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Activation of murine microglial cells by muramyl dipeptide alone and in combination with Toll-like receptor agonists

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Abstract

Meningitis, meningoencephalitis and sepsis are severe diseases causing many deaths all over the world every year. *Escherichia coli* (*E. coli*) is the most common Gram-negative bacterium causing neonatal meningitis and sepsis but also causes meningitis in old and immunocompromised people. Especially immunocompromised patients carry a high risk of developing infections e.g. in the central nervous system (CNS) caused by different pathogens, such as bacteria, viruses and fungi. One cause of this increased susceptibility to CNS infections might be a decreased local immune defense. Microglial cells, the resident immune cells of the brain, constitutively express Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors, which are known to trigger innate immune responses against microbial infection upon pathogen recognition.

This study aimed at three major questions: to detect whether stimulation of microglial cells with (i) the Nod2 ligand muramyl dipeptide (MDP) or (ii) the viral TLR3 agonist poly(I:C) affects phagocytosis and intracellular killing of *E. coli* K1, and (iii) to detect if there is synergism between MDP and the tested TLR agonists on the mentioned parameters. Therefore, primary cultures of murine microglia were stimulated with MDP or a TLR agonist alone as well as in combinations. Phagocytic activity was determined after 30 and 90 min of incubation with *E. coli* K1. To analyze the ability of microglia to kill ingested *E. coli*, bacteria were quantified at different time points after phagocytosis.

In the current work, I was able to demonstrate for the first time that MDP and poly(I:C) alone increased phagocytosis and intracellular killing of *E. coli* K1 in murine microglia. In comparison, activation through the TLR system caused a stronger increase of phagocytosis than stimulation of the Nod2 system alone. Most notably, upon co-stimulation, the Nod2 and TLR systems can synergize to enhance both the phagocytic and bactericidal activities of microglial cells.

In conclusion, microglial innate immune responses to invading *E. coli* K1 are enhanced by stimulation with the TLR3 agonist and Nod2 receptor alone as well as by a dual stimulation with poly(I:C) and MDP. These findings underline the cooperative action of innate immune receptor/signalling systems in fighting infectious threats. These results indicate that more studies should be investigated to find out whether a pre-stimulation with a Nod2 receptor agonist and a TLR agonist could improve CNS resistance to infections in

immunocompromised patients and could therefore help the patients to develop mechanisms of resistance against lethal pathogens.

Abstract

Abbreviations

1 Introduction 1

1.1 The immune system and the special role of microglia 1

1.1.1 Chemo- and Cytokines 2

1.2 Toll - like receptors (TLRs) 3

1.3 Nucleotide-binding oligomerization domain like (NOD) receptors 4

1.4 Characterisation of the receptor ligands 5

1.4.1 TLR agonists 5

1.4.1.1 The TLR1/2 agonist Pam_3CSK_4 5

1.4.1.2 The viral TLR3 agonist poly(I:C) 5

1.4.1.3 The TLR4 agonist LPS 5

1.4.1.4 The TLR9 agonist CpG 6

1.4.2 The NOD2 receptor agonist MDP 6

1.5 Characterisation and pathogenicity of Escherichia coli 7

1.6 Meningitis 7

1.7 Outline 8

2 Material and Methods 9

2.1 Description of the study 9

2.2 Materials 9

2.2.1 Chemicals 9

2.2.2 Equipment and software 11

2.3 Primary mouse microglial cell cultures 12

2.3.1 Preparation of mouse brains 12

2.3.2 Preparation of cell cultures for different assays 12

2.4 Stimulation of murine microglial cells 13

2.5 Measurement of nitric oxide (NO) 13

2.6 Measurement of microglial cell viability 14

2.7 Cytokine and Chemokine release 15

2.8 Bacterial strain 15

2.9 Quantitative plating 15

2.10 Phagocytosis assay 16

2.11 Cytochalosin D inhibition assay 17

2.12 Intracellular survival assay 17

2.13 Statistical analysis *17*

3 Results **18**

3.1 NO release assays *18*

3.1.1 NO release after stimulation with MDP 18

3.1.2 NO release after stimulation with poly(I:C) 20

3.1.3 NO release after stimulation with Pam_3CSK_4 20

3.2 Release of chemo- & cytokines *21*

3.2.1 Release of chemo- and cytokines after stimulation with a single agonist 22

3.2.2 Release of chemo-/cytokines upon co-stimulation with MDP and TLR agonists 23

3.3 Phagocytosis assays *26*

3.3.1 Phagocytosis after stimulation with MDP 26

3.3.2 Phagocytosis after stimulation with poly(I:C) 27

3.3.3 Phagocytosis inhibition studies with CD 28

3.3.4 Comparison of the phagocytic rates after stimulation of the Nod2 and TLR systems 29

3.3.5 Phagocytosis after co-stimulation of MDP and TLR agonists 31

3.3.5.1 Phagocytosis after 30 min of incubation with *E. coli* K1 32

3.3.5.2 Phagocytosis after 90 min of incubation with *E. coli* K1 36

3.4 Intracellular survival assay *41*

4 Discussion **45**

5 Summary of conclusions **50**

6 Publications of current work **52**

6.1 Poster and Papers: *52*

7 References **53**

8 Appendix **61**

8.1 List of figures *61*

8.2 List of tables *63*

Abbreviations

CARD15	Caspase Recruitment Domain 15
CD	Cytochalasin D
CFU	Colony Forming Unit
CpG	Cytosine-phosphate-Guanosine
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CO_2	Carbon Dioxide
CXCL1	Chemokine (C-X-C motif) ligand 1
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
EC50	Half maximal effective concentration
E. coli K1	*Escherichia coli* K1
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Foetal Bovine Serum
h	hour(s)
IFN-γ	Interferon gamma
IL	Interleucin
IRAK	Interleukin receptor-associated kinase
LPS	Lipopolysaccharide
M	Molar mass
MDA5	Melanoma-differentiation-associated gene 5
MDP	N-Acetylmuramyl-L-Alanyl-D-Isoglutamine¸ muramyl dipetide
MHC	Major Histocompatibility Complex
min	Minutes
ml	Millilitre
MyD88	Myeloid differentiation protein 88
NED	Naphthyl-ethylendiamin Dihydrochlorid
NO	Nitric oxide

NOD	Nucleotide-binding Oligomerization Domain (human)
Nod	Nucleotide-binding oligomerization domain (murine)
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate-buffered saline
PGN	Peptidoglycane
Poly(I:C)	Polyinosine–polycytidylic acid
PRR(s)	Pattern recognition receptor(s)
P3C/ Pam_3CSK_4	N-Palmitoyl-S-[2,3bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]
RICK/RIP2	Receptor-interacting serine/threonine kinase
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain–containing adapter protein
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
TRIF	TIR domain-containing adapter inducing IFN-β
WST	Water-Soluble Tetrazolium Salt
°C	Degree in Celsius
μl	Microlitre

1 Introduction

1.1 The immune system and the special role of microglia

The human body is a complex network of multiple interacting organ systems. Each of these has a different function to maintain balance in the individual. The immune system for example plays a pivotal role in protecting the organism from invading pathogens, such as bacteria, fungi, viruses and parasites. Immunity in higher vertebrates can be subdivided into two main parts, the innate and the adaptive arms, both of which offering inducible responses and interacting with each other (Beutler 2000). While adaptive immunity acts specifically against pathogens and has an immunological memory, innate immunity interacts non-specifically but within minutes to few hours upon pathogen encounter thus providing the host's first line of defence. Together with anatomical and humoral barriers, cellular barriers belong to this system and play the most important role in innate immunity in case of inflammation.

In particular, the central nervous system (CNS) needs specific mechanisms of defence as "the neurons of the central nervous system cannot divide and be replenished, and therefore need to be protected against pathogens" (Ransohoff and Cardona 2010; p. 253). A part of this challenge is taken over by microglial cells, which play a special role in the CNS. Besides their immune regulatory function, microglia support communication among astrocytes (Giaume 2010), which maintain homeostasis in the brain. Microglia are the resident innate immune cells of the CNS. They act as first active defence against microorganisms in the brain tissue (Kreutzberg 1995, Mariani and Kielian 2009) and therefore have been of greatest interest for research during the last decades. In the healthy adult CNS, resting microglia constantly scan their environment with highly motile processes (Nimmerjahn et al. 2005). Upon detection of any disturbance of CNS homeostasis, microglia can undergo from the 'resting' to an alerted or 'activated' state (Hanisch and Kettelman 2007). As specialist macrophages, activated microglia can phagocytose and kill pathogens invading the CNS. Once the pathogen is engulfed, it is incorporated and digested in phagosomes that mature by increasing acidification upon fusion with endosomes and later with lysosomes. Amoeboid microglial cells from neonatal rats have been shown to efficiently internalise a non-pathogenic *E. coli* strain in phagosomes one day after a single intracerebral injection (Kaur et al. 2004). In the phagolysosome, *E. coli* is exposed to reactive oxygen species and other hydrolytic enzymes

which eventually results in bacterial lysis. To accomplish pathogen clearance, microglia express pattern-recognition receptors (PRRs) such as the different members of the Toll-like receptor (TLR) family (Akira et al. 2006, Kielian 2006, Hanisch and Kettenmann 2007, Mariani and Kielian 2009) and the nucleotide-binding oligomerization domain (Nod)-like receptors (Sterka and Marriott 2006). PRRs are able to sense a variety of highly conserved structural motifs expressed by microbial pathogens, called pathogen-associated molecular patterns (PAMPs). Upon PAMP recognition, microglia mediate and induce immune responses by releasing chemokines and cytokines (Olson and Miller 2004, Sterka and Marriott 2006), such as tumor necrosis factor-α (TNF-α), chemokine C-X-C motif ligand 1 (CXCL1) and interleukin-6 (IL-6) as well as free radicals leading to further activations of the immune system (Hanisch et al. 2002). Recent studies showed that the activation of murine microglia by ligands of TLR1/2, TLR4 and TLR9 can increase bacterial uptake as well as intracellular killing of Gram-negative and Gram-positive bacteria (Ribes et al. 2009).

Microglial cells also express Nod-like receptors such as Nod1 and Nod2 (Sterka and Marriott 2006). NOD2 and its ligand, muramyl dipeptide (MDP), are known to trigger innate immune responses against microbial infection upon pathogen recognition (Inohara and Nunez 2003). Furthermore, it has been described that Nod2 expression is upregulated when cells are exposed to TLR or MDP and the production of chemo- and cytokines is enhanced (Sterka and Marriott 2006). However, so far there is no evidence that stimulation of the Nod2 system contributes to the elimination of *E. coli* K1 by microglial cells.

1.1.1 Chemo- and Cytokines

The variety and complex functions of chemo- and cytokines will be briefly discussed at this point. Chemo- and cytokines, such as TNF-α, CXCL1 and IL-6 are released by various cell types, hereunder stimulated microglia, and act as transmitters mediating immune responses (Cavaillon 1994, Hanisch 2002, Sterka and Marriott 2006). Microglia can “communicate” and regulate the release of cytokines by themselves (Cavaillon 1994), whereupon the balance of different cytokines seems to be essential for the outcome in case of infection (Hopkins and Rothwell 1995, Rodriguez-Gaspar et al. 2001). Among cytokines, TNF-α plays a central role in the control of local and systemic infection. A neuroprotective effect is described at low concentrations whereas in contrast, an overproduction can cause multiorgan failure, fever, hypotension and tissue damage (Carlson et al. 1999, Hanisch 2002). The same effects are described for IL-6 (Carlson et al. 1999), which has among its great variety of functions, a pro-

inflammatory and an anti-inflammatory effect and is important in mediating fever and acute-phase reactions (Helle et al. 1988).

Chemokines (a shortening of **chemo**attractant cyto**kines**) induce chemotaxis, i.e. they activate cells to migrate to inflammatory sites. They can be divided into four groups depending on their chemical structure or can also be classified according to their function into inflammatory and homeostatic chemokines. An example of a (pro-) inflammatory chemokine is CXCL1 (also known as KC or Gro-α), which has neutrophil chemoattractant activity and plays a central role in inflammatory processes (Hanisch 2002).

1.2 Toll - like receptors (TLRs)

In 1985, Christiane Nüsslein-Vollhard discovered a gene responsible for the appearance of the small fruit fly, called *Drosophila*, to look different or weird. She was so enthusiastic about her discovery that she used the German vocabulary for awesome, which is "toll" to name the gene (Hannson and Edfeldt 2005). Nearly ten years later, Lemaitre et al. (1996) discovered that the gene was not only responsible for a different phenotype but also played a role in the immunity of *Drosophila*. One year later, Medzhitov et al. (1997) were able to clone a human homologue to the Toll receptor, a Toll-like receptor, and reported its ability to activate adaptive immunity. Until today this discovery has encouraged many research groups to perform further investigations to gather more information.

It is known that TLRs are transmembrane proteins playing a central role in innate immunity and belong to PRRs, able to detect highly conserved pathogen structures (PAMPs) which are essential for the survival of pathogens (Takeuchi et al. 2002). On the other hand, the TLR system can also be stimulated by the non-physiological appearance, altered structure or unusual concentration of certain endogenous molecules, which are produced and released by damaged cells. So far there have been identified 10 members of the TLR family in humans and 12 in mice (Takeuchi et al. 2002, Kawai and Akira 2011). TLRs are expressed on antigen-presenting cells such as dendritic cells, macrophages or microglia (Visintin et al. 2001, Kielian 2006). In the present study, TLR1/2, TLR3, TLR4 and TLR9 of microglial cells were stimulated with known TLR agonists (see below).

TLR1, TLR2 and TLR4 are located on the cell surface. TLR1 and TLR2 cooperate through their cytoplasmic domain to form a heterodimer enabling the cell to detect e.g. Gram-negative bacteria, lipoteichoic acids from Gram-positive bacteria and Mycoplasma (Takeda et al. 2003). TLR4 is a dimer that responds to bacterial lipopolysaccharide (LPS), a component of

the outer membrane of Gram-negative bacteria (Akira et al. 2006). In contrast, TLR3 and TLR9 are located in intracellular compartments such as endosome/endoplasmatic reticulum membranes (Kato et al. 2008, p. 1601). While TLR3 responds to double-stranded RNA (dsRNA) (Alexopoulou et al. 2001), TLR9 recognizes bacterial DNA containing CpG motifs (Hemmi et al. 2000).

Once the pathogen is detected the information has to be translated to induce further activation of the immune system (Ozinsky et al. 2000, Takeuchi et al. 2002). Upon recognition of pathogens, TLR can induce responses through a variety of signal cascades. Myeloid differentiation protein 88 (MyD88) is an adaptor protein of interleukin-1 receptor associated kinase (IRAK) that leads to the activation of nuclear factor κB (Medzhitov and Janeway 2000, Akira et al. 2006, Carpenter and O´Neill 2007). All TLR except for TLR3 mediate responses through MyD88 (Yamamato et al. 2004). TLR3 depends on Toll/interleukin-1 receptor domain-containing adapter inducing IFN-β (TRIF) and melanoma-differentiation-associated gene 5 (MDA5), which seem to play a central role in immune defense in case of viral infections (Alexopoulou et al. 2001, Kato et al. 2006, Town et al. 2006). TLR4 mediation needs adaptor molecule MyD88 and TRIF signaling pathways (Kawai et al. 1999; Yamamato et al. 2004).

1.3 Nucleotide-binding oligomerization domain like (NOD) receptors

In recent years, NOD proteins are gaining interest since NOD2, also designated Caspase Recruitment Domain 15 (CARD15), receptor mutations have been identified in patients suffering from Crohn's disease (Hugot et al. 2001). Nod2 is a member of the CED4/APAF1 family of apoptosis regulators (Ogura et al. 2001) which can recognize peptidoglycan (PGN)-derived fragments, including the minimal bioactive PG motif common to all bacteria, i.e. MDP which is also used as a NOD2 synthetic ligand (Girardin et al. 2003). By an activation of the serine/threonine kinase RICK (receptor-interacting serine/threonine kinase; also known as RIP2), MDP triggers the production of pro-inflammatory mediators through NF-κB signalling (Ogura et al. 2001, Strober et al. 2006). NOD proteins have been identified in immune and non-immune cell types (Strober et al. 2006, Ting and Davis 2005). Sterka and Marriott (2006) showed that the expression of Nod2 receptors and the downstream effector molecule Rip2 in microglial cells are increased by stimulation with MDP and agonists of TLR4 and TLR5.

1.4 Characterisation of the receptor ligands

1.4.1 TLR agonists

To stimulate the TLR system of microglial cells, three well-tested ligands, known to enhance phagocytosis and intracellular killing of bacteria (Ribes et al. 2009), as well as one viral agonist, were used. I will briefly introduce these stimuli in the following sections.

1.4.1.1 The TLR1/2 agonist Pam_3CSK_4

Pam_3CSK_4 is a synthetic tripalmitoylated lipopeptide that imitates a virulent factor occurring on bacteria. When the human body is challenged with *E. coli* the expression of TLR2 is upregulated on monocytes, although it does not correlate with the amount of bacteria (Beran et al. 2011).

1.4.1.2 The viral TLR3 agonist poly(I:C)

Polyinosine–polycytidylic acid [poly(I:C)], a synthetic double-stranded RNA analogue, was used as a viral stimulant of the immune system. Poly(I:C) stimulates immunity through different receptors. At first, poly(I:C) was thought to act as specific TLR3 ligand through its associated adaptor protein TRIF in macrophages, dendritic cells and microglia (Alexopoulou et al. 2001, Town et al. 2006). Recently, Kato and collaborators have identified MDA5 as another mechanism how microglial cells can recognize the presence of viral dsRNA (Kato et al. 2006). MDA5 is not only able to recognize poly(I:C), but also plays a central role in the immune system since MDA5 deficient ($MDA5^{-/-}$) mice are more susceptible to viral infections. In response to poly(I:C), $MDA5^{-/-}$ mice did not produce IFN-alpha and IFN-beta, and showed impaired production of IL-6 and IL-12p40 which indicates that TLR3 is not the only receptor poly(I:C) binds to (Kato et al. 2006). Despite these findings, poly(I:C) behaved in this work as a suitable TLR3 agonist as it has been reported to be more selective than alternative compounds, such as poly(A:U) (Hanisch et al., unpublished observations).

1.4.1.3 The TLR4 agonist LPS

For an activation of TLR4, microglial cells were exposed to LPS from *E. coli* serotype 026:B6. LPS consists of chains of sugar, called polysaccharides, and fatty components and is found in the extern all part of the cell membrane contributing to the structural and functional membrane integrity of Gram-negative bacteria (Silhavy et al. 2010, Wang and Quinn 2010). Upon bacterial lysis, these parts of the membrane are released and then called endotoxins (Akira et al. 2006).

