The Diversity of TLR4-triggered Responses in Mouse Microglia

Dissertation

for the award of the degree

"Doctor rerum naturalium" (Dr. rer. nat.)

Division of Mathematics and Natural Sciences

of the Georg-August-University Göttingen

submitted by

Tommy Regen

born in Forst/Lausitz

Göttingen 2010

Reviewer / Members of the Thesis Committee

Supervisor

Prof. Dr. Uwe-Karsten Hanisch
Department of Neuropathology
University Medicine Göttingen, University of Göttingen

Thesis Committee Member (Reviewer)

Prof. Dr. Wolfgang Brück
Department of Neuropathology
University Medicine Göttingen, University of Göttingen

Thesis Committee Member (Reviewer)

Prof. Dr. Fred S. Wouters
Department of Neuro- and Sensory Physiology
University of Göttingen

Thesis Committee Member

Prof. Dr. Mikael Simons
Max Planck Institute for Experimental Medicine, Göttingen

Date of the oral examination: 6th September 2010

Affidavit

I hereby declare that I wrote my doctoral thesis entitled "The Diversity of TLR4-triggered Responses in Mouse Microglia" independently and with no other sources and aids than quoted.

Göttingen, July 2010

_____

(Signature)

Abstract

Microglia are the major immunocompetent effector cells of the CNS. Steadily monitoring their neighborhood they are always ready to transform to activated states upon encounters with danger-spelling signals provided by the sudden presence, abnormal concentration or an unusual molecular format of certain factors. Amongst a variety of receptors, microglia employ Toll-like receptors (TLRs) and their dual function for detecting exogenous as well as endogenous threats. TLR stimulation in microglia causes an innate immune reaction aiming at the protection of the CNS as well as promoting the repair of damaged tissue. Among the TLR family, TLR4 represents a specialized member because of its engagement with both of the major TLR signaling routes depending on the signaling adaptor molecules MyD88 and TRIF, respectively. This study aimed at deciphering the microglial response to bacterial LPS as the prototypic TLR4 agonist. We found microglia to differentially respond to structural variants of LPS as they come with different bacterial strains, thereby revealing a critical role for the co-receptor CD14. We could further show that microglial TLR(4) responses are organized in a complex fashion depending on the 'strength' of the signal. Moreover, these responses are highly sensitive to modulation by secondary immune and non-immune signals. Finally, we demonstrated that microglial TLR(4) signaling undergoes reorganization during CNS maturation and acquires distinct functional profiles in different anatomical regions/populations. Together, these data argue for the versatility of the microglial TLR4 signaling, as being also subject to a high degree of regulation and (re)organization and as being instructed and required by the special conditions of the CNS.

Table of Content

List of Figures

List of Tables

Abbreviations

Aβ	-	amyloid β
AC	-	adenylate cyclase
APC	-	antigen presenting cell
AR	-	adrenergic receptor
AT	-	atenolol
BBB	-	blood-brain barrier
BMDM	-	bone marrow-derived macrophage
CD	-	cluster of differentiation
CNS	-	central nervous system
COX	-	cyclooxygenase
DAMP	-	danger-associated molecular pattern
DAPI	-	4',6-diamidino-2-phenylindole
DC	-	dendritic cell
ECM	-	extracellular matrix
ELISA	-	enzyme-linked immunosorbent assay
Epac	-	exchange protein directly activated by cAMP
ER	-	endoplasmic reticulum
ERK	-	extracellular signal-regulated kinase
FACS	-	fluorescence-activated cell sorting
FITC	-	fluorescein isothiocyanate
Gal	-	galactose
GlcN	-	glucosamine
GlcNac	-	N-acetyl-glucosamine
Glu	-	glucose
Hep	-	heptose
ICI	-	ICI 118,551
IFN	-	interferon
IKK	-	IκB kinase
IL	-	interleukin
ILB4	-	isolectin B4
IP	-	isoproterenol

IRAK - interleukin-1 receptor-associated kinase
IRF - interferon regulatory factor
JNK - c-Jun N-terminal kinase
Kdo - 2-keto-3-desoxyoctonate
ko - knockout
LPS - lipopolysaccharide
LRR - leucine-rich repeat
MAL - MyD88-adaptor-like
ManR - mannose receptor
MAPK - mitogen-activated protein kinase
MD2 - myeloid differentiation protein 2
MDA - melanoma differentiation-associated gene
MEK - MAPK/ERK kinase
MHC - major histocompatibility complex
MKK - MAPK kinase
MS - multiple sclerosis
MyD88 - myeloid differentiation primary response gene 88
NA - noradrenaline
NFκB - nuclear factor κB
NK - natural killer (cell)
NLR - NOD-like receptor
NO - nitric oxide
NOD - nucleotide binding and oligomerization domain
OX - oxymetazoline
oxLDL - oxidized low-density lipoprotein
PAMP - pathogen-associated molecular pattern
PCW - pneumococcal cell wall
PKA - protein kinase A
PM - phentolamine methansulfonate
PR - propranolol
PRR - pattern recognition receptor
RIG - retinoic acid inducible gene
RIP - receptor-interacting protein

RLR	-	RIG-I-like receptor
ROS	-	reactive oxygen species
Rs-LPS	-	*Rhodobacter sphaeroides* LPS
SARM	-	sterile α- and armadillo-motif containing protein
SB	-	salbutamol
S.e.-LPS	-	*Salmonella enterica* LPS
(s)MPLA	-	(synthetic) monophosphoryl Lipid A
TAB	-	TAK1-binding protein
TAK	-	transforming-growth-factor-β-activated kinase
TAM	-	tumor-associated macrophage
TBK	-	TRAF-family-member-associated NFκB-activator-binding kinase
T_H	-	helper T (cell)
TIR	-	Toll/Interleukin-1 receptor
TNF	-	tumor necrosis factor
TRAF	-	tumor necrosis factor-receptor-associated factor
TRAM	-	TRIF-related adaptor molecule
TRIF	-	TIR-domain containing adaptor protein inducing IFNβ
WT	-	wild type

1. Introduction

1.1 The two worlds of the mammalian immune system

In order to fight exogenous as well as endogenous threats, evolution developed the immune system. In higher vertebrates, this system is equipped with a plethora of effector cells, molecules and mechanisms that can be assigned to either the adaptive or the innate arm of immunity. Both present with versatile and also remarkably different modes of action, but depend on each other to organize and coordinate an efficient defense against infections or tumors.

Within the adaptive immunity, the T and B lymphocytes are the major mediators of the response. These cells can recognize virtually any antigenic structure. This is guaranteed by the enormous number of T and B cells with receptors of individual antigen binding features. From this pool, the most appropriate can be chosen during an immune response by mechanisms based on clonal selection. Thereby, these cells are primed with their specific antigen in specialized immunological compartments, i.e. the secondary lymphoid organs (including lymph nodes, spleen and tonsils). Upon repeated encounter of their specific antigen, this time directly at the site of action, T cells can either directly act on infected or damaged host cells ($CD8^+$ cytotoxic T cells) or they arrange and coordinate the response with the help of soluble mediators ($CD4^+$ helper T cells). Following the same principle of activation, B cells can transform into plasma cells which then produce antigen-specific antibodies that can act in various ways to aid the immune response. On top of these immediate mechanisms, activated cells of the adaptive immune system can differentiate into memory cells, thereby 'saving' information about the antigenic nature of the threat. This unique feature enables the host to respond even faster and more efficient to a repeated encounter of the same threat (antigen).

The innate immune system is, in evolutionary terms, much older than its adaptive counterpart. Although innate immune cells, including populations of macrophages and monocytes, granulocytes, dendritic cells (DCs) and natural killer (NK) cells, do not share the feature of clonal selection they can sense a huge variety of molecular structures of both foreign and self-origin, and they employ a complex machinery of soluble mediators that orchestrates the immune response. In addition to the cellular players of innate immunity, the complement system has important roles in supporting both adaptive and innate immune

responses. The innate immunity builds up the host's first line of defense. Its 'troops' are strategically positioned in various external and internal epithelial tissues. They represent the outermost barrier which has to be overcome by invading pathogens. Upon encounter of 'invaders', innate immune cells react within minutes to few hours in order to prevent the spread of the pathogen. The basic mechanisms employed to achieve this goal are direct cytotoxicity towards invading germs, release of soluble mediators to recruit and instruct other immune cells and the ingestion of pathogens (phagocytosis, mainly by macrophages and DCs) – with the consequence of intracellular killing of the pathogen and subsequent presentation of antigenic structures on the cell surface. Only if this initial innate response is overwhelmed or circumvented by the pathogen, an adaptive immune response is required to fight off the challenge. However, the activation of the second (adaptive) line of defense represents by no means the termination of the first, i.e. the innate one. The presentation of antigenic structures in the context of major histocompatibility complex (MHC) molecules expressed on professional antigen presenting cells (APCs, such as DCs and macrophages) is, of course, essential for the initiation of a successful adaptive immune response, since without it, no (antigen-specific) T or B lymphocyte will be able to clonally expand and to reach the required state of activation. Innate immune cells also release soluble factors, like cytokines and chemokines (chemoattractive cytokines), which guide other (innate as well as) adaptive immune cells to the site of infection, instruct effector cells and modulate their executive programs. Thus, innate immune activities are further needed.

Recently, regulatory aspects of innate immune cells have been gaining considerable interest, largely also due to the improved understanding of macrophage diversity. Not only as effector cells for phagocytosis and cytotoxic attacks, but also for the repair-supporting functions, macrophages reveal an impressive spectrum of reactive phenotypes (Gordon and Taylor, 2005; Martinez et al., 2008; Mosser and Edwards, 2008). Moreover, phenotypes as induced by different activating signals and their context may also be further dynamically controlled. They may allow transitions, for example a shift from an initially defense- to a subsequently repair-oriented profile, as upon successful clearance of an infection. Distinct macrophage populations – and especially their dysregulation – have a more and more recognized relevance for inflammation-associated diseases, autoimmune processes and far beyond. In addition to the originally assumed classical effector cell activities contributions to metabolic disorders, atherosclerosis and neurodegenerative diseases get increasingly apparent. This emerging concept of macrophage phenotype diversity illustrates how the

innate immune system has been receiving attention for its – previously underestimated – complexity, effective organization and (patho)physiological impact.

1.2 Microglia represent the innate immune cells of the CNS

The central nervous system (CNS) with the brain, spinal cord and associated formations is considered to be an 'immune-privileged' organ (Galea et al., 2007). Due to its vulnerability and a poor regenerative capacity, the CNS has to be spared from extensive inflammatory processes (generally associated with tissue damage) and the rigorous actions of immune effector cells as they are better tolerated by other parts of the body. The internal milieu of the CNS is largely shielded against the periphery by the vascular structures and functions of the blood-brain barrier (BBB). Constituted by endothelial cells as well as the specialized processes (endfeets) of astrocytes, the BBB does not only control the exchange of nutrients and metabolites, but also the penetration of immune cells. Yet microglia, i.e. the primary resident innate immune cells of CNS, represent a unique population of tissue macrophages. Much of the special and tight regulation of immune processes in the CNS may relate to the way they respond to disturbed homeostasis. Although they can unfold a macrophage-like repertoire of functions, the initiation and execution of these activities are adapted to the special nervous tissue environment.

Unlike their name may indicate, microglia are not of glial but of myeloid origin. They populate the brain in two waves, the first already occurring during fetal development and being followed by a second settlement during early postnatal days (Chan et al., 2007). Starting from this initial population, microglia will have colonized throughout the entire CNS by the end of the postnatal development. The intrinsic microglial turnover in terms of constitutive proliferation is thought to be rather low, and recent data, although still under debate, point to an only minor replenishment of the adult microglial population by blood-borne monocytes under normal, healthy conditions. Only upon pathophysiological events, infiltration of monocytes will contribute to the CNS population of myeloid cells (Mildner et al., 2007). Under pathological conditions, large numbers of monocytes/macrophages can invade the CNS, coming directly from the blood or from the perivascular spaces within the CNS. It is still questionable, however, whether these acutely invading cells will eventually transform to parenchymal microglia proper with indistinguishable properties. Assuming that parenchymal microglia constitute a rather stable population with low turnover rates the

question of their actual lifespan as individual cells or subpopulations unavoidably emerges. Consequently, age-related changes in microglial behavior and their ability to control and preserve CNS homeostasis are gaining attention (Streit and Xue, 2009). Yet a systematic approach to explore functional changes during microglial development and to determine regional adjustments has not been tackled yet.

In the normal healthy brain, microglia present with a ramified morphology, a state in which they are termed 'resting', but which should not be mistaken as 'inactive' microglia (Hanisch and Kettenmann, 2007). Microglia actively and constantly scan their environment for disturbances of the tissue homeostasis (Nimmerjahn et al., 2005). Their fine processes are in motion, while the cell body remains at a fixed position. This intriguing motility of the processes may allow for an efficient scanning without disturbing the neuronal fibers and their circuitry. Indeed, recent follow-up studies provide even evidence for the microglial nursing of synaptic connections (Wake et al., 2009). Upon encounter with a triggering signal, however, microglia will readily transform from the 'resting' to an alerted or 'activated' state.

For many years, microglia activation was believed to be a stereotyped process with the inevitable result of subsequent tissue damage. This was supported by histological findings of neuropathological changes – as they often do correlate with the presence of activated microglia. Indeed, the neurotoxic potential of microglia was demonstrated in diverse scenarios *in vitro* and *in vivo* (Hanisch and Kettenmann, 2007). However, this view of the 'bad guy' might have been biased. Most probably, the majority of microglial activation episodes occur in order to maintain tissue homeostasis. They are hardly noticed simply because of the lack of clinical manifestation.

Experimental settings for the investigation of microglial features often employ triggering signals which associate with infectious agents, e.g. bacterial lipopolysaccharide (LPS), a major cell wall component of Gram-negative strains. LPS is widely used as an *in vitro* stimulus for microglial activation and causes responses also upon delivery *in vivo*. Such microglial activation settings will result in a strong defense-oriented reaction, which covers toxic consequences. These isolated experimental layouts in culture can recapitulate essential cellular processes, even though they do not reflect the *in vivo* situation where activation of microglia occurs within a complex, highly organized, tightly controlled and dynamic environment. On the other hand, even *in vivo* application of LPS may still not

cover all reactive phenotypes inducible by the plethora of bacteria-associated factors with microglia-'activating' potential, such as DNA.

It was only in recent years that the paradigm of microglial activation experienced major conceptual changes (Hanisch and Kettenmann, 2007). Accumulating evidence has weakened the simplified view that the activation process of microglia is a monophasic event. A microglial response rather presents with great variety in reactive profiles and with the dynamic aspect that shifts in reactive profiles seem to occur between its induction and termination. Moreover, microglial responses are tightly controlled and are susceptible to an array of regulatory mechanisms to avoid overshooting, chronic or maladapted reactions – three scenarios causing otherwise detrimental consequences by hyperinflammation or an insufficient protection of the CNS. This new concept of microglia activation led to a more and more accepted view that these cells serve primarily neuroprotection, rather than accounting for CNS damage.

Microglia activation can result by two principles, based on the sorting of triggering 'on' and 'off' signals independently of their (bio)chemical nature (van Rossum and Hanisch, 2004; Biber et al., 2007; Hanisch and Kettenmann, 2007). An 'on' signal thereby represents the classical activation paradigm where a certain ligand binds to its receptor on the target cell, resulting in the induction of signaling cascades and subsequent cellular consequences. Microglia can sense such signals as to their sudden appearance, abnormal concentration or unusual molecular format. On the other hand, disturbed homeostasis can also be indicated by a decline or loss of a constitutive signaling of microglia-calming factors, this scenario representing 'off' signaling. It consequently will lead to microglial activation or enhanced responsiveness to activating factors. For example, CX_3CL1 (also known as fractalkine) is constantly produced by neurons and constitutively binds to its receptor, CX_3CR1, on microglia (Cardona et al., 2006). This interaction keeps the cells in a (calm) surveillance state. CD200/CD200R and SIRP1α/CD47 represent additional examples of such ligand-receptor pairs. Either of these activation principles, i.e. on and off signaling, can thereby lead to microglia activation, probably also in concert and affected by a whole array of co-signals. Depending on the nature of the activating signal and its context, it can result in a wide range of activities, including local proliferation, phagocytosis, induced APC function or the release of a blend of various cytokines and chemokines, which recruit and instruct invading immune as well as neighboring parenchymal cells.

1.3 Microglia shift reactive phenotypes upon encounter of modulatory signals

Much of our current knowledge about phenotypic diversity of (tissue) macrophages has been established based on the experimental employment of extra-neural cell populations. According to the nomenclature used for T helper (T_H) cell subsets, namely T_H1 *versus* T_H2 type of responses, the first distinction of macrophage phenotypes was termed M1 *versus* M2 polarization (Gordon, 2003; Mantovani et al., 2004). Thereby, the so called 'classical' M1 phenotype is induced upon encounter of the prototypic T_H1 cytokine interferon (IFN)γ or a number of other signals indicating an imbalanced homeostasis, including tumor necrosis factor (TNF)α or bacterial LPS. This classical activation typically results in the production of proinflammatory cytokines and chemokines ─ including interleukin-12 (IL-12) as a hallmark of the M1 phenotype ─ as well as other inflammation-related molecules, such as nitric oxide (NO) or reactive oxygen species (ROS). Classical activation also leads to the up-regulation of surface structures, such as MHC II. In contrast, the M2 phenotype of 'alternative' macrophage activation is characterized by the induction of anti-inflammatory cyto/chemokines ─ here with IL-10 serving as hallmark ─ and repair activity-supporting molecules and activities, like fibronectin and arginase. Alternative activation causes also up-regulation of cell surface molecules, like the mannose receptor (ManR).

The M2 phenotype thereby gets induced by the T_H2 'master' cytokines IL-4 and/or IL-13. In more general terms, the M1 *versus* M2 phenotypes can also be described as an orientation toward 'defense' (M1) *versus* 'repair' (M2). Soon after the implementation and acceptance of this nomenclature it became evident that the phenotypic diversity of the differentially activated macrophages would require much more discrimination than simply M1 *versus* M2. Consequently, a number of different non-classical macrophage phenotypes have been described, all of which presenting with partially reciprocal, partially overlapping patterns of induced genes and functional consequences (Gordon and Taylor, 2005). MHC II can, for example be induced on both M1 and M2 cells. Given the steadily growing number of newly discovered phenotypes and taking into account a certain plasticity (in terms of distinct 'biomarkers') of activated macrophages, it might be more suitable to classify these cells with respect to their effector orientations. In more global terms, this classification would roughly discriminate between defense, wound healing/repair and immune regulation (Mosser and Edwards, 2008). As much as has already been learned

from these extra-neural macrophage populations only little is known about the phenotypic diversity of microglia. There is accumulating evidence for a similar response versatility depending on the stimulus, the specific environment and the (patho)physiological context (Hanisch and Kettenmann, 2007; van Rossum et al., 2008; Ransohoff and Perry, 2009). Yet microglia constitute the resident macrophage population of the CNS, a tissue of quite complex and vulnerable nature. Microglia must adapt its reactive phenotypes accordingly.

1.4 Toll-like receptors recognize a huge variety of threats

As cells of the innate immunity, microglia represent the CNS' parenchymal first (and probably only) line of defense against invading pathogens. To fulfill this duty, microglia employ a number of germline-encoded receptors belonging to the complex class of pattern recognition receptors (PRRs). These receptors sense a range of conserved structural motifs of pathogens which are generally termed pathogen-associated molecular patterns (PAMPs). PRRs thus cover the recognition of a wide range of bacteria, viruses, fungi and protozoa. It must be emphasized that this PRR-PAMP interaction is not a nonspecific one, as the term 'pattern' may imply. The recognition of these essential microbial components should rather be understood as an extremely effective way to sense a huge variety of exogenous threats (treacherous structures) by only a limited number of receptors.

There are three major families of PRRs: (i) the Toll-like receptors (TLRs, described in more detail below), (ii) the nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) with more than 20 family members and (iii) the retinoic acid inducible gene (RIG)-I-like receptors (RLRs) comprising their three members RIG-I, melanoma differentiation-associated gene (MDA)5 and Lgp2 (Creagh and O'Neill, 2006; Kawai and Akira, 2010). In addition, a larger number of lectin-type receptors, like the ManR or β-glucan receptors carry PRR functions. These receptors are strategically located either on the cellular surface or within endosomal compartments, depending on their specific 'target structures'. Those include lipoproteins and -peptides, (glyco)lipids and glycan or nucleic acid structures. Despite of coming with specific ligand recognition and, in large parts, distinct signaling mechanisms, there is also some considerable cooperation between PRRs of the different families. One of the best investigated examples regards the production of IL-1β and IL-18. Both are induced as pro-forms by TLR-dependent mechanisms, but then

require subsequent processing by caspase 1, an enzyme activated and controlled by NLRs (Martinon, 2008).

TLRs are type I transmembrane receptors. Due to their homology in the cytosolic portions, i.e. the Toll/IL-1 receptor (TIR) domain, they are grouped together with the IL-1 receptor I (IL-1RI) and its homologues in the IL-1R/TLR superfamily. Consequently, the underlying signaling adaptors and signal-conveying molecules are common to members of both subfamilies (see further below). The extracellular domains of TLRs, however, differ significantly from those found in IL-1RI-like receptors. The latter employ immunoglobulin domains for ligand binding, whereas TLR ectodomains are characterized by leucine-rich repeat (LRR) units.

The name-defining protein 'Toll' was originally described in the fruit fly *Drosophila melanogaster,* where it controls the dorsoventral polarity during embryogenesis. Toll in *Drosophila* regulates the transcription factor Dorsal by the help of the protein kinase pelle (Belvin and Anderson, 1996). Interestingly, Dorsal is a member of the nuclear factor (NF)κB family and pelle shares homology with the IL-1 receptor-associated kinase (IRAK), both NF-κB and IRAK being essential elements of TLR signaling as well. Later, Toll itself was shown to have a critical role in the resistance of *Drosophila* to fungal pathogens (Lemaitre et al., 1996), thereby initiating the search for human Toll homologues. The first was then described in 1997 (Medzhitov et al., 1997). To date, 10 human and 12 murine functional TLRs have been discovered, with TLR1 to TLR9 being conserved across the two species (Kawai and Akira, 2010). The constant progress in identifying and characterizing new TLRs has also considerably renewed the general interest in innate immunity.

1.5 TLR signaling is highly complex and controlled in diverse ways

According to their cellular localization, TLRs can be divided into two groups. The group of receptors that are incorporated into the cell membrane comprises TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11. They mainly recognize microbial membrane components. The other group is defined by TLR3, TLR7, TLR8 and TLR9, which sense microbial nucleic acids and exclusively locate in intracellular compartments, like the endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes. Restriction to be expressed in defined cellular compartments assures optimal ligand (PAMPs) accessibility and, at the same time,

helps sustaining a tolerance to self-molecules. In addition, this specific localization assures close proximity to the downstream signaling elements, including a still poorly understood cross-talk between TLR and non-TLR pathways, which creates another level of regulation and control.

