complex (Lamb et al., 2003). In our experiments *Listeria* LO28 infection and treatment with a JNK inhibitor resulted in increased survival of macrophages compared to non-treated cells, leading us to the conclusion that in our approach the JNK pathway has a pro-apoptotic role.

Interferons lead to the activation of JAK-STAT signaling. Depending on the type of IFN Stat homo- or heterodimers are formed via phosphotyrosin-SH2 domain interaction (Decker et al., 2005). The activation of some genes also depends on the oligomerisation of Stat proteins, where the N-Terminus plays a crucial role. The Stat1 F77A mutant fails to form Stat1 tetramers, due to the mutation in the N-terminal region (Meyer et al., 2004; Vinkemeier et al., 2005).
In our studies we adredded the question wether this mutation influences the intracellular replication of *L. monocytogenes* LO28 in BMDM. Our results showed that the replication of *Listeria* in IFN-γ-treated Stat1 F77A BMDM is stronger compared to the replication in IFN-γ-treated wildtype BMDM up to 9 hours of infection. This shows that the mutation decreases the ability of IFN-γ to activate macrophages.

Consistent with this the induction of IFN-responsive genes by *Listeria* LO28 infection or after treatment with IFN-γ was strongly reduced in Stat1 F77A BMDM. In both cases we could see a strong decrease in IRF1, Gbp2 and Tap1 expression. IRF1 contains a single GAS but no ISRE in its promoter (Kimura et al., 1996). Our results thus indicate that Stat interactions, crucially involving the Stat1 N domain, are mandatory for full transcriptional activity of promoters containing only one GAS site. Published data showed that the ability to form Stat tetramers on DNA did not require multiple GAS sites (Meyer et al., 2004). Gbp2 and Tap1 have ISRE in their promoters in addition to GAS DNA (Min et al., 1998; Ramsauer et al., 2007). Because of the decreased induction of these genes, we wanted to determine, if the expression of Mx2, which is an ISRE driven gene (Asano et al., 2003), is also influenced by Stat1 F77A macrophages.
We observed that the induction of this gene was also significantly reduced 2 and 4 hours after infection with *Listeria* LO28.
RT-PCR analysis of IFN-α dependent target genes (ISG-15 and ISG-56k), demonstrated reduced transcriptional activity in Stat1 F77A expressing U3A cells (Meyer et al., 2004).
This leads us to the conclusion that there is not only a defect in Stat1 oligomerization, but that the mutation also influences the formation of ISGF3. An alternative explanation could be that the N-terminal domain also plays a role in the recruitment of transcriptional co-activators or the recruitment of interacting transcription factors.
In vitro studies showed that a defect in interaction of Stat dimers with the promoter DNA can be excluded (Meyer et al., 2004).

Investigation of the cell death of Stat1 F77A macrophages after infection with *L. monocytogenes* did not show any differences to wildtype cells, indicating that the mutation does not influence the expression of any pro-apoptotic or pro-survival genes.

7 Materials

7.1 Bacteria

***Listeria monocytogenes* LO28 wildtype (LO28 wt)**

The strain was kindly provided by Pascale Cossart (Institute Pasteur, Paris).

7.2 Cells

All cells were routinely grown at 37°C, 5% CO_2 and a humidity of 95%.

RAW 264.7 Macrophages

This cell line was cultured in DMEM containing 10% of FCS.

Primary Macrophages

Bone marrow derived macrophages were generated from wildtype mice or from a knock-in variant carrying the mutation F77A in the Stat1 gene with the genetic background of C57Bl/6.

The Stat1 F77A bone marrow samples were provided by Uwe Vinkemeier (Queen's Medical Centre, University of Nottingham).

The mice were housed under special, pathogen free conditions.

The cells were grown at 37°C, 5%CO_2 and 95% humidity and used after 10-14 days of cultivation.

7.3 Common reagents and solutions

Media

LB

10g Trypton

5g yeast extract

10g NaCl

fill up to 1 liter with H_2O and adjust ph to 8

BHI

37g BHI

fill up to 1 liter with H_2O

Solutions and antibiotics used in cell culture

10x PBS (phosphate-buffered saline)

1,4M NaCl

25mM KCl

81mM Na_2HPO_4 x $2H_2O$

15mM KH_2PO_4

pH 7,4

1000x Penicillin/Streptomycin

0,6g Penicillin

1g Streptomycin

add H_2O to 10ml

filter sterilize and store at -20°C

Gentamycin

Gentamycin was diluted in water to a stock concentration of 50mg/ml and stored at 4°C.