In case of a systemic infection, LPS is able to reach the brain directly fixed on LPS binding protein. This complex ligates to a special co-receptor of TLR4, its prototypic associate glycosylphosphatidylinositol-anchored cluster of differentiation **(**CD) 14, placed on the surface of microglial cells (Poltorak 1998, Akira et al. 2006). The interaction of LPS with CD14 leads to the recruiting of intracellular MyD88, an adaptor protein of IRAK leading to an activation of a cascade of immune reactions (Medzhitov and Janeway 2000, Akira et al. 2006, Carpenter and O´Neill 2007). There are also findings indicating that TLR4 additionally needs MD-2 to form a complex to detect LPS (Shimazu et al. 1999).

1.4.1.4 The TLR9 agonist CpG

Cytosine-phosphate-guanosine (CpG) is a part of the DNA from different organisms. While viral and bacterial DNA contain a high proportion of unmethylated CpG dinucleotides, mammalian DNA has less and mostly methylated CpG (Hemmi et al. 2000). These differences concerning the structure of CpG in the DNA are detected by TLR9 and enable the immune system to distinguish foreign DNA from self-DNA. Underlining this thesis, Hemmi et al. (2000) showed that wild-type dendritic cells express more CD40, CD80, CD86 and major histocompatibility complex (MHC) class II after exposure to CpG than TLR9-deficient cells. This indicates that TLR9 is essential to detect CpG (Hemmi et al. 2000). Today, synthetic unmethylated CpG is used as a PAMP to simulate invading pathogens and induce an immune reaction. Clinical research with CpG focuses on e.g. improving vaccination using it as an immunoadjuvant (Rothenfußer et al. 2001).

1.4.2 The NOD2 receptor agonist MDP

N-Acetylmuramyl-L-Alanyl-D-Isoglutamine also known as MDP is “the minimal bioactive peptidoglycan motif common to all bacteria” (Girardin et al. 2003, p. 8869) that can be recognized by NOD2, inducing different signal cascades. MDP derivatives enhanced phagocytic and microbicidal activities of monocytes and macrophages (Cummings et al. 1980). In human dendritic cells, MDP treatment augmented the expression of major histocompatibility complex class II (MHC class II) antigens (Cooney et al. 2010) and resulted in a weak but consistent up-regulation of CD80 and CD86 (Kramer et al. 2006). The essential structure of MDP has been used as an adjuvant component in vaccines (Chedid 1983).

1.5 Characterisation and pathogenicity of *Escherichia coli*

E. coli is a rod-shaped Gram-negative bacterium which belongs to the *Enterobacteriacea* family. Although it is part of the human gut flora, *E. coli* can cause inflammation when invading other organ systems. Besides infections which often can be treated without sequela (e.g. infections of the urinary tract), *E. coli* is the leading cause of Gram-negative neonatal bacterial sepsis and meningitis with a high associated fatality rate and permanent neurological dysfunction in more than half of the survivors (Dawson et al. 1999, Mittal et al. 2011). *E. coli* also causes meningitis in older (Cabellos et al. 2009) and immunocompromised patients (Roos 2009). To determine which type of *E. coli* was most commonly causing meningitis, cerebrospinal fluid (CSF) of infants with meningitis caused by *E. coil*- were studied. In approximately 80% of these *E. coli* strains the polysaccharide capsule K1 was present (McCracken et al. 1974). Later it has been shown that the presence of this capsule allows *E. coli* strains to survive in the bloodstream and ultimately to cross the blood-brain barrier by penetrating the brain's micro-vascular endothelial cell layer and entering the CNS (Kim 2002) which makes it highly virulent.

1.6 Meningitis

The brain and the spinal cord are surrounded by layers of connective tissue with a protective and metabolic function, the so-called meninges. The inflammation of these layers is defined as meningitis, which can be lethal. Meningitis can be induced by infection with bacteria, viruses or other microorganisms. Clinically, patients suffer from headache, high sensitivity to light, stiffness of the neck, hyperesthesia, fever, nausea, vomiting and confusion (van de Beek et al. 2004). As complications, encephalitis and sepsis with lethal outcomes are possible. Until nowadays, meningitis is a severe disease causing thousands of victims all over the world even in countries with high medical standards and diverse schemes of antibiotic treatments (Hoffmann and Weber 2009). Especially immunocompromised patients such as prematurely born infants and elderly carry a high risk of developing CNS infections including *E. coli* meningitis and meningoencephalitis (Teng et al. 2004, Roos 2009). One cause of this increased susceptibility to CNS infections might be a decreased local immune defence. However, even people with an intact immune system can suffer or even die of the consequences of meningitis, e.g. septic shock or brain oedema, in particular, when the therapy is not immediately started (Pfister et al. 1993, Proulx et al. 2005).

1.7 Outline

Infections with *E. coli* K1 can lead to serious diseases like sepsis, meningitis or meningoencephalitis which are often lethal, especially in immunocompromised patients. Therefore, many studies aimed to strengthen the immune system and its ways to communicate. Many studies have focused on bacterial TLR agonists and their effect on microglial cells but there are no reports about the effect of the viral TLR3 agonist poly(I:C) on the phagocytic activity of microglia.

In addition to TLRs, microglia express different kind of receptors, such as NOD receptors. So far it is known that mutations of NOD2 receptor are associated with chronic inflammatory bowel diseases such as Crohn´s disease. The question whether infections of the brain correlate with abnormalities this receptor is a matter of interest. We do not know whether activation of NOD receptor increases microglial response in case of infection. Therefore following questions should be addressed.

Does stimulation of murine microglial cells with

1. MDP alone
2. Poly(I:C) alone
3. Co-stimulation of MDP and a TLR agonist

enhance

a) Cytokine and chemokine release?
b) Phagocytosis of *E. coli* K1?
c) Intracellular killing of *E. coli* K1?

The aim of this study was to investigate whether the single stimulation of microglial cells with the agonist of the Nod2 receptor (MDP) or the TLR3 [poly(I:C)] have an effect on chemo-/cytokine production and phagocytosis and intracellular killing of *E. coli* K1. In a second set of experiments it was tested whether co-stimulation of MDP with TLR1/2, TLR3, TLR4 or TLR9 agonists influence the previously mentioned parameters.

2 Material and Methods

2.1 Description of the study

In the present study, primary microglial cell cultures from mice aged one to three days were stimulated with agonists of the Nod and TLR systems alone or in combination. The aim was to investigate whether a stimulation of microglial cells with MDP or poly(I:C) alone or the co-stimulation of MDP and different TLR agonists [Pam_3CSK_4, poly (I:C), LPS and CpG] increases chemo- and cytokine release and enables microglial cells to phagocytose and eliminate higher amounts of ingested bacteria in comparison to unstimulated cells. This could improve CNS resistance to infections and therefore especially have impact in immunocompromised patients.

2.2 Materials

Chemicals were used from BioLegend (San Diego, USA), Braun (Melsungen, Germany), Eppendorf (Hamburg, Germany), Greiner bio-one (Solingen, Germany), Invitrogen (Karlsruhe, Germany), InvivoGen (San Diego, USA), Merck (Darmstadt, Germany), R&D-Systems (Wiesbaden, Germany), Roche (Mannheim, Germany), Sarstedt (Nümbrecht, Germany), Sigma-Aldrich Biochemistry (Saint Louis, USA), Thermo Scientific, TECAN (Crailsheim, Germany). They were purchased in commercially available form.

2.2.1 Chemicals

Acetic acid (100%)	Merck, Darmstadt, Germany
Cell Proliferation Reagent WST-1	Cat.No. 11 644 807 001, Roche, Mannheim, Germany
Cytochalasin D (CD)	Sigma-Aldrich, St. Louis, USA
Cytosine-phosphate-guanosine (CpG)	CpG oligodesoxynucleotide 1668 (TCC ATG ACG TTC ATG CT; 6,383 Da; TIB Molbiol, Berlin, Germany)

Chemokine (C-X-C motif) ligand 1 (CXCL1)	DuoSet ELISA Development Kit R&D Systems, Wiesbaden, Germany
Dulbecco's Modified Eagle Medium DMEM + GlutaMAX –I	Gibco, Karlsruhe, Germany
DuoSet ELISA Development Kits	R&D Systems, Wiesbaden, Germany
Escherichia coli K1 *(E. coli* K1)	gift of Dr. G. Zysk, Institute of Medical Microbiology and Virology, Heinrich-Heine-University, Düsseldorf, Germany
Ethyl alcohol	Merck, Darmstadt, Germany
Foetal bovine serum (FBS)	Gibco, Karlsruhe, Germany
Gentamicin	Sigma-Aldrich, St. Louis, USA
Interleucin-6 (IL-6) DuoSet ELISA Development Kit	R&D Systems, Wiesbaden, Germany
Lipopolysaccharide (LPS): *Escherichia coli* serotype 026:B6	Sigma-Aldrich, St. Louis, USA
Muramyl dipetide (MDP)	InvivoGen, San Diego, USA
Naphthyl-ethylendiamin Dihydrochlorid (NED)	Sigma-Aldrich, St. Louis, USA
Pam_3CSK_4 (910.5 Da)	EMC Microcollections, Tübingen, Germany
Penicillin – Streptomycin	Gibco, Karlsruhe, Germany
Phosphate-buffered saline (PBS):	Invitrogen, New York, USA
Polyinosine–polycytidylic acid [Poly(I:C)] (1.5 – 8kb)	InvivoGen, San Diego, USA
Sterile saline	Braun, Melsungen, Germany
Sulfonamid	Sigma-Aldrich, St. Louis, USA
Tumor necrosis factor α (TNF-a) DuoSet ELISA Development Kit	BioLegend San Diego, USA
Trypan blue	Sigma, St. Louis, USA

2.2.2 Equipment and software

Blood agar plates	Microbiology, University of medicine, Göttingen, Germany
Centrifuge	Eppendorf, Hamburg, Germany
Coverslips	Menzel, Braunschweig, Germany
Eppendorf cups	Eppendorf, Hamburg, Germany
Extractor hood	Heraeus, Thermo Scientific
Falcon tubes, 15ml and 50ml	Sarstedt, Nümbrecht, Germany
Genios multiplate reader	Tecan, Crailsheim, Germany
GraphPad Prism 4.0 Software	GraphPad, San Diego, USA
Incubator	Thermo Scientific, Braunschweig, Germany
Microscope (magnification: 40 x):	Olympus, Hamburg, Germany
Neubauer Counting Chamber	LO-Laboroptik, Bad Homburg, Germany
Petri dishes	Greiner bio-one, Solingen, Germany
Pipettes	Eppendorf, Hamburg, Germany
Pipette tips	Eppendorf, Hamburg, Germany
T75 culture flask	Corning Costar, Wiesbaden, Germany
Vortexer	Heidolph REAX top, Schwabach, Germany
Waterbath	GFL, Burgwedel, Germany
24- and 96-well-plates	Greiner bio-one, Solingen, Germany

2.3 Primary mouse microglial cell cultures

2.3.1 Preparation of mouse brains

In advance 70% alcohol was prepared by mixing 700 ml of 99% ethyl alcohol with 300 ml distilled water. To prepare phosphate-buffered saline (PBS), 23,875 g of PBS Dulbecco-powder was mixed with 2.5 l distilled water and mixed by using magnet-rotors. Thereafter the solution was autoclaved. Mixed cultures of astrocytes and microglial cells were prepared as previously described by Ebert et al. (2005). Brains of wild type newborn C57Bl/6 mice, aged one to three days, were used to prepare primary cultures of microglial cells. Mice were killed by decapitation and disinfected with 70% alcohol. The brains were put into a petri dish with iced PBS. Meninges and blood vessels were removed under the microscope with the help of forceps. All material used was sterile. The prepared brains were collected in 50ml falcon tubes filled with 10ml iced PBS, mechanically dissociated and additionally centrifuged (conditions: 1000rpm for 10min at 4°C). The supernatants were removed with the help of a 1ml pipette and the pellet was suspended in 500 μl of complete culture medium [Dulbecco's modified Eagle medium (DMEM) with Glutamax I supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100U/ml penicillin and 100 μg/ml streptomycin] per brain. It will be referred from this point on as complete cell/culture medium. Cells were plated at a density of two brains per T75 culture flask and 11 ml culture medium, which was previously added. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. Culture medium was changed twice a week.

2.3.2 Preparation of cell cultures for different assays

After 10 to 14 days of culture, culture flasks were examined under the microscope to ensure growth and the absence of fungi. Microglial cells were isolated from the mixed glial (astrocytes and microglia) cultures by shaking 200times/min for 30min as previously described by Ebert et al. (2004). Additionally, cell medium with the resuspended microglial was collected in 50ml falcon tubes and centrifuged for 10min (conditions: 250rpm at 20°C) Supernatants were removed with the exception of 5ml; cell sediment was resuspended by gently mixing with a 1ml pipette. For quantification, 10 μl of cell suspension was mixed with 90 μl tryptan blue and counted with the Neubauer Counting Chamber (twice in each harvest). Microglial cells were re-plated in 24-well-plates (for intracellular survival studies) and 96-well plates (for phagocytosis and nitrite assays) at a density of approximately 60,000cells/well and cultured in complete medium. Cells were incubated at 37°C in a

humidified atmosphere with 5% CO_2 for 24 h before stimulation. The harvested cell cultures were fed with 10 ml of complete cell culture medium per flask. After 10 to 14 days the next harvest was done. Flasks were not harvested more than 4 times.

2.4 Stimulation of murine microglial cells

Microglial cell cultures were incubated for 24 h in a humidified atmosphere at 37°C with 5% CO_2. Afterwards, the supernatants were removed with the help of a 1ml pipette and discarded. Microglial cells were exposed to 250 µl/well (in phagocytosis and nitrite assays) or 500 µl/well (in survival assays) of complete culture medium containing a TLR-agonist alone, MDP alone or a combination of MDP and a TLR agonist at different concentrations for additional 24 h. A control group with unstimulated cells, exposed to complete medium alone, was included in all experiments. After 24 h of stimulation, supernatants were removed using a 1ml pipette and collected in 96-well plates to directly perform nitrite assays or stored at –20°C until chemo-/cytokines were measured.

2.5 Measurement of nitric oxide (NO)

Activation of microglia can be measured by the release of nitric oxide (NO). Ebert et al. (2005) have previously shown that CpG and LPS stimulated microglial cells to release NO in a dose-dependent manner. In this work, dose response curves of NO release on microglial cells stimulated with MDP, poly(I:C) and Pam_3CSK_4 were constructed. These data were crucial at deciding which concentrations of ligands of the Nod2/TLR systems were used for later experiments. NO release was quantified in the supernatants of cells stimulated with different concentrations of MDP and poly(I:C) (0.1, 0.3, 1, 3, 10, 30 and 100 µg/ml) as well as with Pam_3CSK_4 (0.000001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1 and 10 µg/ml).

It has been reportet that interferon gamma (IFN-γ) enhances the NO release of microglial cells (Häusler et al. 2002, Ebert et al. 2005). Therefore, cells which were afterwards used for measurement of NO release were stimulated with the TLR1/2, TLR3 or Nod2 ligand in complete medium supplemented with 100 U/ml IFN-γ as co-stimulant. As “positive” control of maximal NO release (Ebert et al. 2005), microglial cells were stimulated with LPS at 1 µg/ml (also with 100 U/ml IFN-γ as co-stimulant).

NO release was quantified by the measurement of nitrite (NO_2^-) accumulation in the supernatants of stimulated microglia. Nitrite, a product of living tissue, leads to a colorimetric

change, when adding the Griess reagent. After 24 and 48h of stimulation, 50 μl of supernatants were taken from each well and put into a 96-well plate. Thereafter, 50 μl of Griess reagent was added without mixing the samples to prevent bubbles (since this would have affected the measurement). After short incubation time (5 – 10 min), the optical density of each sample was measured with the Genios multiple reader at a filter of 570 nm. Concentrations were calculated by the comparison of absorptions with a standard curve.

Griess reagent was prepared as follows:

Solution A: 1g Sulfonamid + 30ml 100% acetic acid + 70ml sterile water

Solution B: 0.1g Naphthyl-ethylendiamin Dihydrochlorid (NED) + 60ml 100% acetic acid + 40ml sterile water

Solution A and B were used at equal volumes and gently mixed to obtain the undiluted solution (=100%).

A standard curve was prepared at following concentrations:

100%; 50%; 25%; 12.5%; 6.25%; 3.125%; 1.5625% and 0% depending on the amount of Griess reagent in the solution. In each dilution step, the same amount of sample and sterilised water was used. For example: 2ml of 100% reagent + 2ml sterilised water led to a concentration of 50% of the solution.

2.6 Measurement of microglial cell viability

To verify microglial cell viability and exclude toxicity of the used stimuli, the water soluble tetrazolium salt (WST)-1 cell proliferation reagent was used. The test is based on a chemical reaction in the respiratory chain which only occurs in viable cells. The splitting of WST-1 by active mitochondria produces a soluble formazan. This reaction leads to a colorimetric change of the solution.

One test for each stimulant was performed. Microglial cells were stimulated with different concentrations of MDP and poly(I:C) (0.1, 0.3, 1, 3, 10, 30 and 100 μg/ml) as well as with Pam_3CSK_4 (0.000001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1 and 10 μg/ml).

A WST-1 solution containing one part of WST-1 reagent solved in nine parts of DMEM at 37°C was prepared (e.g. 500 µl of WST-1 reagent + 4500 µl of DMEM). After 24 h of stimulation, cell supernatants were removed and either used for further tests or stored in 96-well plates at –20°C until the measurement of chemo- and cytokines. Then, 100 µl of WST-1 solution was added per well and cells were incubated for 2 up to 4 h at 37°C in a humidified atmosphere with 5% CO_2. Thereafter, the optical density at 490 nm using a Genios multiplate reader was measured to quantify the formazan dye formed. The metabolic activity of the cells was directly correlated with the absorbance (Ebert et al. 2005).

2.7 Cytokine and Chemokine release

The levels of TNF-α, IL-6 and CXCL1 were measured by using DuoSet ELISA Development Kits according to manufacturer´s instructions and Regen et al. (2010).

2.8 Bacterial strain

For all experiments, a strain of *E. coli* with the antiphagocytic capsule K1, isolated from a child with neonatal meningitis was used (gift of Dr. G. Zysk, Institute of Medical Microbiology and Virology, Heinrich-Heine-University, Düsseldorf, Germany). The *E. coli* strain was suspended in a medium consisting of DMEM supplemented with 10% FBS. For each assay, the concentration of the bacterial inoculum was determined by ten-fold serial dilutions in 0.9% saline and quantitative plating on sheep blood agar plates. A final bacterial concentration of approximately 6×10^6 CFU/well (approximately 100 bacteria per microglial cell) was used for all experiments.