Like it is known for many other receptors, TLRs dimerize upon ligand recognition, with the majority forming homodimers. TLR2 makes an exception from this rule as it forms heterodimers with either TLR1 or TLR6, respectively. Additionally, the group of TLR7, TLR8 and TLR9 presents with more complexity as all of them, despite forming homodimers, can interact with each other, thereby antagonizing the signaling of their heterodimeric partners (Wang et al., 2006a). Although already previously indicated, only recently the principle of heterodimerization got also demonstrated for a combination of TLR4 and TLR6 (Stewart et al., 2010). Ligand binding and dimerization will ultimately bring the two intracellular TIR domains in close proximity, resulting in a conformational change necessary for the recruitment of signaling adaptors. There are five TIR domain-containing adaptors (O'Neill and Bowie, 2007). These are (i) myeloid differentiation primary response gene 88 (MyD88), (ii) MyD88-adaptor-like (MAL, also known as TIRAP), (iii) TIR-domain containing adaptor protein inducing INFβ (TRIF, also known as TICAM1) as well as (iv) TRIF-related adaptor molecule (TRAM, also known as TICAM2). The fifth TIR domain-containing adaptor protein, i.e. sterile α- and armadillo-motif containing protein (SARM), was shown to interact with TRIF, thereby interfering with TRIF signaling (Carty et al., 2006).

By use of the signaling adapters MyD88 or TRIF, TLR signaling has been divided into two main routes, distinguished as MyD88-dependent and MyD88-independent (in here referred to as TRIF-dependent) signaling. With the exception of TLR3, which solely uses TRIF, all TLRs rely on the MyD88-dependent pathway, recruiting MyD88 either directly or indirectly – via the sorting adaptor MAL – to their respective TIR domain. MyD88 then recruits members of the IRAK family of protein kinases, which in turn recruit and activate TNF-receptor-associated factor (TRAF)6. Activated TRAF6 then interacts with a complex consisting of transforming-growth-factor-β-activated kinase (TAK)1 in association with the regulatory components TAK1-binding protein (TAB)2 and TAB3. Ultimately, TAK1 will simultaneously activate the (early phase) NFκB system and members of the mitogen-activated protein kinase (MAPK) families, including the c-Jun N-terminal kinase (JNK),

p38^{MAPK} and p44/42^{MAPK} (also known as ERK1/2). Subsequently activated transcription factors account for the production of inflammatory factors (Fig. 1.1).

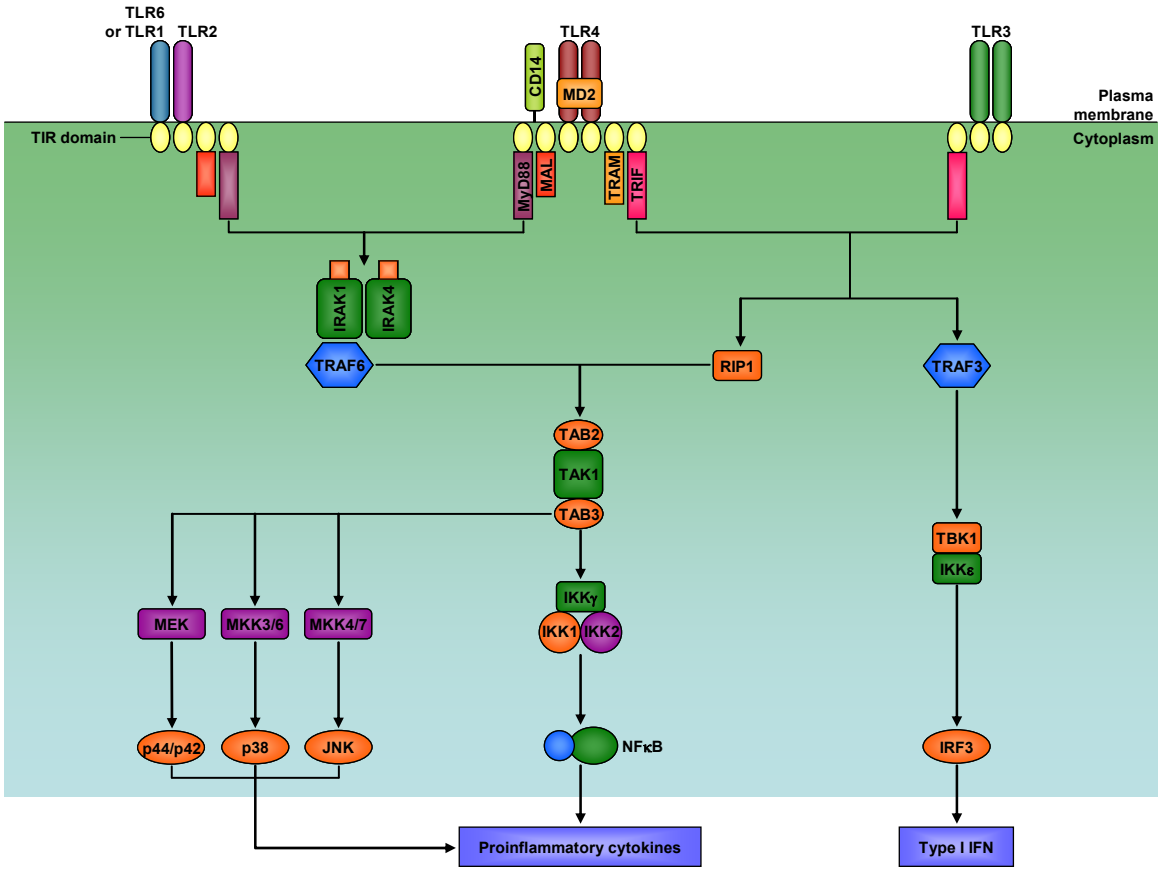


Fig. 1.1: Schematic representation of TLR signaling pathways. The majority of TLR-mediated responses get induced through the MyD88-dependent signaling pathway, which is used by all TLRs, except for TLR3. In contrast to the other TLRs, TLR2 (in heterodimeric association with either TLR1 or TLR6, respectively) and TLR4 recruit MyD88 to the plasma membrane indirectly via the sorting adaptor MAL. MyD88 then recruits members of the IRAK family which, in turn, activate TRAF6. Activated TRAF6 interacts with a TAK1/TAB2/TAB3 complex, which ultimately leads to the activation of NFκB and MAPK family members, all of which inducing the production of proinflammatory cytokines, like TNFα. TRIF is recruited directly to TLR3 or indirectly (via the sorting adaptor TRAM) to TLR4 to subsequently activate RIP1, which will in turn activate the TAK1/TAB2/TAB3 complex leading to the consequences as described for MyD88 signaling. In parallel, TRIF activates TRAF3, resulting in the ultimate activation of IRF3. This pathway will induce the production of type I interferons (IFN), like IFNβ. Of note, TLR4 is the only TLR family member that enjoys association to both, MyD88-dependent and TRIF-dependent signaling routes. For recognition of its prototypic exogenous agonist – bacterial LPS – TLR4 also recruits the co-receptors MD2 and CD14. IKK, IκB kinase; IRAK, interleukin-1 receptor-associated kinase; IRF, IFN regulatory factor; JNK, c-Jun N-terminal kinase; MAL, MyD88-adaptor-like; MAPK, mitogen-activated protein kinase; MD2, myeloid differentiation protein 2; MEK, MAPK/extracellular-signal-regulated kinase (ERK) kinase; MKK, MAPK kinase; MyD88, myeloid differentiation primary response gene 88; NFκB, nuclear factor κB; RIP, receptor-interacting protein; TAB, TAK1-binding protein; TAK, transforming-growth-factor-β-activated kinase; TBK, TRAF-family-member-associated NFκB-activator-binding kinase; TIR, Toll/Interleukin-1 receptor; TLR, Toll-like receptor; TRAF, tumor necrosis factor-receptor-associated factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain containing adaptor protein inducing INFβ.

The TRIF-dependent signaling is employed by TLR3, but also by TLR4, the latter thus representing the only family member enjoying access to both of the major signaling pathways. The signaling adaptor TRIF is thereby recruited to the receptor either directly (TLR3) or indirectly (TLR4), via the sorting adaptor TRAM. TRIF signaling activates (late phase) NFκB and MAPKs involving a similar signaling cascade as described for MyD88-dependent signaling as well as an alternative pathway involving the receptor-interacting protein (RIP)1. In addition, TRIF recruits a signaling complex involving TRAF-family-member-associated NFκB-activator-binding kinase (TBK)1 and IκB kinase (IKK)ε. This complex, in a TRAF3-dependent manner, then activates the transcription factor interferon-regulatory factor (IRF)3, which ultimately leads to the production of type-1 interferons (Fig 1.1). It should be noted, however, that this sorting by MyD88- *versus* TRIF-dependent pathways should not distract from the fact that both cooperate and cross-regulate each other, more than originally anticipated, and that these two prominent TLR-characteristic pathways are likely requiring contributions from additional signaling systems.

1.6 TLR4 shows the highest versatility within the family of TLRs

Among the various TLRs discovered thus far, TLR4 appears to have some unique features concerning its signaling complexity as well as ligand acceptance and discrimination. TLR4 is the only family member that uses all four above mentioned signaling adaptor molecules. Consequently, the receptor initiates both MyD88- as well as the TRIF-dependent pathways (Yamamoto et al., 2003). Of note, TLR4 engages with the signaling adaptors indirectly by making use of the sorting adaptors MAL (to MyD88) and TRAM (to TRIF). This feature is thought to introduce a rather upstream level of regulatory control. Although TLR4 uses both signaling pathways, this is not happening as a coincidental, but as a rather sequential series of events. After ligand binding, TLR4 initially recruits MAL/MyD88 to the plasma membrane for an early activation of NFκB (i.e. early phase) and MAPK-based signaling. Only afterwards, the receptor-ligand complex undergoes dynamin-dependent endocytosis to continue with signaling by recruiting TRAM/TRIF to the newly formed endosome and to thereby initiate further NFκB (i.e. late phase) and IRF3 signaling (Kagan et al., 2008; Rowe et al., 2006).

Although a few genes can be induced independently through only one of the two signaling pathways, the majority of genes needs the cooperation of both pathways to be

effectively induced (Hirotani et al., 2005). This phenomenon stands in contrast to all other TLRs for which activation of either the MyD88- or TRIF-dependent pathway is sufficient to induce the full spectrum of inflammatory factors. It might be explained by a stabilization of NFκB activation which – when induced through MyD88 only – shows an oscillatory (unstable) behavior (Covert et al., 2005). Given the enormous inflammatory potential of the TLR4 activation, the phenomenon of sequential usage of the two signaling cascades might add yet another principle of possible regulatory interference – to avoid overshooting responses with the potential risk of a systemic inflammatory reaction. Indeed, there is some evidence for also negative cross-regulation.

The unique position of TLR4 is not only manifested by its complex signaling but also by its ability to sense a wide variety of ligands, including a number of endogenous molecules referred to as danger-associated molecular patterns (DAMPs, also known as 'alarmins') (Beg, 2002; Rifkin et al., 2005; Bianchi, 2007). The concept of an endogenous molecule triggering an immune response was proposed by Matzinger, who also introduced the 'danger model' of immune activation (Matzinger, 2002; Seong and Matzinger, 2004; Matzinger, 2007). The danger signal concept has thereby been complementing the classical 'stranger model' of immune activation by non-self molecules, as proposed by Medzhitov and Janeway (2002). DAMPs themselves present as most diverse molecules, often but not exclusively of protein nature. They serve diverse functions under normal physiological conditions, such as acting as chaperones, chromatin components or plasma factors (Lotze et al., 2007; Kono and Rock, 2008; Milanski et al., 2009). According to the current theory, these molecules acquire a DAMP property when they are presented in an unphysiological compartment or format, for example when they are released into the extracellular space by dying cells or into parenchymal compartments by vascular leakage (or BBB impairment), when they are shed off the extracellular matrix (ECM) upon tissue damage or massive cell migration or when they are modified in their conformation, aggregation, glycosylation or oxidization (Lotze et al., 2007; Pineau and Lacroix, 2009; Stewart et al., 2010).

Consequently, cells of the innate immunity would sense such molecules as they occur in pathophysiological scenarios only. DAMPs could thereby amplify the immune response to PAMPs, as infection most often associates with tissue damage. This way, the DAMP response could prepare or accelerate the subsequent or even modulate an ongoing PAMP response. However, an immune response to DAMPs can also occur in the absence of inflammation, a scenario referred to as 'sterile inflammation'. Indeed, TLR4 (either alone

or in cooperation with TLR2 or TLR6) could be shown to bind ECM degradation products, like hyaluronic acid or fibronectin (Okamura et al., 2001; Jiang et al., 2005a), cellular components, like members of the high-mobility group box (HMGB) family, heat-shock proteins (Hsp), S100A8/S100A9 (Apetoh et al., 2007; Tsan and Gao, 2009; Vogl et al., 2007) or structure-modified proteins with links to degenerative or metabolic diseases, such as amyloid-β (Aβ) and oxidized low-density lipoprotein (oxLDL) in Alzheimer's disease and atherosclerosis (Stewart et al., 2010).

This ability of TLR4 to accept such a wide range of different ligands is most likely made possible – or supported – by the engagement of ligand-specific co-receptors. The GPI-anchored CD14 is the prototypic associate of TLR4 in the recognition of LPS. It also reveals more and more importance for some discriminative signaling consequences of the complex (Gangloff et al., 2005). Yet the list of proposed or proven TLR4 partners keeps growing and includes Fc receptors (FcRII/III, also known as CD32/16), integrins (CD11b), chemokine receptors (CXCR4) and many more (e.g. like the scavenger receptor CD36, or CD55) (Triantafilou and Triantafilou, 2002; Triantafilou et al., 2008). In this regard, the most recent report on the recognition of oxLDL and Aβ convincingly shows that TLR4 can chose among TLR and non-TLR surface molecules to create varying assemblies (Stewart et al., 2010). TLR4 in homodimeric form and partnership with CD14 would accept LPS and some DAMPs, whereas the newly described TLR4-TLR6-CD36 receptor complex can sense oxLDL and Aβ.

1.7 Structural variations of LPS are differentially recognized by TLR4

Bacterial LPS represents one of the primary targets of the host innate immune system to recognize a Gram-negative bacterial infection. Upon LPS encounter, the subsequent innate immune response is characterized by the release of proinflammatory mediators (including TNFα and IL-6), which is beneficial in initiating and orchestrating the elimination of the infection by means of the innate as well as the adaptive immunity. However, in the case of an excessive (systemic) exposure to LPS, the body will react with a systemic inflammatory reaction leading to multi-organ failure, with a high risk of death, a condition often referred to as septic shock.

Positioned in the outer membrane of Gram-negative bacteria, LPS serves as a most critical component guaranteeing both the structural and functional membrane integrity.

Biochemically, the molecule was identified to consist of lipid and carbohydrate moieties, thereby defining the common name 'lipopolysaccharide'. The term 'endotoxin' is also still in use. Structurally, all known LPS variants share a common architecture, comprising three major building blocks, i.e. the Lipid A portion, a core polysaccharide as well as the O-polysaccharide (Fig. 1.2A). Modifications of this basic structure give rise to a huge range of variants in the different bacterial strains. LPS molecules containing all three sub-structures are referred to as 'wild type' or 'smooth' LPS. In contrast, molecules that lack the O-polysaccharide are known as 'rough' mutants, which can be further classified according to the level of completeness of their core polysaccharide structures. This way, rough LPS mutants are termed from 'Ra', with a complete core, to 'Re', having only the basic sugar residues attached to their Lipid A portion (see also below). All together, these structural variants are generally termed LPS 'chemotypes'. The classification as to smooth (S) and rough (R) derives from the appearance of the bacterial colonies made by strains expressing either of the two LPS versions as their major cell wall components. The discrimination as to S and R chemotypes is, however, better based on molecular differences in the LPS – and these structural versions come with distinct functional properties.

The Lipid A portion of the molecule was shown to be the carrier of the endotoxic activity, as synthetic Lipid A preparations exhibited biological activities identical to those of *E.coli* Lipid A (Tanamoto et al., 1984; Galanos et al., 1985). This portion of the LPS molecule is typically composed of a bisphosphorylated diglucosamine backbone which is substituted with up to four acyl chains. These acyl chains can be further substituted with fatty acids leading to a Lipid A that carries up to seven acyl substituents. Depending on the bacterial strain, these fatty acid substitutions vary by number, length, order and saturation. In terms of biological activity, it appears that *E.coli* Lipid A, with its hexa-acylated and diphosphorylated diglucosamine backbone (Fig. 1.2B), represents the structure optimally recognized by the respective mammalian receptors and that any modification of this 'ideal' structure will result in reduced endotoxicity (Rietschel et al., 1994).

The core polysaccharide represents a relatively defined carbohydrate structure, with only a limited number of different sugars being incorporated. This consequently results in a high degree of conservation among bacterial strains, regarding this partial element. Structurally, the core polysaccharide can be formally divided into an inner and an outer core, the latter being generally more variable by composition. The inner core is especially characterized by the presence of rather unusual sugars, such as 3-deoxy-D-manno-

octulosonic acid (Kdo) and heptose. Kdo is found in almost every known LPS. It links the core polysaccharide to the carbohydrate backbone of Lipid A. It might be this particular function – assuring bacterial viability – which makes the Kdo residue an indispensible constituent of virtually any LPS structure. Indeed, an essential role is supported by findings where the smallest saccharide component found in naturally occurring bacteria consisted of only one to three Kdo residues (Brade et al., 1987; Helander et al., 1988).

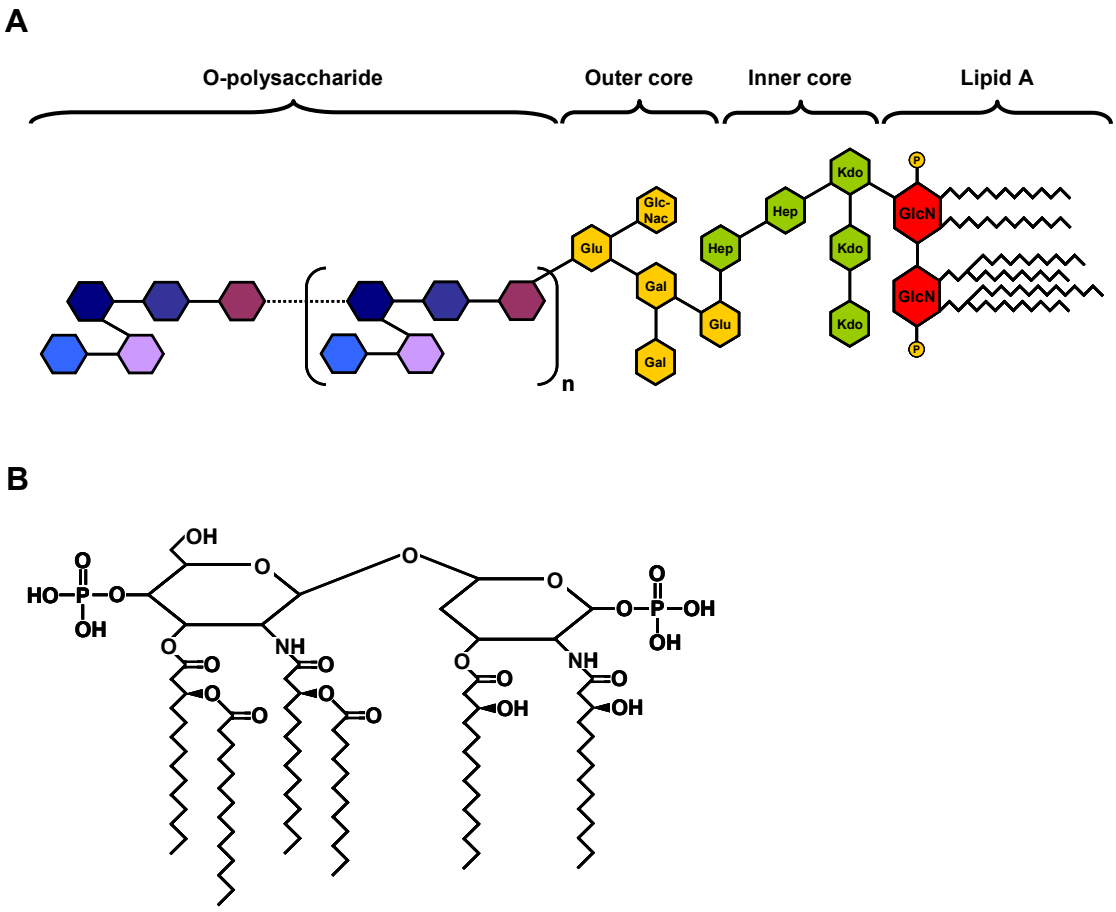


Fig. 1.2: Structure of LPS. (A) Principle structure of 'wild type' LPS as divided into O-polysaccharide, core polysaccharide and Lipid A portion. Gal, galactose; GlcN, glucosamine; GlcNac, N-acetyl-glucosamine; Glu, glucose; Hep, heptose; Kdo, 2-keto-3-desoxyoctonate; P, phosphate. **(B)** Chemical structure of *E.coli* Lipid A as having the format widely believed to be optimally recognized by mammalian TLR4. Adapted from Erridge et al. (2002).

The O-polysaccharide consists of 1 to 50 repeating units with each of them being composed of 1 to 8 sugar residues. Thereby, a given bacterial strain shows individual O-polysaccharide characteristics by virtue of the set of sugar units (monosaccharides), their sequence and chemical linkage, substitutions and ring formats. By combination, these variables lead to an almost limitless diversity of O-polysaccharide structures, which is

reflected by the appearance of hundreds of serotypes for particular Gram-negative species. Due to its positioning at the outermost part of the LPS molecule, the O-polysaccharide is also the major target for host antibody responses, which is the reason for its alternative name 'O-antigen'.

At this point, it should be stressed that, despite the fact of Lipid A being the carrier of the molecules' endotoxic activity, the nature and number (length) of the attached sugar chains both have considerable impact on modulating this activity (Erridge et al., 2002). Indeed, several studies already demonstrated some relationship between LPS structures (chemotypes) and function (Gangloff et al., 2005; Jiang et al., 2005b; Huber et al., 2006). These observations were, however, made on mast cells and extra-neural macrophages. With the growing understanding of differences in the TLR4 organization by individual cell types and a concomitant variation in agonist action, the situation may differ for microglia, which have not been studied yet in this regard. Thus, some of the 'rules' of chemotype signaling though TLRs, namely TLR4, may reveal variation when focusing on other cell types.

2. Aim of the Study

Microglia are the resident tissue macrophages of the CNS and as such they take care of the tissue homeostasis. Based on histopathological findings, microglia are engaged in virtually any CNS pathology, which is why they have been considered for a long time as 'toxic cells'. However, microglia did not simply evolve as 'risk factors' for the CNS. Evidence is accumulating that their reactions aim primarily (if not exclusively) at neuroprotection. Misinterpretations of microglial actions might originate from the still limited knowledge about the versatility of their reactive programs and functional options. Like other (tissue) macrophages, also microglia are equipped with a large 'tool set' of molecular antennas, i.e. receptors, for the detection of tissue disturbances. These receptors are linked to a complex machinery for the initiation and propagation of appropriate consequences. Among the huge number of receptors, microglia employ the family of TLRs to recognize exogenous as well as endogenous threats. Based on a general microglial proinflammatory response to TLR stimulation in the context of 'stranger' signals we addressed the following questions:

(1) Is the (mouse) microglial TLR4 signaling capable of discriminating between different structural variants of bacterial LPS (chemotypes), like it was shown for other extra-neural macrophage populations?

(2) How is the microglial TLR signaling organized and what are critical determinants for the efficient induction of the response?