PCR reagents

10mM dNTP mix

10mM dATP

10mM dCTP

10mM dGTP

10mM dTTP

RNAse/DNAse free water was purchased from Sigma

TAE

242g Tris base

57,1ml acetic acid

100ml 0,5M EDTA

pH 8

DNA loading dye

10mM Tris pH 7,6

60% Glycerol

60mM EDTA

0,15% OrangeG

0,03% Xylene Cyanol

fill up with H_2O to the desired volume

25x RNA running buffer:

0,25M Na_2HPO_4

0,25M NaH_2PO_4

RNA loading dye

80% (v/v) Formamid

1mM EDTA

add bromphenolblue until desired color is reached

10.000x EtBr

5mg ethidium bromide/ml H_2O

Solutions for protein isolation

Frackelton buffer

10mM Tris

50mM NaCl

30mM NaPPi

50mM NaF

1% Triton X-100

pH: 7-7,5

store at 4°C

1M DTT

Dithiothreitol

0,01M sodium acetate (pH 5,2)

100mM PMSF

100mM Penylmethylsulfonylfuoride in 2-Propanol

store at 4°C

10mM sodium vanadate

18mg Na_3O_4V

10µl HCl:H_2O in the ratio 1:1

add H_2O to 20ml

Boil the solution in a water bath at 85°C until the yellow color of the solution disappears and store at 4°C (solution can be used for 14 days) or at -20°C

Protease inhibitors

Complete EDTA-free inhibitor protease inhibitor cocktail tablets were purchased from Roche (Cat.Nr.: 11 873 580 001).

1 tablet was dissolved in 1ml H_2O

Mixture for protein extraction

Frackelton buffer	1ml

Freshly added:

1mM PMSF	10µl
100µM Na_3VO_4	10µl
1mM DTT	1µl
Protease inhibitor	50µl

Solutions for SDS-PAGE

APS 20% (w/v)

20g Ammoniumpersulfate solved in 100ml H_2O

store at -20°C

stacking gel (4%)

0,5mL Acrylamide/Bisacrylamide-solution

2,5mL H_2O

1ml stacking gel buffer (1,5M Tris-HCl pH 8,8)

40µL 10 % SDS

12µL TEMED

12µl APS

resolving gel	**7,5%**	**10%**
Acrylamide/Bisacrylamide-solution	2ml	1,3ml
H_2O	2ml	1ml
stacking gel buffer (0,5M Tris-HCl pH 6,8)	4ml	1,7ml
10 % SDS	80µl	40µl
TEMED	24µl	12µl
APS	24µl	12µl

4x stacking gel buffer

1,5M Tris HCl

pH 8,8

autoclave

4x resolving gel buffer

0,5M Tris-HCl

pH 6,8

autoclave

10% (w/v) SDS

dissolve 10g sodiumdodecylsulfate in H_2O with gentle stirring and fill up to 100ml H_2O

10x running buffer for SDS PAGE

0,25M Tris

1,92M glycin

1% SDS (w/v)

4x SDS sample buffer

13,5ml H_2O

12,5ml 0,5M Tris HCl pH 6,8

10ml Glycerol

8ml 10% SDS

4ml β-Mercaptoethanol

2ml 0,05% (w/v) Bromphenolblaulösung

Solutions for Western Blotting and protein detection

Blotting buffer

25mM Tris

192mM Glycin

15% Methanol

10x TBST

12g Tris

88g NaCl

5mL Tween 20

add H_2O to 1L

pH 8

Ponceau staining solution

0,2% (w/v) Ponceau S

3% (w/v) Trichloraceticacid TCA

TBST

10mM TrisHCl pH 8,0

150mM NaCl

0,05% Tween 20

Blocking solution

10% milk in 1x TBST

Stripping buffer

200mM Glycin

150mM NaCl

0,05% Tween 20

Add water to 1L

pH 2,8

autoclave the solution

Reagents for cell death analysis

10x Citratbuffer

1,35M KCl

0,15M NaCitrate

autoclave and store at 4°C

FACS buffer

0,2% BSA

0,2% sodium azide

in sterile PBS

filter sterilize

7.4 Antibodies

antibody	spezies	dilution	size (kDa)	company	Order number
pan-ERK	mouse	1:2000	42/44	BD Biosciences	610123
IκBα (C-21)	rabbit	1:1000	~40	Santa Cruz Biotechnology	sc-371
Stat1 C-Terminus	rabbit	1:1000	84	(Kovarik P. et al, 1998)	
Phospho-Stat1 (Tyr701)	rabbit	1:1000	84/91	Cell Signaling	9171L
Phospho-p38 (Thr180/Tyr182)	rabbit	1:1000	43	Cell Signaling	9215S
p38α (N-20)	rabbit	1:1000	43	Santa Cruz Biotechnology	sc-728
p-AKT (Thr308)	rabbit	1:1000	60	Cell Signaling	9275
TBK1/NAK	rabbit	1:1000	80	Cell Signaling	3013
AKT1	rabbit	1:1000	60	Cell Signaling	2967
SAPK/JNK	rabbit	1:1000	46/54	New England Biolabs	9252
Phospo-SAPK/JNK (Thr183/Tyr185)	rabbit	1:1000	46/54	New England Biolabs	9251S

The antibodies were diluted in TBST with 2% BSA and 0,05% (w/v) NaN_3 and stored at 4°C.

For the detection of the primary antibody, anti-mouse antiserum conjugated to Alexa Fluor 680 (Invitrogen detection technologies,

Cat.Nr.:38879A) or IRDy800 (Rockland, Cat.Nr.: 610-132-121) or anti-rabbit antiserum conjugated to IRDye700DX or IRDy800 (Rockland, Cat.Nr.: 611-130-122, 611-132-122) were used in a dilution of 1:20.000.