2.9 Quantitative plating

To quantify the number of bacteria in the inoculum, supernatants and lysates, 10 µl of each sample were taken and added to 90 µl sterile saline in sterile 1ml Eppendorf-cups. Additionally 10 µl of this suspension were taken and plated on sheep blood agar plate with a 1ml pipette. For each dilution step, 10 µl were taken from the new dilution and mixed with 90 µl sterile saline. This was done for dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}.

Serial dilution:

$$\begin{aligned} A = \text{dilution of } 10^{-1}: &\quad 10\,\mu l \text{ sample} + 90\,\mu l \text{ saline} \\ \text{dilution of } 10^{-2}: &\quad 10\,\mu l \text{ of } A + 90\,\mu l \text{ saline} \end{aligned}$$

Once the surface on the plate was dry, the plates were incubated at 37°C for 12-24 hours upside down to prevent further dilution from dropping condensate. After incubation, colony-forming units (CFUs) were counted and bacterial concentrations were expressed as CFU/ml.

2.10 Phagocytosis assay

After 24 h of incubation with a TLR agonist alone, MDP alone or a combination of both, supernatants were removed and stored at –20°C until measurement of chemo- and cytokines. Thereafter, cells were washed with warm PBS by using a 1ml pipette. PBS was added and removed carefully without touching the ground of the well plate to prevent destruction of the cell cultures. The pipette was changed after each well. Ribes et al. (2009) have shown that a multiplicity of infection of about 100 bacteria per microglial cell was optimal for phagocytosis assays involving *E. coli* strains. On the basis of these results, this concentration was used for all following experiments. Therefore a bacterial suspension with *E. coli* K1 at a concentration of about 2.5×10^7 CFU/ml was prepared and 0.25 ml of the solution was added to each well of the 96-well plate. Bacteria were co-incubated for 30 and 90 min with microglial cells (co-incubation at 37°C and 5% CO_2). Afterwards, the supernatants were removed, serially diluted in 0.9% saline and plated on sheep blood agar plates to determine the number of extracellular bacteria in the supernatants. Microglial cells were washed twice with warm PBS and incubated for 1h in a solution of DMEM containing 200 μg/ml gentamicin (Sigma-Aldrich, St. Louis) to kill all extracellular nonphagocytosed bacteria (Nazareth et al. 2007). Supernatants were removed and the cell monolayers were washed twice with warm PBS before lysing them with 100 μl of distilled water. Quantitative plating of the lysates on sheep blood agar was performed to enumerate the intracellular surviving *E. coli* K1. To confirm the bactericidal effect of gentamicin, supernatants were plated after 1h of antibiotic incubation. The extracellular amount of bacteria was found to be below 10 CFU/well in each experiment.

2.11 Cytochalosin D inhibition assay

The re-organization of the actin cytoskeleton is essential for the process of phagocytosis (May and Machesky 2001). Cytochalasins are fungal metabolites inhibiting actin polymerization (Cooper 1987) and therefore inhibit the process of phagocytosis. To verify whether bacteria found on microglia are phagocytosed or invaded the cells, cytochalasin D (CD) was used. Nazareth et al. (2007) showed that CD inhibits phagocytosis of *E. coli* for up to 99%. In the current work, CD was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 4 nM and stored at –20°C until use. Microglial cells cultures were prepared and stimulated with the Nod2 and TLR agonists for 24 h as previously described. Afterwards, a final concentration of 10 μM CD/well was added to the cell monolayers for 30 min prior to the addition of bacteria and was present until the end of the experiment (Ribes et al. 2009). The number of bacteria in the supernatants from microglia treated with and without CD was quantified after 90 min of bacterial incubation to assure that CD did not influence bacterial replication.

2.12 Intracellular survival assay

Cells were harvested, plated into 24-well-plates and stimulated as previously described. To control whether phagocytosed *E. coli* K1 were able to survive or even replicate inside microglial cells, microglia were given 90 min to phagocytose bacteria (co-incubation at 37°C and 5% CO_2). Thereafter, 500 μl of DMEM containing 200 μg/ml of gentamicin were added, and cells were incubated for up to 6 h. After 60, 150, 240, 330 min of incubation, supernatants were removed with a 1ml pipette and stored at –20°C. Thereafter, cells were washed twice with warm PBS. Finally, PBS was removed and cells were lysed with 300 μl of distilled water by mixing with a 1ml pipette. The lysates were quantitatively plated on sheep blood agar plates to determine the number of intracellular surviving *E. coli* K1.

2.13 Statistical analysis

GraphPad Prism software was used to perform statistical analysis and graphical presentation. Kolmogorov-Smirnov statistical test showed that some data were not normally distributed. Therefore, all data were analysed by two-tailed Kruskal-Wallis test. When Kruskal-Wallis showed a p value of ≤ 0.05, selected comparisons between two groups were performed by using two-tailed Mann-Whitney U-test. To correct for repeated testing we used the Bonferroni-Holm method (Holm 1979).

3 Results

3.1 NO release assays

As previously described, NO was used as a parameter to measure and correlate microglial activation of the different agonists used in this study. A stimulation time of at least 24 h was chosen as previous experiments had shown that measurements after shorter incubation times with TLR ligands caused milder NO release (Ebert et al. 2005). Since previous experiments had shown the NO dose response curves for TLR4 and TLR9 agonists (Ebert et al. 2005) this work focused on studying the NO release upon stimulation of microglial cells with MDP, Pam_3CSK_4 and poly(I:C) (agonists of Nod2, TLR 1/2 and TLR3, respectively).

The WST-1 test showed no cytotoxic effect of MDP, Pam_3CSK_4 or poly(I:C) at any concentration studied in microglial cells.

Ebert et al. (2005) described LPS as the strongest stimulants of NO release. To compare the potency in terms of NO release of the tested stimuli [MDP, Pam_3CSK_4 and poly(I:C)] I compared my results with the magnitude of NO release by LPS described by Ebert et al. (2005). For an optimised comparison, I chose the results found after stimulation with $0.1\,\mu g/ml$ of each agonist [MDP, Pam_3CSK_4, poly(I:C), LPS and CpG] as this concentration was tested in all groups.

3.1.1 NO release after stimulation with MDP

Stimulation of microglial cells with MDP induced the production of low amounts of NO. The release of NO after 24 h of stimulation were not potent enough to determine the half-maximum stimulating effect on microglial cells (EC_{50}) assessed from the dose-response curve at chosen concentration (Figure 3.1). I did not test higher concentrations as those would be unphysiologically high. MDP at $0.1\,\mu g/ml$ and $10\,\mu g/ml$ induced, respectively, approximately 7.5% and 9.2% of the NO released after stimulation with $0.1\,\mu g/ml$ LPS. Additionally I measured NO release after 48 h of stimulation with MDP at same concentrations. Here, marginal higher amounts of NO release were measured (Figure 3.2).

For further experiments, I decided to use a concentration of $10\,\mu g/ml$ MDP as the lowest concentration inducing the maximum NO release attainable with MDP as well as a submaximum concentration of $0.1\,\mu g/ml$ MDP to demonstrate the synergistic effect of MDP in combination with TLR agonists.

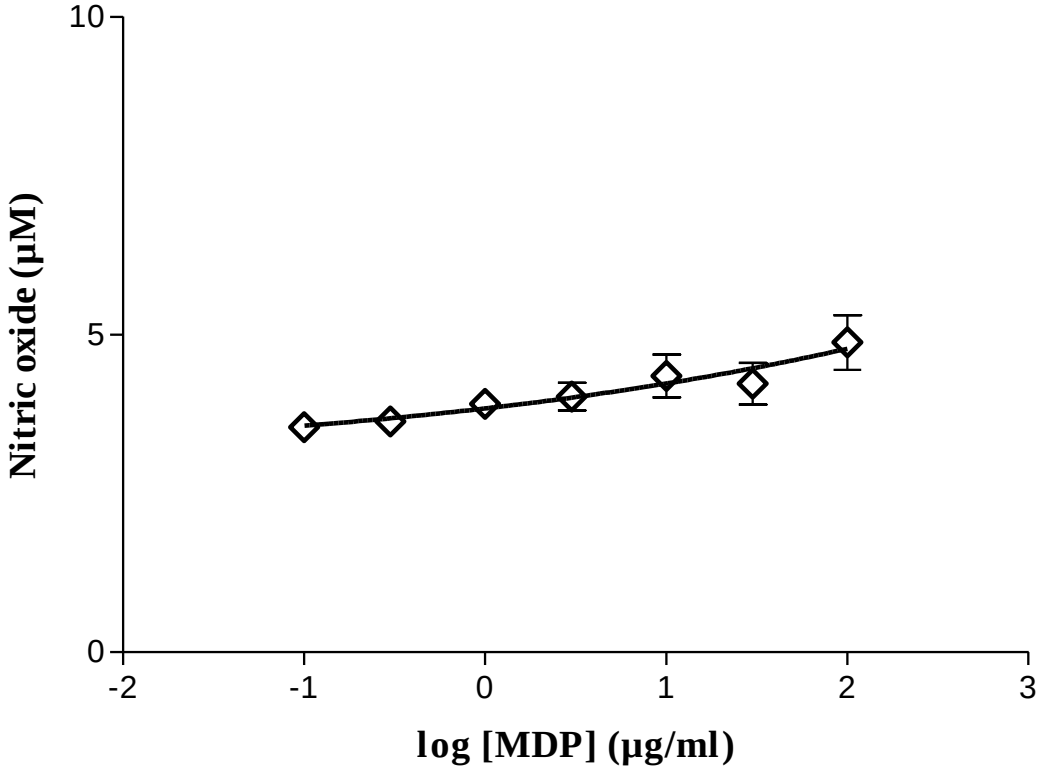

Figure 3.1 NO concentrations after **24h** incubation with MDP at 0.1, 0.3, 1, 3, 10, 30 and 100 μg/ml. Data are shown as mean ± SD.

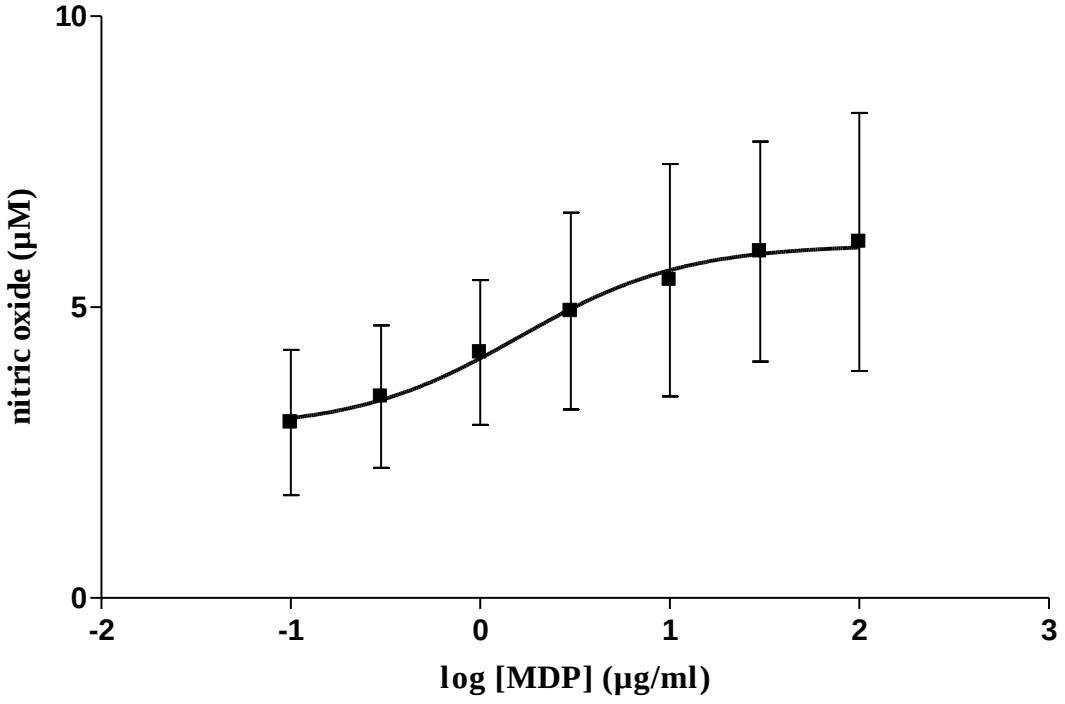

Figure 3.2 NO concentrations after **48h** incubation with MDP at 0.1, 0.3, 1, 3, 10, 30 and 100 μg/ml. Data are shown as mean ± SD.

3.1.2 NO release after stimulation with poly(I:C)

To determine the appropriate concentration of the TLR3 agonist poly(I:C) to stimulate microglial cells, NO assays were performed as previously described. The EC50 assessed from the sigmoidal dose response curve of NO release after 24 h of stimulation was 4.3 μg/ml (Figure 3.3). Poly(I:C) at 0.1 μg/ml and 10 μg/ml induced, respectively, 7.2% and 11.4% of the NO release measured in cells stimulated with 0.1 μg/ml LPS. These results are comparable to the levels of NO measured after stimulation with MDP at the same concentrations.

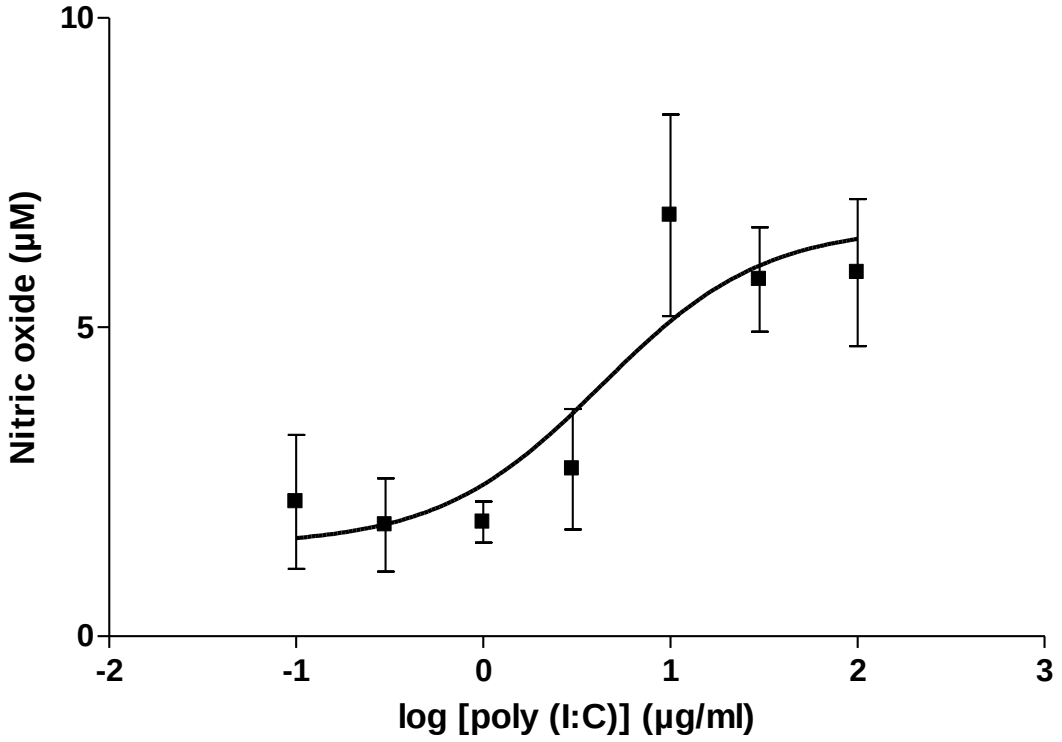

Figure 3.3 NO release after **24 h** of microglial stimulation with poly(I:C) at 0.1, 0.3, 1, 3, 10, 30 and 100 μg/ml. Data are shown as mean ± SD.

3.1.3 NO release after stimulation with Pam_3CSK_4

The EC50 assessed from the sigmoidal dose response curve of NO release after 24 h stimulation with different concentrations of Pam_3CSK_4 was 0.002 μg/ml as demonstrated in Figure 3.4. Pam_3CSK_4 at 0.001 μg/ml and 0.1 μg/ml induced, respectively, 21% and 36.5% of the NO release reached after stimulation with 0.1 μg/ml LPS. Compared to cells stimulated with MPD or poly(I:C), microglia stimulated with Pam_3CSK_4 released about five times more NO at a concentration of 0.1 μg/ml.

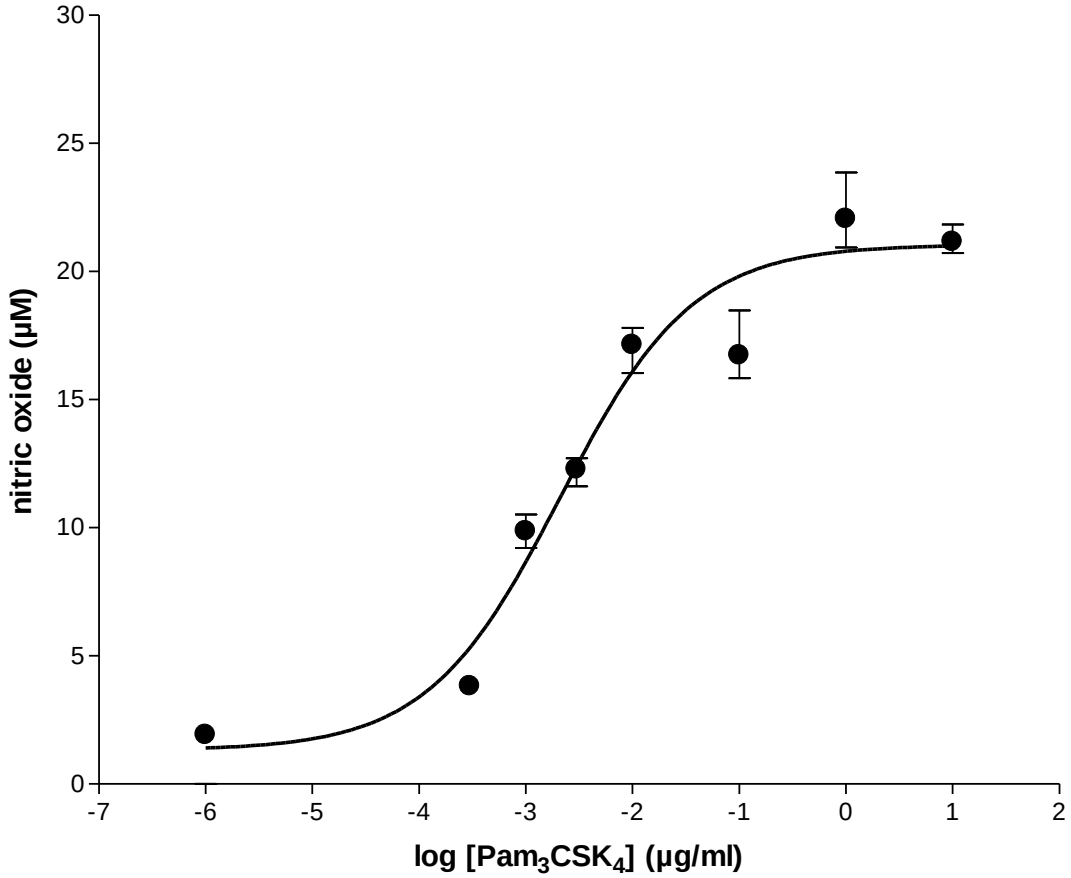

Figure 3.4 NO release after **24h** of microglial stimulation with Pam_3CSK_4 at 0.000001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 10 μg/ml. Data are shown as mean ± SD.