(3) To which extent are microglial TLR responses sensitive to modulation by secondary (signaling) events and what are the consequences of such interference?

(4) Does the microglial population comprise a rather inhomogeneous population of cells with respect to developmental changes as well as populational diversity?

Following recent conceptual changes in the perception of diverse microglial reactive phenotypes, this work thus aimed at substantiating this rather new concept by means of deciphering the diverse and fine-tuned microglial responses to TLR stimulation *in vitro*, thereby also trying to complement respective *in vivo* studies.

3. Material and Methods

3.1 Animals and cells

All animals were routinely bred by and obtained from the central animal facility of the University Medicine Göttingen, Germany. NMRI and C57Bl/6J wild type strains were housed under normal conditions. The maintenance of mutant strains with a deficiency for CD14 ($CD14^{-/-}$), MyD88 ($MyD88^{-/-}$ and $MyD88^{+/-}$), TLR4 ($TLR4^{-/-}$) and TRIF ($TRIF^{-/-}$) was carried out under specific pathogen-free (SPF) conditions. All animals were housed and treated according to the guidelines for animal care of the University Medicine Göttingen.

Primary microglial cell cultures were prepared from whole brains of newborn (P0) mice from the above mentioned strains and cultured in complete medium [Dulbecco's modified Eagle's medium (DMEM; Invitrogen/Gibco, Karlsruhe, Germany) supplemented with 10 % fetal calf serum (FCS; Invitrogen/Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (both Biochrom, Berlin, Germany)] as previously described (Hanisch et al., 2001). In brief, brains were liberated from meninges and blood vessels, washed with Hank's balanced salt solution (HBSS; Biochrom) and incubated with 2.5 % trypsin (Biochrom) for 10 min at 37°C. Enzymatic reaction was stopped by addition of complete medium supplemented with 0.4 mg/ml DNAse (CellSystem, St. Katherine, Swizerland), followed by another incubation at 37°C for 5 min. Afterwards, remaining cell clusters were mechanically separated and the suspension was centrifuged at 200 x g at 4°C for 10 min. The supernatant was removed and cells were resuspended in fresh complete medium to be seeded in 75 cm^2, poly-L-lysine (PLL)-coated culture flasks. Subsequent cultures were carried out in a humidified atmosphere with 5 % CO_2 at 37°C. The next day, primary mixed-glial cultures were washed 3 times with PBS (Invitrogen/Gibco) and received fresh complete medium. Culture medium was then changed every other day. After 5 days of primary cultivation, microglial proliferation was stimulated by adding complete medium supplemented with 30 % of L929-conditioned cell culture supernatant (see below). After another 3-5 days, microglial cells were harvested by shaking, making use of the cells different adhesion properties. Cells were harvested, washed with complete medium and counted. The purity of microglia preparations was determined to be routinely >98 %, based on nuclear and cell type-specific immuno- and lectin-cytochemistry (data not shown).

For stimulation experiments cells were plated in 96-well tissue culture plates at a density of 15,000 cells/well, in 12-well plates at a density of $3x10^5$ cells/well, or in Petri dishes (diameter 34 mm) at a density of $1x10^6$ cells/dish.

Immediately before being used for primary culture, cell culture flasks were incubated with 100 µg/ml PLL (Invitrogen/Gibco) for 30 min at RT. Excess of PLL was washed off by rinsing flasks 3 times with sterile ddH_2O.

Bone marrow-derived macrophages (BMDM) from 10.5 weeks old NMRI wild type mice were kindly prepared and provided by Eva Rietkötter from the Department of Hematology and Oncology at the University Medicine Göttingen.

3.2 *In vitro* culture of *ex vivo* isolated postnatal microglia

For the *in vitro* culture of postnatal microglia the protocol for culturing neonatal microglia (section 3.1) was adjusted as follows. Mice of the indicated strain and age (in days) were sacrificed and the head was immediately separated from the body by decapitation. The brain was prepared out of the skull and roughly dissected into brain stem, cerebellum and cerebrum, the latter being further separated into the two hemispheres. All parts were liberated from meninges, large blood vessels and the choroid plexus (where applicable). Afterwards, the tissue was mechanically dissociated resulting in tissue chunks of approximately 1 mm^3. Tissue chunks of the formerly separated parts were collected into a single tube to be washed and enzymatically processed as described for neonatal microglia. Before being seeded the cell suspensions were passed through a cell strainer with a pore size of 40 µm (BD Biosciences, Heidelberg, Germany) in order to remove remaining tissue chunks and large cell clumps.

Cell suspensions were seeded into 75 cm^2 tissue culture flasks containing a confluent monolayer of neonatal astrocytes. These 'feeder cultures' were derived from regular neonatal mixed glial cultures as described in section 3.1. After the astrocytic monolayer of these cultures had reached confluence loosely attached microglia were shaken off and harvested to be discarded. The culture was then incubated with 200 µg/ml clodronate (Merck/Calbiochem, Darmstadt, Germany) in complete medium for 48 h at 37°C and 5 % CO_2. This bisphosphonate is taken up primarily by phagocytosing cells (like microglia), thereby inducing apoptosis of these cells. Accordingly, clodronate was used here to deplete the neonatal mixed glial cultures from (neonatal) microglia. After the

incubation period cultures were vigorously shaken over night at 37°C to detach as much as possible residual microglia. The supernatant was discarded and the culture washed once with PBS and once with complete medium to be then left in the incubator (37°C, 5 % CO_2) until used (usually within 24-48 h). Immediately before the addition of the postnatal cell suspension the astrocytic feeder culture got another medium change. After seeding, the postnatal mixed glial cultures were treated as described for neonatal preparations (with the appropriate washes and medium changes). After addition of L929-conditioned cell culture supernatant postnatal microglia were harvested approximately every 7 days for a maximum of 4 harvests. Postnatal microglia were plated and stimulated exactly the same way than described for neonatal cells. Contamination of postnatal microglia by neonatal cells was tested to be less than 5 % (not shown).

For region-specific neonatal preparations the cerebellum was prepared as described above and then treated and cultured separately. In order to achieve a good yield of postnatal cerebellar microglia, 2-3 cerebella were prepared for one T75 flask, depending on the original brain sizes. Similarly, cortical microglia were prepared as regional sub-population. Here, differing from the preparation of whole postnatal brains, the cortical portion of each cerebral hemisphere was flapped off, separated and liberated from any visible (white) myelin. Usually one complete cortex preparation (2 hemispheres) was used for one culture. In addition, spinal cord microglia were prepared in parallel to the other region-specific populations. For this purpose, the vertebral column was prepared from the sacrificed animal and from this the spinal cord was dissected carefully. Meninges were removed and the tissue was further processed as described above. Depending on the original size (of the animal) 1-2 spinal cord preparations were used for one culture.

3.3 L929 mouse fibroblast cultures

In parallel to primary microglial cultures, L929 mouse fibroblasts were routinely cultured in complete medium (see 3.1) and passaged (1:5) every 2 weeks. After 14 days of continues cultivation (no medium change in between), culture supernatants were isolated and stored at -20°C until used for the stimulation of microglial proliferation. After 30 passages fresh L929 cultures were established from a stock stored in liquid nitrogen.

3.4 Co-culture of microglia and tumor cell lines

Co-culture experiments involving tumor cell lines were performed in collaboration with Prof. Claudia Binder and Dr. Tobias Pukrop from the Department of Oncology and Haematology, University of Göttingen. The human breast cancer cell line MCF-7 and the two murine mammary carcinoma cell lines M410.4 (herein described as 410.4) and 4T1, respectively, were routinely cultured and provided by the above mentioned collaborators. For co-culture experiments $1x10^5$ tumor cells (of the respective cell line) were plated per well in 24-well tissue culture plates and cultured for 24 h. For indirect co-culture $2x10^5$ neonatal (P0) microglial cells were seeded in 24-well culture plate inserts on a polycarbonate membrane with a pore size of 0.4 µm (Merck/Millipore, Darmstadt, Germany) and cultured for 24 h as well. After this initial culture period the microglia-containing inserts were transferred to the tumor cell-containing 24-well plates and the co-cultures were stimulated as indicated. As controls, tumor cells (in 24-well plates) and microglia (in inserts) were stimulated in the absence of the respective second cell type, in parallel to the co-culture stimulation. For direct co-culture $2x10^5$ microglial cells were seeded on top of the respective tumor cells ($1x10^5$ in 24-well plates) and the (direct) co-cultures were cultured for 24 h before getting stimulated as indicated. As controls tumor cells (in 24-well plates) and microglia (in 24-well plates) were stimulated in the absence of the respective second cell type, in parallel to the co-culture stimulation. All experiments were performed using complete medium. Cells were cultured in a humidified atmosphere at 37°C and 5 % CO_2.

3.5 *In vitro* stimulation experiments

S-LPS [smooth chemotype lipopolysaccharide (LPS), *E.coli*, serotype O55:B5], Ra-LPS (Ra chemotype LPS, *E.coli*, serotype EH100), Rc-LPS (Rc chemotype LPS, *E.coli*, serotype J5), Re-LPS (Re chemotype LPS, *E.coli*, serotype R515) and Lipid A (E.coli, serotype R515) were purchased as ultra-pure (TLR*grade*™) preparations from Axxora/Alexis Biochemicals (Grünberg, Germany). *S.e.*-LPS (presumably Ra chemotype LPS, *Salmonella enterica*, serotype minnesota) was from Sigma-Aldrich (Taufkirchen, Germany). MPLA (Monophosphoryl Lipid A, *Salmonella minnesota*, serotype R595), sMPLA (synthetic MPLA), and Rs-LPS (LPS of undefined chemotype, *Rhodobacter*

sphaeroides) were purchased from InvivoGen (San Diego, CA, USA). Monoclonal rat-anti-mouse CD14 (clone 4C1) was from BD Pharmingen (Heidelberg, Germany). Recombinant mouse IFNγ, IL-4 and IL-10 were purchased as carrier-free preparations from R&D Systems (Wiesbaden, Germany). Carrier-free mouse IFNβ was from PBL Biomedical Laboratories (Piscataway, NJ, USA). Atenolol (AT), ICI 118,551 hydrochloride (ICI), DL-noradrenaline hydrochloride (NA), oxymetazoline hydrochloride (OX), phentolamine methansulfonate salt (PM), (S)-(-)-propranolol hydrochloride (PR) and salbutamol (SB) were all purchased from Sigma-Aldrich. Isoproterenol bitartrate (IP) was provided by Prof. Michael T. Heneka, University of Bonn, Germany. Forskolin was from Sigma-Aldrich. The PKA inhibitors 14-22 amide and H-89 dihydrochloride were both from Merck/Calbiochem. All working solutions containing single or the combination of two or more substances were freshly prepared from frozen (-20°C/-80°C) or refrigerated (4°C) stocks by dilution in complete medium immediately prior to addition to the cells. Unless otherwise specified, cells were incubated for 18 h in a humidified atmosphere at 37°C with 5 % CO_2. After the incubation period cell culture supernatants were isolated and stored at -20°C until used for the measurement of released factors. In addition, after each stimulation experiment (carried out in the 96-well dimension) cells were routinely tested for their viability. For this purpose cells received fresh complete medium supplemented with 10 % WST-1 reagent (Roche Applied Science, Mannheim, Germany) to be incubated for another 3 h. After that incubation the resulting color reaction was measured at 450 nm (with subtraction of a 655 nm reference wavelength) on a microplate reader (BioRad, Munich, Germany). The measured values served as direct indication for the cells viability and were used for the interpretation of individual results.

When working with blocking antibodies (anti-CD14) or inhibitors (14-22 and H-89, respectively), cells received a pre-incubation with the respective antibody/inhibitor concentration for 30 min. Antibody/inhibitor preparations were routinely tested for their intrinsic stimulation capacity (with respect to cyto/chemokine release) and were found to be inactive on microglial cells.

3.6 Induction efficacy assay

For this particular microglia stimulation experiment cells received the indicated stimuli for an initial period A of 15, 30, 60, 120 or 180 min, respectively. Afterwards, the stimulus

was removed and cells were carefully washed 3 times with complete medium to receive fresh complete medium (without stimulus) for an intermediate incubation period B of varying length, depending on the duration of the corresponding period A. In each case the combined incubation time for periods A and B was 180 min, e.g. 15 + 165 min, 60 + 120 min or 180 + 0 min, respectively. After these first 3 experimental hours all supernatants were removed and cells again were carefully washed 3 times with complete medium. After receiving fresh complete medium (without stimulus) cells were incubated for a collection period C of 21 h. After this final incubation period cell culture supernatants were isolated and stored at -20°C until further used for the measurement of released factors. In addition, the experiment employed 3 control groups: (i) cells receiving complete medium (without stimulus) for the entire 24 h; (ii) cells receiving stimulus containing medium for 3 + 21 h; (iii) cells receiving stimulus containing medium for the entire 24 h. All results were normalized to the results obtained from control group (iii). For an illustration of the experimental layout see also Fig. 4.6B.

3.7 Induction stability assay

Cells received the indicated stimuli for an initial stimulation period of 24 h. After that period cell culture supernatants were isolated and stored at -20°C until further used for the measurement of released factors. At the same time, cells were carefully washed 3 times with complete medium and received fresh complete medium (without stimulus) for an additional incubation period of 12, 24, 36 or 48 h, respectively (as indicated). After this second incubation period, again cell culture supernatants were isolated and stored as above. In parallel, a group of control cells received stimulus containing medium for the maximum incubation period of 72 h. All results were normalized to the results obtained from this control group.

3.8 Sub-threshold pre-conditioning stimulation assay

Parallel groups of cells received the indicated stimulus in various non-saturating doses (as previously determined in dose-response studies) for an initial stimulation period of 24 h. After that period cell culture supernatants were isolated and stored at -20°C until further used for the measurement of released factors. At the same time, cells were carefully

washed 3 times with complete medium and received fresh complete medium now containing a saturating dose of the respective stimulus for an additional incubation period of 24 h. After this second incubation period, again cell culture supernatants were isolated and stored as above. In parallel, a group of control cells received the respective stimulus at a saturating dose for the entire incubation period of 48 h. All results were normalized to the results obtained from this control group.

3.9 Quantification of soluble factors in cell culture supernatants

Collected cell culture supernatants were analyzed for the release of cyto- and chemokines by microglia with the help of commercial enzyme-linked immunosorbent assay (ELISA) test systems. Levels of IL-1β, IL-6, IL-10, IL-12p70, CCL2 (monocyte chemoattractant protein [MCP]-1), CCL3 (macrophage inflammatory protein [MIP]-1α), CCL5 (regulated upon activation normal T-cell expressed and presumably secreted [RANTES]), CCL11 (Eotaxin), CCL17 (thymus and activation regulated chemokine [TARC]), CCL22 (macrophage derived chemokine [MDC]), CXCL1 (KC, the mouse equivalent of human growth regulated protein [GRO]α), CXCL2 (MIP-2, the mouse equivalent of human GROβ) and CXCL10 (IFNγ inducible protein [IP]10) were determined using DuoSet ELISA Development Kits (R&D Systems). The amount of secreted IFNβ was measured using a sandwich ELISA kit from PBL Biomedical Laboratories, TNFα levels were measured using a sandwich ELISA kit from BioLegend (San Diego, CA, USA), and total IL-12p40 (covering the detection of monomeric p40, homodimeric $p40_2$ and heterodimeric IL-12/IL-23) levels were determined using a sandwich ELISA kit from eBioscience (San Diego, CA, USA). All ELISA procedures were performed according to the manufacturer's instructions. Absorbance was measured at 450 nm (with subtraction of a 540 nm reference wavelength) on a microplate reader. Based on individual standardization of each ELISA assay with recombinant proteins, results were calculated with the help of a Microsoft Excel program (macro), which was developed by Jörg Scheffel at the Institute of Neuropathology, University Medicine Göttingen.

3.10 Analysis of MHC I up-regulation and myelin phagocytosis by flow cytometry

Cells ($2x10^5$ per well in 12-well plates) were incubated with the indicated stimuli for 48 h in a humidified atmosphere with 5 % CO_2 at 37°C. After the incubation period cells were carefully washed twice with complete medium and once with PBS. For detaching from the culture dish surface cells received 0.05%/0.02% Trypsin/EDTA (Biochrom) and were incubated for 3-5 min at 37°C. The enzymatic reaction was stopped by adding complete medium and cells were carefully scraped off to be collected in microcentrifuge tubes. Afterwards, cells were centrifuged at 700 x g for 10 min at 4°C and resuspended in FACS buffer (PBS supplemented with 2% FCS, 0.01 M EDTA pH 8.0 and 0.1 % NaN_3). Cells were washed once and resuspended in FACS buffer before 0.5 µg rat anti-mouse CD16/CD32 antibody (Fc Block™, clone 2.4G2, BD Pharmingen) was added. After incubation on ice for 5 min 0.5 µg of Alexa647-conjugated anti-mouse MHC I antibody (H-$2K^b$/H-$2D^d$, clone 34-1-2S, BioLegend) was added and cells were incubated for 30 min on ice in the dark. Cells were washed once with FACS buffer to be than incubated with 0.2 µg of phycoerythrin (PE)-conjugated anti-mouse CD11b antibody (clone M1/70, eBioscience) for 30 min on ice in the dark. Finally, cells were washed once and resuspended in FACS buffer to be aquired on a FACSCalibur flow cytometer (BD Biosciences). Only $CD11b^+$ events were analyzed for their MHC I surface expression. Data was analyzed using FlowJo (Tree Star, Ashland, OR, USA) and WinMDI software.

For the myelin phagocytosis assay cells ($2x10^5$ per well in 12-well plates) were incubated with the indicated stimuli for 24 h before 5 µg fluorescein isothiocyanate (FITC)-conjugated mouse myelin was added to the cells for another 2 h of incubation. Afterwards, cells were washed and harvested from the culture dish as described above. Cells were centrifuged at 800 x g for 8 min at 4°C, washed once with FACS buffer and incubated with 0.2 µg of allophycocyanin (APC)-conjugated anti-mouse CD11b antibody (clone M1/70, eBioscience) for 30 min on ice in the dark. Samples were washed once with FACS buffer and acquired and analyzed as described above. Only $CD11b^+$ events were analyzed for their myelin uptake characteristics.

3.11 Analysis of intracellular COX2 by flow cytometry

Cells ($2x10^5$ per well in 12-well plates) were incubated with 10 ng/ml Re-LPS (or medium) for 24 h in a humidified atmosphere with 5 % CO_2 at 37°C. After the incubation period cells were harvested from the tissue culture plate as described in section 3.10. After a centrifugation at 800 x g for 12 min at 4°C, cells were washed once with PBS and resuspended in 250 µl Cytofix/Cytoperm™ solution (BD Biosciences) while vortexing (to avoid cell aggregation). Cells were incubated for 20 min at 4°C in the dark before 500 µl of Perm/Wash buffer (2 % FCS and 0.1 % saponin in PBS) were added and the mixture was centrifuged as before. Cells were then resuspended in FACS buffer (section 3.10) and incubated with 0.5 µg Fc Block™ for 5 min at RT. Next, cells were incubated with 0.2 µg of APC-conjugated anti-mouse CD11b antibody (eBioscience) for 20 min at RT in the dark. After the incubation cells were washed with FACS buffer using centrifugation as before. Cells were then resuspended in 100 µl Perm/Wash buffer and incubated again with Fc Block™ (as before). Next, cells were incubated with 0.1 µg of rabbit anti-mouse COX2 polyclonal antibody (Cayman Chemical, Ann Arbor, MI, USA) for 30 min at RT in the dark. After another wash with Perm/Wash buffer cells were resuspended in 100 µl Perm/Wash buffer and incubated with 1.1 µg of FITC-conjugated anti-rabbit antibody (Sigma-Aldrich) for 20 min at RT in the dark. Finally, cells were washed with Perm/Wash buffer, resuspended in FACS buffer and acquired and analyzed as described in section 3.10.

3.12 Analysis of intracellular cytokines by flow cytometry

Cells ($2x10^5$ per well in 12-well plates) were incubated with 10 ng/ml Re-LPS (or medium) for 5 h before 50 µl of 1x Monensin solution (BioLegend) were added for another 3 h. All incubations were carried out in a humidified atmosphere with 5 % CO_2 at 37°C. After the incubation period cells were harvested from the tissue culture plate as described in section 3.10. After a centrifugation at 800 x g for 10 min at 4°C cells were washed once with FACS buffer (section 3.10) and resuspended in 100 µl FACS buffer to be incubated with 0.25 µg of FITC-conjugated anti-mouse CD11b antibody (clone M1/70, eBioscience) for 20 min at RT in the dark. Cells were then washed once with PBS and resuspended in 200 µl Cytofix/Cytoperm™ solution while vortexing (to avoid cell aggregation). Cells were

incubated for 20 min at 4°C in the dark before being washed once with FACS buffer. Cells were then stored in FACS buffer overnight at 4°C. The next day, cells were washed once with Perm/Wash buffer (section 3.11) and resuspended in 100 µl Perm/Wash buffer to be incubated with 0.5 µg Fc Block™ for 5 min at RT. Next, cells were incubated with 250 ng of PE-conjugated anti-mouse TNFα monoclonal antibody (clone MP6-XT22, R&D Systems) or APC-conjugated anti-mouse MIP-1α monoclonal antibody (clone 39624, R&D Systems) or the combination of both antibodies, respectively, for 45 min at RT in the dark. Finally, cells were washed once with Perm/Wash buffer and once with FACS buffer to be resuspended in FACS buffer and acquired and analyzed as described in section 3.10.

3.13 Measurement of phosphorylated signaling proteins

Cells ($1x10^6$ per Petri dish) were stimulated with the respective stimuli for the indicated times. After the stimulation periods cells were washed once with ice-cold PBS, incubated with cell lysis buffer [New England Biolabs (NEB)/Cell Signaling Technology (CST), Frankfurt/Main, Germany] for 5 min on ice. Next, cells were scraped off, harvested and sonicated for further dissociation. Final lysates were stored at -80 °C until assayed. For measurement of phosphorylated (activated) NFκB p65, $p38^{MAPK}$ and $p44/42^{MAPK}$ (ERK1/2) levels, lysates were analyzed with appropriate sandwich ELISA kits from NEB/CST. All ELISA procedures were carried out according to the manufacturer's instructions. Absolute levels of measured signals were normalized to the respective experimental maxima.

3.14 Analysis of microglial adrenergic receptor expression

Cells ($1x10^6$ per Petri dish) were stimulated with 1 ng/ml Re-LPS or were left unstimulated for 18 h. After the stimulation period cells were washed once with PBS. Then, cells were lysed, harvested and total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), thereby following the manufacturer's instructions. Total RNA was used for reverse transcription of mRNA to cDNA using SuperScript® RT (Invitrogen). PCR was then performed with the resulting cDNA using the primers as listed in Tab. 3.1. PCR products were separated on a 2 % agarose gel and visualized with the ChemiDoc gel documentation system (BioRad), using ethidium bromide.

Tab. 3.1: Primer specifications for the analysis of microglial adrenergic receptor (AR) expression.