7.5 Inhibitors

Inhibitor	**inhibitory function on**	**endconc.**	**company**	**order number**
BX795	TBK1/IKKε	1µM	Axon Medchem	Axon 1390
BIX02550	IKKβ	6µM	Boehringer-Ingelheim	-
SB203580	p38	4µM	Sigma Aldrich	S8307
SP600125	JNK	17,5nM	Sigma Aldrich	S5568

All inhibitors were added to the cells one hour before infection or transfection and remained on the cells until the samples were taken.

7.6 Primer

Primers for Reverse Transcription PCR

InlAdown_for: 5’ - ACG CGT CGA CGC CAC TTC ATC TGT TGC AGG CTT A - 3’

InlAdown_rev: 5’ - ACA TCT AGC TCT TTA CAC TAC TTT ATA TAC ACT CCT TTT TCA ATA GT - 3’

InlAup_for: 5’ - GCA AGA TCT AGG TTT AGG TGC AGT TAT CCG CGT - 3’

InlAup_rev: 5’ - GCA AGA TCT AGG TTT AGG TGC AGT TAT CCG CGT - 3’

bgal: 5' - CAT CTT CAT ACC AAA TTT CCT CTG G - 3'

InlAdownfor_Nco: 5’ – CAT GCC ATG GGC CAC TTC ATC TGT TGC AGG CTT A –3’

down190_NcoI: 5´- CATG CCATGG TGC TGG AAC GAA CGA GAT GAA GGA - 3´

down_rev_lig: 5’ –CCA TAC ATA TCG TTT TTT TCT CAC TAT ATA CAC TCC TTT TTC AAT AGT-3’

up_for_lig: 5’ – TAA AAA AGC ACG TGC TAG TAA ATA GAA GTA GTG TAA AGA GCT AGA TGT G-3’

InlAuprev_SalI: 5’ - CAG TGT CGA CAG GTT TAG GTG CAG TTA TCC GCG T – 3’

up224_SalI: 5´- ACG CGT CGA CAT TTG CTT GAT TGG CGT TGG CAC G - 3´

InlA_for_lig: 5’- ACT ATT GAA AAA GGA GTG TAT ATA GTG AGA AAA AAA CGA TAT GTA TGG-3’

InlA_rev_lig: 5’- CAC ATC TAG CTC TTT ACA CTA CTT CTA TTT ACT AGC ACG TGC TTT TTT A-3’

InlA_AflII_for: 5’ – CGT GAC GCA GCC ACT TAA GGC AAT – 3’

InlA_AflII_rev: 5’ - CAT GCC ATG GAT TGC CTT AAG TGG CTG CGT CAC G – 3’

InlA_2_rev: 5’ – AAC TTG GTC TGG ATC CGT TTG TGA GAC – 3’

InlA_3_for: 5’ – ATG AAC GCT TAG GAT CCT TAT AAT TCA – 3’

Start_lig_rev: 5`-ATT ATA AGG GTC ATA AGC GTT CAT AAC TTG GTC TAG ATC TGT TTG TGA GAC-3’

Stop_lig_for: 5’-TCA CAA ACA GAT CTAGAC CAA GTT ATG AAC GCT TAT GAC CCT TAT AAT TCA-3’

down_rev: 5’ - TAT ATA CAC TCC TTT TTC AAT AGT - 3’

up_for: 5’ – AAG TAG TGT AAA GAG CTA GAT GTG - 3’

InlA_for: 5’ – GTG AGA AAA AAA CGA TAT GTA TGG - 3’

InlA_rev: 5’ – CTA TTT ACT AGC ACG TGC TTT TTT A - 3’