The lowest concentration inducing maximum NO release (from now on referred as the **maximum concentration of the agonist**) was chosen according to published data in case of agonists of TLR4 (LPS) and TLR9 (CpG) (Ebert et al. 2005, Ribes et al. 2009) and NO assays described in the current work in case of ligands of Nod2 (MDP), TLR1/2 (Pam_3CSK_4) and TLR3 [poly(I:C)]. To investigate further studies on synergism of the combination of MDP and a TLR ligand, for each stimulant a concentration inducing a factor of 10^{-2} NO release compared to each maximum concentration was chosen. From now on I will refer to that as the **submaximum concentration of the agonist**.

3.2 Release of chemo- & cytokines

Ribes et al. (2009) showed that murine TLR-stimulated microglial cells (TLR agonists used as the maximum concentration in terms of NO release) released significantly higher levels of chemo- and cytokines compared to unstimulated cells. Stimulation with TLR1/2 (Pam_3CSK_4), TLR4 (LPS), TLR9 (CpG) agonists led to comparable amounts of TNF-α release, whereas the

levels of CXCL1 differed significantly among the stimulated groups. The maximum amounts of CXCL1 release were measured after stimulation with $0.1\,\mu$g/ml Pam_3CSK_4, followed by $0.1\,\mu$g/ml CpG. The lowest amounts of CXCL1 were measured in cells previously stimulated with $0.01\,\mu$g/ml LPS (Ribes et al. 2009).

In the current work, concentrations of TNF-α, CXCL1 and IL-6 were measured in the supernatants of microglial cells ($n \geq 6$ wells/group) after 24 h of incubation with either the different TLR agonists at submaximum concentration or MDP at sub/maximum concentrations alone (Figure 3.5) as well as with MDP at $0.1\,\mu$g/ml and $10\,\mu$g/ml in combination with the submaximum concentrations of TLR agonists [$0.001\,\mu$g/ml Pam_3CSK_4, $0.1\,\mu$g/ml poly(I:C), $0.0001\,\mu$g/ml LPS, $0.01\,\mu$g/ml CpG] (data shown in Figure 3.6, Figure 3.7, Figure 3.8).

3.2.1 Release of chemo- and cytokines after stimulation with a single agonist

Unstimulated cells and cells stimulated with $0.1\,\mu$g/ml MDP or $0.01\,\mu$g/ml CpG did not release measurable amounts of TNF-α, CXCL1 and IL-6 (Figure 3.5). Compared to measurements with higher concentrations of CpG (Ribes et al. 2009), these results led to the conclusion that the concentration of CpG used in this work was too low to evoke chemo- and cytokine release by microglial cells.

Stimulation with maximum MDP concentration as well as stimulation with submaximum concentrations of Pam_3CSK_4, poly(I:C) and LPS alone induced significantly higher release of TNF-α compared to the unstimulated control group [$p < 0.001$ for MDP, $p < 0.001$ for Pam_3CSK_4, poly(I:C) and $p < 0.01$ for LPS] (Figure 3.5). Significantly higher CXCL1 levels were detected after incubation with Pam_3CSK_4 ($p < 0.001$) or LPS ($p < 0.01$) alone compared to unstimulated cells, whereas all other tested groups were devoid of release. The only single stimulant causing significantly higher concentration of IL-6 compared to the control group of cells was LPS ($p < 0.0001$).

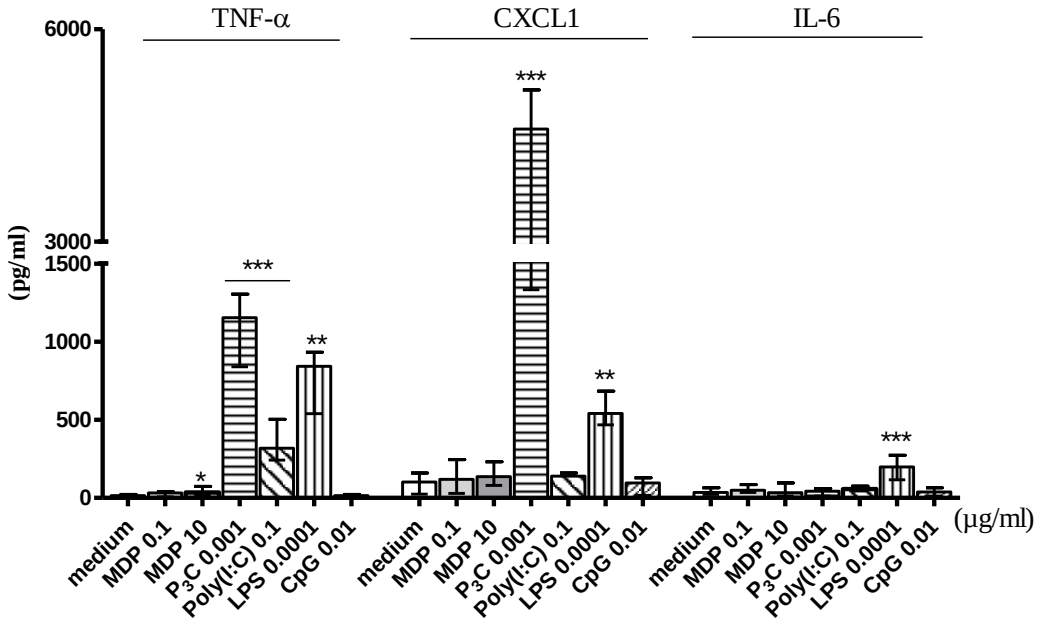

Figure 3.5 Chemo- and cytokine release after stimulation with one compound [MDP, TLR1/2 (Pam_3CSK_4), TLR3 [poly(I:C)], TLR4 (LPS) and TLR9 (CpG)] agonists.

Data are shown as medians (25%/75% interquartile ranges). $n = 6-17$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method. $*p < 0.05, ** p < 0.01, *** p < 0.001$ compared to the unstimulated group.

3.2.2 Release of chemo-/cytokines upon co-stimulation with MDP and TLR agonists

The next step was to find out whether microglial chemo-/cytokine release increased if cells were co-stimulated with MDP and one TLR agonist at the same time. Therefore, cells were incubated in medium containing MDP at $0.1\,\mu$g/ml or $10\,\mu$g/ml in combination with submaximum concentrations of TLR ligands for 24 h and then measurement of TNF-α, CXCL1 and IL-6 levels was performed in the supernatants (Figure 3.6, Figure 3.7, Figure 3.8 respectively).

Microglia co-stimulated with CpG at submaximum concentration and MDP did not release measurable amounts of chemo- and cytokine release. Significantly increased concentrations of TNF-α were observed after co-incubation of poly (I:C) with $0.1\,\mu$g/ml MDP ($p < 0.01$) as well as for Pam_3CSK_4 or poly(I:C) with $10\,\mu$g/ml MDP ($p < 0.001$). The greatest impact on

TNF-α release was seen when cells were stimulated with LPS in combination with sub/maximum doses of MDP ($p < 0.001$) (Figure 3.6).

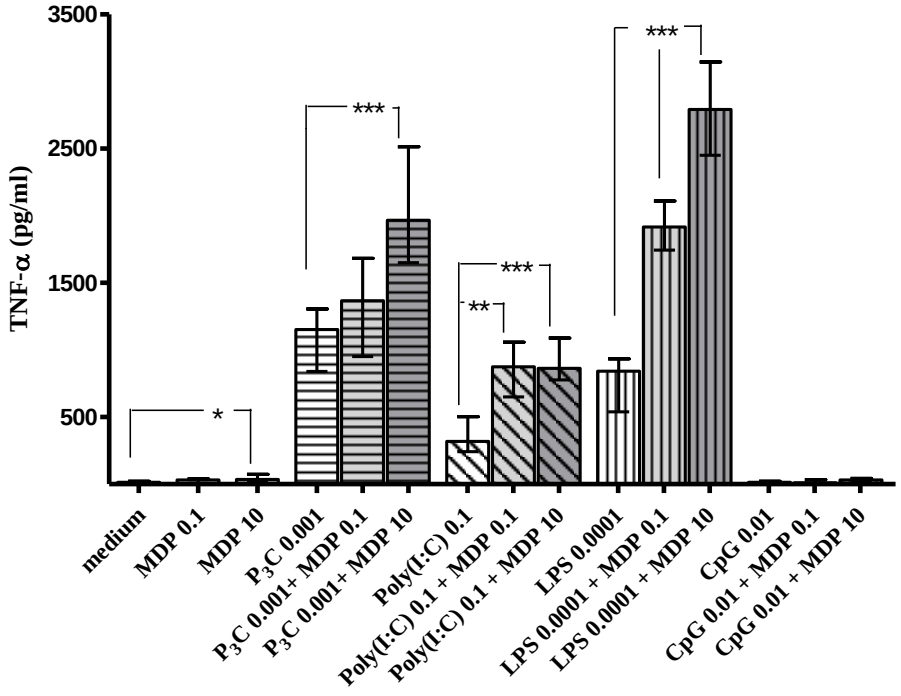

Figure 3.6 **TNF-α release** after stimulation with **0.1 μg/ml** and **10 μg/ml MDP** and **submaximum** concentrations of TLR1/2 (Pam_3CSK_4), TLR3 [poly(I:C)], TLR4 (LPS), TLR9 (CpG) agonists.

Data are shown as medians (25%/75% interquartile ranges); $n = 6-18$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method. $*p < 0.05$ compared to unstimulated control, $**p < 0.01$, $***p < 0.001$ compared to TLR agonists alone.

Similarly, CXCL1 levels (Figure 3.7) increased significantly after co-stimulation of Pam_3CSK_4 or LPS with 0.1 μg/ml MDP ($p < 0.05$ and $p < 0.001$, respectively) and 10 μg/ml MDP ($p < 0.01$ and $p < 0.001$, respectively) compared to stimulation with each agonist alone. The combination of MDP with either TLR3 [poly(I:C)] or TLR9 (CpG) agonists failed to reach any significant differences in comparison to the release of CXCL1 in the control group.

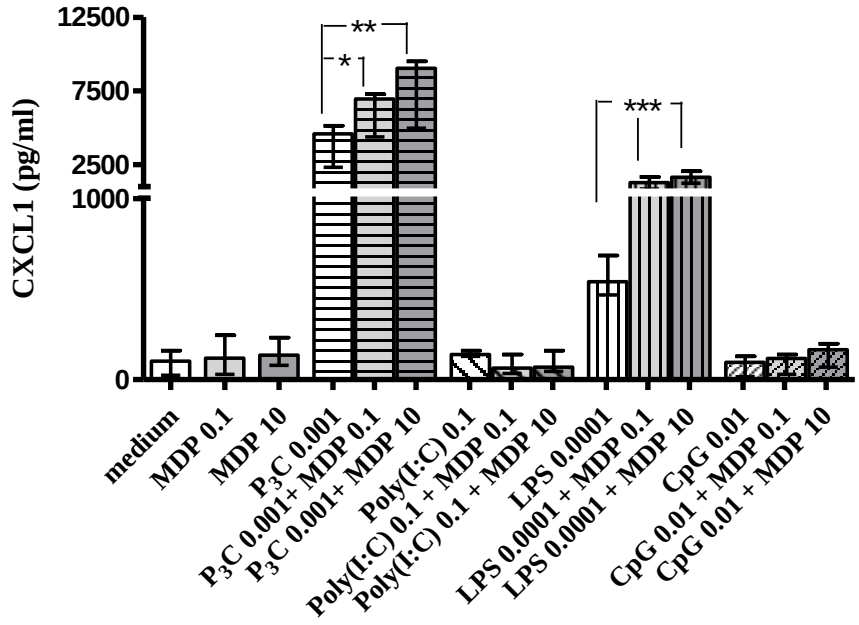

Figure 3.7 **CXCL1 release** after stimulation with **0.1 μg/ml** and **10 μg/ml MDP** and **submaximum** concentrations of TLR1/2 (Pam_3CSK_4), TLR3 [poly(I:C)], TLR4 (LPS), TLR9 (CpG) agonists.

Data are shown as medians (25%/75% interquartile ranges); $n = 6-18$ wells/group. Data were by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to TLR agonist alone.

As mentioned in Figure 3.8, the release of IL-6 was increased when LPS was combined with either low ($p < 0.01$) or high concentrations of MDP ($p < 0.001$). Whereas single stimulation with Pam_3CSK_4 and poly(I:C) failed to cause significant release of IL-6, significantly higher levels were found in the supernatans of microglia stimulated with poly(I:C) combined with submaximum MDP concentration ($p < 0.05$) and Pam_3CSK_4 in combination with 10 μg/ml MDP ($p < 0.01$) or 0.1 μg/ml MDP ($p < 0.05$).

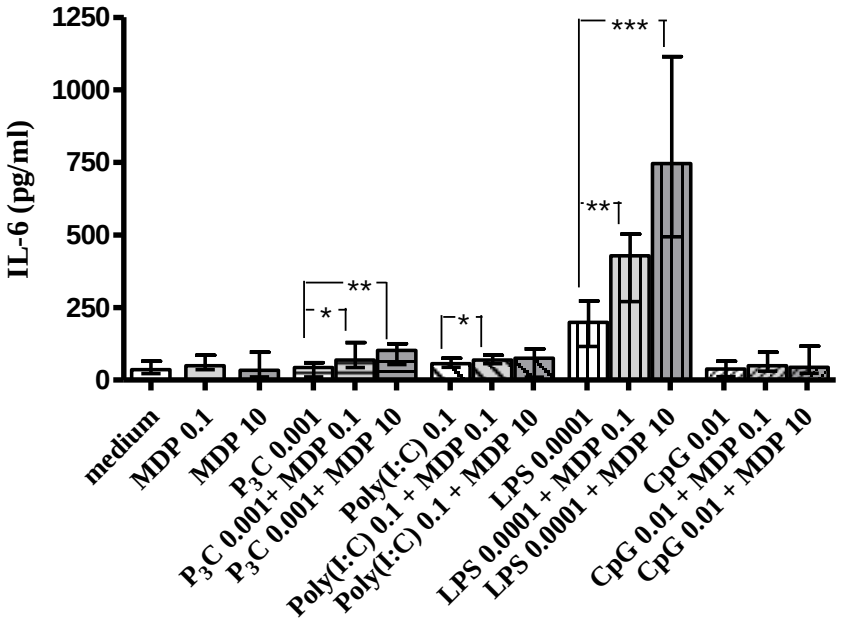

Figure 3.8 **IL-6 release** after stimulation with **0.1 µg/ml** and **10 µg/ml** MDP and **submaximum** concentrations of TLR1/2 (Pam_3CSK_4), TLR3 [poly(I:C)], TLR4 (LPS), TLR9 (CpG) agonists.

Data are shown as medians (25%/75% interquartile ranges); $n = 6-18$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to TLR agonist alone.

3.3 Phagocytosis assays

After microglial stimulation, a bacterial suspension with *E. coli* K1 at a concentration of 2.5×10^7 CFU/ml was added for 30 min or 90 min to allow phagocytosis. Quantitative plating of the lysates on sheep blood agar was performed to enumerate *E. coli* K1 surviving intracellularly. For each independent experiment, the phagocytic rate of *E. coli* K1 quantified in unstimulated cells was considered to be 100%. Thereafter, the number of bacteria ingested by the stimulated groups was calculated as the percentage of the amount of *E. coli* K1 ingested by the unstimulated group. The n-values in every experiment differ because control groups and MDP stimulated groups were included in every experiment and therefore have higher n-values.

3.3.1 Phagocytosis after stimulation with MDP

Microglial cells were incubated with sub/maximum concentrations of MDP. Thereafter, cells were co-incubated for 30 min and 90 min to phagocytose bacteria as shown in Figure 3.9. Compared to the unstimulated group, microglial cells previously stimulated with maximum

concentration of MDP, phagocytosed more bacteria than cells stimulated with submaximum concentration of MDP after **30 min** of co-incubation with E. coli K1 ($p < 0.001$; $p < 0.05$, $n = 69 - 77$ wells/group).

The same tendency could be observed when microglia were allowed to phagocytose bacteria for **90 min** ($p < 0.001$ for MDP at $0.1\,\mu$g/ml and $10\,\mu$g/ml with respect to the unstimulated group), with the highest rate of phagocytosis calculated in cells previously stimulated with $10\,\mu$g/ml MDP ($n = 89 - 93$ wells/group).

At both tested time points, maximum concentrations of MDP caused significantly higher phagocytosis rates compared to submaximum concentration ($p < 0.05$ at 30 min; $p < 0.0001$ at 90 min).

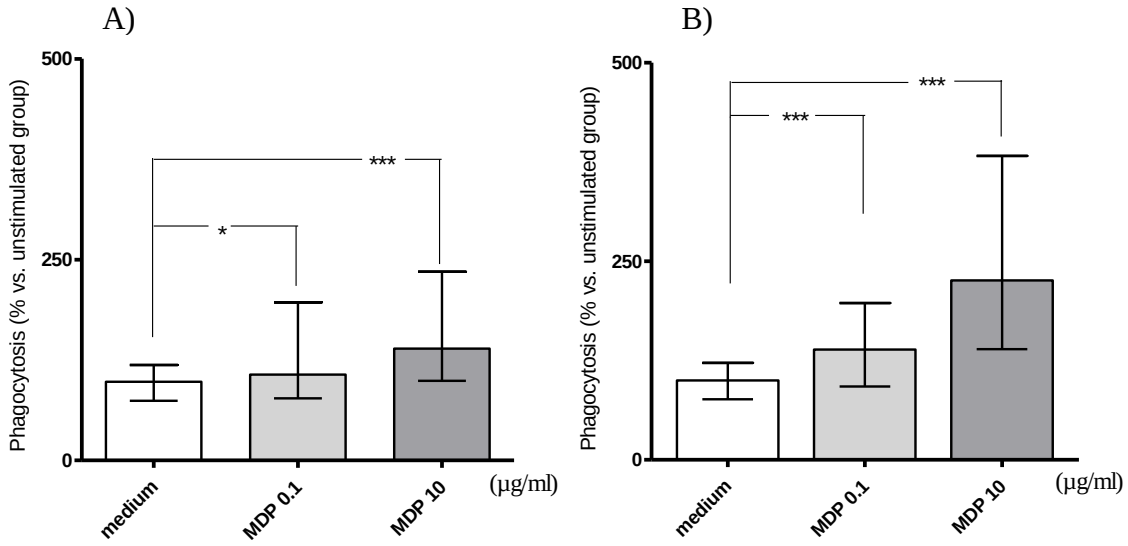

Figure 3.9 MDP stimulation enhanced phagocytosis of *E. coli* K1 after **30 min** (A) and **90 min** (B) of incubation.