AR subtype	Primer sequence
$\alpha 1_A$ (forward)	5' – GTA GCC AAG AGA GAA AGC CG – 3'
$\alpha 1_A$ (reverse)	5' – CAT CCC ACC ACA ATG CCC AG – 5'
$\alpha 1_B$ (forward)	5' – GCT CCT TCT ACA TCC CAC TGG – 3'
$\alpha 1_B$ (reverse)	5' – AGG GGA GCC AAC ATA AGA TGA – 3'
$\alpha 2_A$ (forward)	5' – GCT CCC CAA AAC CTC TTC CTG GTG – 3'
$\alpha 2_A$ (reverse)	5' – GAG TGG CGG GAA GGA GAT GAC AGC – 3'
β1 (forward)	5' – TCG TGT GCA CAG TGT GGG CC – 3'
β1 (reverse)	5' – AGG AAG CGG CGC TCG CAG CTG TCG – 3'
β2 (forward)	5' – ACC TCC TTT TTG CCT ATC CA – 3'
β2 (reverse)	5' – TAG GTT TTC GAA GAA GAC CT – 3'
β3 (forward)	5' – ATG GCT CCG TGG CCT CAC – 3'
β3 (reverse)	5' – CCC AAG GGC CAA TGG CCA GTC AGC G – 3'

3.15 Quantification of intra- and extracellular cAMP levels

For the measurement of cAMP levels, induced after microglial stimulation with noradrenaline (NA), culture supernatants and cell lysates were analyzed using a cAMP Parameter™ assay kit (R&D Systems). In brief, cells ($1x10^6$ per Petri dish) were stimulated with 10 µM NA for the indicated periods of time. After the incubation supernatants were isolated and centrifuged at 600 x g for 10 min at 4°C in order to remove any particulates. The remaining cells were rinsed 3 times with ice-cold PBS before 200 µl of cell lysis buffer (provided with the kit) were added per well. Cells were then frozen at -20°C and thawed immediately afterwards. This freeze-thaw cycle was repeated once before the cell material was collected and centrifuged at 600 x g for 10 min at 4°C. The cleared lysates and supernatants were then analyzed by ELISA according to the instructions provided with the kit. Absorbance was measured at 450 nm (with subtraction of a 540 nm reference wavelength) using a microplate reader. Results were calculated based on a cAMP reference provided with the kit.

4. Results

Part A – Mouse microglia respond to LPS of various chemotypes

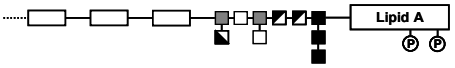
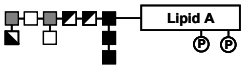
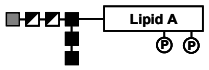
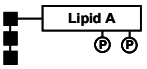
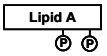
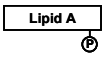
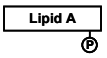
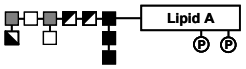
LPS, also commonly termed 'endotoxin', represents the prototypic exogenous ligand for TLR4. As a major cell wall component of Gram-negative bacteria, the complex glycolipid comes with structural variations by strain. Common to all molecules, the Lipid A part as a multi-acylated glucosamine structure carries the basic pathogenicity of LPS (Fig. 1.2B). LPS structures can vary by acylation and phosphorylation. Moreover, the molecules are classified into structural variants depending on the additional glycosylation, giving rise to different 'chemotypes' (Tab. 4.1). From studies on various cell types, including macrophages, it is known that the various LPS chemotypes have distinct properties in terms of the response they can induce. So far, this has not been proven for microglia.

4.1 LPS chemotypes trigger similar patterns of released cyto- and chemokines

To answer the question whether mouse microglia would recognize different chemotypes of pathologically relevant bacterial strains, e.g. *E. coli* and *Salmonella* species, primary cell cultures were stimulated with the compounds listed in Tab. 4.1 and respective dose-response relations were monitored (Fig. 4.1A).

In brief, microglial cells were capable of recognizing all substances tested as they released a similar pattern of proinflammatory cyto- and chemokines. However, there was a clear 'sensitivity gradient' with the highest sensitivity towards the members of the rough LPS type, followed by S-LPS, Lipid A and *S.e.*-LPS, all of which needed a 10-fold higher concentration to elicit a response. Furthermore, the monophosphoryl Lipid A (MPLA) and its synthetic counterpart (sMPLA) needed a 100-fold and 1000-fold, respectively, higher concentration to elicit a substantial release response by microglia. This gradient could similarly be observed for the induction of IFNβ (Fig. 4.1B). In addition, some of the LPS variants, especially the *S.e.*-LPS (presumably of the Ra chemotype), showed a bell-shaped dose-response behavior before reaching a more plateau-like level, whereas others reached a plateau at already lower concentrations, which then remained at its level even with higher concentrations.

Tab. 4.1: LPS chemotypes vary in their biochemical characteristics. Structural variants of LPS derived from various Gram-negative bacterial strains are discriminated by the presence of an O-polysaccharide structure (S-LPS) and the composition of the core polysaccharide (R-LPS variants). Additional structural variations reside in the Lipid A part and come with the number of phosphate groups and acyl chain substitutions (structural schematics modified from Huber et al., 2006; see also Erridge et al., 2002)

Full name (bacterial strain, serotype)	Abbrev.	Structure
smooth LPS (*E.coli*, O55:B5)	S-LPS	
rough Ra LPS (*E.coli*, EH100)	Ra-LPS	
rough Rc LPS (*E.coli*, J5)	Rc-LPS	
rough Re LPS (*E.coli*, R515)	Re-LPS	
Lipid A (*E.coli*, R515)	Lipid A	
monophosphoryl Lipid A (*S.minnesota*, R595)	MPLA	
synthetic monophosphoryl Lipid A	sMPLA	
presumably rough Ra LPS (*S.enterica*, Minnesota)	*S.e.*-LPS	

■ 2-keto-3-desoxyoctonate; ◩ heptose; ■ glucose; □ galactose; ◪ N-acetyl-glucosamine;
▭ O-polysaccharide unit of undefined carbohydrate composition

Tab. 4.2 summarizes the absolute values of released cyto- and chemokines (from Fig. 4.1A) that were obtained with stimulations at maximal concentrations. Interestingly, (s)MPLA, despite being recognized by microglial cells only at relatively high concentrations, was able to induce the highest absolute amounts of most cytokines, compared to the other LPS compounds. On the other hand, S- and R-LPS structures presented with a very similar pattern of the released factors, also in terms of absolute amounts. Taken together, microglial cells were able to react to all tested LPS chemotypes but, at the same time, could discriminate between them in terms of sensitivity. Moreover, the quantitative nature of the induced proinflammatory cytokine responses varied, in part significantly, depending on the stimulus. Of note, C57Bl/6 wild type microglial cells exhibited responses with quite similar dose-response profiles as established for NMRI wild type cells (Fig. 4.1). Only minor differences in the absolute production capacity were observed for individual cyto/chemokines (data not shown).

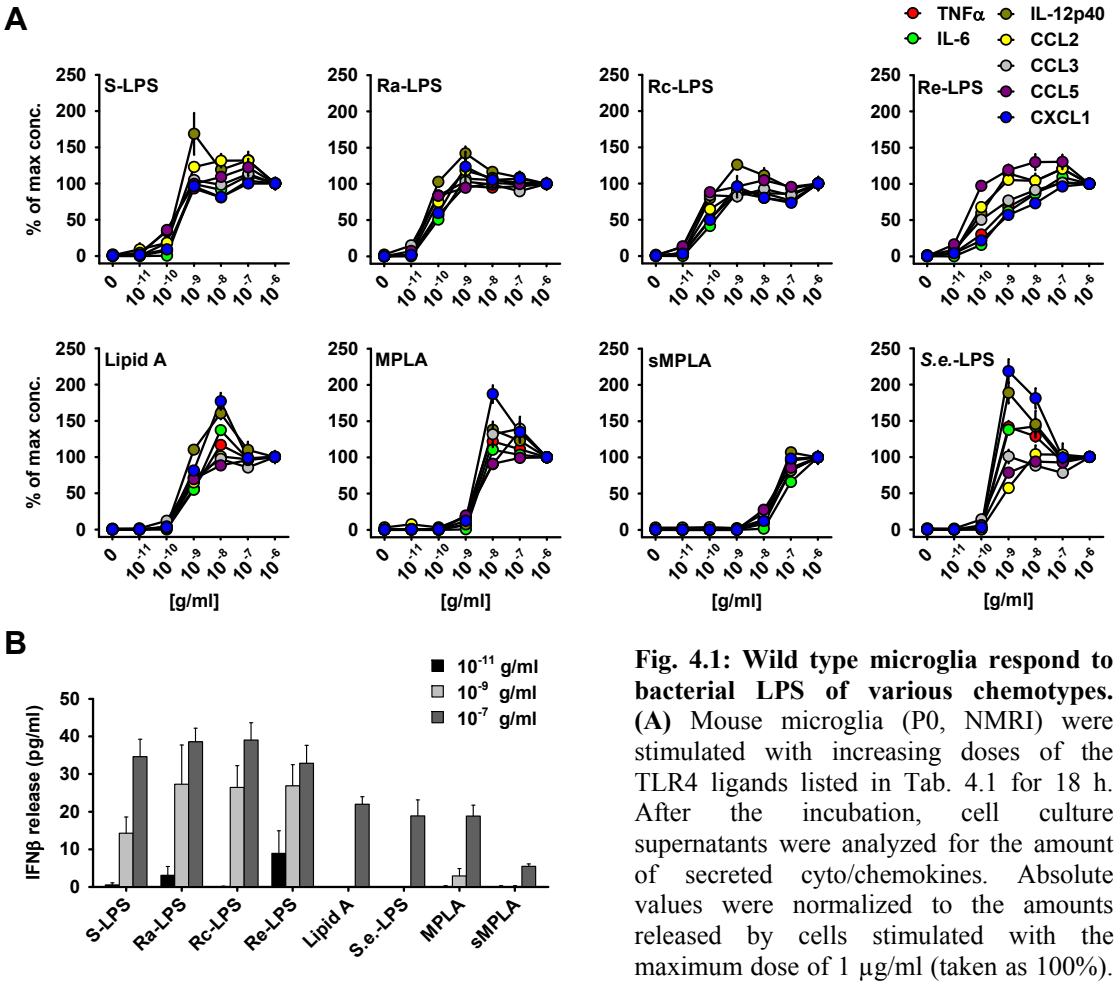


Fig. 4.1: Wild type microglia respond to bacterial LPS of various chemotypes. **(A)** Mouse microglia (P0, NMRI) were stimulated with increasing doses of the TLR4 ligands listed in Tab. 4.1 for 18 h. After the incubation, cell culture supernatants were analyzed for the amount of secreted cyto/chemokines. Absolute values were normalized to the amounts released by cells stimulated with the maximum dose of 1 µg/ml (taken as 100%). Data are given as mean ± SEM with n=8 (S- and R-LPS, Lipid A and *S.e.*-LPS) and n=12 (MPLA and sMPLA), respectively, from 2 individual experiment. **(B)** As described in (A) but with a limited number of doses (as indicated), another set of cells was stimulated and the release of IFNb was determined in cell culture supernatants. Data are given as mean ± SEM with n=3.

Tab. 4.2: Various LPS chemotypes trigger varying absolute amounts of cytokines and chemokines. Microglia from NMRI wild type mice were stimulated as described in Fig. 4.1. Values represent the release [pg/ml] obtained with the LPS variants at the maximal concentration tested (1 µg/ml).

	TNFα	IL-6	IL-12p40	CCL2	CCL3	CCL5	CXCL1
S-LPS	2469	4409	4937	979	8209	16564	10183
Ra-LPS	2474	3817	4618	1008	7862	16639	8920
Rc-LPS	2763	4616	4937	1441	10150	17451	11672
Re-LPS	2451	4213	4582	861	11181	15480	9830
Lipid A	2296	3875	4402	892	10916	18163	7785
MPLA	3572	5947	6269	1538	16477	28661	9176
sMPLA	3929	6338	6378	1730	17932	28056	12266
***S.e.*-LPS**	2763	4415	5236	1125	13471	18216	10445

4.2 LPS chemotype signaling varies by the dependence on the co-receptor CD14

Another key aspect to clarify for microglial cells was the question whether there would be a correlation between LPS chemotypes and their dependence on the LPS co-receptor CD14, as variable contributions were shown for other macrophage populations (Gangloff et al., 2005; Jiang et al., 2005b). Therefore, microglial cells with a deficiency in CD14 were stimulated like described above for the wild type cells. Fig. 4.2A summarizes the resulting dose-response relations. The lack of CD14 had the most striking effect on the microglial responses to S-LPS, as there was a 100-fold higher dose necessary to elicit a response, as compared to the wild type situation. Within the dose range used here (up to 1 µg/ml of S-LPS), the production of neither of the cytokines measured did reach a clear plateau. The rough LPS group (Ra-, Rc- and Re-LPS, respectively) similarly needed a 100-fold higher dose for the response initiation, as compared to the wild type situation. Thereby, Ra- and Rc-LPS, respectively, were barely able to reach a release plateau. In contrast, Re-LPS could manifest its response as the release reached a plateau within the given dose range. When stimulated with Lipid A, $CD14^{-/-}$ microglia only needed a 10-fold higher dose to mount a response comparable to wild type cells. Surprisingly, monophosphoryl Lipid A (including both the natural and the synthetic version) showed a dose-response behavior almost identical to that seen with wild type cells. The *Salmonella* LPS (presumably Ra-LPS) completed and confirmed the observations made so far as it showed a dose-response curve similar to that of the *E.coli* Ra-LPS.

Taken together, the results indicated a clear gradient of CD14-dependence starting from S-LPS with the biggest shift in the dose-response relation and ending at (s)MPLA showing to be least affected by the lack of CD14. CCL3 represented the only exception. This particular chemokine was produced already at concentrations similar to the wild type situation. In case of stimulation with MPLA (as well as sMPLA), CCL3 was induced even at lower doses than it could be observed with wild type cells. This unexpected phenomenon implied a general CD14-independent induction of CCL3, regardless of the LPS chemotype. These findings suggest that the impact of CD14 to the TLR4 signaling of structural LPS variants can differ with the cell type, indicating in turn that the receptor/signaling complex comes with different organization. Moreover, the signaling to inducible genes can also present with individuality.

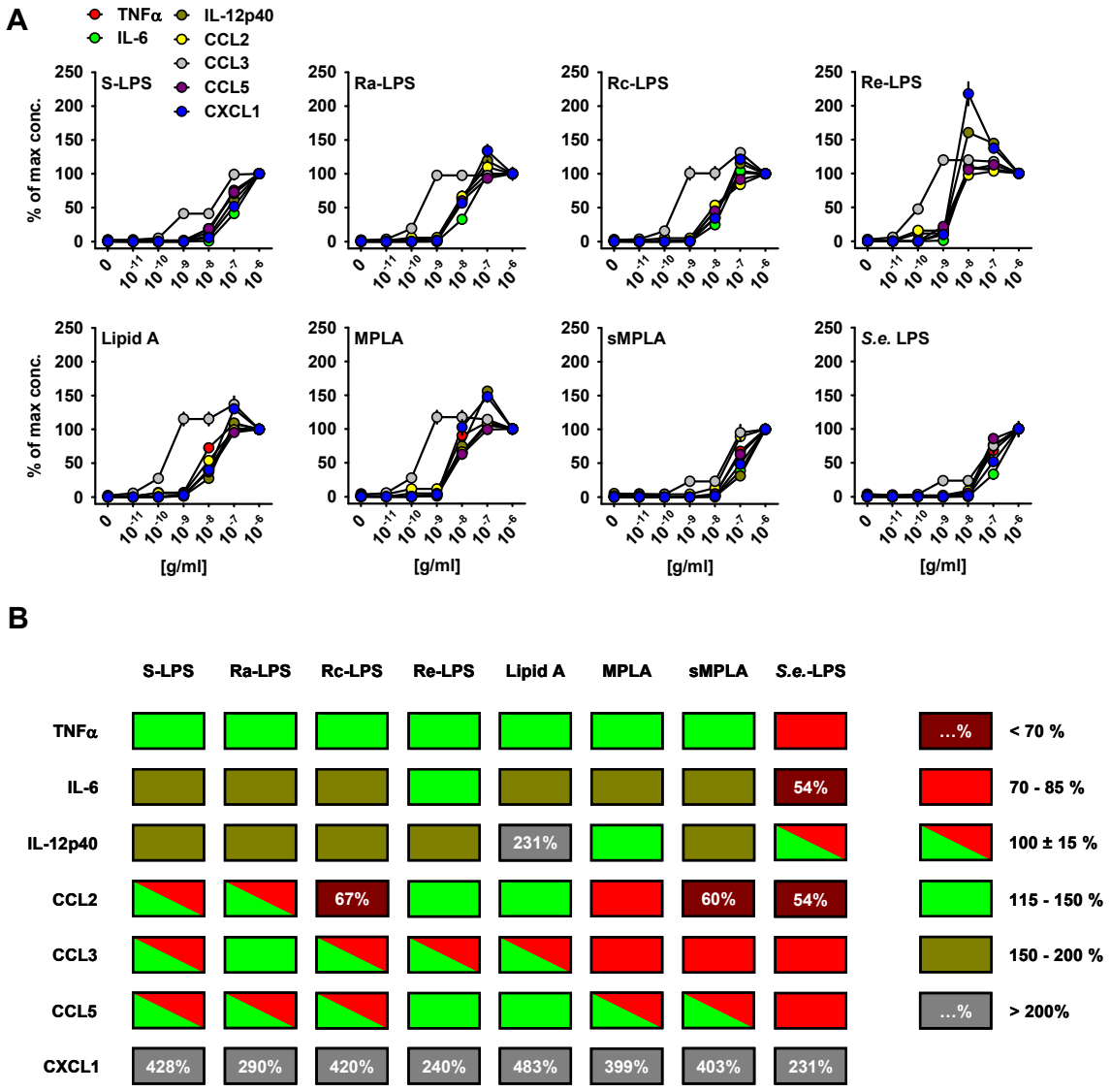


Fig. 4.2: LPS chemotypes show specific dependence on TLR4/CD14 co-signaling. (A) Mouse microglia (P0) with a deficiency in CD14 were stimulated with increasing doses of the TLR4 ligands listed in Tab. 4.1 for 18 h. After the incubation, cell culture supernatants were analyzed for the amount of secreted cyto/chemokines. Absolute values were normalized to the amounts released by cells stimulated with the maximal concentration of 1 µg/ml (taken as 100%). Data are mean ± SEM with n=8 from 2 individual experiments. **(B)** Absolute amounts from (A) at 1 µg/ml of the respective stimulus were compared to absolute amounts produced by equivalently stimulated C57Bl/6 wild type cells. Results expressed as relative release by $CD14^{-/-}$ cells were classified according to the color scheme. Numbers give the actual values in addition to the classification.

When interpreting data from knockout (ko) studies, it is indispensable to also compare the quantitative aspects of the response between the wild type and genetically modified cells. Therefore, Fig. 4.2B complements the dose-response profiles (Fig. 4.2A) by comparing absolute amounts of cyto- and chemokines as released after stimulation with the maximum dose (1 µg/ml) of the respective LPS preparations. The overview summarizes the response of $CD14^{-/-}$ cells as percentage of the response measured for corresponding C57Bl/6 wild

type microglia, the genetic background of the ko system. The majority of the factors, including TNFα, IL-6 and IL-12p40, were produced in substantially higher amounts by $CD14^{-/-}$ cells, when compared to the wild type. Most impressively, there was an up-regulation in the production of CXCL1, a neutrophil attractant, which was produced by the ko cells in up to 5-fold higher amounts. The remaining factors were released in similar amounts (the T cell attractants CCL3 and CCL5) or at slightly lower level (the monocyte/macrophage attractant CCL2) by the ko compared to the wild type cells. Again there was an exception, as $CD14^{-/-}$ cells stimulated with *S.e.*-LPS produced lower absolute amounts of all the factors measured, except for CXCL1.

4.3 Block of CD14 unmasks the TLR4 discrimination between LPS chemotypes

The involvement of CD14 as co-receptor of TLR4 was further tested for the LPS chemotypes by using a blocking antibody against CD14. This clone (4C1) had proven effective in our studies on endogenous TLR4 agonists to also inhibit microglial responses to LPS. As expected, it was able to suppress the cyto/chemokine production induced by Re-LPS, shown here for TNFα and CXCL1 release (Fig. 4.3A). For LPS concentrations of 0.1 and 1.0 ng/ml (the latter giving already maximal responses, see Fig. 4.1A), the release was completely or substantially reduced. Unexpectedly, this suppressive capacity was reversed when using a higher LPS concentration. When microglia were stimulated with LPS at 10 ng/ml, presence of the anti-CD14 antibody resulted in enhanced production of cyto- and chemokines (Fig. 4.3A). The phenomenon may have an explanation by some low-affinity binding sites for LPS, which can take over effects on the cells when the access to the high-affinity sites is blocked. Indeed, LPS has been reported for complex interactions affecting the TLR4 signaling directly (in both CD14-dependent and -independent ways) or indirectly (by affecting accessory components of a TLR4-centered receptor complex or the membrane environment), as it will be considered in the Discussion.

We took advantage of this differentiating influence of an antibody-based CD14 block and compared wild type microglia as to their responses to the various LPS chemotypes. Cells were stimulated with the structural LPS variants at 10 ng/ml in the presence of increasing concentrations of the anti-CD14 antibody and the consequences for a panel of cytokines and chemokines were determined (Fig. 4.3B). The enhancing effect of

a CD14 block was confirmed for the Re-LPS, and also other LPS chemotypes presented with augmented release inductions, such as for CXCL1. On the other hand, not all factors inducible by a LPS chemotype revealed up-regulation as the CXCL1 or TNFα did. Ra-, Rc- and Re-LPS, for example, induced CCL5 in amounts unaffected by anti-CD14. The *S.e.*-LPS even showed quite some inhibition with a CD14 block, such as for CCL2. There was no simple 'common theme' throughout the various LPS chemotypes following an order of S to R to Lipid A classification as it was seen in the $CD14^{-/-}$ situation. Accordingly, there was not even a strong similarity of presumably similar R structures, like Ra-LPS and *S.e.*-LPS (see Tab. 4.1). Yet the CD14 block unraveled again that the signaling of the various TLR4 agonists comes with individual properties. Profiles for the chemotypes did not differ simply by quantity (potency), but by the quality of induced gene patterns ─ and this individuality became even more prominent when the TLR4 signaling was manipulated. This principle will be shown for other experimental settings again below.