Sequencing primers

InlAEGD_for1: 5′ – CAG GTG TAA CAA TCG GCG AA – 3′

InlAEGD_rev1: 5′ – AGC GGG TCT ATA TCC GTT ATC TG – 3′

InlAEGD_for2: 5′ – ACT TTC AAA GGC TGG TAT GAC GA – 3′

InlAEGD_rev2: 5′ – ATC GTA TCG GTC TAA GGA AAG GTT – 3′

InlAEGD_int1: 5′ – TCG TTT ACG TTG ACG TCT TCG GGT – 3′

InlAEGD_int2: 5′ – GTG CCA CTT GCA GGT TTA ACT – 3′

flInlA_3neu: 5′ – CAC AAC AAG TAC AAT GAA – 3′

seqPrInlA5: 5′ – TGC CTG AGC ATT TGT CCC GTT ACT – 3′

PCRInlALO28_for: 5′ – AAA TAC CAT AGG AAC AAT TCG TGG G – 3′

PCRInlALO28_rev: 5′ – CGG TGA TAG TCT CCG CTT GTA CTT – 3′

seqInlALO28_1: 5′ – CGC ATT ATC GCT ATC GCC AGT TGT – 3′

seqInlALO28_2: 5′ – CCC AGC TTC CAC TTC TTT GGT TGT – 3′

seqInlALO28_3: 5‘ – GGT TTG TTA AAC TCG CCA ATG TGC C – 3′

seqInlALO28_4: 5′ – AGA ACC AAA GGC ACC AAC GAA AGC – 3′

seqinsert3: 5′ – CGC CGG AAG CGA GAA GAA TCA TAA – 3′

seqinsert_1neu: 5‘ – CCG TTC AAG GAA TTC AGT ATT TAC CC – 3‘

seqinsert_2neu: 5‘ – TTT GGC GGT AAG AGT GCG G – 3‘

Primers for control of the *inlA* knock-out

#1 seqInlALO28_1: 5′ – CGC ATT ATC GCT ATC GCC AGT TGT – 3′

#2 seqInlALO28_4: 5′ – AGA ACC AAA GGC ACC AAC GAA AGC – 3′

#3 PCRInlALO28_for: 5′ – AAA TAC CAT AGG AAC AAT TCG TGG G – 3′

#4 PCRInlALO28_rev: 5′ – CGG TGA TAG TCT CCG CTT GTA CTT – 3′

#5 InlAEGD_for1: 5′ – CAG GTG TAA CAA TCG GCG AA – 3′

#6 InlAEGD_rev1: 5′ – AGC GGG TCT ATA TCC GTT ATC TG – 3′

#7 InlAEGD_for2: 5′ – ACT TTC AAA GGC TGG TAT GAC GA – 3′

#8 InlAEGD_rev2: 5′ – ATC GTA TCG GTC TAA GGA AAG GTT – 3′

#9 pMAD_for630: 5′ – TCG CTA ACG GAT TCA CCA CTC CAA – 3′

#10 pMAD_rev630: 5′ – TTG GTC ACT TGA TGC CTC CGT GTA – 3′

Real Time Primers

GAPDH_forward: 5' - CAT GGC CTT CCG TGT TCC TA – 3'
GAPDH_reverse: 5' - GCG GCA CGT CAG ATC CA – 3'

IFNb_forward: 5' - TCA GAA TGA GTG GTG GTT GC – 3'
IFNb_reverse: 5' - GAC CTT TCA AAT GCA GTA GAT TCA – 3'

TAP1_forward: 5'- CTG GCA ACC AGC TAC GGG T – 3'
TAP1_reverse: 5' – TGA GAA TGA GGA TGT GGT GGG – 3'

GBP2_forward: 5' - TGC TAA ACT TCG GGA ACA GG – 3'
GBP2_reverse: 5' - GAG CTT GGC AGA GAG GTT TG – 3'

IRF1_forward: 5' – CCG AAG ACC TTA TGA AGC TCT TTG – 3'
IRF1_reverse: 5' – GCA AGT ATC CCT TGC CAT CG – 3'

Mx2_forward: 5' - CCA GTT CCT CTC AGT CCC AAG ATT – 3'
Mx2_reverse: 5' – TAC TGG ATG ATC AAG GGA ACG TGG – 3'

7.7 Transfection reagents

Opti-MEM® I reduced serum medium (Invitrogen)
Lipofectamine™ RNAiMAX (Invitrogen)
PolyFect® Transfection Reagent (Quiagen)

Poly(dA-dT) acid sodium salt (Sigma, Cat.No P0883-10UN)
Poly(dA-dT) was used in a concentration of 1µg/µl.

siRNA: **ON-TARGET*plus* Non-targeting pool**
(Dharmacon, Cat.No D-001810-10-05)

TBK1 ON-TARGET*plus* SMART pool
(Dharmacon, Cat.No L-063162-00-0010)

siRNAs were used in a concentration of 2µM.

8 Methods

8.1 Cell culture

8.1.1 Culturing cell lines

RAW 264.7 Macrophages

This cell line was cultured in DMEM containing 10% of FCS. For splitting, the medium was sucked off, the cells were scraped in fresh medium and then seeded out into new 10cm dishes. These cells grow very fast. Therefore they have to be split 1:5-1:10 every second or third day.

8.1.2 Isolation of bone marrow derived macrophages

For the generation of bone marrow derived macrophages the femur and tibia of the hind legs of mice were isolated with scissors and forceps and put into PBS.

The epiphyses were cut off and the bones were flushed with DMEM containing 10% FCS, 10-20% L-conditioned medium and Pen/Strep using a syringe with a needle of size 18.

Bone marrow cells were cultivated in DMEM with 10% FCS, 10-20% L-conditioned medium and Pen/Strep on 10cm square dishes. The cells of one mouse were split to three to four dishes. The cells were fed every second or third day and after five to seven days they were split in a ratio of 1:2 or 1:3.

The differentiated macrophages were used after 10-14 days in culture.

L-cell conditioned medium was generated in the lab. The L-cells were cultured in starvation medium without FCS for 10 days and then the supernatant was sterile-filtered.

8.1.3 Freezing and thawing of bone marrow

For freezing bone marrow, the bones were flushed as described before and centrifuged at 300g for 5-10min. The pellet was resuspended in a mixture of 500µl 10% dimethylsulfoxide (DMSO) and 90% DMEM. The cell suspension was transferred into a cryotube and stored at -80°C.
For thawing, the frozen cells were warmed quickly and transferred into a tube containing DMEM. The cell suspension was centrifuged at 300g, the pellet was resuspended in DMEM containing 10% FCS and 10-20% L-cell conditioned medium and the cells were plated on 3-4 10cm square dishes per mouse.