Data are shown as medians (25%/75% interquartile ranges); A) $n = 69 - 77$ and B) $n = 83 - 93$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method. $*p < 0.05$, $***p < 0.001$ compared to unstimulated microglial cells.

3.3.2 Phagocytosis after stimulation with poly(I:C)

To determine whether microglia ingest more bacteria after co-incubation with the viral TLR3 agonist poly(I:C), cells were stimulated for 24 h with poly(I:C) at submaximum ($0.1\,\mu$g/ml)

and maximum ($10\,\mu$g/ml) concentrations. Thereafter, cells were challenged for 30min (Figure 3.10 A) and 90min (Figure 3.10 B) with *E. coli* K1.

After 30min of exposure, cells stimulated with poly(I:C) at $0.1\,\mu$g/ml and $10\,\mu$g/ml ingested more bacteria than unstimulated cells ($p < 0.05$, $p < 0.01$, respectively). An increase in the time of co-incubation of poly(I:C)-stimulated cells and bacteria up to 90min led to higher phagocytic rates compared to the unstimulated group ($p < 0.001$).

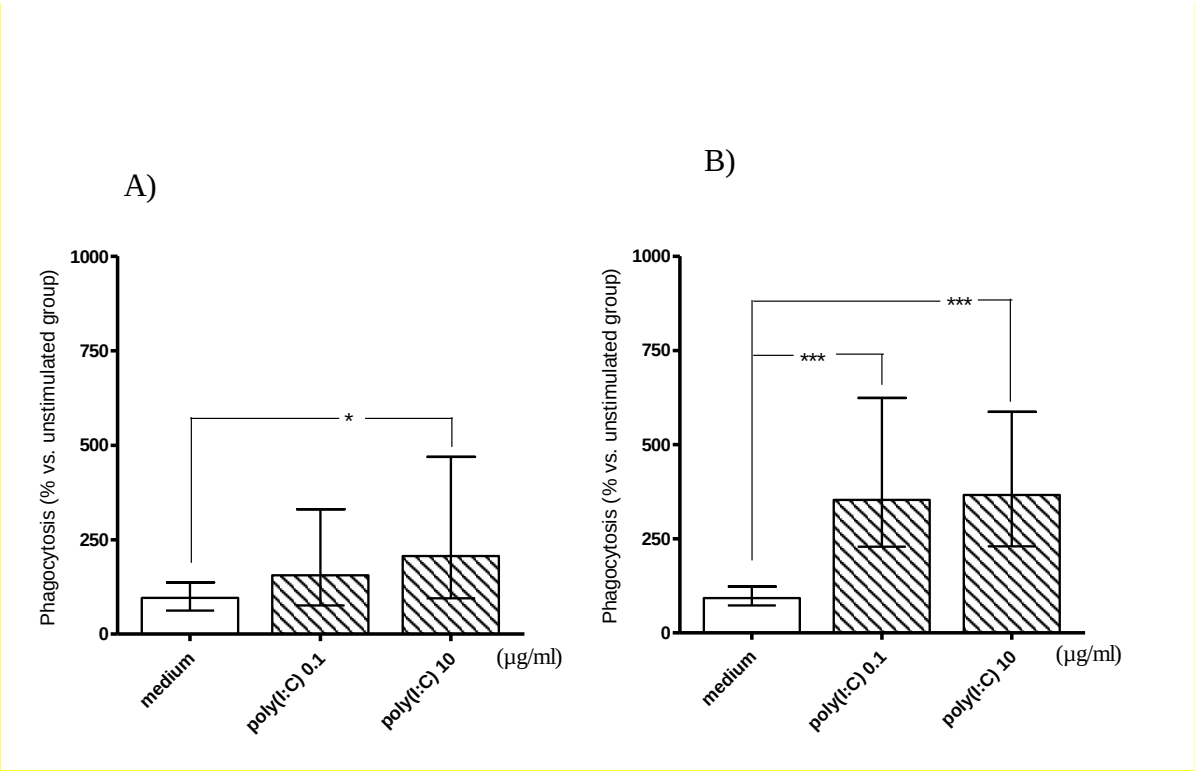

Figure 3.10 Phagocytic rates in poly(I:C)-stimulated microglia in (A) **30min** and (B) **90min** phagocytosis assays

Data are shown as medians (25%/75% interquartile ranges); A) $n = 14-20$ wells/group and B) $n = 19-20$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method. $*p < 0.05$, $***p < 0.001$ compared to unstimulated microglial cells.

3.3.3 Phagocytosis inhibition studies with CD

The ingestion of *E. coli* K1 was reduced by $>90\%$ after 90min of phagocytosis in cells treated with CD compared to the group without CD as presented in Figure 3.11. This indicates that the uptake of *E. coli* K1 by poly(I:C) and MDP-stimulated cells depends on the

rearrangement of the actin cytoskeleton. The inhibitory effect of CD on the action of TLR1/2 (Pam_3CSK_4), TLR4 (LPS), TLR9 (CpG) agonists were already shown by Ribes et al. (2009).

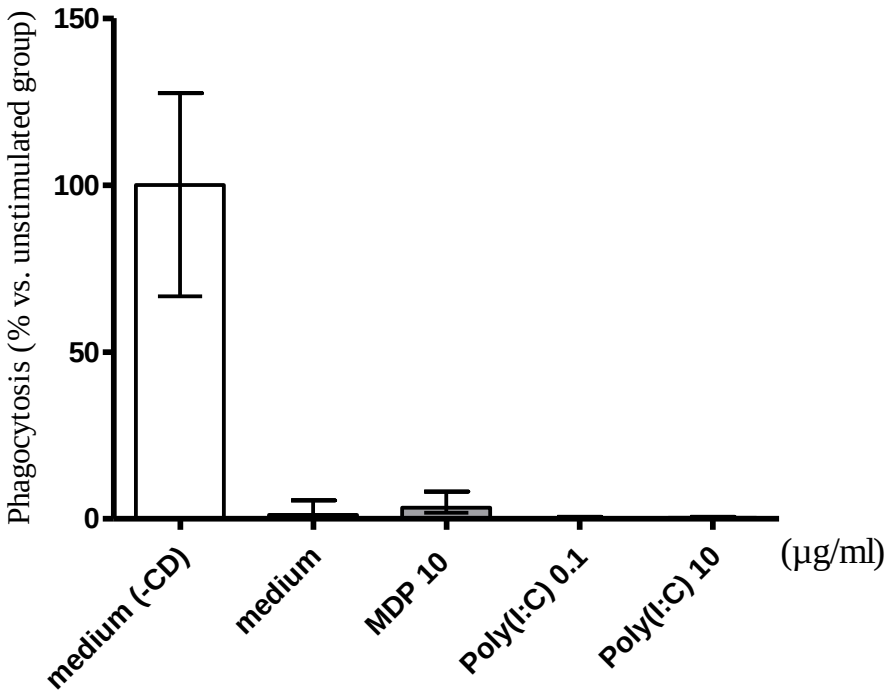

Figure 3.11 Inhibitory effect of cytochalasin D (CD) on phagocytosis of *E. coli* K1.

Data are shown as medians (25%/75% interquartile ranges). $n = 5-16$ wells/group. Phagocytosis of unstimulated cells that were not treated with CD was considered 100%.

3.3.4 Comparison of the phagocytic rates after stimulation of the Nod2 and TLR systems

Prior experiments had shown that MDP and poly(I:C) enhanced phagocytosis of *E. coli* K1. It is known that Pam_3CSK_4, LPS and CpG activate murine microglial cells to ingest more bacteria (Ribes et al. 2009). Therefore, the next question addressed was to compare the magnitude of the responses achieved with poly(I:C)- and MDP-stimulated cells compared to cells activated with the other TLR agonists. Therefore I compared the phagocytic rate of microglial cells previously exposed to each agonist (MDP, Pam_3CSK_4, poly(I:C), LPS or CpG) at submaximum and maximum concentrations. Microglial cells were stimulated for 24 h and then incubated with *E. coli* K1 for 30 min and 90 min.

In all groups and time points, cells stimulated with either Nod2 or TLR ligands had a higher phagocytic rate than unstimulated microglia. Stimulation with most of the tested TLR agonists alone induced stronger microglial phagocytosis than the Nod2 system alone after 30 min (Figure 3.12) and 90 min (Figure 3.13) of challenge with *E. coli* K1. Except for poly(I:C)-stimulated cells, microglia stimulated with agonists at maximum concentration ingested more bacteria than cells stimulated with agonists at submaximum concentration (Figure 3.13).

In the 30 min phagocytosis assays (Figure 3.12), higher numbers of ingested bacteria were counted in cells stimulated with submaximum concentrations of CpG ($p < 0.001$) and Pam_3CSK_4 ($p < 0.01$) than in groups incubated with submaximum concentrations of poly(I:C), MDP or LPS. The highest numbers of ingested bacteria were quantified in cells stimulated with maximum concentrations of CpG, LPS and followed by Pam_3CSK_4 and MDP (all $p < 0.001$) and poly(I:C) ($p < 0.01$).

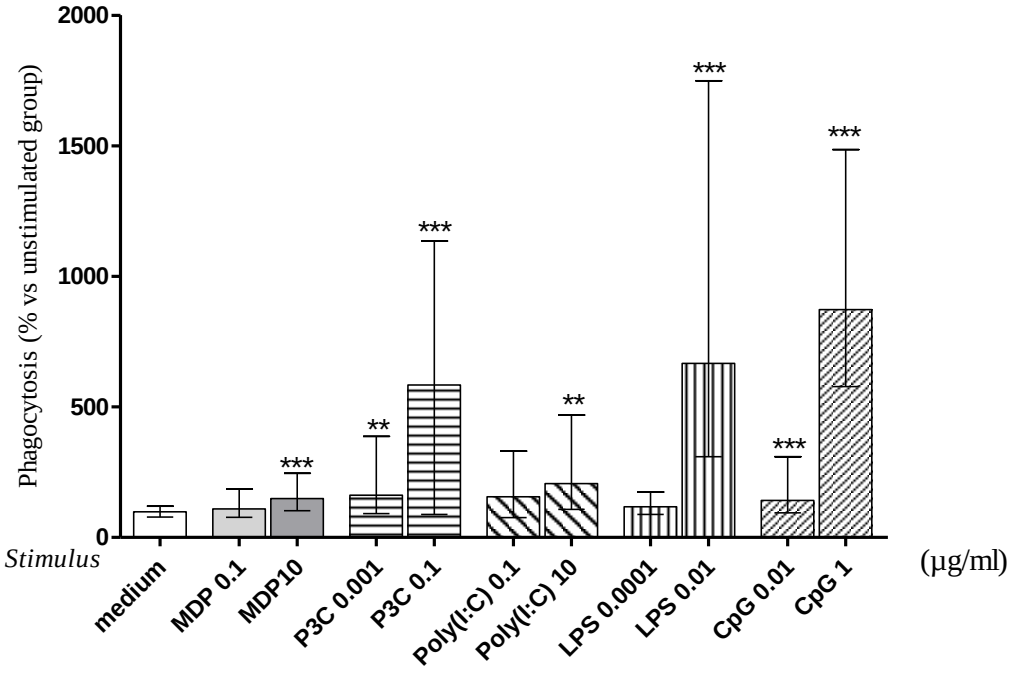

Figure 3.12 Stimulatory effect of agonists of the MDP and TLR systems on phagocytosis after **30min** of co-incubation of microglia and bacteria.

Data are shown as medians (25%/75% interquartile ranges). $n = 10 - 78$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method. $** p < 0.01$, $*** p < 0.001$ compared to unstimulated control.

When bacteria were incubated for 90 min with cells stimulated with submaximum concentrations, phagocytic rates were higher in the Pam_3CSK_4, poly(I:C) and LPS groups ($p < 0.001$) than in the MDP or CpG groups ($p < 0.01$) (Figure 3.13). For groups of cells stimulated with maximum concentrations, the highest phagocytic rates were achieved in the CpG, LPS groups followed by the Pam_3CSK_4, MDP or poly(I:C) groups (all $p < 0.001$). In contrast to the co-incubation for 30 min, stimulation with LPS at submaximum concentration led to a higher phagocytic rate than the rate observed in unstimulated cultures ($p < 0.001$) (Figure 3.13).

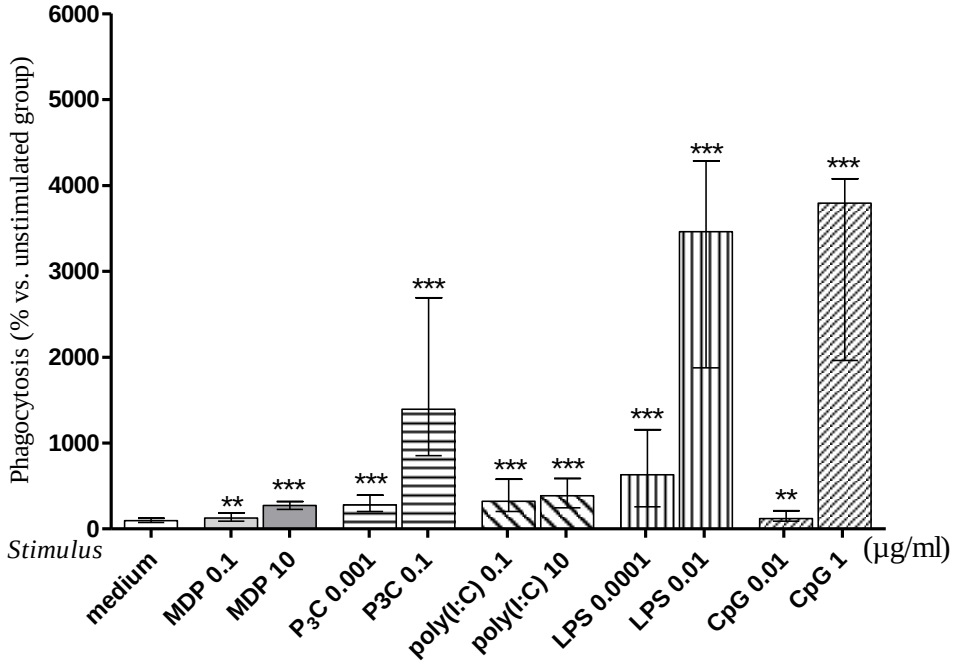

Figure 3.13 Stimulatory effect of MDP and TLR after **90 min** of phagocytosis.

Data are shown as medians (25%/75% interquartile ranges). $n = 11 - 60$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method. $** p < 0.01$, $*** p < 0.001$ compared to unstimulated control.

3.3.5 Phagocytosis after co-stimulation of MDP and TLR agonists

In all experiments, unstimulated microglia cells ingested the lowest number of bacteria. After having shown that MDP had a stimulatory effect on microglial cells, I chose MDP as the reference control group instead of unstimulated cells for all graphs showing co-stimulation of MDP and TLRs, as the aim of the current work was to find out whether there is a synergistic

effect between these two pathways. A synergistic effect of a combination on *E. coli* phagocytosis was assumed when the phagocytic rate by the combination was significantly higher ($p < 0.05$) than the phagocytic rate by each agonist alone.

3.3.5.1 Phagocytosis after 30 min of incubation with *E. coli* K1

The addition of MDP enhanced the phagocytic rates of cells treated with submaximum concentrations of TLR ligands [poly(I:C), LPS, CpG] as shown in Figure 3.14 for $0.1\mu g/ml$ MDP and Figure 3.15 for $10\,\mu g/ml$ MDP. A synergistic bacterial uptake was observed when sub/maximum concentrations of MDP were tested with submaximum and maximum concentrations of LPS ($p < 0.0005, p < 0.0001$ respectively). Also, the addition of $10\,\mu g/ml$ MDP to submaximum concentration of poly(I:C) or CpG had a synergistic effect on the phagocytic rate ($p < 0.05$) (Figure 3.15). The addition of MDP at $0.1\mu g/ml$ or $10\,\mu g/ml$ to Pam_3CSK_4 at submaximum concentration did not increase phagocytosis.

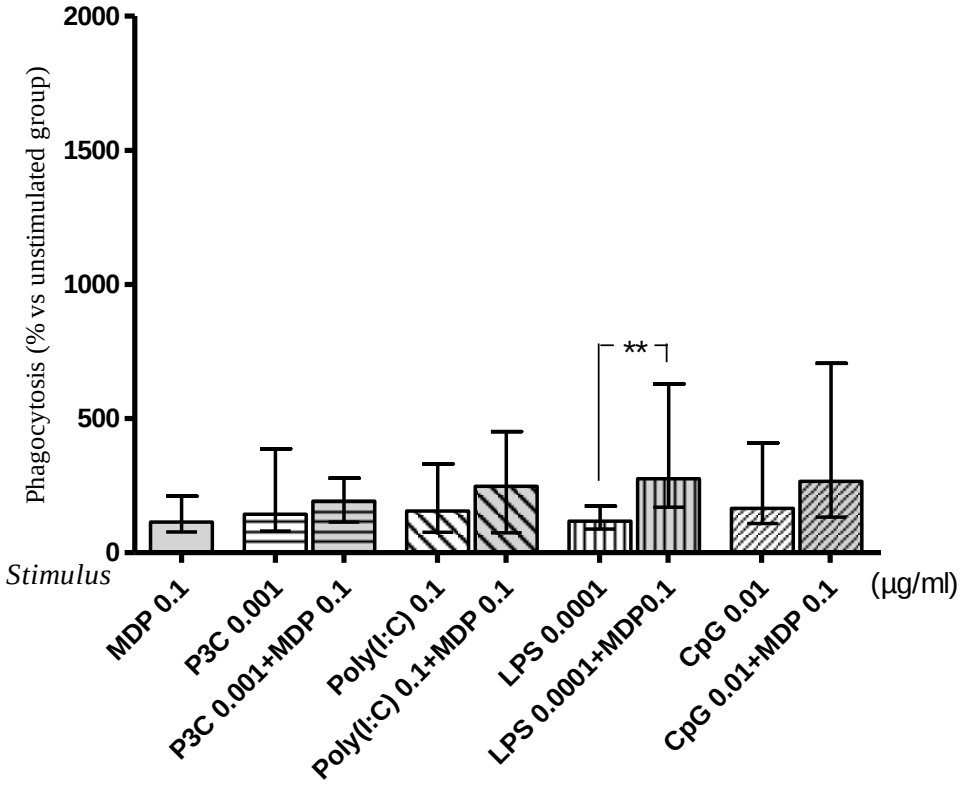

Figure 3.14 Synergistic effect on **30 min** phagocytosis after stimulation with **submaximum** concentrations of **MDP and TLR agonists**.