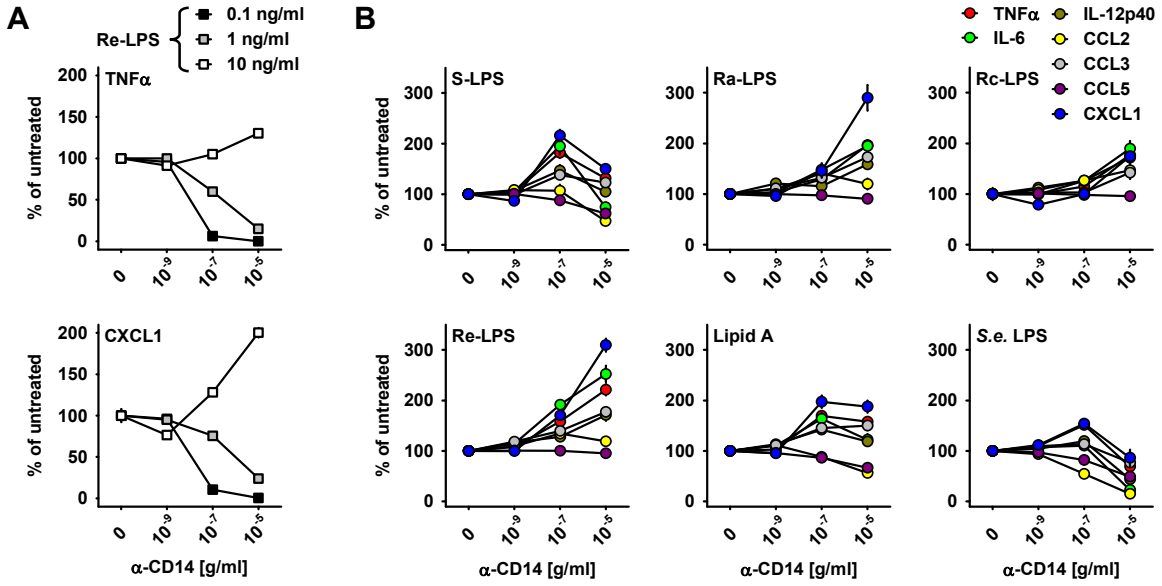


Fig. 4.3: LPS chemotypes are differentially affected by an antibody blockade of CD14. (A) Mouse microglia (P0, NMRI) were stimulated with varying concentrations of Re-LPS (as indicated) in the absence or presence of an anti-CD14 antibody (clone 4C1) at different concentrations for 18 h. Cells also received a 30 min pre-incubation with the antibody at the respective concentration. The release of cyto- and chemokines (shown for TNFα and CXCL1 as representatives) was determined in the supernatants. Absolute amounts were expressed as percentage of the release by control cells stimulated with the TLR4 ligand but without the antibody (untreated set to 100 %). Data are given as mean ± SEM with n=6 from 2 individual experiments. **(B)** Cells were stimulated with 10 ng/ml of each of the TLR4 ligands as listed in Tab. 4.1 in the absence or presence of increasing concentrations of an anti-CD14 antibody for 18 h, as in (A). Data are mean ± SEM with n=10 from 2 individual experiments.

Even though the CD14$^{-/-}$ situation indicated in part overshooting responses, as in the case of Re-LPS-triggered CXCL1 (Fig. 4.2A), it could not match the pattern obtained with antibody blockade. The two experimental approaches differ by more than an interference with CD14. A system with a CD14$^{-/-}$ background had to develop TLR4 signaling in complete absence of the co-receptor, whereas the binding of an antibody introduces sterical hindrance in a receptor complex which otherwise contains an intact CD14 molecule. The critical finding is thus the unmasking of the agonist-discriminating capacity of the microglial TLR4.

4.4 MyD88 *versus* TRIF-dependent signaling without LPS chemotype discrimination

In contrast to all other known members of the TLR family TLR4 is in a unique situation concerning its associated signaling pathways, since this receptor uses both known TLR signaling adaptors, i.e. MyD88 and TRIF. Own studies could show that TLR4 can selectively use either one or both adaptors to induce certain genes upon LPS challenge (see part B of the Results section). Here, the question was whether TLR4 could discriminate between individual LPS chemotypes in terms of the signal conveyance through its associated adaptor molecules. Therefore, microglia with a deficiency in MyD88 or TRIF, respectively, were stimulated with the various LPS chemotypes at a fixed dose of 100 ng/ml. This rather high dose was used to be certain that (wild type) TLR4 would be saturated with its respective ligand, thereby ruling out any misleading interpretations resulting from differential dose-response relations among the various LPS chemotypes. Microglial cells could be induced to mount a selective pattern of cyto- and chemokines depending on the signaling route they were 'forced' to use, i.e. signaling through TRIF in the case of MyD88$^{-/-}$ and *vice versa* (Fig. 4.4). Furthermore, despite minor variations in the absolute produced amounts of individual cyto- and chemokines, the resulting patterns were almost identical when comparing microglial stimulations with the various LPS chemotypes. This led to the conclusion that in terms of the selective usage of the two TLR4 adaptor molecule pathways for the induction of cyto/chemokines microglia would *per se* not discriminate between different LPS chemotypes – as long as no additional manipulation would be imposed.

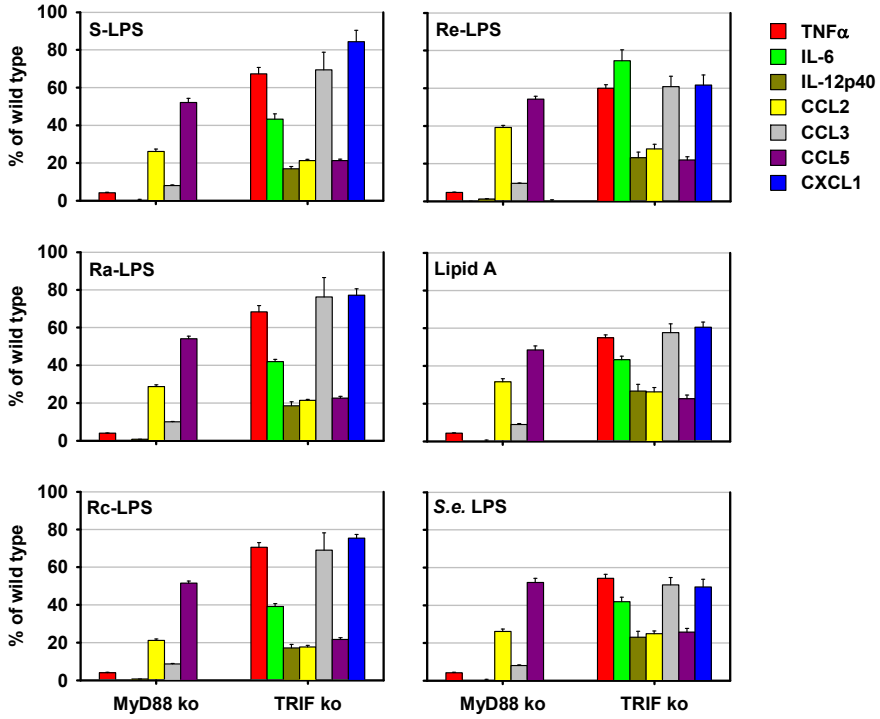


Fig. 4.4: Microglial TLR4 does not differentiate between LPS chemotypes in terms of a signaling adaptor usage. Mouse microglia (P0) with a deficiency in either MyD88 or TRIF, respectively, were stimulated with 100 ng/ml of the respective TLR4 ligands, as listed in Tab. 4.1, for 18 h. After the incubation, cell culture supernatants were analyzed for the amount of secreted cyto/chemokines. Absolute values were normalized to the release obtained by C57Bl/6 wild type cells, i.e. the genetic background strain of the knockouts MyD88$^{-/-}$ and TRIF$^{-/-}$. Data are mean ± SEM with n=4 for MyD88$^{-/-}$ cells and n=8 from 2 individual experiments for TRIF$^{-/-}$ cells, respectively.

4.5 A potential TLR4 antagonistic LPS fails to inhibit the cyto/chemokine induction

The LPS chemotypes tested thus far all originated from pathologically relevant *E.coli* and *Salmonella* species. Pathogenicity and immunogenicity of these compounds reside mainly in the respective Lipid A compartment. In this context, the number of Lipid A-associated fatty acids directly correlates with the pathological impact. It was shown that underacylated Lipid A structures (with five or less fatty acid moieties) have substantially lower pathogenic potential or even exert antagonistic properties with respect to TLR4 binding. One of these potential antagonistic structures derives from LPS isolated from the photosynthetic bacterium *Rhodobacter sphaeroides* (Rs-LPS), which was shown to antagonize *E.coli* LPS in a very potent fashion. The antagonistic potential was found to vary among macrophages of different mammalian species, but was described for mouse cells and the induction of TNFα (Strittmatter et al., 1983; Qureshi et al., 1991). To test

whether this effect could be reproduced for microglial TLR4 stimulation, cells were incubated with saturating concentrations of *E.coli* Re-LPS in the presence of increasing concentrations of Rs-LPS. Surprisingly, Rs-LPS was not able to antagonize the Re-LPS-induced release of proinflammatory cyto- and chemokines, regardless of the concentration that was set for the Re-LPS as the agonist (Fig. 4.5A). Moreover, Rs-LPS dose-dependently enhanced the Re-LPS effect. Interestingly, Rs-LPS itself had even a strong agonistic effect on microglia (Fig. 4.5B). At maximal concentration, the Rs-LPS-induced release even exceeded the levels induced by equivalent concentrations of Re-LPS.

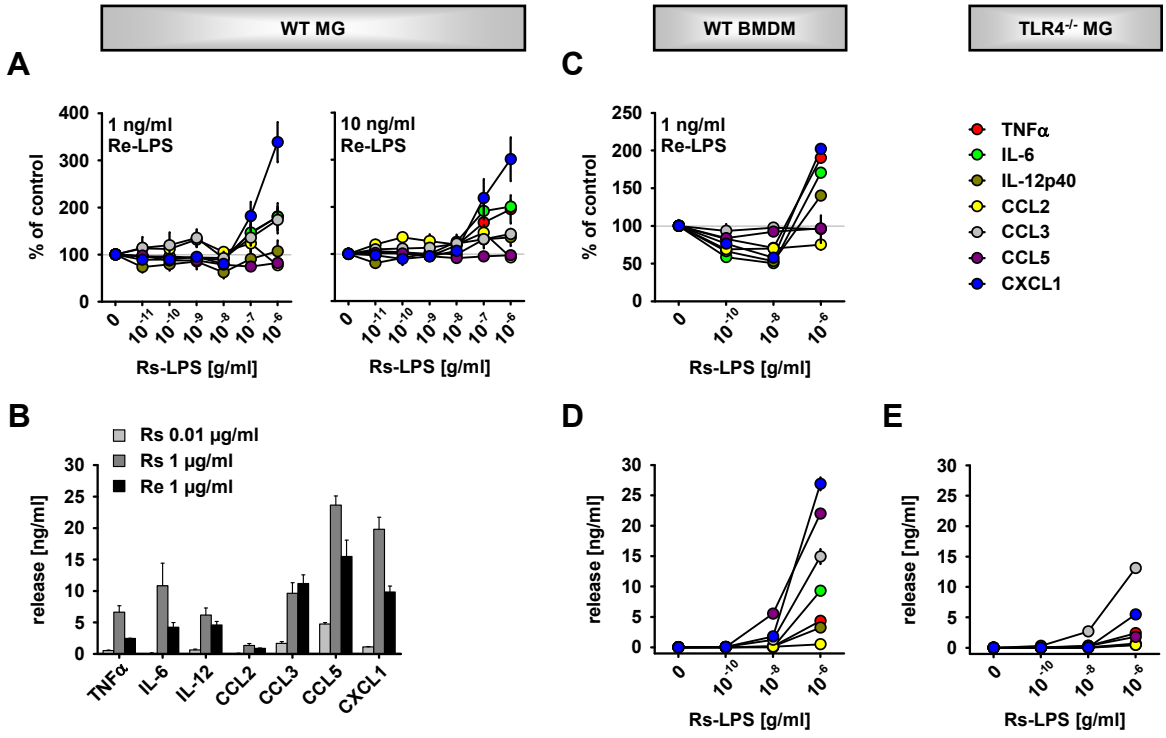


Fig. 4.5: Rs-LPS exerts agonistic rather than antagonistic effects on microglial cells. (A) Mouse microglia (P0, NMRI) were stimulated with the indicated concentrations of Re-LPS in the presence of increasing concentrations of the TLR4-antagonist Rs-LPS for 18 h. After the incubation, cell culture supernatants were analyzed for the amount of secreted cyto/chemokines. Absolute amounts were normalized for the release obtained by control cells stimulated with Re-LPS only (set to 100 %). Data are mean ± SEM with n=8 from 2 individual experiments. **(B)** Absolute amounts of cyto/chemokines produced by microglia in (A) as stimulated individually with the indicated LPS structures and concentrations. **(C)** As in (A) for microglia, experiments were performed here with BMDM, revealing a dose-dependent, partially antagonistic effect of Rs-LPS in combination with Re-LPS. **(D)** Release induction in BMDM by Rs-LPS alone. Data in (C) and (D) are mean ± SEM with n=3. **(E)** Microglia (P0) deficient in TLR4 were stimulated with Rs-LPS for 18 h at indicated concentrations and the resulting release was determined as above. Data are mean ± SEM with n=3.

In order to clarify whether this was a microglia-specific phenomenon the same experimental layout was applied to bone marrow-derived macrophages (BMDM). Here, despite having agonistic properties at high doses as well, Rs-LPS was able to at least partially antagonize the Re-LPS induced response (Fig. 4.5C+D). The findings suggest that, in contrast to extra-neural macrophage populations, microglial TLR4 stimulation with pathogenic LPS is not as much sensitive to an antagonization by Rs-LPS. Conceivably, the structural and functional organization of the microglial TLR4 differs from that in other macrophage populations within the same species. Binding of Rs-LPS to TLR4 complexes would occur in both cases, but with distinct signaling consequences. In microglia and BMDM, Rs-LPS would have some own agonistic potential, but with different competitive outcome for Re-LPS. Rs-LPS could be thus considered as a partial agonist.

Nevertheless, the question had to be addressed whether the remarkable agonistic properties of Rs-LPS were solely associated with TLR4 signaling. Therefore, microglial cells deficient in TLR4 ($TLR4^{-/-}$) were stimulated with Rs-LPS and pathogenic LPS chemotypes, as described above. As expected, $TLR4^{-/-}$ microglia were not able to respond to any of the compounds listed in Tab. 4.1 (data not shown). However, despite the lack of TLR4, Rs-LPS could still induce substantial amounts of proinflammatory cyto- and chemokines (Fig. 4.5E). This response showed certain similarities with the microglial wild type response to Rs-LPS, but at the same time, the absolute and relative amounts of the individual factors came with a discrete pattern. Taken together, Rs-LPS did not exert antagonistic properties on microglial stimulation with pathogenic LPS. Instead, its agonistic potential might contain some TLR4-independent contribution based on a 'second receptor target'.

4.6 LPS chemotypes vary by the induction of surface MHC I expression

Besides the direct influence on neighboring cells and the recruitment of other immune cells through the release of cyto- and chemokines microglia also serve as antigen-presenting cells (APCs) within the CNS. From (our) previous studies it was known that, upon LPS challenge, microglia would up-regulate the expression of surface MHC class I molecules. In contrast, LPS treatment would neither induce MHC class II structures, nor interfere with their up-regulation by other factors, such as IFNγ (data not shown). To test whether this feature could be correlated with the LPS chemotype microglia were stimulated with the

LPS variants listed in Tab. 4.1. Flow cytometric analysis revealed the enhanced expression, as in the case of Re-LPS (Fig. 4.6A). A common concentration of 10 ng/ml was chosen for a comparison between the LPS chemotypes, because it gave already maximal responses of cyto/chemokine release (see Fig. 4.1) and it was found optimal for the MHC I regulation. Wild type microglia responded with a similar up-regulation of MHC I when stimulated with S-LPS or any of the R-LPS variants, respectively (Fig. 4.6B). In contrast, Lipid A as well as its monophosphorylated counterparts displayed a substantially lower ability to induce MHC I. This pattern was modified in a $CD14^{-/-}$ situation. Here, S-LPS showed a dramatically lower capacity for MHC I induction. This drop by the lack of CD14 was less pronounced for the R-LPS family and was even reversed for the Lipid A group. Especially in the case of sMPLA, the ability to induce MHC I was substantially increased when CD14 was absent. This gradient of CD14-dependence from S-LPS to Lipid A appeared very similar to that seen in cyto/chemokine release studies (see Fig. 4.2), underlining the importance of the co-receptor in the potential distinction between the agonists. Importantly, this time, the potential TLR4 antagonist Rs-LPS had virtually no agonistic effect as it could not induce a clear up-regulation of the surface MHC I (Fig. 4.6C). However, when wild type microglia were incubated with a mixture of Re- and Rs-LPS, the capacity of Re-LPS alone to induce MHC I expression was diminished by approximately 60 % – indicating a clear antagonistic effect of Rs-LPS. This antagonism appeared to be independent of a CD14 involvement. When microglia with a deficiency in TRIF were subjected to incubation with the LPS structural variants, the cells ability to up-regulate MHC I was more or less abolished, regardless of which LPS preparation was tested (Fig. 4.6B+C). Importantly, in the $TRIF^{-/-}$ situation IFNγ still was able to up-regulate MHC I (not shown), underlining the essential role of TRIF to mediate the TLR(4)-induced up-regulation of this molecule. Obviously, the signaling of TLR4 to cyto/chemokines and MHC I reveals a distinct dependence on the LPS-structural variation. Chemotypes are recognized but their interpretation in terms of inducible genes may differ. Of course, an apparent lack of microglial release antagonism by Rs-LPS was thought to relate in part to some independent agonistic contribution based on a 'non-TLR4 receptor target', this phenomenon missing completely in the case of MHC I regulation (as there was no induction by Rs-LPS at all). Yet data below rather support a notion that interference by Rs-LPS does unmask differential agonist recognition and signaling.

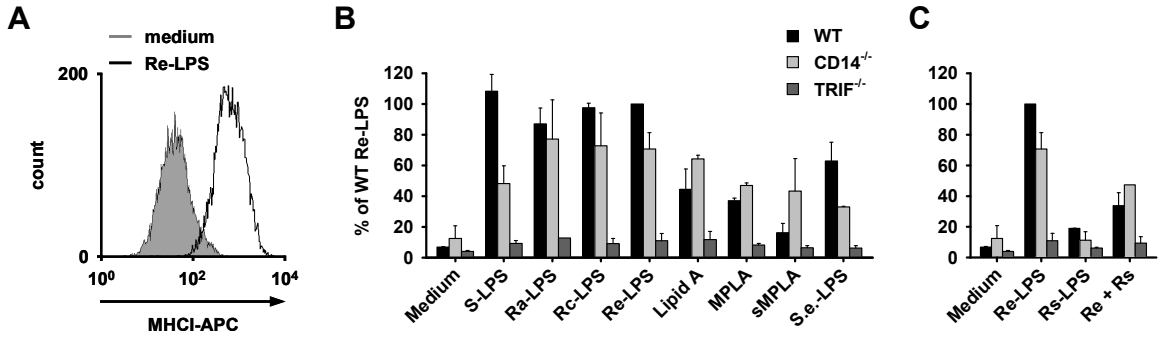


Fig. 4.6: Microglial MHC I expression is differently regulated by LPS chemotypes. (A) Representative flow cytometry histogram for the Re-LPS-induced up-regulation of MHC I in C57Bl/6 (P0) wild type cells, as studied in (B+C). **(B, C)** Microglia (P0) of the indicated genotypes were stimulated with 10 ng/ml of the respective compounds for 48 h. Subsequently, cells were removed from the culture dish and stained with anti-MHC I as well as anti-CD11b antibodies for acquisition by flow cytometry. For data analysis, only the $CD11b^+$ events were included. For the experimental details, refer to the section Material and Methods. The graphs show the mean fluorescence intensities with respect to the MHC I staining. Absolute amounts were normalized to the amount induced by Re-LPS stimulation of wild type cells (WT Re-LPS set to 100 %). Data are mean ± SEM with n=2 from 2 experiments for each genotype. Note that the combinatory experiments in (C) were performed at a higher concentration of Rs-LPS (1 µg/ml) to ensure that no own agonistic effect was missed.

4.7 LPS chemotypes vary by the suppression of myelin phagocytosis

Phagocytosis of microbes, apoptotic cells or tissue debris is another important property of microglia. It serves either the clearance of the particular matter during development and tissue maturation and upon injuries or the processing and subsequent presentation of material on the cell surface to activate cells of the adaptive immune response as an APC. In the context of the CNS, phagocytosis of myelin, especially by microglia, is a critical issue in demyelinating diseases, such as multiple sclerosis (MS). Debris clearance promotes remyelination attempts, but would also be a prerequisite for antigen presentation. Otherwise unstimulated microglia readily phagocytosed myelin *in vitro*, a process which could be dramatically suppressed by exposing cells to Re-LPS (Fig. 4.7A). Of course, measurement of intracellular myelin amounts at a given time point represents a snap shot of the steady-state affected by phagocytotic uptake and degradation (equivalent to the loss of the fluorescence signal). Such an analysis cannot resolve the effect of a modulating agent as to its contributions to the two processes. However, our previous work with analyses at different time points had indicated that myelin uptake is likely to be affected.

To determine whether such an inhibitory effect of LPS varies between the different chemotypes, microglia were stimulated with the various LPS preparations and then

allowed to incorporate fluorescence-labeled mouse myelin. Except for sMPLA, all compounds were able to inhibit myelin phagocytosis by wild type microglia in a similar fashion. This property was only partially dependent on CD14. In the respective ko situation, a noticeable effect was seen only for *S.e.*-LPS, showing a partial reduction of the inhibitory effect. The same was true when microglia deficient in TRIF were stimulated with the various LPS. In addition, the lack of TRIF also negatively influenced the members of the Lipid A group in their ability to inhibit myelin phagocytosis. Together, however, there was no such obvious gradient of either CD14- or TRIF-dependence as it was seen for the induction of MHC I or the release of proinflammatory cyto- and chemokines, respectively. Finally, Rs-LPS had very little effect on the microglial phagocytosis of myelin. Moreover, when combined with Re-LPS, there was no antagonistic effect observed, neither on wild type microglia nor on $CD14^{-/-}$ cells. In contrast, the lack of TRIF could provoke an at least partial antagonistic effect of Rs-LPS as the inhibition of myelin phagocytosis by Re-LPS could be diminished by half. In a comparison with the data on cyto/chemokine release and MHC I regulation, Rs-LPS would differently affect the consequences of other LPS-derived agonists, also in a rather TLR4-confined context. In other words, it revealed that the TLR4 ligand recognition and signaling has the potential for differentiation – with distinct functional downstream consequences.

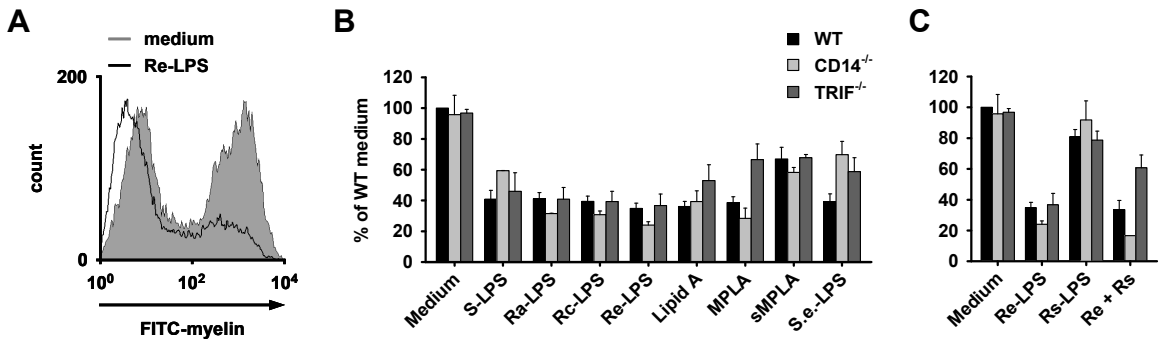


Fig. 4.7: Microglial myelin phagocytosis is differentially regulated by LPS chemotypes. (A) Representative flow cytometric histogram for the Re-LPS-induced inhibition of myelin phagocytosis in C57Bl/6 (P0) wild type cells. **(B, C)** Microglia (P0) of the indicated genotypes were pre-incubated with the respective compounds for 24 h before FITC-conjugated myelin was added to the cells for additional 2 h. Subsequently, cells were removed from the culture dish and stained with an anti-CD11b antibody for acquisition by flow cytometry. For data analysis only $CD11b^{+}$ events were included. The graphs show the mean fluorescence intensities with respect to the FITC-myelin signal. Values for the incorporated myelin were normalized to the signal measured in unstimulated wild type cells (WT medium set to 100 %). Data are mean ± SEM with n=4 from 4 individual experiments for wild type microglia and n=2 from 2 individual experiments for $CD14^{-/-}$ and $TRIF^{-/-}$ microglia, respectively.