8.1.4 Transfection of BMDM and RAWs

For the siRNA transfection, $0{,}5x10^6$ BMDM were seeded into 3,5cm tissue culture dishes. On the next day the transfection was performed. 250µl Opti-MEM® I reduced serum medium (Invitrogen) were mixed with 15µl siRNA (2µM) and 250µl Opti-MEM® I reduced serum medium were mixed with 5µl Lipofectamine™ RNAiMAX (Invitrogen). The two solutions were combined, resuspended and incubated for 15min.
The medium of the cells was changed and the siRNA duplex-Lipofectamin complexes were added dropwise to the cells.

48h post transfection the medium was sucked off and replaced by 2,5ml fresh medium. On the next day the cells were infected.

For the transfection with poly(dA-dT), 10^6 RAW macrophages or $1,6*10^6$ primary macrophages were seeded into 6-well plates. Before adding the transfection reagents, the medium was reduced to 1,5ml. 140µl DMEM, 10µl poly(dA-dT) (1µg/µl) and 25µl polyFect per sample were mixed, vortexed and incubated for 5min at room temperature. The solution was added dropwise to the cells. Samples were taken 2 or 3 hours and 4 hours after transfection.

8.2 Working with bacteria

8.2.1 Cultivation of bacteria

For the cultivation of *Listeria monocytogenes* 5ml of Brain-Heart infusion (BHI) medium purchased from BD (Cat.Nr.: 237200) were inoculated with a glycerol bacteria stock (stored at -80°C), and incubated over night at 37°C on a shaker.
Escherichia coli was cultured in 5ml Luria Broth medium at the same temperature.
The *Escherichia coli* strain DH5α, containing the temperature sensitive shuttle vector pMAD or pAUL-A, was incubated in 5ml Luria broth medium containing 100µg/ml ampicillin.
Listeria monocytogenes strains, containing the plasmids pMAD or pAUL-A, were incubated in 5ml Brain-Heart infusion medium containing 3µg/ml erythromycin.

To determine the cell number, the optical density at 600nm was measured using a visible spectrophotometer from Amersham Biosciences. Thereafter the over night cultures were diluted 1:10 in the respective medium.
For *Listeria monocytogenes* an OD_{600} of 1 is equivalent to $2x10^9$ bacteria/ml.

8.2.2 Infection of cells with *Listeria monocytogenes*

For infection experiments with *Listeria monocytogenes*, the host cells were seeded one day before onset of the experiment.
Thereafter the medium containing Pen/Strep was sucked off and the cells were washed with sterile PBS. 4ml of medium without antibiotics were added to the dish and the cells were scraped off with a rubber policeman. The cells were pooled and counted in a 1:10 dilution, using the Vi cell™ cell viability analyzer (Beckman Coulter).
The respective amount of cells was seeded in medium without Pen/Strep and incubated over night.
On the next day the cells were infected with the appropriate amount of an over night culture to get a multiplicity of infection (MOI) of 10-30, according to the requirements.
One hour after infection the medium was replaced by medium containing 50µg/ml gentamycin to kill all the extracellular bacteria, which were not taken up by the cells. After an additional hour the medium was again sucked off and medium containing 10µg/ml gentamycin was added.

8.2.3 Generating competent bacteria

Escherichia coli

To obtain competent DH5α bacteria, an O/N-culture was diluted 1:100 and the bacteria were incubated at 37°C until they reached an OD between 0,35 and 0,4. The cellsuspension was centrifuged at approximately 4700g for 15min at 4°C and the pellet was resuspended in 10ml 100mM $CaCl_2$ solution. The cells were incubated for 20min on ice and then they were again centrifuged for 15min at 4692g and 4°C. The pellet was resuspended in 2ml 100mM $CaCl_2$ solution with 15% Glycerol, aliquoted in 100µl and frozen at -80°C.

Listeria monocytogenes

2ml of an overnight culture of *Listeria monocytogenes* were used to inoculate 100ml of BHI medium containing 0,5M sucrose. The cells were incubated on a shaker at 37°C until they reached an OD_{600} of about 0,2.

Ampicillin was added to the culture to get a final concentration of 10µg/ml and the bacteria were again incubated on a shaker at 37°C for two more hours. Then the culture was centrifuged at 7000rpm for 10 minutes at 4°C. All the following steps were carried out with cold solutions and on ice. The supernatant was poured off and the pellet was resuspended in 90ml 1mM Hepes (pH 7) containing 0,5M sucrose.

The centrifugation step was repeated and the pellet was again washed with 45ml Hepes/sucrose solution. Finally the suspension was centrifuged one last time, the pellet was resuspended in 400µl Hepes/sucrose, aliquoted into 100µl and stored at -80°C.

8.2.4 Transformation of bacteria

Escherichia coli

For transformation, the plasmid and 100µl of the competent DH5α cells, which were thawed on ice, were mixed. They were incubated for 15-30min on ice, transferred 1min to 42°C in a waterbath and placed for 2min on ice again. 1ml LB was added, the cell suspension was transferred into a round bottom tube and incubated for about 1 hour at 37°C. 100µl of the cell suspension were plated onto an LB dish containing 500µg/ml erythromycin.