Data are shown as medians (25%/75% interquartile ranges); $n = 12 - 79$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method. $** p < 0.01$ compared to TLR agonist alone.

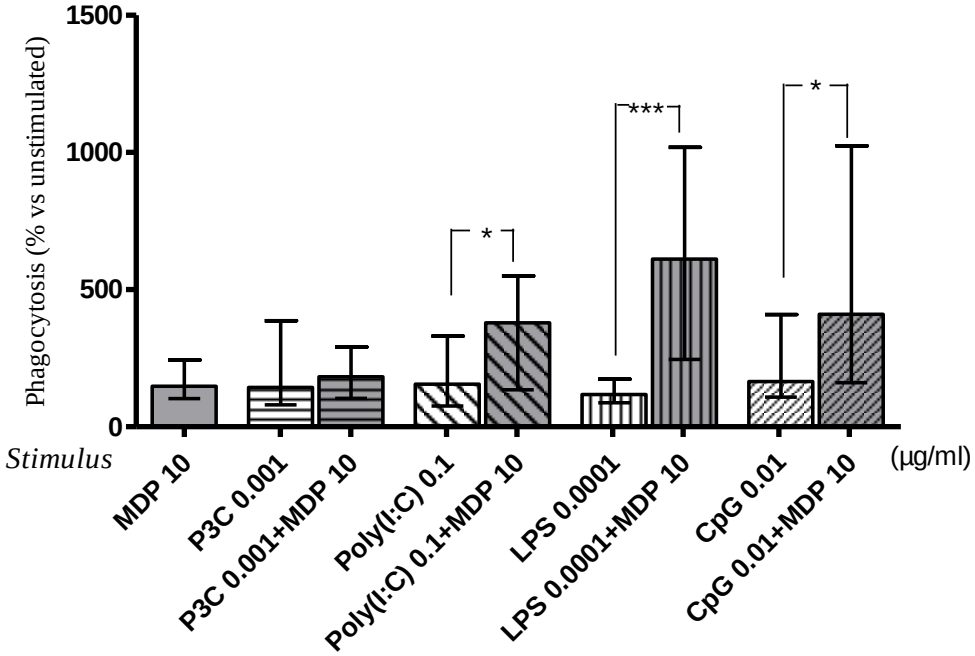

Figure 3.15 Synergistic effect on **30 min** phagocytosis after stimulation with **10 µg/ml MDP** and **submaximum** concentrations of **TLR agonists**.

Data are shown as medians (25%/75% interquartile ranges); $n = 12 - 78$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method. $*p < 0.05$, $***p < 0.0001$ compared to TLR agonist alone.

Co-stimulation with TLR agonists at maximum concentration and $10\mu g/ml$ MDP (Figure 3.16) resulted in nearly same amounts of phagocytosed bacteria compared to combinations with $0.1\mu g/ml$ MDP (Figure 3.17). However, none of the tested combinations showed a synergistic effect.

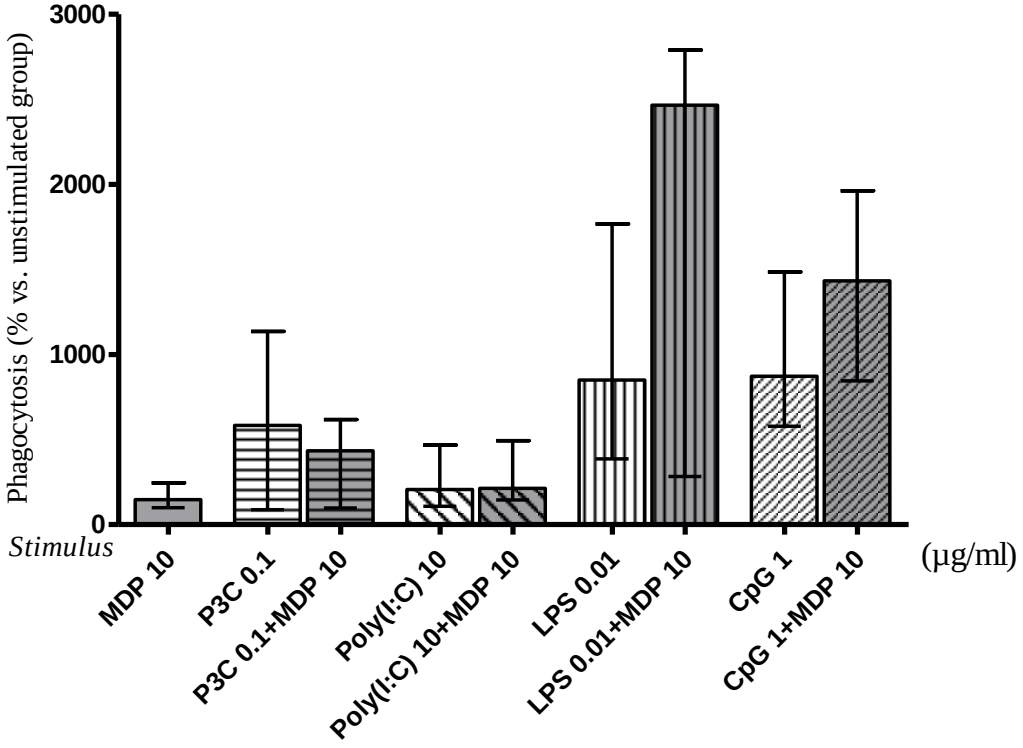

Figure 3.16 Synergistic effect on **30 min** phagocytosis after stimulation with **maximum** concentrations of **MDP and TLR agonists**.

There was no increasing effect on phagocytosis after co-stimulation of TLR1/2, 3, 4 and 9 agonists and MPD at maximal concentration. Data are shown as medians (25%/75% interquartile ranges), $n = 10 - 82$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method. Although data show high ranges of differences it was not statistically different because of the statistical spread.

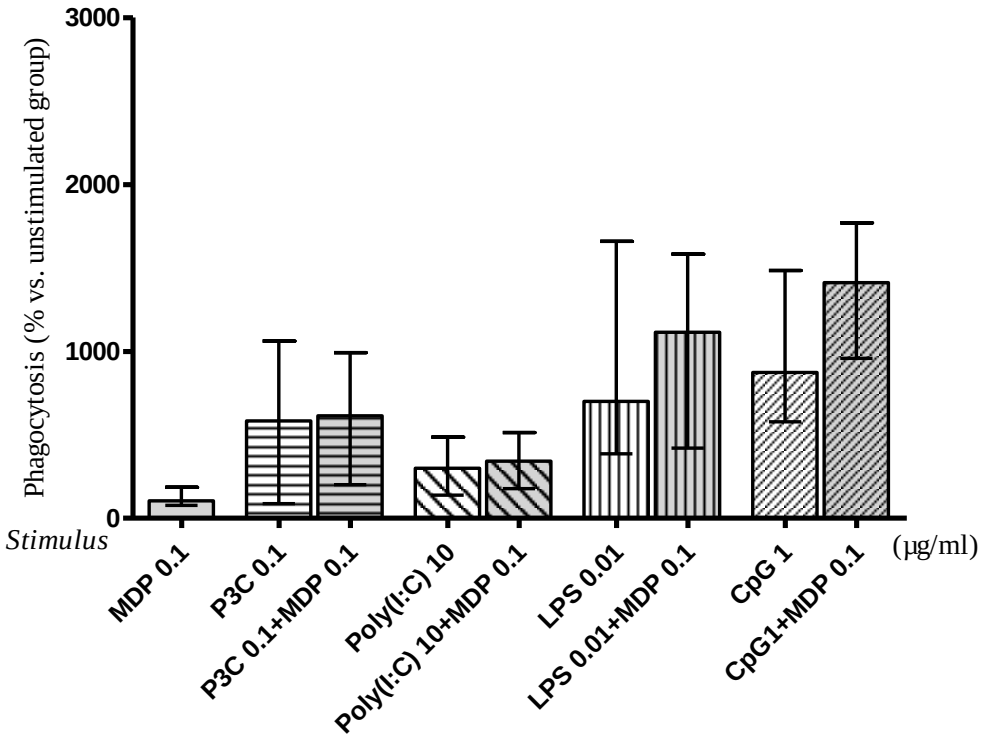

Figure 3.17 Synergistic effect on **30 min** phagocytosis of *E. coli* K1 after stimulation with **0.1 µg/ml MDP** and **maximum** concentrations of **TLR agonists**.

Data are shown as medians (25%/75% interquartile ranges). $n = 10 - 72$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method. Although data show high ranges of differences it was not statistically different because of the statistical spread.

3.3.5.2 Phagocytosis after 90 min of incubation with *E. coli* K1

Similar to the results in the 30 min phagocytosis assays, the addition of MDP enhanced the phagocytic rates of cells treated with submaximum concentrations of TLR ligands after 90 min of *E. coli* K1 challenge. Synergism was observed when sub/maximum concentrations of MDP were co-incubated with submaximum concentrations of Pam_3CSK_4 ($p < 0.05$) and CpG ($p < 0.01$) (Figure 3.18 and Figure 3.19). In contrast to the observations in 30 min phagocytosis assays, the combination of MDP and submaximum doses of LPS did not show a synergistic effect on phagocytosis (Figure 3.20 and Figure 3.21) whereas those combinations let to a significant rise of the release of tested chemo- and cytokines in the cultures supernatans (Figure 3.6,Figure 3.7 and Figure 3.8).

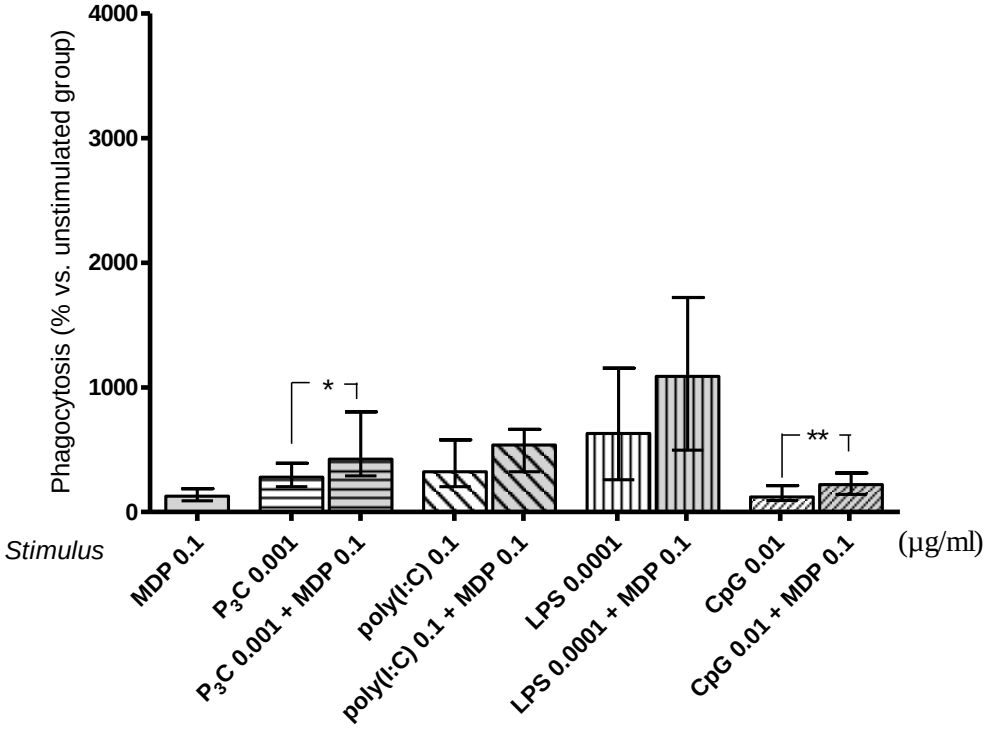

Figure 3.18 Co-stimulation of Nod2 and TLR agonists at **submaximum** concentration after **90 min** of exposure to *E. coli* K1.

Data are shown as medians (25%/75% interquartile ranges). $n = 11-53$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method. $*p < 0.05$, $**p < 0.01$ compared to TLR agonist alone.

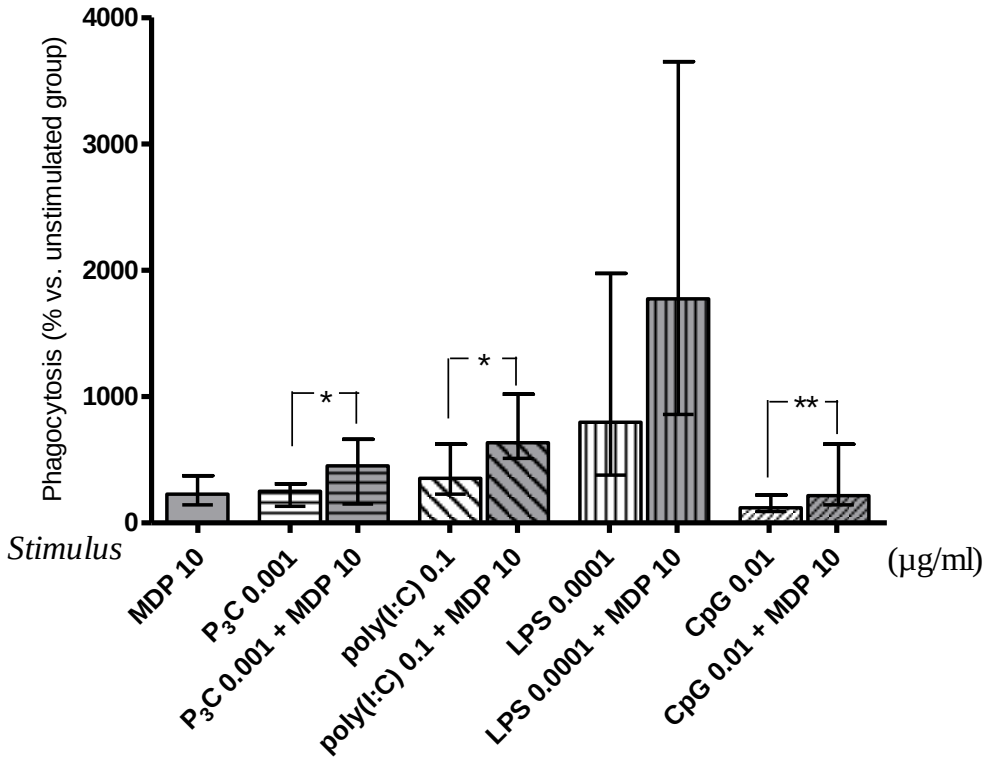

Figure 3.19 Stimulatory effect of **10 µg/ml MDP** and **TLR agonists** at **submaximum** concentration after **90 min** of exposure to *E. coli* K1.

Synergistic effect could be seen after adding MDP to TLR1/2, TLR3 and TLR9 agonists [Pam_3CSK_4, poly(I:C) and CpG]. Data are shown as medians (25%/75% interquartile ranges); $n = 15 - 62$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method. $*p < 0.05$, $**p < 0.01$ compared to TLR agonist alone.

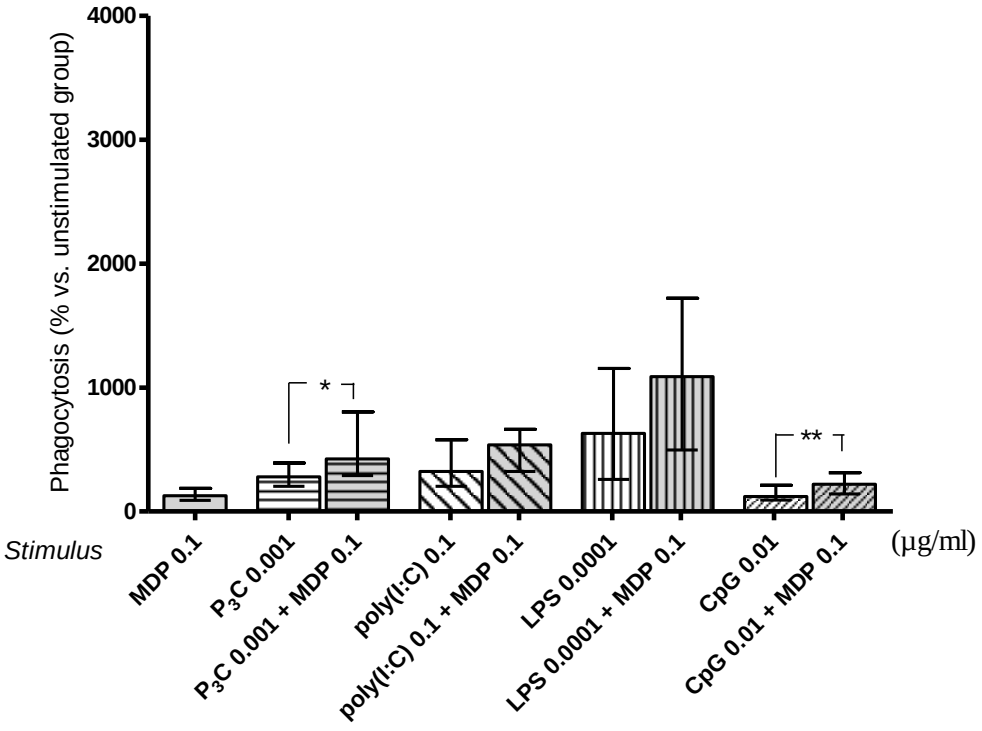

Figure 3.20 Co-stimulation of MDP and TLR agonists at **submaximum** concentration after **90 min** of exposure to *E. coli* K1.

Data are shown as medians (25%/75% interquartile ranges). $n = 11-55$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method. $*p < 0.05$, $**p < 0.01$ compared to TLR agonist alone.

A synergistic effect of the phagocytic rate was found when cells were co-stimulated with MDP in combination with maximum concentrations of Pam_3CSK_4 ($p < 0.05$) (Figure 3.21) and poly(I:C) ($p < 0.05$) (Figure 3.21 and Figure 3.22). Addition of MDP to LPS and CpG failed to reach any statistical significance.