Part B – Microglial responses to TLR stimulation are tightly organized

Microglial activation episodes are most likely not monophasic events, initial reactive phenotypes may adjust over time and eventually the response needs to deescalate (Hanisch and Kettenmann, 2007). In the context of the tissue, a multitude of factors would influence and control phenotype switches as well as the return to a (more or less) pre-activation state. In addition, some cell-autonomous regulations may help preventing reactions to overshoot. The probably simplest mechanism by which an induced reaction could get terminated is the disappearance (neutralization) of the triggering stimulus – or desensitization to its presence at the receptor or the cellular level.

4.8 Microglial TLR stimulation reveals a pattern of early *versus* late gene inductions

In vitro studies of macrophage/microglia activation very often concentrate on short time windows, thereby focusing on the initial phase of the induced response. However, such activation processes are likely to be of high dynamics and tightly (self)regulated, including phenomena as taking place longer after the response was initiated. To investigate such an activation process on a longer run, microglial cells were stimulated with saturating doses of TLR agonists for up to 72 h, with a 12 h analysis interval. Taking parameters, such as cell viability, evaporation and exhaustion of culture medium with changing concentration of nutrients, metabolites and inducible factors into account, the total time of 72 h as used here represented the maximal possible duration for this kind of 'static' *in vitro* stimulation.

Accordingly, microglial cells were stimulated with Pam_3CSK_4 (TLR1/2 agonist), Re-LPS (TLR4 agonist) and MALP-2 (TLR6/2 agonist), respectively, and time profiles of released proinflammatory cyto- and chemokines were determined (Fig. 4.8A). In general, the resulting profiles were qualitatively comparable between microglia isolated from either C57Bl/6 or NMRI wild type mice. In quantitative terms, there were differences between the two strains depending on the respective factor (Fig. 4.8B). The similarities between the responses induced through a TLR1/2 and a TLR6/2 stimulation (later in part summarized as TLRX/2 responses) could be expected and explained by the share of the TLR2 signaling. They thus served at the same time as a validation of the results, especially when cellular responses to TLR4 were found to differ. In all cases, the release of factors, like TNFα, CCL3 and CXCL1, reached an early plateau within the first 12 to 24 h of

stimulation, whereas IL-6, CCL2 and CCL5 accumulated more constantly over time, sometimes even not reaching a plateau within the 72 h monitoring. Most strikingly, there was a rather late induction of IL-10. The immunosuppressive cytokine – mainly produced by alternatively activated (M2-like) macrophages or B cells – could thus play a role as a self-limiting signal and, therefore, was investigated in more detail (see below).

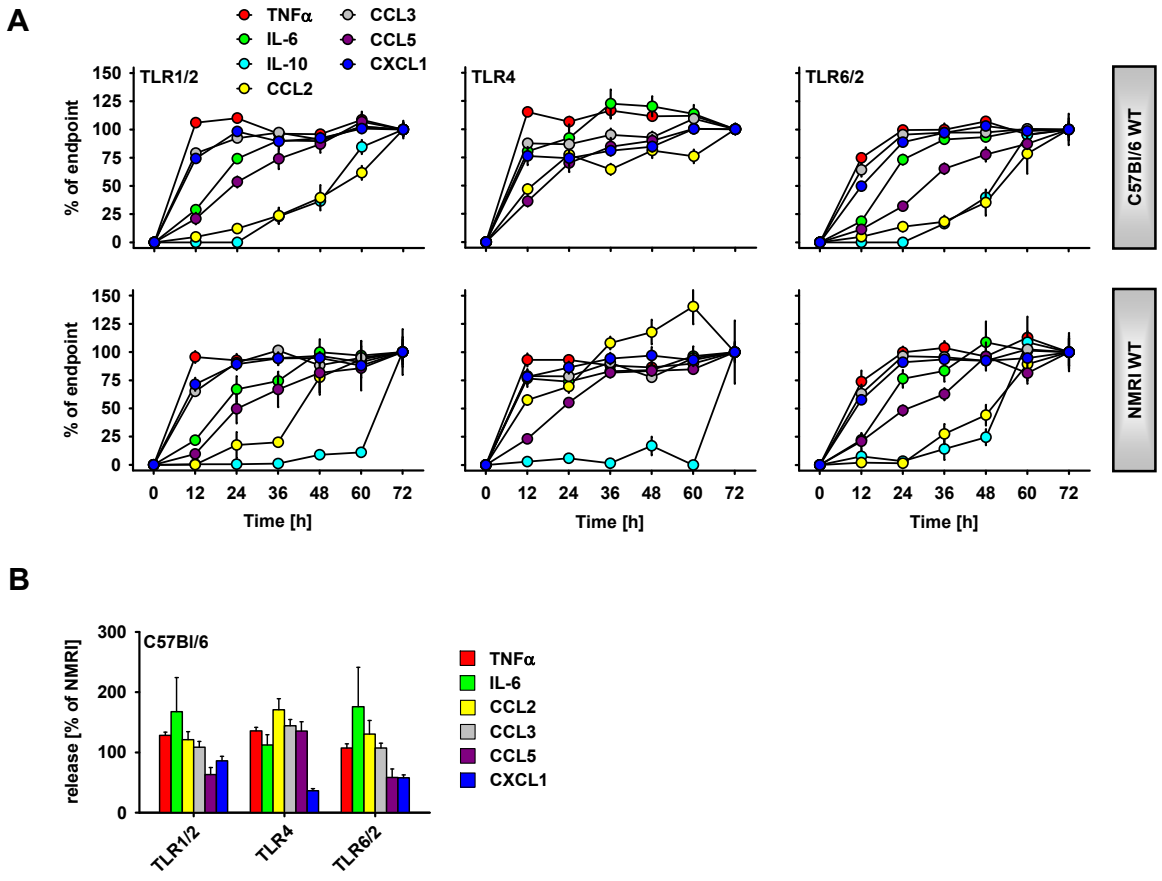


Fig. 4.8: Microglial TLR stimulation reveals time courses of early *versus* late gene inductions. (A) Mouse microglial cells (P0) were stimulated with 10 ng/ml of the TLR1/2 agonist Pam_3CSK_4, 1 ng/ml of the TLR4 agonist Re-LPS and 10 ng/ml of the TLR6/2 agonist MALP-2, respectively, for 12 to 72 h. These concentrations were determined before to have a saturating effect on the microglial cyto- and chemokine release. Supernatants were isolated at the respective time points of analysis from independent cell cultures to avoid repeated exchanges of medium for the same cells. Secreted cyto- and chemokines were determined and the absolute amounts were normalized for the total release obtained by control cells stimulated over 72 h (endpoint set to 100 %). Data are mean ± SEM with n=12 for each group (treatment period) from 2 individual experiments. **(B)** Microglia (P0) of either NMRI or C57Bl/6 origin were stimulated with the TLR agonists as described above for 18 h and cyto/chemokine production was determined. Absolute amounts secreted by C57Bl/6 cells were normalized to the release obtained with NMRI cells. Data are mean ± SEM with n=18 from 6 individual experiments.

When concentrating on the early (fast) induced genes by zooming into the first 12 h of the stimulation period, there were several critical observations made (Fig. 4.9). First of all, there was a clear dissociation of the profiles seen for TLR1/2- *versus* TLR6/2-stimulated microglia, even though both heterodimeric systems otherwise resembled each other in their overall profiles. When stimulated with Pam_3CSK_4 (TLR1/2), microglial cells responded with a fast induction of TNFα which could be detected already after 2 h of stimulation. This response was followed in time by the production of CCL3 and CXCL1 and then IL-6 and CCL5. Only minor amounts of CCL2 were produced within the first 12 h of this TLR1/2 stimulation. TLR6/2-stimulated microglia responded with an identical sequence of induced factors, but their production lagged behind. The first measurable amounts of any of the cyto/chemokines were detected only as of 6 h of stimulation. In other words, the two TLR systems – required for responses to Gram-positive bacteria – induce effective release at different speed. On the other hand, microglia stimulated through the Gram-negative bacteria-recognizing TLR4 responded in the early phase with a timed profile comparable to that induced by TLR1/2 stimulation. Here, the major difference was the relatively early production of CCL2 which felt into a same group with IL-6 and CCL5.

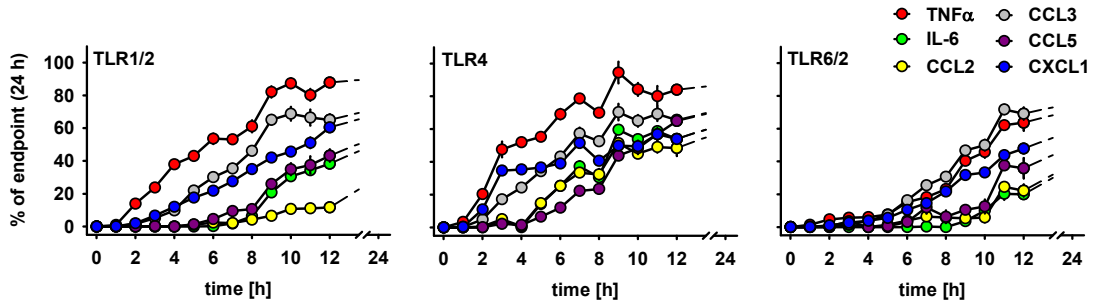


Fig. 4.9: Initial phases of the cyto/chemokine production vary with the response-triggering TLR. Mouse microglia (P0, NMRI) were stimulated with saturating concentrations (as described in Fig. 4.8) of TLR1/2, TLR4 and TLR6/2 agonists, respectively, for the indicated 1 to 12 h. Cyto-and chemokines were determined in the supernatants and the absolute amounts were normalized to the release obtained with control cells stimulated for 24 h (endpoint set to 100 %). Data are mean ± SEM with n=6.

4.9 Early interference by IL-10 can effectively suppress cyto/chemokine production

A special feature of the 72 h time profile was the late induction of IL-10. As the majority of the factors was already released at that time point an autocrine self-limiting effect of this

late IL-10 was not very likely. However, within the dynamics of an inflammatory reaction *in vivo*, earlier production of IL-10 by other cell populations would certainly affect an ongoing response. To simulate such a paracrine effect, microglial cells were stimulated with TLR agonists in the simultaneous presence of 10 ng/ml of recombinant mouse IL-10 (Fig. 4.10). This concentration is known to cause relevant effects on macrophages. As it was seen before, effects for TLR1/2- and TLR6/2-stimulated cells presented with very similar patterns. Among the factors measured, CCL2 was the least affected by IL-10 addition. Similarly, the time profile for CCL3 was slightly delayed in the beginning but was able to reach control levels towards the end of the 72 h stimulation period. In contrast, TNFα, CCL5 and CXCL1 were significantly suppressed from the beginning and barely reached 30 to 40 % of control levels throughout the whole period. The profile of TLR4-stimulated microglia revealed, however, some difference. Here, all factors were suppressed throughout the time profile. There was, on the other hand, no such strong suppression as seen, for example, for CCL5 in the TLR2-stimulated cells. The IL-10-induced suppression of the TLR4 response ranged between 25 to 50 % for all factors, as compared to control levels.

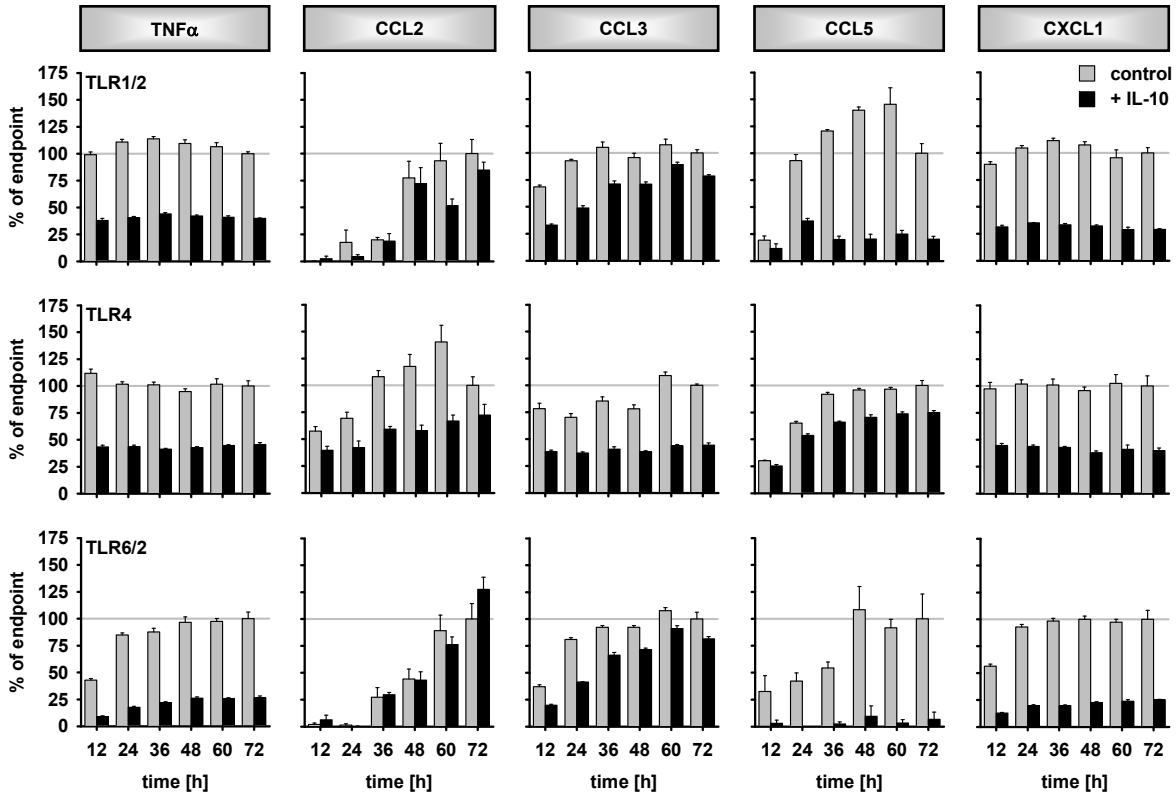


Fig. 4.10 (previous page): Simultaneous presence of IL-10 can suppress the microglial TLR response. Mouse microglia (P0, NMRI) were stimulated with saturating concentrations (as described in Fig. 4.8) of TLR1/2, TLR4 and TLR6/2 agonists, respectively, either alone (grey bars) or in the presence of 10 ng/ml of IL-10 (black bars) for the indicated time periods. Secreted cyto- and chemokines were determined in the supernatants at the respective time points. Absolute amounts were normalized to the release obtained by control cells (no IL-10) reached within 72 h (endpoint set to 100 %). Data are mean ± SEM with n=6.

The comparison between the different TLRs indicated a situation-specific 'importance' of certain factors which would make an individual response more or less prone to be suppressed by paracrine IL-10. Simultaneous presence of paracrine IL-10 with an arrival of TLR agonists represents the opposite extreme of an autocrine IL-10 induced late upon a TLR challenge. The question thus was whether IL-10 would influence an ongoing microglial response. Therefore, cells were stimulated with Re-LPS as described before and 10 ng/ml of IL-10 were added at 12 h or 24 h, respectively, after the LPS response initiation. In fact, cells could develop similar responses to TLR4 stimulation, regardless of the absence or presence of the later added IL-10 (Fig. 4.11). Absolute amounts of produced cyto- and chemokines were also comparable between the three conditions (data not shown). Taken together, with the results from the simultaneous presence of IL-10 (Fig. 4.10), there was a clear indication that presence of IL-10 can modulate the microglial response to TLR stimulation with long-lasting consequences for the ongoing response. However, this only applies when IL-10 can influence the activation process early or before its initiation.

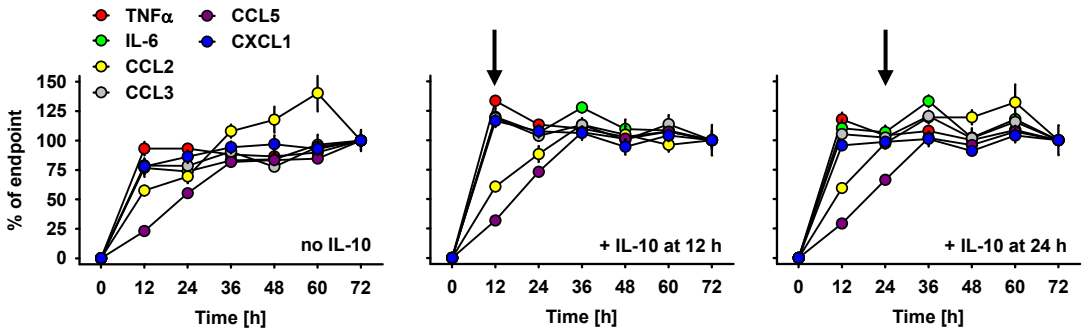


Fig. 4.11: Late IL-10 addition during an ongoing TLR4 stimulation has no effect on the response. Mouse microglia (P0, NMRI) were stimulated with 1 ng/ml of Re-LPS either alone or in the presence of 10 ng/ml of IL-10, added at the indicated time points (arrows). Secreted cyto- and chemokines were determined in the supernatants at the respective time points and amounts were normalized to the release obtained by control cells (no IL-10) stimulated for 72 h (endpoint set to 100 %). Data are mean ± SEM with n=12 from 2 individual experiments (no IL-10) and n=6 (IL-10 at 12/24 h), respectively.

4.10 MyD88 and TRIF reveal unexpected cooperativity for cyto/chemokine inductions

In order to determine the roles of the TLR signaling adaptor molecules, MyD88 and TRIF, microglial cells with a deficiency in either of the two molecules were stimulated as described for wild type cells. To incorporate both the response kinetics as well as the absolute amounts Fig. 4.12 shows the release of selected factors (TNFα, CCL3, CCL5 and CXCL1) in relation to the release by wild type cells over 72 h of stimulation. As expected, MyD88$^{-/-}$ microglia stimulated with either TLR1/2 or TLR6/2 agonists were not able to release any of the measured factors, except for some negligible levels of CXCL1, since both heterodimeric receptor complexes are thought to signal via MyD88. In contrast, TLR4-stimulated microglia deficient in MyD88 still released small amounts of TNFα (and CCL3) and, most strikingly, CCL5 at absolute levels comparable to the wild type situation. This could be explained by the unique situation of TLR4 which can use both adaptor molecules. The results suggest that TLR4 induces CCL5 preferably through the TRIF-dependent signaling route, whereas other factors mandatorily depend on MyD88.

However, creating the opposite signaling situation by a stimulation of TRIF-deficient microglia, the previous assumption needed some correction. First, TLR4-stimulated TRIF$^{-/-}$ microglia were able to induce all of the factors, yet also including CCL5 (note the scaling of the y axis in Fig. 4.12). So, CCL5 revealed an organization of its induction by both TRIF and MyD88. Second, the TLR4-induced profile of factors came with significant lower absolute amounts, compared to wild type cells. These findings indicated a cross-dependence of the two signaling pathways downstream of TLR4. Third, even more surprising, the resulting profiles of TLRX/2-stimulated TRIF$^{-/-}$ microglia also indicated some cooperation among the adaptor protein pathways. As expected, these cells could induce all factors. However, absolute levels varied significantly, ranging from lower (CXCL1) to comparable (TNFα and CCL3) to even several-fold higher levels (CCL5), when compared to the wild type situation. By theory, lack of TRIF should be ignored by TLR1/2 and TLR6/2 signaling. Unexpectedly, this conclusion was not true. On the other hand, it is conceivable that the two TLR signaling pathways would also communicate. The present data thereby give a hint to mechanisms by which multiple TLR activations could get integrated. Such interactions may not only affect the total amounts of inducible cyto/chemokines in a selective manner (in/decrease), but also the timing of their induction. In this regard, TLR6/2-induced CCL5 under TRIF$^{-/-}$ may serve as an example.

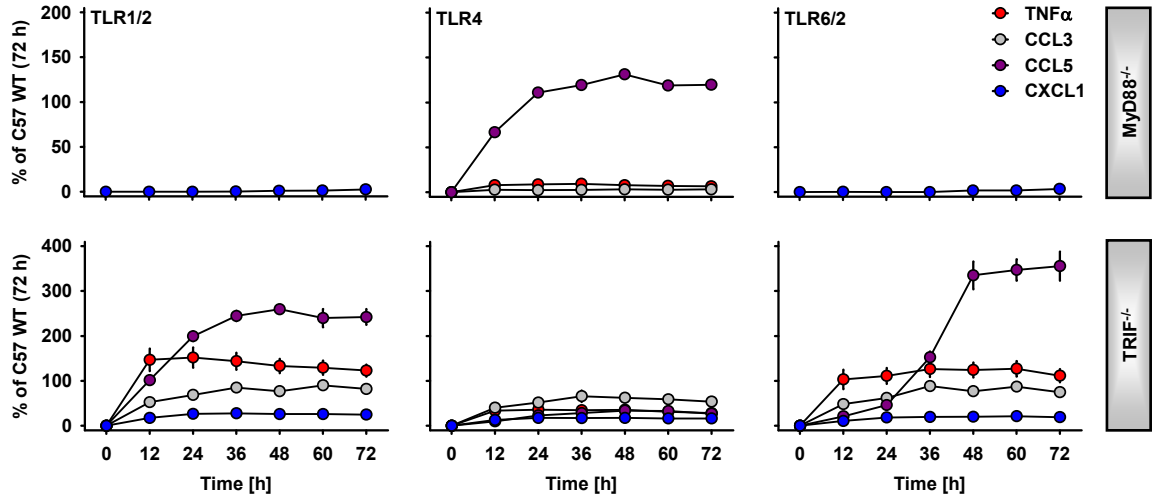


Fig. 4.12: Microglial cells selectively use signaling adaptor molecules upon TLR stimulation. Mouse microglial cells (P0) with a deficiency in MyD88 or TRIF, respectively, were stimulated with saturating concentrations (as described in Fig. 4.8) of TLR1/2, TLR4 and TLR6/2 agonists, respectively, for 12-72 h. Secreted cyto- and chemokines were determined in the supernatants at the respective time points and amounts were normalized to the release obtained by control cells (C57Bl/6 wild type) stimulated for 72 h (endpoint set to 100 %). Data are mean ± SEM with n=4 for MyD88$^{-/-}$ and n=12 from 2 individual experiments for TRIF$^{-/-}$.