Listeria monocytogenes

For electroporation a 0,2cm electorporation cuvette was washed with water and ethanol and dried at 70°C for some minutes. Remaining DNA was crosslinked to the cuvette for 2 times with 360 Joule using the UV Stratalinker 2400 from Stratagene.
About 1µg of plasmid was added to competent *Listeria monocytogenes* cells and the electroporation was accomplished with a Gene pulser (BioRad) under the following conditions: 1,5kV/400Ω/25µFD and a pulse duration of about 5ms.
1ml of BHI medium containing 0,5M sucrose was added and incubated for two hours at 30°C for temperature-sensitive plasmids or for 1 hour at 37°C without shaking.
100µl of the electroporated culture were plated on a BHI agarose plate containing the appropriate antibiotic and incubated at the proper temperature. The colony formation took about 2-3 days.

8.3 Recombinant DNA technology

8.3.1 Plasmid DNA purification

The plasmid DNA purification was accomplished by using the 5prime PerfectPrep Spin Mini Kit of Eppendorf (Cat.Nr.: 2300110) according to the instructions. The plasmid was eluted with 50µl of DNAse free water and the concentration was measured with a Nano-Drop system.

8.3.2 Polymerase chain reaction (PCR)

For the polymerase chain reactions, proof-reading DNA polymerases were used (PFU, Fermentas; AccuPol, Ampliqon).
The mastermixes were prepared as follows:

5µl	buffer-$MgSO_4$ (10x) PFU or
	ammonium reaction buffer (10x) AccuPol
4µl	$MgSO_4$ for PFU polymerase
1µl	10mM dNTPs
1µl	forward primer (100pmol/µl)
1µl	reverse primer (100pmol/µl)
*µl	template DNA
1µl	Pfu or AccuPol
_____	H_2O
50µl	

* depending on the concentration of the DNA

The PCR was performed with the following program:

1x	95°C for 5min
30x	95°C for 30sec
	* for 30sec *(primer annealing)*
	72°C for * sec *(Pfu extention time 2min/kb)*
1x	72°C for 7min

* Primer annealing temperature depends on the primer length and the A-T/G-C percentage. The extention time depends on the length of the DNA fragment that should be amplified. The extention time for the Fermentas proof reading polymerase is about 2min per kilobase, for the Ampliqon AccuPol about 1-2 kilobases per minute.

8.3.3 Phenol-Chloroform extraction

5ml of an O/N culture were centrifuged at 8000rpm and 4°C for 10min. The pellet was resuspended in 500µl TE buffer, 25µl of lysozyme (50mg/ml) were added and incubated at 37°C for 30-40min on a shaking heating block.
120µl 0,5M EDTA, 160µl 10% SDS and 50µl RNAseA (10mg/ml) were added and again incubated under the same conditions.
20µl of proteinaseK (20mg/ml) were added and incubated at 37°C for 30min with occasional tumbling to mix the suspension. 1 volume (about 875µl) of phenol:chloroform:isoamylalcohol (25:24:1) were added to the extract and strongly mixed. The emulsion was centrifuged at 15000rpm at room temperature for 10min and the upper phase was carefully transferred into a new sample tube. The last step was repeated at least once, until there was no white layer at the interface.
1 volume of chloroform (about 400µl) was added and again mixed well. The centrifugation was repeated and the upper phase was

transferred into a new sample tube. To each tube, 2 volumes of 100% EtOH were added, gently mixed by inverting the tube several times, centrifuged at 15000rpm at room temperature for 10min and the supernatant was carefully removed. The pellet was washed with 70% ethanol and air-dried. Finally the DNA was resuspended in 50µl sterile H_2O and stored at -20°C.

8.3.4 Precipitation of DNA

One volume of isopropanol was added to the sample and incubated at -20°C for 30 minutes to 1 hour. Then the tube was centrifuged at 4°C and maximum speed in a table top centrifuge.
The pelleted DNA was washed with 500µl 70% EtOH and dried at 55°C on a heating block for some minutes. Then 30-50µl of DNAse-free water were added and the sample was again inucubated at 55°C up to one hour and finally resuspended.
Concentrations were mesuared with a Nano-Drop system.

8.3.5 Agarose gel electropheses and extraction of DNA

For the agarose gel electrophoreses a 0,7-1% Bioyzme LE Agarose gel (2% for very small DNA fragments) was used, containing 0,5µg/ml ethidiumbromide. The gels were run at 80-110V in TAE buffer.
DNA was extracted using the 5prime DNA extraction kit from Fermentas according to the manufacturer's protocol. DNA was eluted with RNAse/DNase free water and measured with a Nano-Drop system.

8.3.6 Purification of PCR products

For DNA purification the 5prime Perfect Prep Spin Mini Kit (Eppendorf, Cat.Nr.: 2300600) was used. All the steps were performed following the manufacturer's manual.
DNA was eluted using 30-50µl of DNAse free water.

8.3.7 Restriction digest

Restriction digests were usually performed with 5% restriction enzyme in the mastermix.
For double digests the following buffers were used.