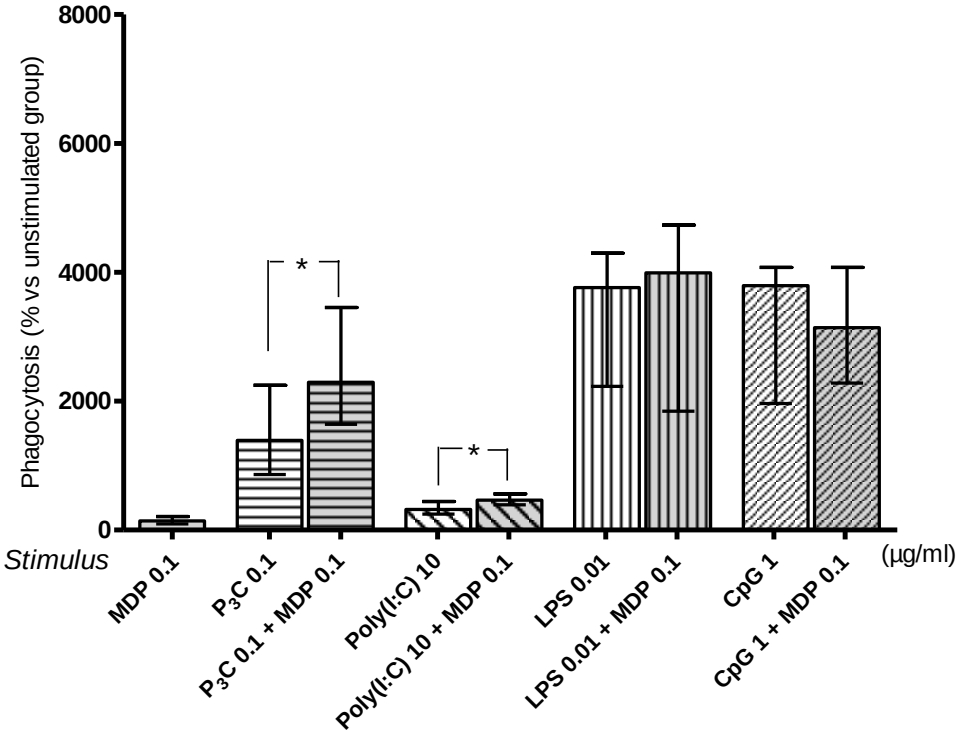

Figure 3.21 Phagocytosis after co-stimulation with **0.1 µg/ml MDP** and **TLR agonists** at **maximum** concentration after **90 min** of exposure to *E. coli* K1.

Data are shown as medians (25%/75% interquartile ranges); $n = 11 - 55$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method; $*p < 0.05$ compared to the TLR agonist alone.

Co-stimulation of MDP and TLR agonists both at maximum concentrations led to a significant increase of bacterial uptake only in case of poly(I:C) (Figure 3.22) whereas the combinations with Pam_3CSK_4 and LPS failed to stimulate phagocytosis significantly compared to the phagocytic rates achieved by the TLR agonist at maximum concentration. In case of a co-stimulation with MDP and maximum concentrations of CpG, the phagocytic rate was slightly lower in the combination than in CpG alone ($p > 0.05$).

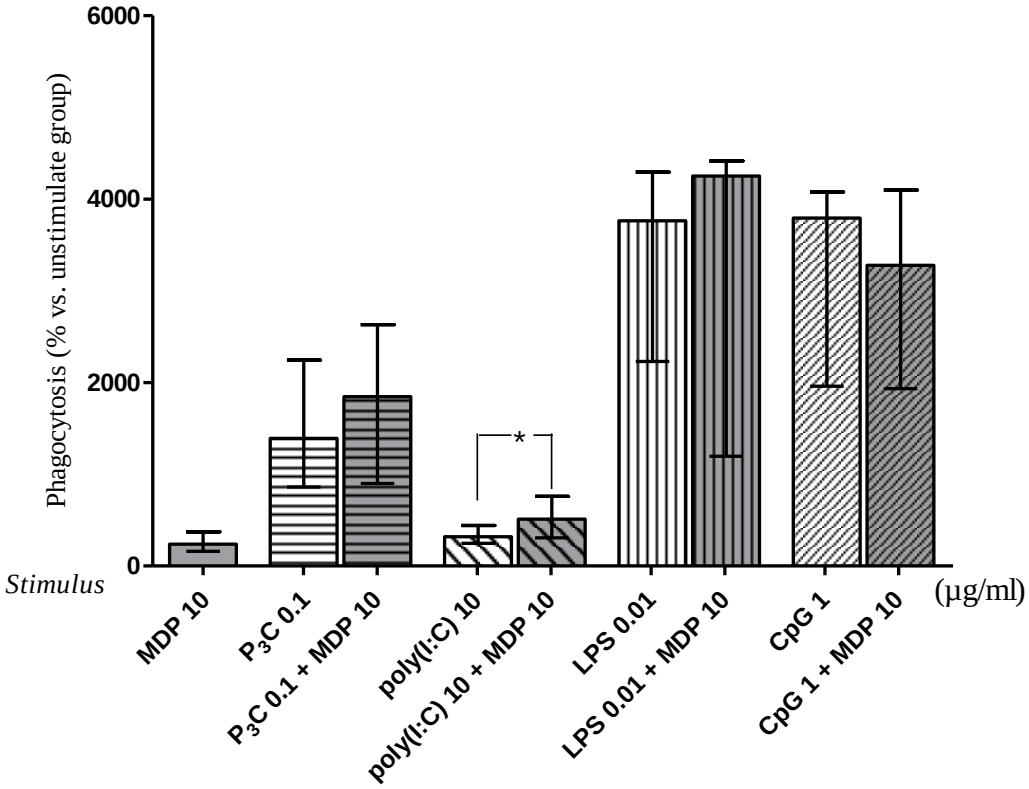

Figure 3.22 Stimulation with Nod2 and TLR agonists at **maximum** concentration after **90 min** of exposure to *E. coli* K1.

Data are shown as medians (25%/75% interquartile ranges); $n = 12 - 50$ wells/group. Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method; $*p < 0.05$ compared to TLR agonist alone.

3.4 Intracellular survival assay

After demonstrating the enhanced ability of microglial cells to phagocytose bacteria upon stimulation with MDP or poly(I:C) alone, as well as with the combination of MDP and certain TLR agonists, I studied whether stimulated microglia could eliminate ingested bacteria at higher rates than unstimulated cells. The intracellular survival assays were performed with 10 μg/ml MDP and submaximum concentrations of TLR agonists alone (Figure 3.23) and with 10 μg/ml MDP in combination with submaximum concentrations of TLR agonists (Figure 3.24). A control group with unstimulated cells was included in all experiments. The numbers of surviving intracellular bacteria were determined by quantitative plating at

different time points ($60, 150, 240, 330\,\text{min}$). In all groups, the amount of intracellular surviving bacteria decreased throughout the experiment (Figure 3.23 and Figure 3.24). To determine whether there is a synergistic effect on intracellular killing, statistical analyses were shown in Table 3.1. The n-values in every experiment differ because control groups and MDP stimulated groups were included in every experiment and therefore have higher n-values.

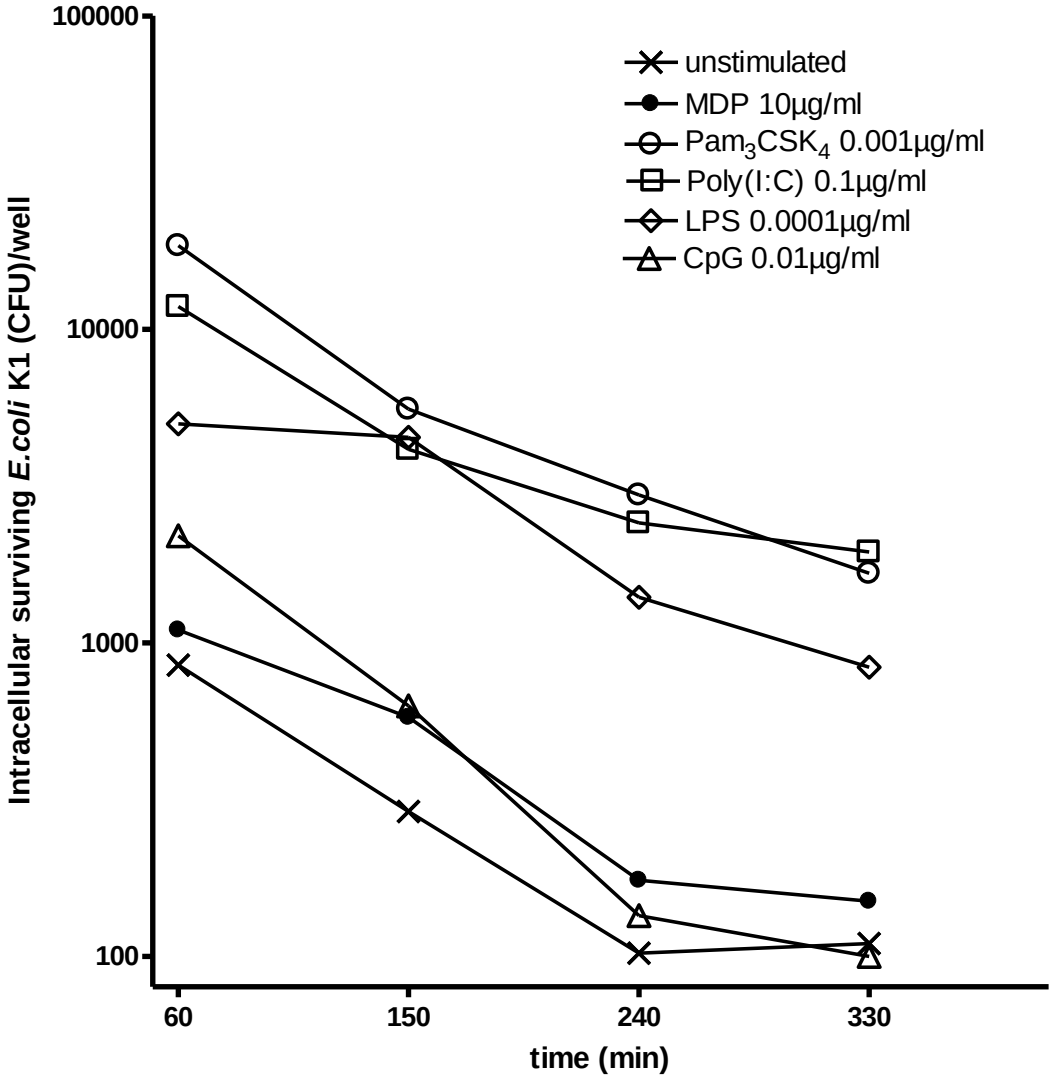

Figure 3.23 Time course of bacterial killing of *E. coli* K1 after single stimulation with **10 µg/ml MDP** and **submaximum** concentrations of **TLR agonists**.

For each group, intracellular survival is expressed as the number of recovered bacteria expressed in logarithmic scale at the different time points. Data at different time points is shown as median; $n = 6 - 30\,\text{wells/group}$.

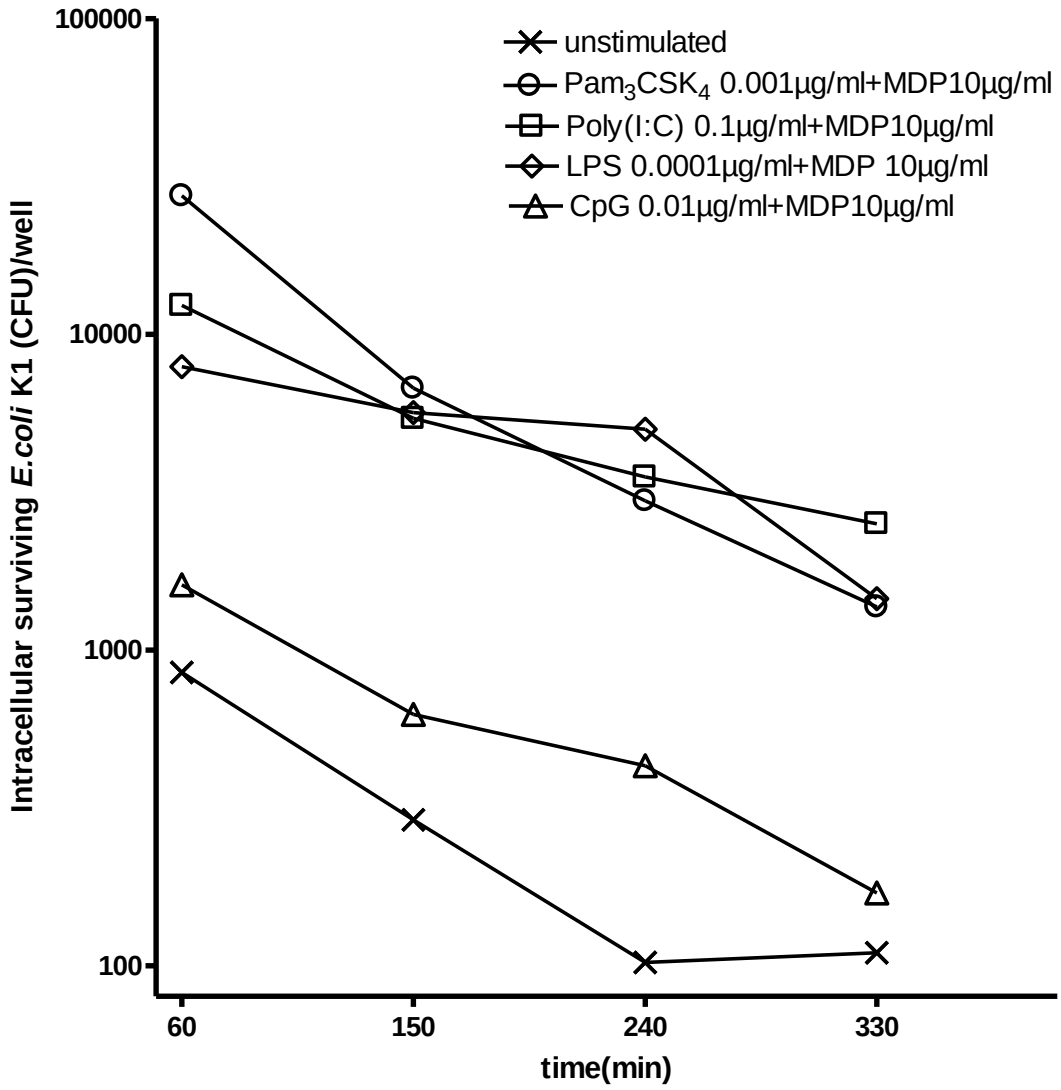

Figure 3.24 Time course of bacterial killing after **combined** stimulation with **10 µg/ml MDP** and **submaximum** concentrations of **TLR agonists**.

For each group intracellular survival is expressed as the number of recovered bacteria expressed in logarithmic scale at the different time points. Data at different time points are shown as median; $n = 6 - 30 \text{wells/group}$.

For each group, the absolute amount of killed bacteria was calculated as the difference between each individual value at 330 min and the median of ingested bacteria of the respective group after 60 min of gentamicin exposure, which I defined as initial point in Table 3.1. Stimulation of microglia with either MDP or one TLR agonist alone increased the number of phagocytosed bacteria (initial point) and killed bacteria in comparison to unstimulated cells (after 330 min) ($p < 0.001$) (Table 3.1). The combined stimulation of microglial cells with MDP and Pam_3CSK_4 or LPS resulted in significantly higher intracellular killing of *E. coli* K1, compared to cells stimulated with each agonist alone ($p < 0.001$). In contrast, the combinations of CpG with MDP and MDP with poly(I:C) slightly reduced the number of intracellular killed bacteria ($p > 0.05$) (Table 3.1).

Table 3.1 Intracellular survival

	Phagocytosed *E. coli* K1 at initial point	**Intracellularly killed *E. coli* K1 after 330 min**
medium	835 (612 / 1218) ††	743 (687 / 784) ††
MDP 10	1350 (1000 / 1830)	1195 (1045 / 1260)
P3C 0.001	16000 (12200 / 18500)	14957 (14330 / 15411)
P3C0.001+MDP10	20000 (16500 / 27500)	18873 (18629 / 19020)***
Poly(I:C)0.1	11834 (9017 / 13062)	9883 (9184 / 10044)
Poly (I:C)0.1+MDP10	12359 (10113 / 13967)	9849 (8896 / 10121)
CPG 0.01	2200 (1288 / 6375)	2100 (1733/2148)
CPG 0.01 + MDP10	1610 (1325 / 14275)	1440 (1064 / 1478)
LPS 0.0001	5650 (4738 / 8708)	4390 (3800 / 4830)
LPS 0.0001+MDP10	7900 (6450 / 9167)	6920 (5825 / 7027)***

Data are shown as medians (25 % / 75 % interquartile ranges). Data were analysed by Mann–Whitney U-test and corrected for repeated testing with the Bonferroni–Holm method; n=6–30 wells/group. Data were from at least three independent experiments. Difference between unstimulated and every stimulated group were statistical significant. ††$p < 0.0001$ compared to stimulation with all agonists, ***$p < 0.001$ compared to TLR agonists alone. Difference between unstimulated and every stimulated group alone was statistical significant.

4 Discussion

TLRs and Nod-like are examples of germline-encoded PRRs, which enable innate immunity to detect PAMPs (Medzhitov and Janeway 2000, Akira et al. 2006). Microglial cells are the local macrophages of the CNS (Hanisch and Kettenmann 2007) and express different members of the TLR family (TLR1-9), which sense diverse PAMPs such as lipopeptides, virus-derived double-stranded DNA and LPS (Olson and Miller 2004). Furthermore, they express Nod2 and low levels of Nod1 (Sterka and Marriott 2006). NOD2 functions as an intracellular receptor of the minimal PGN motif common to all bacterial PGN: MDP (Girardin et al. 2003). After the recognition of pathogens, microglias have various functions and abilities such as the release of NO, chemo- and cytokines or the transformation into a phagocytic phenotype (Hanisch and Kettenmann 2007, Ribes et al. 2009).

Measurements of NO release were performed to assess microglial activation. In case of LPS and CpG, the minimal concentration inducing maximum NO release were chosen according to previously published data (Ebert et al. 2005), where LPS at a concentration of 1µg/ml was defined as the strongest stimulant inducing maximum NO release (100%). In the present work, NO assays were performed to detect the lowest concentration of Pam_3CSK_4, poly(I:C) and MDP inducing maximum NO release. As submaximum concentration inducing NO release, a concentration 100 times lower was chosen. The results showed that the viral TLR3 agonist poly(I:C) and the Nod2 ligand MDP elicited nearly the same amounts of NO when microglia were stimulated with the same concentrations of each stimulant. MDP and poly(I:C) achieved only about 7% of the maximum NO release whereas a stimulation with Pam_3CSK_4 elicited about 36% compared to cells stimulated with $0.1\,\mu l/\mathrm{ml}$ LPS.