4.11 The signal duration is critical for the TLR-mediated microglial responses

The experiments described above assumed a constant presence of the stimulus. However, bacterial infections can be cleared very rapidly, and the local stimulus concentration sufficient to induce an inflammatory response might only be of short-term duration or might reach some distant cell populations at critical levels only transiently. On the other hand, it would also be of interest to understand the organization of TLR4 signaling through its adaptor pathways. MyD88 and TRIF are thought to signal with a time shift, as MyD88 is activated through the plasma membrane-localized TLR4, whereas TRIF signaling requires receptor internalization. To determine the period during which the presence of extracellular LPS would be required to cause effective signaling with gene induction consequences, microglia were stimulated only for short time periods and the thereby instructed release of cyto/chemokines was measured. For the interpretation of these experiments it is important to clarify that the proteins released within the first 3 h of the experiment were discarded with a medium change and that only those amounts as accumulated in the culture supernatants between experimental hours 3 to 24 were harvested for quantification and comparison. As an exception, one type of control cells constantly received the respective stimulus over 24 h. Their cyto/chemokine production

was used to normalize the release values obtained with shorter stimulation. Another control was based on a 3+21 h presence of the stimulus. The culture supernatant with the stimulus was replaced with by medium also containing the stimulus. In other words, the two controls would differ in the factors released over the whole 24 h period or during the last 21 h. The factors measured as in Fig. 4.13 were thereby effectively secreted in the latter period only, but other – unknown – release products could affect within the first 3 h their production and export (see the scheme in Fig. 4.13; for experimental details refer to the Material and Methods section).

These experiments, indeed, revealed substantial differences in the induction efficacy for the individual cyto- and chemokines, as shown for the example of microglial stimulation with Re-LPS (Fig. 4.13A). TNFα, IL-6 (not shown) or CXCL1 could not be effectively induced when the stimulus was present for periods up to 180 min. Control data for the induction of TNFα show also very nicely that within the first 3 h (unidentified) factors were produced (and then removed by the medium exchange at 3 h) that were important for the ongoing production of the respective cytokine (compare the groups '3+21h' and '24 h'). This phenomenon, at the same time, illustrates the importance of including the two controls as reference options in the experimental set up.

In contrast to the TNFα type, there were also factors, especially CCL5, that were induced very efficiently. Even when the stimulus was present for no longer than 15 min the cells were sufficiently instructed to produce CCL5 with absolute amounts close to those obtained with constantly stimulated control cells. Again other factors, like IFNβ, CCL2 and CCL3, were also induced quite efficiently (e.g. by short-term stimulation), but the thereby instructed absolute amounts ranged only between 20 to 40 % of the control levels or revealed some biphasic dependence on the stimulus presence period. In summary, the induction efficacy by agonist presence could be classified into three groups: (i) factors needing long stimulation to be effectively induced (represented by TNFα), (ii) factors with an intermediate (CCL3) and finally those (iii) with a high induction efficacy (CCL5), the latter ones needing only short-term stimulation to be substantially induced – and to be later on produced and released.

To investigate whether the varying induction efficacy could also be associated to downstream signaling elements microglial cells were stimulated with TLR ligands either constantly or for only 5 min with the adequate medium change after the stimulation period. The pulse of 5 min stimulation was sufficient to increase the levels of phosphorylated (and

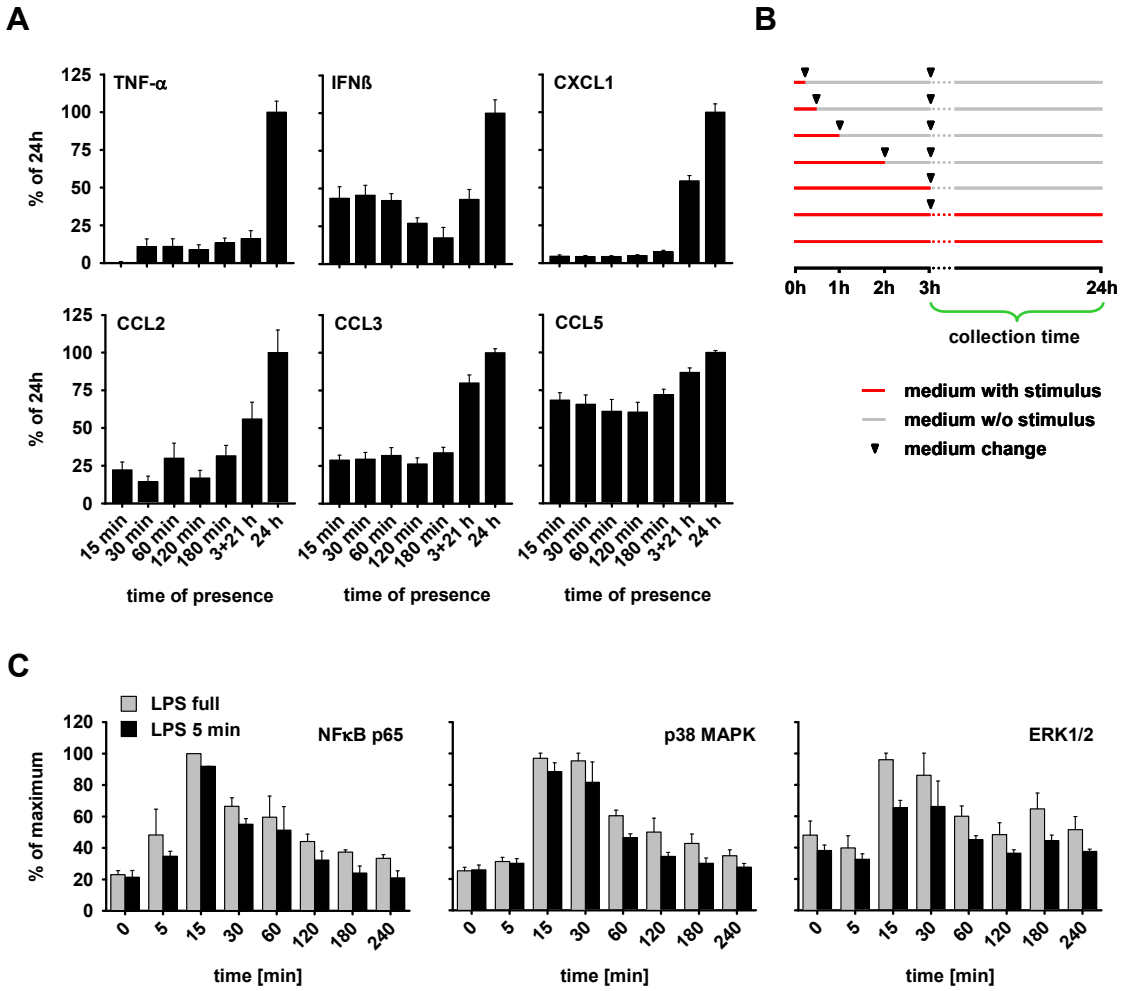


Fig. 4.13: TLR4 stimulation reveals differences in the induction efficacy of individual genes. (A) Mouse microglia (P0, NMRI) were stimulated with 1 ng/ml of Re-LPS for the indicated periods of time, varying from 15 to 180 min. The LPS-containing supernatant was thereafter replaced by fresh medium. At 180 min, the medium was changed again and cells were incubated for additional 21h (see (B) and Material and Methods for experimental details). Secreted cyto- and chemokines were determined in the supernatants and the absolute amounts were normalized to the release obtained by control cells stimulated throughout the complete 24 h (set to 100 %). Another set of controls received LPS as the stimulus for 3 h and, after a medium change, again for another 21 h. The regime allowed for comparing cells stimulated for varying periods up to 3h in terms of the release executed for the time window between 3 h and 24 h. Data are mean ± SEM with n=12 from 2 individual experiments. **(B)** The scheme illustrates the stimulation, incubation and supernatant collection periods as used in (A). **(C)** Another set of cells was stimulated with Re-LPS for either the full 240 min period (grey bars) or only the first 5 min and medium for the remaining time (black bars) in order to determine the consequences for NFκB p65, $p38^{MAPK}$ and ERK1/2 ($p44/42^{MAPK}$) activation as reflected by their phosphorylation status at the indicated time points. The time point '0' refers to cells which were not stimulated (no LPS administration). Cell lysates were prepared at the indicated time points and analyzed for the amount of phosphorylated proteins (refer to Material and Methods). Values were normalized to the respective experimental maximum. Data are mean ± SEM with n=2 from 2 individual experiments.

therefore activated) NFκB p65, $p38^{MAPK}$ and ERK1/2 ($p44/42^{MAPK}$), which organize for a substantial number of proinflammatory genes induced by TLR(4) stimulation (Fig. 4.13C). As these signaling elements are rather proximal to the transcriptional level the data clearly indicated that the decision about a low, intermediate or high induction efficacy of a

particular factor is probably made downstream of (or after) the initial signaling cascade. Especially for the cyto/chemokines requiring ongoing presence of the extracellular signal, i.e. TLR4-agonistic LPS, the fast activation wave through NFκB p65, $p38^{MAPK}$ and ERK1/2 must be followed by additional signals from the receptor to establish the productive gene activation.

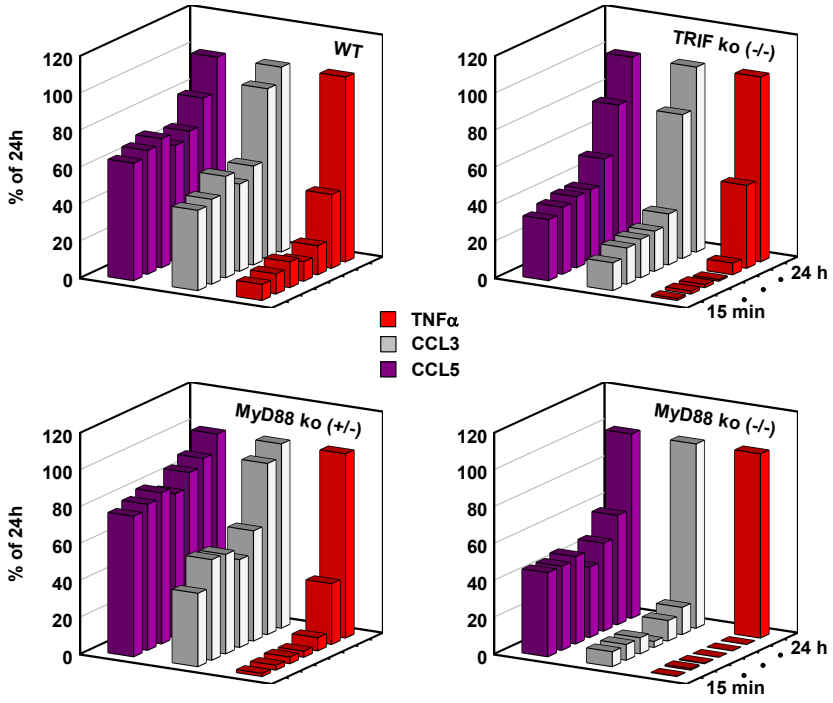


Fig. 4.14: Induction efficacy after TLR4 stimulation is associated with the selective usage of signaling routes. Mouse microglia (P0) with a deficiency in MyD88 or TRIF, respectively, were stimulated with 1 ng/ml of the TLR4 agonist Re-LPS for the indicated periods ranging from 15 to 180 min (as in Fig. 4.13A/B), with a respective replacement of the stimulus-containing supernatant by fresh medium until 180 min. Afterwards, all cells received a medium change and were cultured for additional 21 h (see also Material and Methods for details). Secreted cyto- and chemokines were determined in the supernatants and the absolute amounts were normalized to the release obtained by control cells stimulated throughout the complete 24 h (set to 100 %). The comparison shows also results from wild type microglia. Note that the figures compare relative release consequences and not absolute values. Data are mean with n=12 from 2 individual experiments for each genotype.

Surprisingly, the induction efficacy of the three representative factors also associated with the signaling adaptor proteins through which they were induced after TLR4 stimulation (Fig. 4.14). When the TLR4 signaling was limited to either one of the two pathways ($MyD88^{-/-}$ *versus* $TRIF^{-/-}$ microglia), the induction efficacy also dropped from high to intermediate for CCL5 and from intermediate to low for CCL3. TNFα, slowly induced already in wild type microglia, even got further decreased, in terms of induced amounts

upon shorter periods of LPS presence. The impairments were most pronounced for MyD88$^{-/-}$ cells, whereas much of the wild type situation was maintained in the heterozygous situation (MyD88$^{+/-}$). Lack of TRIF, on the other hand, revealed also an impact on all of the factors. Once again, the outcomes demonstrated that the two signaling pathways downstream of TLR4 act highly cooperative, rather than as 'signaling alternatives'. Not only do they exert a control over their respective gene sets by quantities *per se*, apparently also the kinetic of induction depends on their contributions.

The unique signaling situation of TLR4 was further demonstrated when the induction efficacy of certain factors was compared between TLR4 and TLRX/2-stimulated microglia (Fig. 4.15). The situation of TLR1/2 as well as TLR6/2 to be 'limited' to MyD88-associated signaling directly correlated with the inability to induce any of the representative factors with a high efficacy. In other words, short presence of an agonist translates into an already productive gene induction and subsequent release output when TLR4 is used to trigger the response, whereas the TLRX/2 heterodimers need longer presentation of the extracellular signal to install their full cyto- and chemokine program. The TLR4 options of dual pathway access thus revealed another facet.

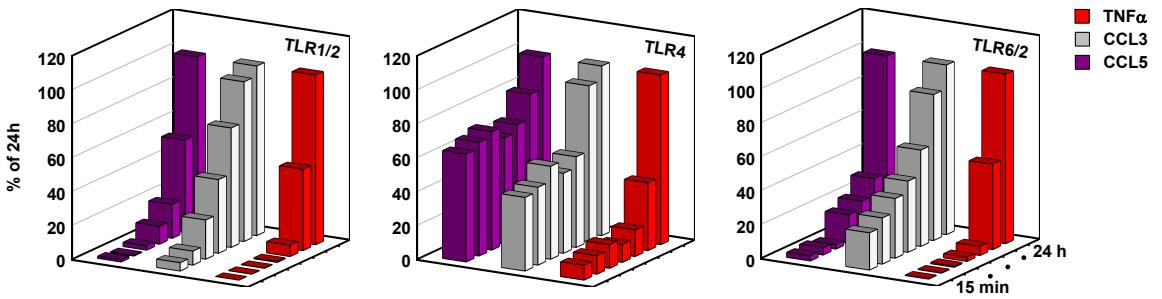


Fig. 4.15: Induction efficacy for individual genes differs among TLR members. Mouse microglia (P0, C57Bl/6) were stimulated with saturating concentrations (as described in Fig. 4.8) of TLR1/2, TLR4 and TLR6/2 agonists, respectively, for the indicated periods from 15 to 180 min, with a further treatment and supernatant collection regime as well as data expression as described in Fig. 4.14. Data are mean with n=12 from 2 individual experiments.

4.12 TLRs reveal a rapid rundown of microglial responses upon agonist removal

A different scenario than those applied in Fig. 4.8 and Figs. 4.13 to 4.15 was applied to determine the effect of an agonist removal after a TLR response got established. In Fig.

4.8, cyto/chemokine production was followed over time in the continuous presence of the agonists. In Figs. 4.13 to 4.15, short-term presence of the extracellular stimulus was followed as to the subsequent output. In the following, microglia were allowed to mount an effective response before the agonists were taken away (Fig. 4.16).

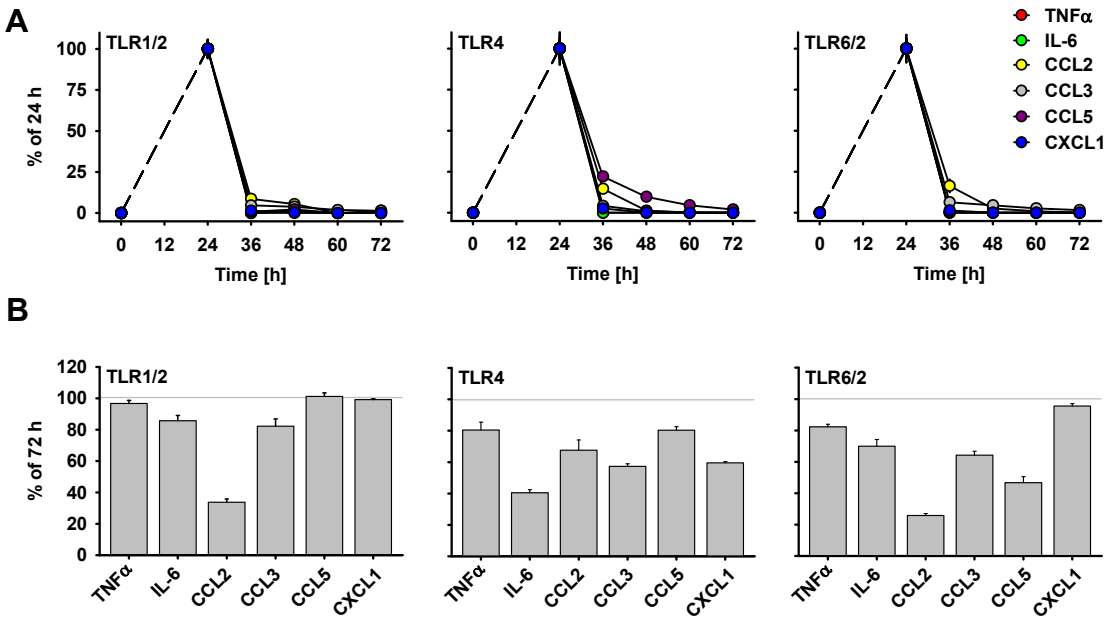


Fig. 4.16: Production of cyto/chemokines declines shortly after stimulus removal. **(A)** Mouse microglia (P0, NMRI) were stimulated with saturating concentrations (as described in Fig. 4.8) of TLR1/2, TLR4 and TLR6/2 agonists, respectively, for 24 h. Subsequently, the supernatants were removed, cells received fresh medium without TLR agonists and the incubation continued for additional 12 to 48 h. Secreted cyto- and chemokines were determined in the supernatants at the indicated time points and normalized for the amounts determined at 24 h, the time point at which the stimulation was terminated. **(B)** Additionally, absolute amounts of cyto/chemokines as determined after the 24 h stimulation period in (A) were normalized to another set of control cells that have been incubated for 72 h with constant presence of the respective TLR agonist. This was done to estimate how much of the maximal response (after 72 h) has already been reached within the 24 h period. In some cases, the maximal total release was thus already executed at 24 h, in other cases not. Data in (A) and (B) are mean ± SEM with n=6.

To test such a sudden disappearance for a subsequent, i.e. probably lasting release activity, cells were stimulated with saturating doses of Pam_3CSK_4, Re-LPS and MALP-2, respectively, for 24 h. Afterwards, the stimulus (as well as the released factors) were removed by a supernatant replacement with medium. Cells continued in the cultures devoid of any TLR ligand for another 12 to 48 h, thereby monitoring their ability to continue producing cyto- and chemokines. The cells were barely able for further release activity upon stimulus withdrawal (Fig. 4.16A). Even though certain factors had not yet reached at the 24 h time point their total release output – the amount that cells could mount over 72 h – the missing amounts could not be delivered anymore (Fig. 4.16B, see also Fig.

4.8). For example, the TLR1/2-driven CCL2 production was only at a third of the maximal output, but the release activity got terminated upon removal of Pam_3CSK_4. This dampening was observed in a similar fashion for TLRX/2- and TLR4-stimulated microglia. The findings from these rundown experiments suggested that the production of certain factors over an extended period would depend on the persistent presence of the TLR stimulus. Once it was gone, the 'production program' got turned off.

The results of Fig. 4.16A may also include the information that negative feedbacks of released factors are unlikely to explain the autonomous termination of further release as seen in Fig. 4.8, although auto/paracrine influences seem to play some role (Fig. 4.13A). Taken all the findings together, microglial responses to TLR challenges revealed to be highly sensitive concerning the duration of agonist presence and receptor signaling, with lasting consequences for the induced proinflammatory response.

4.13 Microglia can be (de)sensitized for specific TLR stimuli

Besides timing of presentation and presence of a stimulus, its concentration is another critical aspect which determines the cellular consequences of TLR stimulation. Local concentrations of TLR ligands can be highly variable *in vivo*, but they must reach a threshold to efficiently activate microglia. On the other hand, concentrations below such threshold may not simply be ignored. First, a sub-threshold concentration would be defined on the basis of a measurable reaction. Interestingly, dose-response relations may vary depending on the cellular activity. A given agonist dose may thus not yet trigger detectable effects for one function, while being already effective for another. Second, exposure to a sub-threshold concentration could even set some concealed effect for the very same cellular activity – not inducing it immediately but changing its magnitude upon a subsequent supra-threshold stimulation. Such 'priming' may organize for enhanced as well as attenuated reactions, as our group had previously shown for the complex impact of IFNγ on microglial challenges through TLRs (Häusler et al., 2002).

To address the 'self-conditioning' of TLR-inducible responses, microglial cells were stimulated for 24 h with various concentrations of the TLR ligands and their response was monitored in terms of release of proinflammatory cyto- and chemokines (Fig. 4.17, grey bars to the left). Cells received then fresh medium, now containing saturating doses of the respective TLR ligands. After another 24 h, the induced amounts of cyto/chemokines were

determined in the supernatants (Fig. 4.17, black bars to the right). Thus, cells underwent two 24 h stimulation periods, the first one even with very low (sub-threshold) and the second with effective agonist concentrations. Data were then analyzed for two aspects: First, the release output – if any – was determined for both periods to compare whether a partial first response would, e.g., reduce the second response, i.e. the amount of released factors, simply by its own amount. Measured amounts of the cyto/chemokines were thus expressed as percentage of the total amount collected over the complete 48 h. Second, in the case of a lack of any cyto/chemokine induction by the first agonist exposure, the question was whether the output of the subsequent period with a full stimulation would be affected – either in terms of an enhancing 'priming' or as a kind of 'homodesensitization' (homotolerance).

In microglial TLR1/2 stimulations (Fig. 4.17, upper panel, also being representative for TLR6/2 stimulation), there were factors which were produced efficiently within a first period (e.g. TNFα, IL-6). With the agonist concentration being sufficiently high, this first release period accounted for all the secreted protein which was determined for reference cells over the total of 48 h with an effective (saturating) stimulation (see the legend of Fig. 4.17). In other words, the first period did not leave any release activity for the second stimulation. If less was induced in the first stimulation, often more release was left for the second (TNFα, CCL3 and CXCL1). However, the total balance did not necessarily always stay in the same range. In some cases, the former response was only moderate and still the second release was impaired so much that the total 48 h production would be diminished (TNFα and IL-6 at 10^{-9} g/ml, also CCL2 and CCL5). Yet within a certain limit, the pre-conditioning concentration roughly correlated with its inhibitory effect, i.e. the lower the concentration the lower the subsequent inhibition. The only exception was seen with CCL2. Interestingly, at moderate concentrations (10^{-10} g/ml), the truly sub-threshold pre-stimulation caused a clear suppression of the full challenge.

The outcomes for microglial TLR4 stimulations presented with a different pattern. Here, basically all factors were affected by the sub-threshold pre-incubation, but the overall inhibition of the subsequent response was not as pronounced as it was seen, in part, for TLR1/2-stimulated microglia. In some cases, the inhibition of the subsequent response was even stronger with lowering the concentration in the first challenge. This dose dependency could be in the opposite way for another factor (compare CCL2 and CXCL1).