SalI		buffer O+
BglII		
SalI		2x Tango
NcoI	2-fold excess	
BamHI		1x Tango
NcoI		
BamHI		buffer BamHI
SalI	2-fold excess	
BglII		2x Tango
BamHI	2-fold excess	

All restriction enzymes were purchased from Fermentas.
The restriction digest was accomplished at 37°C for 1-3 hours or at 16°C over night. For incubation longer than one hour, additional enzyme was added after 1 or 1,5 hours.

8.3.8 Ligation

For ligation of DNA fragments a T4 ligase, purchased from Fermentas, was used.
The ligation was accomplished at 22°C for 1 hour or at 16°C over night. The enzyme was inactivated by heating to 65°C for 10min.

8.4 Colony forming units (CFU) assay

For the CFU assays $5x10^4$ BMDM cells per well were seeded in a 96-well plate in medium without Pen/Strep.
The cells were infected with *Listeria monocytogenes* LO28 at an MOI of 10.
After 2 hours the medium was replaced by prewarmed medium containing 50µg/ml gentamycin and after an additional hour the medium was again exchanged by medium containing 10µg/ml.
The inoculum was serially diluted 1:10 and the dilutions with about 10, 100 and 1000 bacteria were plated on BHI plates for the calculation of the real MOI.
For each timepoint the medium was sucked off, the cells were washed with 100µl sterile PBS for two times, 100µl sterile H_2O were added per well and resuspended to detach the cells. The cell lysats were transferred into sample tubes, again 100µl sterile H_2O were added, resuspended and transferred in the same sample tube.
The cell lysates were diluted 1:10 and 100µl of the dilutions with an expected value of 10-10.000 bacteria were plated out.
The BHI plates were incubated at 37°C over night and on the next day the colonies were counted.

8.5 Analysis of proteins

8.5.1 Protein extracts

For the protein extracts $1,5x10^6$-$2x10^6$ macrophages were seeded out one day before on 6-well plates or 6cm tissue culture dishes (Nunc). The cells were infected with *Listeria monocytogenes* LO28 at an MOI of 10 or 20.

All the following steps were performed on ice and in the presence of protease inhibitors and vanadate to prevent dephosphorylation of proteins.

The medium was sucked off and the cells were washed with cold PBS, which was then completely sucked off. 80µl of the Frackelton buffer, including the other reagents (see chapter 7.3; solutions for protein isolation), were added and the cells were scraped off with a cell scraper, and transferred into a reaction tube. The cells were centrifuged for 5min at 12.000rpm and 4°C in a table top centrifuge and the supernatant was transferred into a new Eppendorf tube. 30µl of SDS sample buffer were added. The solutions were mixed and cooked for 5-10min at 95°C on a heating block and stored at -20°C.

8.5.2 SDS-PAGE (polyacrylamid gel electrophoresis)

The separating gel was poured and overlaid with waterlogged 1-butanol to get a straight and air bubble free surface. After polymerization, the alcohol was removed, the stacking gel was poured and the combs were immediately inserted.

The polymerized gels were fixed in the electrophoresis apparatus and running buffer was added. 5µl of protein marker (Page ruler prestained protein ladder (Cat.Nr.: SM0671) or Page ruler plus

prestained protein ladder (Cat.Nr.: SM1811), purchased from Fermentas) and 20-30µl of the thawed samples were loaded onto the gel.
The gels were run at 80-90V until the proteins passed the borderline between the stacking gel and the separating gel. Then the voltage was increased to about 110.

8.5.3 Western Blotting

For western blotting we used a semi-dry transfer cell from Biorad. The separating gel and the blotting paper (BioRad, Protean® xi size) were equilibrated in the blotting buffer for some minutes and the nitrocellulose membrane (Optitran BA-S83 reinforced NC, Schleicher and Schuell) was equilibrated in water.
The blotting sandwich was assembled in the following way:

Anode (+)
Blotting paper
Nitrocellulose membrane
Separating gel
Blotting paper
Cathode (-)

Air bubbles were removed and the blotting was performed at 32mA per gel for 1,5 hours.

8.5.4 Immunostaining

For immunostaining of the membranes the antibodies listed in chapter 7.4 were used.

After blotting, the membranes were stained with Ponceau S for some minutes to ensure the protein transfer. Then the membrane was rinsed with water and blocked with TBST containing 10% of non fat milk powder for 30 minutes.
After the blocking, the membrane was washed three times with TBST for 10 minutes.
Then the primary antibody solution was added and incubated over night at 4°C (or at room temperature for 1 to 2 hours) on a shaker.
On the next day the antibody solution was transferred back to a falcon tube and stored at 4°C for reuse. The membranes were washed again three times with TBST for 10 minutes and then the secondary antibody, coupled with a fluorescence dye and diluted 1:20.000 in TBST, was added and incubated for 1 hour at room temperature protected from light.
After the incubation, the membrane was again washed 3 times for 10 minutes with TBST and finally the membrane was analysed with an Odyssey scanner.