In a previous report microglia stimulated with $0.01\,\mu l/\mathrm{ml}$ LPS and $1\,\mu l/\mathrm{ml}$ CpG, the lowest concentrations inducing maximum NO release, (Ebert et al. 2005) ingested comparable numbers of *E. coli* K1 in $30\,\mathrm{min}$ and $90\,\mathrm{min}$ phagocytosis assays (Ribes et al. 2009). These findings indicated that the ability of stimulated microglia to ingest bacteria might correlate with NO release. However, in the present study this correlation was not seen with all tested stimuli. Whereas cells previously stimulated with e.g. the viral TLR3 agonist poly(I:C) and the Nod2 ligand MDP released the same amount of NO when tested at same concentration, the number of ingested bacteria after $90\,\mathrm{min}$ of exposure to *E. coli* K1 was about three times higher in poly(I:C)-stimulated cells (Figure 3.9 and Figure 3.10). These findings were observed for other stimuli and concentrations as well. Therefore, I assume that NO can be

considered as one indicator of the magnitude of cell activation promoted by each single stimulant but does not allow comparisons among different stimuli. Possibly an experimental setting for phagocytosis and intracellular killing with concentrations of stimuli that elicit the same amount of NO would have allowed a more accurate conclusion concerning the potency of low concentrations. The same observation can be made in terms of release of pro-inflammatory molecules such as TNF-α, CXCL1 and IL-6. These are known indicators of cell activation that support the immune system in pathogen clearance (Sterka et al. 2006, Ribes et al. 2009) but in case of overstimulation of the immune system could lead to fatal consequences (Aloisi 2001). Figure 3.5 shows that Pam_3CSK_4-stimulated cells released the highest amount of TNF-α and CXCL1, whereas cells stimulated with MDP and CpG at submaximum concentrations were nearly devoid of chemo- and cytokine release. Concerning MDP, similar results were found in further investigations on the release of chemo- and cytokines (Ribes et al. 2012). Whereupon a stimulation with the viral TLR3 agonist poly(I:C) elicited only significant levels of TNF-α. Chemo- and cytokine release showed that, in case of stimulation with submaximum concentrations, the TLR system elicits stronger activation of microglial cells than the Nod2 system. The low chemo-/cytokine release observed after stimulation with submaximum concentrations of MDP ($0.1\mu l/ml$) and CpG ($0.01\mu l/ml$) could be interpreted as these tested concentrations failed to cause a significant level of stimulation necessary for the elimination of invading pathogens. However, further experiments studying in more detail pathogen phagocytosis and survival assays revealed that this was not true. Therefore the question is whether measurement of NO release is the only way to determine cell activity.

The next question addressed was to find out whether stimulated microglia were able to kill more ingested bacteria than unstimulated cells. Stimulation with TLR1/2, TLR4 and TLR9 agonists enhanced phagocytose and intracellular killing of *E. coli* (Ribes et al. 2009).

Phagocytosis and intracellular survival assays showed that microglia exposed to MDP or poly(I:C) ingested and killed higher numbers of a pathogenic *E. coli* K1 strain compared to unstimulated cells although cells stimulated with MDP "did not significantly augment the basal expression of cell surface molecules for antigen presentation and T cell stimulation" (Ribes et al. 2012, p 21). Microglial stimulation with submaximun and maximum concentrations of MDP and poly(I:C) increased phagocytosis as well as intracellular killing of *E. coli* K1 despite the low release of tested chemo- and cytokines. However, the percentages of bacterial uptake in MDP and poly(I:C)-treated cells were lower than in cells stimulated with maximum concentrations of Pam_3CSK_4, LPS or CpG similarly to the previously

described release of chemo- and cytokines. The number of surviving intracellular *E. coli* K1 decreased after stimulating murine microglia with agonists of TLR1/2 (Pam_3CSK_4), TLR4 (LPS) and TLR9 (CpG) at maximum concentration in terms of NO release (Ebert et al. 2005, Ribes et al. 2009). Here, I was able to show that this is not only true for cells stimulated with submaximum concentrations of the above mentioned agonists but also for poly(I:C) and MPD. Furthermore, the addition of maximum concentrations of MDP to cells stimulated with TLR agonists led to higher numbers of intracellular killed bacteria in case of LPS-MDP and Pam_3CSK_4-MDP combinations. This suggests a possible augmentation of selected microglial functions upon stimulation with these particular combinations. In contrast, the absolute number of intracellular killed bacteria was lower when maximum concentrations of MDP were added to submaximum concentrations of poly(I:C) and CpG compared to those found after stimulation with the agonists alone.

In case of infection, different PRRs located either on the cell surface or in intracellular compartments might recognize the invading pathogen at the same time. Sterka and Marriott (2006) showed an upregulation of Nod2 expression after exposure to Gram-negative bacteria, MDP and TLR4 and TLR5 ligands. The next aim was to find out whether a potential collaboration of the NOD and TLR system enhanced microglial responses. Guo et al. (2006) showed an increased expression of P2X4R, a purinergic ion channel receptor having a central role in CNS inflammation in rat microglia (Guo and Schluesener 2005) after stimulation with MDP plus TLR agonists. Experiments with murine peritoneal macrophages, human mononuclear (Netea et al. 2005) and dendritic cells (Kramer et al. 2006) showed an increase of TLR2 mediated pro- and anti-inflammatory molecules through co-stimulation with MDP as well as modulator effects of NOD2 on TLR3 and TLR4 signalling, whereas no effect could be found for TLR9. The same could be found with respect to the release of chemo- and cytokines in the present work. Watanabe et al. (2004) described an inhibitory effect on the release of IL-12 and IFN-γ when dendritic cells were stimulated with Pam_3CSK_4 in combination MDP. Contrarily, I found an increase of TNF-α, CXCL-1 and IL-6 release after stimulation with MDP plus Pam_3CSK_4 similar to the findings of Netea et al. (2005) concerning IL-10 and TNF-α. An increase of TNF-α, CXCL-1 and IL-6 release could also be found after co-stimulating with MDP plus LPS and in case of IL-6 and TNF-α for poly(I:C) plus MDP. In the present study, cells showed the highest release of chemo- and cytokines after co-stimulation with MDP and TLR agonists as shown in Figure 3.6, Figure 3.7 and Figure 3.8. While the release of TNF-α and IL-6 were enhanced after the combined stimulation with MDP and Pam_3CSK_4 (TLR1/2 agonist), poly(I:C) (TLR3 agonist) and LPS (TLR4 agonist),

the level of CXCL1 increased significantly after simultaneous stimulation with MDP and LPS or Pam_3CSK_4, whereas the addition of MDP to CpG (TLR9 agonist) did not increase chemo- or cytokine release. Although cells stimulated with CpG at submaximum concentration alone or in combination with $0.1\,\mu g/ml$ and $10\,\mu g/ml$ MDP failed to elicit significant amounts of the chemo- and cytokines measured, microglial cells previously stimulated with CpG ingested significant higher amounts of bacteria. This leads to the question whether this stimulation caused the release of chemo- and cytokines not measured in this work. Co-stimulation of microglial cells with the Nod2 ligand MDP and TLR agonists showed an increase of bacterial uptake compared to stimulation with the TLR agonists or MDP alone, especially when bacteria were co-incubated for $90\,min$. Whereas the addition of submaximum or maximum concentration of MDP to cells stimulated with the different TLR agonists at maximum concentration did not enhance phagocytosis after $30\,min$ of challenge with *E. coli* K1, bacterial ingestion was significantly higher after $90\,min$ of challenge. These results indicated that there is a crosstalk between NOD2 and TLR systems, which may need a certain time to start signal cascades that result in an increase of bacterial uptake. This could explain the lower number of bacterial uptake after $30\,min$ of exposure to *E. coli* K1. In accordance to these cross-talks, in case of a nasopharyngeal infection with encapsulated *Haemophilus influenza*e TLR and NOD systems were simultaneous needed to fight effectively against the pathogen; furthermore the absence of one system resulted in a prolonged infection (Zola et al. 2008). This finding supports the results of my experiments indicating benefit from both systems, although one has to consider that Zola et al. (2008) focused on stimulation of the NOD1 system.

One important point to take into account is that only microglial cells from newborn mice were used. We do not know whether microglia cells from old mice have similar mechanisms and are capable of ingesting/killing the same number of bacteria. Immunocompromised patients including the elderly are at higher risk of suffering infections than immunocompetent people. Old people show an impaired immune system. One of the questions that arose after the observations of this thesis was to study how MDP alone and in combination influenced microglial responses in microglia from old mice. Therefore, further experiments were performed with microglia from adult mice (49 days, P49) and parts of these data are published together with main parts of the present work (Ribes et al. 2012). The next step would be to transfer the findings of the current work to human cells, for which further experiments would be necessary. Concentrations of endotoxins, like LPS, found in patients suffering from sepsis were described as $0{,}0001\,\mu g/ml$ (Shenep et al. 1988, van Deventer et al.

1988) which is the submaximum concentrations I used to stimulate microglia before *E. coli* K1 challenge and could represent physiological concentrations.

Beran et al. (2011) described an upregulation of TLR2 expression when the human organism was challenged with low concentrations of *E. coli*, whereas a slighter increase of TLR2 and an inhibition of TLR4 expression were measured with higher concentrations of *E. coli*. Contrary results were found when cells were exposed to Gram-positive bacteria (Beran et al. 2011). On the other hand, mutations causing a loss of function of TLR4 predisposed to Gram-negative sepsis, whereas the remaining immune system stays intact (Poltorak et al. 1998). This generates the question whether the concentration of *E. coli* K1 chosen for my experimental settings represents an "adequate" level. Furthermore the impression occurs that microglia express lower numbers of receptors to prevent the immune system from an overreaction, before this would lead to further damages or can even adapt to modulate immunological consequences (Beran et al. 2011, Hanisch and Kettenmann 2007).

MDP is known as adjuvant inducing antigen-specific T and B cell responses, delayed-type hypersensitivity as well as antibody production (Takada and Kotani 1995). Mašek et al. (2011) described MDP as one component of an experimental proteoliposome-based vaccine formulation against *Candida* species, since important immune responses were induced without toxic side effects. Similarly, administration of biodegradable microparticles comprising MDP with bacterial DNA from *Propionibacterium acnes* (MIS416) resulted in a nontoxic Th1 cellular adjuvant with desirable immunostimulatory properties in mice and rabbits (Girvan et al. 2011). The synergism of the NOD and the TLR systems may help to develop strategies for vaccination and immunotherapy to protect the CNS against invading pathogens, especially in immunocompromised patients with a poor local defense. The risk of neuronal injury or further progress of selfsame, in acute (Iliev et al. 2004) and chronic CNS ailments with a phlogistic component including neurodegenerative diseases (Perry et al. 2007; Cunningham et al. 2009) rises with prolonged microglial activation. The aim of future experiments is to find a substance which enhances the microglial potential to protect the CNS against invading pathogens (e.g. via phagocytosis/clearance) without the consecutive increase of damage to dendrites, neuronal somata, or axons.

5 Summary of conclusions

The aim of the current work was to study whether murine microglial cells stimulated with either the Nod2 ligand MDP or the viral TLR3 agonist poly(I:C) phagocytosed and thereafter were enabled to kill higher numbers of *E. coli* K1 than unstimulated cells. Furthermore, a possible synergism between the Nod and TLR systems in terms of release of nitrit and chemo-/cytokines, phagocytosis and intracellular killing was investigated.

Meningitis, meningoencephalitis and sepsis are still severe diseases causing many victims, especially immunocompromised people, all over the world. Among many pathogens, *E. coli* is the leading cause of Gram-negative neonatal bacterial sepsis and meningitis. Patients suffering from those illnesses have severe limitations concerning their well-being, fitness and mind. Many of the mentioned problems do not derive directly from invading bacteria but from the host response against pathogen invasion. For example, an increasing production of chemo- and cytokines induced by compounds released upon bacterial lysis.
The host response which usually prevents the body from the spread of invading bacteria and induces efficient pathways to eliminate the pathogen can become harmful for the organism itself. This could be the case, if the organism produces high amounts of mediators resulting in an overreaction which consecutive leads to a dysregulation of homoeostasis. Fever or major decrease of body temperature might lead to a loss of function of pivotal enzymes. Furthermore, blood vessels can dilate resulting in a decrease of blood pressure. It is clear then, why it is so important to develop strategies to help the organism to cope with pathogen invasion. In the best case scenario the pathogen could be eliminated before the organism had triggered signaling pathways and caused a maximum alert of the immune system which would lead, in worst case, to a lethal ending. In case of the brain, this part is taken over by microglial cells.

The release of chemo- and cytokines by murine microglial cells pre-stimulated with MDP and/or TLR agonist were measured. These data indicated that murine microglial cells can be stimulated by MDP alone, as well as in combinations with TLR agonists, whereupon the co-stimulation of MDP and TLR agonists led to the highest amounts of chemo- and cytokine release. On the one hand, these measurements indicated that stimulation of microglia was successful. On the other hand, MDP and poly(I:C) and CpG could be considered as stimuli which enable cells to ingest more bacteria without a strong reaction of the immune system.

In the current work, I was able to demonstrate for the first time that a stimulation with the Nod2 ligand MDP as well as the viral TLR3 agonist poly(I:C) not only enables murine microglia to phagocytose significantly higher numbers of pathogenic *E. coli* K1 bacteria but also enhances intracellular bacterial killing compared to unstimulated cells. However, the potency of microglial responses elicited by MDP and poly(I:C) was not as strong as the ones achieved by agonists of the TLR system recognizing PAMPs from bacterial origin (TLR1/2, TLR4 and TLR9). Furthermore, I could demonstrate that a crosstalk between Nod2 and TLR systems can enhance the phagocytosis and the intracellular killing of a pathogenic *E. coli* K1 strain. The synergism of the NOD2 and the TLR systems may help to develop strategies for vaccination and immunotherapy to protect the organism against invading pathogens, especially in immunocompromised patients.

6 Publications of current work

Essential parts of the dissertation have been published as followed.

6.1 Poster and Papers:

Adam N, Ribes S, Ebert S, Regen T, Bunkowski S, Hanisch U-K, Nau R. Poly(I:C) stimulates phagocytosis and intracellular killing of *Escherichia coli* by microglial cells. Poster at the 20th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Vienna, Austria, April 10–13, 2010.

Ribes S, Adam N, Ebert S, Regen T, Bunkowski S, Hanisch UK, Nau R (2010): The viral TLR3 agonist poly(I:C) stimulates phagocytosis and intracellular killing of *Escherichia coli* by microglial cells. Neurosci Lett 482: 17-20.

Ribes S, Adam N, Schütze S, Regen T, Redlich S, Janova H, Borisch A, Hanisch U-K, Nau R (2012): The nucleotide-binding oligomerization domain-containing-2-ligand muramyl dipeptide enhances phagocytosis and intracellular killing of *Escherichia coli* K1 by Toll-like receptor agonists in microglial cells. J Neuimmol: 252, Issue 1: 16-23.

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8 Appendix

8.1 List of figures

Figure 3.1 NO concentrations after **24h** incubation with MDP at 0.1, 0.3, 1, 3, 10, 30 and 100 μg/ml. Data are shown as mean ± SD. 19

Figure 3.2 NO concentrations after **48h** incubation with MDP at 0.1, 0.3, 1, 3, 10, 30 and 100 μg/ml. Data are shown as mean ± SD. 19

Figure 3.3 NO release after **24h** of microglial stimulation with poly(I:C) at 0.1, 0.3, 1, 3, 10, 30 and 100 μg/ml. Data are shown as mean ± SD. 20

Figure 3.4 NO release after **24h** of microglial stimulation with Pam_3CSK_4 at 0.000001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 10 μg/ml. Data are shown as mean ± SD. 21

Figure 3.5 Chemo- and cytokine release after stimulation with one compound [MDP, TLR1/2 (Pam_3CSK_4), TLR3 [poly(I:C)], TLR4 (LPS) and TLR9 (CpG)] agonists.....23

Figure 3.6 TNF-α release after stimulation with **0.1 μg/ml** and **10 μg/ml** MDP and submaximum concentrations of TLR1/2 (Pam_3CSK_4), TLR3 [poly(I:C)], TLR4 (LPS), TLR9 (CpG) agonists.....24

Figure 3.7 CXCL1 release after stimulation with **0.1 μg/ml** and **10 μg/ml** MDP and submaximum concentrations of TLR1/2 (Pam_3CSK_4), TLR3 [poly(I:C)], TLR4 (LPS), TLR9 (CpG) agonists.....25

Figure 3.8 IL-6 release after stimulation with **0.1 μg/ml** and **10 μg/ml** MDP and submaximum concentrations of TLR1/2 (Pam_3CSK_4), TLR3 [poly(I:C)], TLR4 (LPS), TLR9 (CpG) agonists. 26

Figure 3.9 MDP stimulation enhanced phagocytosis of *E. coli* K1 after **30min** (A) and **90min** (B) of incubation. 27

Figure 3.10 Phagocytic rates in poly(I:C)-stimulated microglia in (A) **30min** and (B) **90min** phagocytosis assays.....28

Figure 3.11 Inhibitory effect of cytochalasin D (CD) on phagocytosis of *E. coli* K1. 29

Figure 3.12 Stimulatory effect of agonists of the MDP and TLR systems on phagocytosis after **30min** of co-incubation of microglia and bacteria. 30

Figure 3.13 Stimulatory effect of MDP and TLR after **90min** of phagocytosis. 31

Figure 3.14 Synergistic effect on **30min** phagocytosis after stimulation with submaximum concentrations of MDP and TLR agonists. 33

Figure 3.15 Synergistic effect on **30min** phagocytosis after stimulation with **10 μg/ml** MDP and submaximum concentrations of TLR agonists. 34

Figure 3.16 Synergistic effect on **30min** phagocytosis after stimulation with maximum concentrations of MDP and TLR agonists. 35

Figure 3.17 Synergistic effect on **30 min** phagocytosis of *E. coli* K1 after stimulation with **0.1 µg/ml** MDP and maximum concentrations of TLR agonists. 36

Figure 3.18 Co-stimulation of Nod2 and TLR agonists at submaximum concentration after **90 min** of exposure to *E. coli* K1. 37

Figure 3.19 Stimulatory effect of **10 µg/ml** MDP and TLR agonists at submaximum concentration after **90 min** of exposure to *E. coli* K1.....38

Figure 3.20 Co-stimulation of MDP and TLR agonists at submaximum concentration after **90 min** of exposure to *E. coli* K1. 39

Figure 3.21 Phagocytosis after co-stimulation with **0.1 µg/ml** MDP and TLR agonists at maximum concentration after **90 min** of exposure to *E. coli* K1. 40

Figure 3.22 Stimulation with Nod2 and TLR agonists at maximum concentration after **90 min** of exposure to *E. coli* K1. 41

Figure 3.23 Time course of bacterial killing of *E. coli* K1 after single stimulation with **10 µg/ml** MDP and submaximum concentrations of TLR agonists. 42

Figure 3.24 Time course of bacterial killing after combined stimulation with **10 µg/ml** MDP and submaximum concentrations of TLR agonists. 43

8.2 List of tables

Table 3.1 Intracellular survival 44

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