8.5.5 Stripping of the membrane

Nitrocellulose membranes can be reused for detection of other proteins with a different antibody.
For this purpose the membrane has to be stripped. The membrane is shortly rinsed with water and then stripping buffer is added for about 10 minutes. After the incubation on the shaker the membrane is

again rinsed with water and the immunostaining procedure, including the blocking step and all the washing steps, can be repeated.

8.6 Analysis of mRNA levels

8.6.1 RNA extraction of whole cell lysates

For the RNA extracts $1x10^6$ macrophages were seeded one day before the infection in 6-well plates (Nunc, Cat.Nr.:140675).
On the next day they were infected with *Listeria monocytogenes* LO28 according to the procedure described in chapter 8.2.2 with an MOI of 10.
The medium was sucked off and the macrophages were washed with ice-cold PBS.
The cells were scraped in 400µl RA1 lysis buffer (Nucleospin RNA II kit, Macherey and Nagel) containing 4µl of β-mercapthoetanol, transferred into a reaction tube and stored at -80°.
For the further RNA preparation, the Nucleospin RNA II kit was used according to the manufacturer's instructions.
The RNA was eluted with 60µl of RNAse free water.

8.6.2 Preparation of cDNA from RNA samples

For the reverse transcription approximately 1µg of RNA was used and filled up to 10µl with RNAse free water.
1µl of oligo dT primer was added and the mixture was incubated for 5 minutes at 70°C. Then 4µl 5x reaction buffer (Fermentas), 2µl 10mM

dNTPs and 2µl RNAse free water were added and incubated for 5 minutes at 37°C.
1µl of Reverse Aid Reverse Transcriptase (Fermentas) was added and the cDNA was generated in 1 hour at 42°C. The reverse transcriptase was inactivated for 10 minutes at 70°C.
For real time PCR the cDNA samples were further diluted 1:10 in RNAse free water and stored at -20°C.

8.6.3 Real Time PCR with Sybr Green

For real time PCR experiments an iCycler IQ real time PCR machine (Biorad) or a Mastercycler® ep realplex (Eppendorf) were used.
Sybr Green is a dye, which intercalates into double stranded DNA and can be measured at 490nm.
For the normalisation of the gene of interest the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified.
The Sybr Green was diluted 1:20 in DMSO and stored at -20°C. This solution was further diluted 1:100 in RNAse free water. The final dilution needs to be prepared freshly.
As a calibration dye FITC (purchased from Biorad, 1mM) was used in a dilution of 1:1000 in RNAse free water.The dilutions have to be stored in a light protective reaction tube at -20°C.
The real time PCRs were performed using the Redmix (ampliqonIII, Cat.Nr.: 52003009G14), which already contains the polymerase.

The PCR mastermix was prepared as following:

12,5µl	Redmix (2x)
0,1µl	forward primer (100pmol/µl)
0,1µl	reverse primer (100pmol/µl)
0,25µl	FITC (1:1000)
1µl	Sybr Green (1:2000)
6,05µl	RNAse/DNAse free water
5µl	template DNA
25µl	

The PCRs were performed with the following programmes:

I cycler:

1x	95°C for 3min
40x	95°C for 10sec
	60°C for 15sec
	72°C for 15sec
1x	95°C for 1min
80x	60°C + for 10sec

Real Plex (Eppendorf):

1x	95°C for 4min
40x	95°C for 15sec
	60°C for 15sec
	68°C for 20sec
1x	95°C for 15sec
80x	60°C-95°C for 15sec
1x	60°C for 2min

8.7 Cell death analysis

8.7.1 Propidium iodide (PI) staining

$5x10^5$-$1x10^6$ cells were seeded one day before infection on 6-well plates, 3,5cm tissue culture dishes or 6cm tissue culture dishes.
The cells were infected with an MOI of 10 to 30.
In preparation for the PI staining, 1ml PBS was added to a FACS tube (BD Falcon, Cat.Nr.: 352052). The medium of the cells, which could contain dead cells, was transferred to the tube with the PBS.
An appropriate volume of the 10x stock solution of citrate buffer was diluted 1:10 with sterile water and 1ml of the prewarmed (37°C) dilution was added to each well/plate and incubated at 37°C for a maximum of 5 minutes.
The cells were detached by pipetting up and down for several times and also transferred to the same FACS tube.
The cell suspension was centrifuged for 10 minutes at 300g and 4°C and the supernatant was sucked off. The pellet was washed with 1ml FACS buffer and again centrifuged under the same conditions.
Finally the cell pellet was resuspended in 500µl FACS buffer.
Directly before flow cytometric analysis 5µl of PI with a concentration of 500µg/ml were added.
The FACS experiments were performed with a FACSCalibur (BD Biosciences).

9 Acknowledgements

I would like to thank Thomas Decker for giving me the opportunity to do my diploma thesis in his lab and for his motivation throughout my work.

I also want to thank all members of the Decker lab for a nice working atmosphere and many helpful discussions, especially Renate Kastner, who supervised me.

Stephanie Füreder, Gerhard Sponder and Chris de Graffenried are gratefully acknowledged for their advice concerning my main project.

I would like to thank my boyfriend Stefan for his support during the last year, for his understanding when I had to work on weekends and for the time we spend together.

Finally, I would like to thank my family for their love and support throughout my life and for giving me the opportunity to complete a study.

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