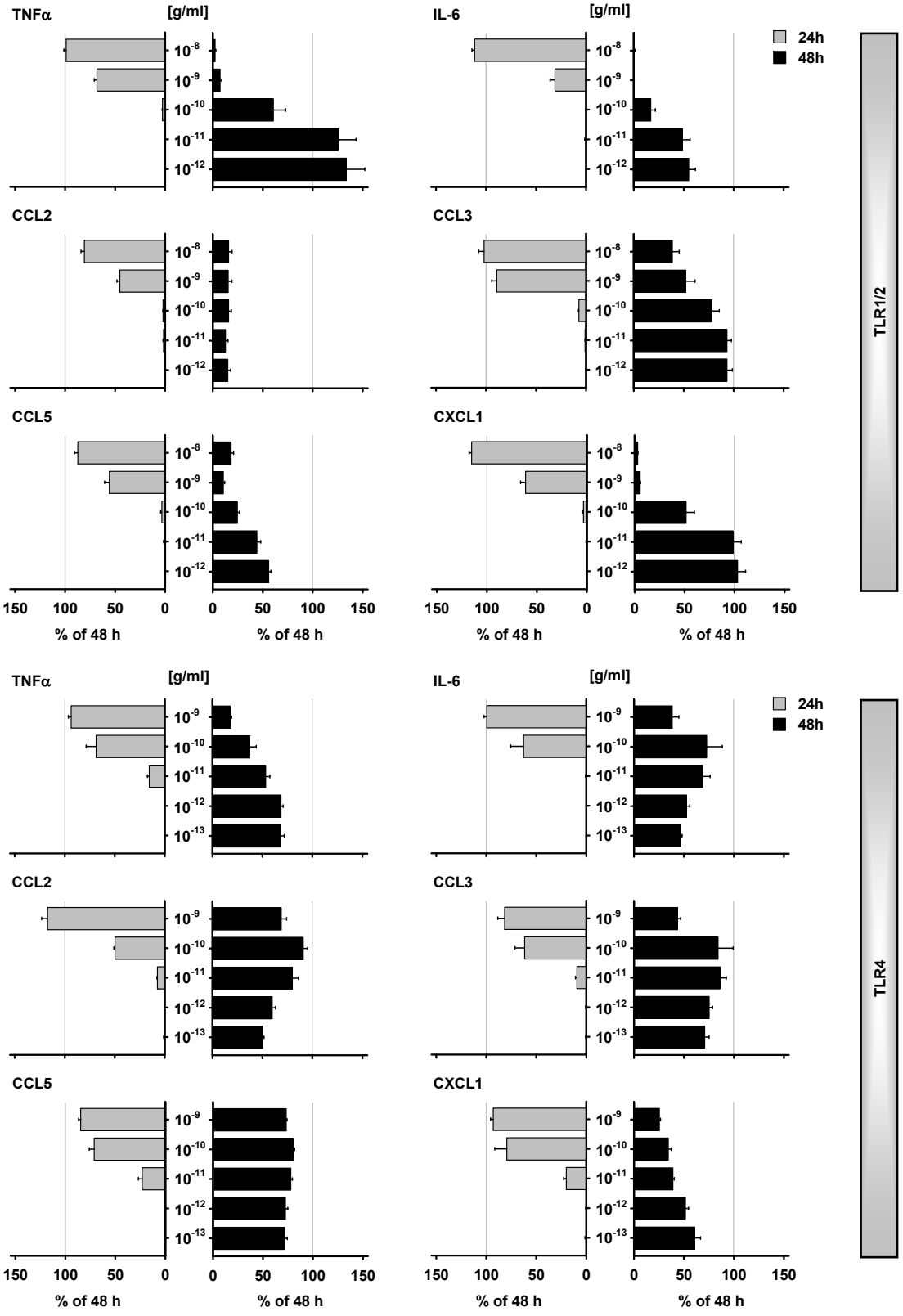

TNFα
[g/ml]
IL-6
[g/ml]
24h
48h
CCL2
CCL3
CCL5
CXCL1
TLR1/2
TLR4
% of 48 h
% of 48 h
% of 48 h
% of 48 h

Fig. 4.17 (previous page): Microglial responses to TLR stimulation can be influenced by pre-conditioning with sub-threshold TLR agonist concentrations. Mouse microglia (P0, NMRI) were stimulated with the indicated non-saturating doses of the TLR1/2 agonist Pam_3CSK_4 and the TLR4 agonist Re-LPS, respectively, for 24 h (grey bars). Afterwards, the culture medium was replaced with fresh medium containing a saturating dose of the respective agonists (10 ng/ml Pam_3CSK_4 and 1 ng/ml Re-LPS, respectively). Cells were then cultured for additional 24 h. Cyto- and chemokines released during the first 24 h and the second 24 h stimulation periods were determined in the respective supernatants. Absolute amounts were normalized to the release obtained by control cells stimulated throughout the complete 48 h with the saturating concentration of the respective TLR ligand (set to 100 %). Thus, release data in the first and second stimulation reflect fractions of the maximal output under a full-blown challenge. Data are mean ± SEM with n=12 from 2 individual experiments.

In summary, it appears that microglial TLRs could be desensitized by their own agonist (homotolerance) already at concentrations that by themselves would not elicit a (or any) response. Importantly, such a phenomenon is not a global effect for all genes driven by this TLR. Individual cyto- and chemokines can reveal substantial independence. Pre-conditioning would thus not only affect the quantity, but also the quality of the later challenge, i.e. the pattern of the inducible genes.

Part C – Microglial TLR responses are modulated in diverse scenarios

4.14 Cytokine co-stimulation reveals fine-tuned ligand discrimination by TLR(4)

It is obvious that in the context of an immune reaction *in vivo* the microglial response to TLR stimulation is not an isolated event. There are numerous influences that can control the microglial response which potentially originate from autocrine feedback mechanisms, signals from neighboring CNS cells, like astrocytes, or invading immune cells including monocytes/macrophages as well as T and B lymphocytes. To mimic such scenarios *in vitro* microglia were stimulated with saturating doses of TLR1/2, TLR4 and TLR6/2 ligands, respectively, in the presence of increasing doses of one out of four immunomodulatory cytokines: (i) the prototypical T_H1 cytokine IFNγ, (ii) the T_H2 'master' cytokine IL-4, (iii) the generally anti-inflammatory IL-10 and (iv) IFNβ, a type I interferon which is typically induced upon viral infection. IFNβ is also of special interest as this cytokine is used as an immunotherapeutic drug in MS. Importantly, it is controlled by the TRIF signaling pathway and we had observed that respective TLR(4) agonists were able to trigger its induction when they were of PAMP nature, whereas DAMPs virtually failed to do so. On the other hand, IFNβ had a very strong influence on DAMP-induced TLR4 functions, suggesting that it could represent a critical mean by which PAMPs exert a control over DAMP activity.

As Fig. 4.18 shows, all of the above mentioned modulators were capable of influencing microglial responses to TLR stimulation in terms of the release of proinflammatory cyto- and chemokines, yet with clear distinctions. In general, modulations of TLR1/2- *versus* TLR6/2-triggered responses appeared rather similar – which is not surprising considering the sharing of TLR2 signaling – whereas TLR4 responses revealed a different pattern.

Effects of IFNγ on TLRX/2 (summarizing TLR1/2 and TLR6/2) responses presented with a clear up-regulation of CCL5, accompanied by a down-regulation of CXCL1 and CXCL2, while having varying and less potent effects on other cyto/chemokines. This could be interpreted as an enhanced call for T cells, whereas chemoattraction of neutrophils (and in part also macrophages) would be suppressed. This shift agrees with our earlier observations made with pneumococcal cell walls (Häusler et al., 2002). The Gram-positive bacteria-derived material contained proteoglycans and

lipoteichoic acid, two agents now known to signal via TLR1/2 and TLTR6/2, respectively. IFNγ had similar effects also on the TLR4 response, yet without the enhancement of CCL5 production. An attenuated release of CCL3, a chemokine for T_H1 cells, along with an increased or not affected production of CCL5, thought to attract both T_H1 and T_H2 populations, suggests a bias of the chemoattraction towards T_H2 cells. Again, this would agree with previous findings (Häusler et al., 2002) and indicate an IFNγ-instructed complex reorganization of TLR-triggered chemoattractive profiles with a 'self-limiting' outcome on the T_H1 subpopulation as a major source of IFNγ production.

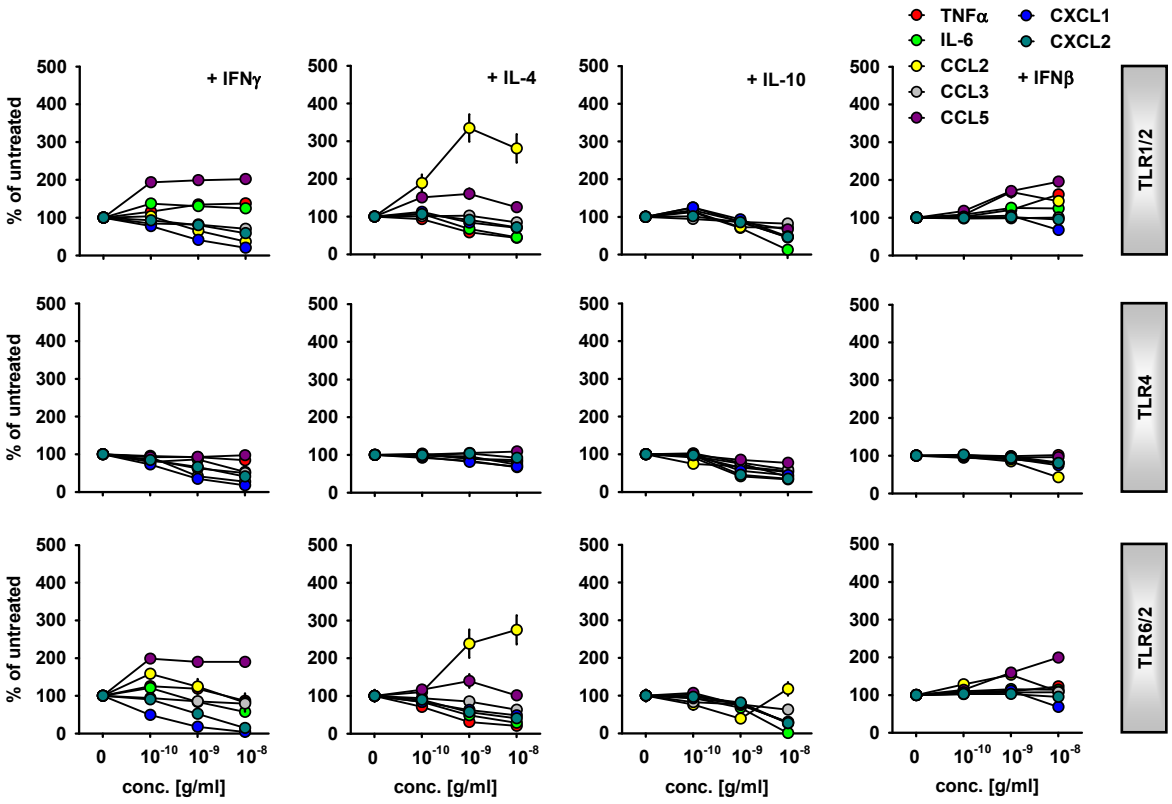


Fig. 4.18: Microglial responses to TLR stimulation are modulated by critical cytokines. Mouse microglia (P0, NMRI) were stimulated with saturating concentrations (as described in Fig. 4.8) of TLR1/2, TLR4 and TLR6/2 agonists, respectively, in the presence of increasing doses of the indicated cytokines for 18 h. Cyto- and chemokines were determined in the supernatants and the absolute amounts were normalized to the amounts obtained by cells stimulated with the respective TLR agonist only (untreated cells set to 100 %). Data are mean ± SEM with n=6 for TLR1/2 and TLR6/2, respectively, and n=14-26 from 2-4 individual experiments for TLR4.

The addition of IL-4 caused a general down-regulation of proinflammatory cytokines by TLRX/2-stimulated microglia. However, while the call for T cells (CCL5) was spared from

this dampening effect the recruitment of monocytes/macrophages was strongly favored, as shown by the marked increase in CCL2 protein. IL-4 had no such effect on the TLR4 response which was basically not modulated by this T_H2 cytokine. Only IL-10, a cytokine with a general anti-inflammatory and immunosuppressive activity, had similar effects on all TLR responses. Here, the (dose-dependent) dampening of signals for T cells, monocytes and macrophages was less potent than for the other cyto- and chemokines.

The effect of IFNβ on TLRX/2 responses was characterized by a clear up-regulation of CCL5 and a partial up-regulation of TNFα, a scenario similar to that induced by IFNγ. CCL2 regulation varied from that seen with IFNγ, while CXCL1 and CXCL2 were almost spared from changes – a clear difference to the regulation obtained with IFNγ. Notably, there was not much of an influence of IFNβ on the TLR4-triggered response, except for a partial down-regulation of CCL2. In other words, TLR4 stimulation could lead to IFNβ induction, but this would, in turn, not modify the profile of the other cyto/chemokines.

While these combined stimulations tell about the control of TLR-driven responses by additional signals and reveal shifts in the reactive phenotypes of microglia under influences by, for example, T_H1 and T_H2 cells, they can also indicate subtle differences in TLR signaling as triggered by different agonists. As mentioned, we found TLR4 responses to the PAMP Re-LPS and several DAMPs to differ dramatically when a second stimulus was allowed to enrich the signaling in microglia. Although the PAMP and DAMP factors all shared TLR4 as receptor the presence of IFNγ, IFNβ or IL-4 had surprisingly distinct outcomes on the respective cyto- and chemokine patterns. We considered the differential impact of the same 'second' stimulus (like IFNγ) on the release profiles of the individual TLR4 agonists as a hint to their distinct signaling through TLR4. If all the agonists would come with a completely identical signaling, i.e. meaning that they would be indistinguishable agonists, then the modulatory impact of the second stimulus should be the same as well. In contrast, if the modulation by the second stimulus would turn out different, then it would most likely be due to a difference in the primary TLR4 signaling. Indeed, the latter scenario was indicated by comparing DAMPs to Re-LPS and among each other. In these studies, we had chosen a 'standard' LPS to represent the PAMP stimulation.

To determine a potential individuality in the regulation of cyto/chemokine profiles as induced by different LPS chemotypes, the same modulation paradigm was applied on

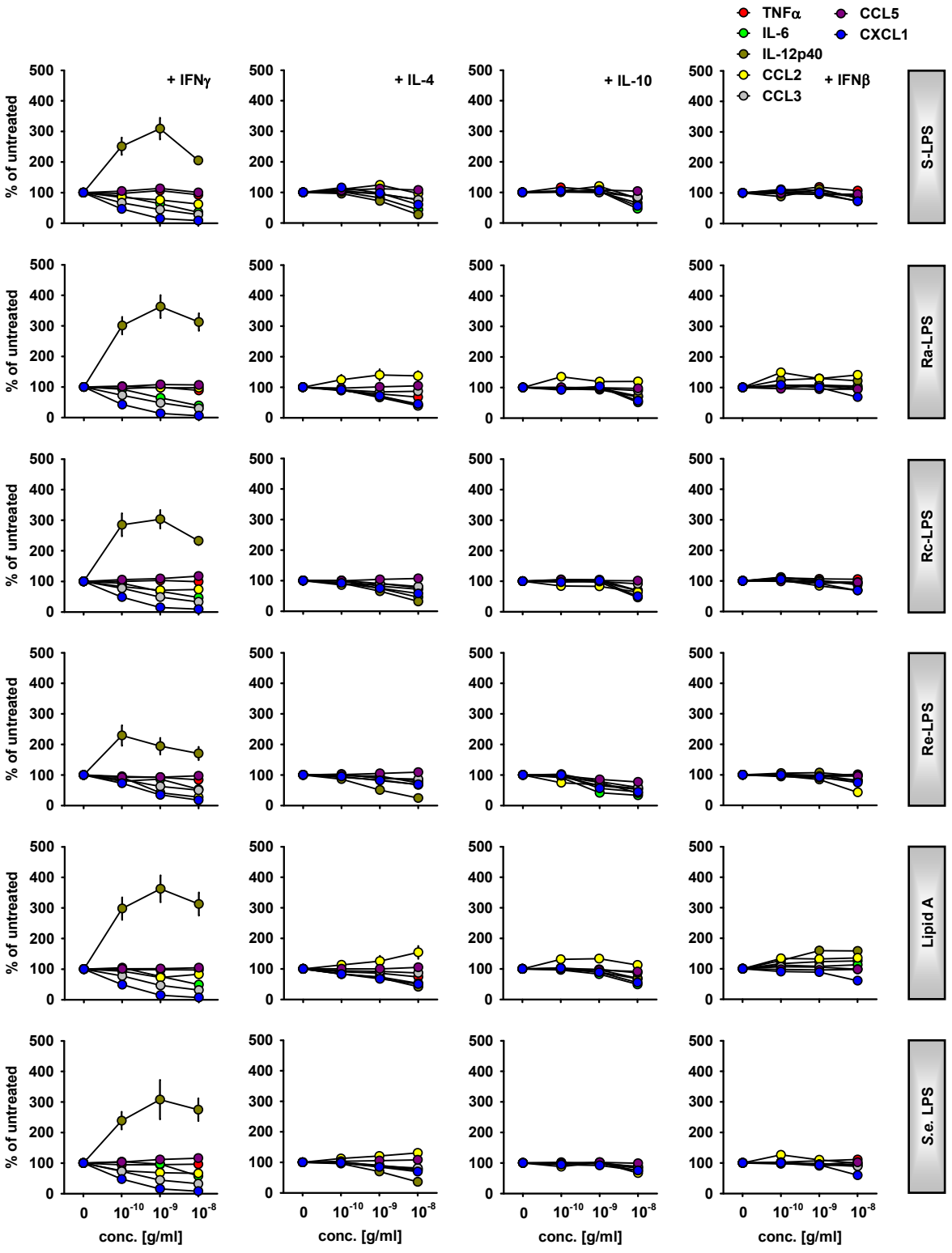


Fig. 4.19: Microglial responses to different LPS chemotypes are similarly modulated. Mouse microglia (P0, NMRI) were stimulated with saturating doses (10 ng/ml for each) of the various LPS structural variants (as listed in Tab. 4.1) in the presence of increasing doses of the indicated cytokines for 18 h. Cyto-and chemokines were determined in the supernatants and the absolute amounts were normalized to the amounts obtained by cells stimulated with the respective LPS variant only (untreated cells set to 100 %). Data are mean ± SEM with n=8 from 2 individual experiments for S-, Ra-, Rc-LPS, Lipid A and *S.e.*-LPS, respectively, and n=14-26 from 2-4 individual experiments for Re-LPS.

the microglial responses to various LPS variants (see Part A of the Results section). As expected, all the chemotypes showed a similar pattern of modulation under the co-stimulations. However, differences were noted especially in the responses to co-stimulations with IFNβ and IL-4. Focusing on these two cytokines, the modulation of Ra-LPS and Lipid A responses appeared very similar but with some distinction from the remainder of chemotypes. Such similarities in functional outcomes between different chemical agonist structures again suggest that the sequence from S- to R-LPS and Lipid A in terms of carbohydrate complexity may not truly translate into a gradient of features in their TLR4 signaling. A remarkable effect was noticed throughout the various LPS. There was a strong up-regulation of IL-12p40, when co-stimulating LPS with IFNγ. This marked regulation agreed with earlier observations (Häusler et al., 2002) and indicated, at first glance, a deviation from the self-limiting role of the T_H1-produced interferon on the chemoattraction. Yet IL-12p40 can come in different monomeric, homo- and heterodimeric formats. In association with the IL-12p35 subunit, it builds up IL-12p70, the agonistic IL-12 proper, and in combination with p19, it constitutes the family member IL-23. The homodimer IL-12p40$_2$, however, has been reported to serve as an antagonist for IL-12p70, although own effects as an agonist were discussed as well. Since IL-12p70 is a T_H1-supporting cytokine, large amounts of IL-12p40$_2$ may rather interfere with the promotion of T_H1 responses. Indeed, we never observed significant inductions of IL-12p70 (or IL-23), and the detection of IL-12p40 may thus reflect a sole production of the homodimer. In other words, its up-regulation would go in line with a kind of negative feedback of T_H1 cells via IFNγ.

In conclusion, these findings showed that within the context of an ongoing complex inflammatory process there could be some minor variations in terms of what a specific LPS chemotype is activating in microglia – with regard to the cyto/chemokine profile. Distinct reactive phenotype features could develop under the influence of the signaling context, as it was mimicked here by co-stimulation. Compared to the scenarios with DAMP challenges, LPS chemotypes behaved as a clearly different class of agonists with little variation among themselves. Consequently, the data on the set of LPS chemotypes support the notion of a clear discrimination of PAMPs and DAMPs, regardless of the fact that all of them bind to as well as signal through the microglial TLR4.

4.15 Noradrenaline bears the capacity to regulate microglial TLR signaling

In contrast to immunomodulatory cytokines originating largely from invading immune cells or auto/paracrine (micro)glial mechanisms, noradrenalin (NA) is a typical CNS molecule. Having important transmitter and hormone-like activities in the autonomous nervous system it is, within the CNS, one of the diffuse modulatory neurotransmitters. In addition, NA is well known for some immunosuppressive effects on peripheral macrophages. There are thus also immune functions of a 'non-immune' messenger. Recently, the link between microglia and the noradrenergic system was demonstrated in the context of facilitated Aβ clearance and attenuated inflammation (Heneka et al., 2010). On the other hand, a link between Aβ and microglial TLR was shown in several studies. Therefore, arguments for a general modulatory capacity of NA over microglial TLR responses already existed.

In principle, microglia are capable to sense NA in their environment as the transmitter is also released to extra-synaptic sites and since these cells express a wide range of adrenergic receptors on their surface (Fig. 4.20A). To test for the influence of NA on TLR(4) signaling *in vitro*, microglia were stimulated with a saturating dose of the TLR4 agonist Re-LPS in the simultaneous presence of increasing doses of NA. Based on the capacity of microglial cells to release proinflammatory cytokines upon stimulation, NA at higher – yet physiologically relevant – doses revealed to be a potent inhibitor of the response to LPS (Fig. 4.20B). Of note, this inhibition appeared to be selective for some factors, e.g. TNFα and IL-12p40, whereas others, like CCL5, remained almost unaffected by the NA addition.

To identify the type of adrenergic receptor (AR), a set of specific agonists and antagonists was employed (Fig. 4.20B). Co-stimulation of microglia with the α_1/α_2-AR agonist oxymetazoline (OX) had no (or not much) influence on the LPS-induced cytokine profile. However, both the β_1/β_2-specific isoproterenol (IP) and the β_2-specific salbutamol (SB), respectively, could mimic the selective modulation of the LPS response as seen with NA co-stimulation. To confirm and further specify the responsible β-AR subclass, cells were co-stimulated with LPS, a sufficiently high concentration of NA to establish the inhibitory effects and increasing concentrations of AR-specific antagonists in order to reverse the NA-induced inhibition (Fig. 4.20C). As expected, the β_1/β_2-specific

propranolol (PR) was capable to reverse the NA effect. However, only the β_2-specific ICI118,551 (ICI), but not the β_1-specific atenolol (AT) could reverse NA effects. As a control, the α_1/α_2-specific phentolamine methansulfonate (PM) was tested, but failed to reverse the NA effects. In conclusion, the combined data on AR agonist as well as antagonist activities revealed that microglial TLR(4) signaling is modulated by NA via a β_2-AR mechanism.

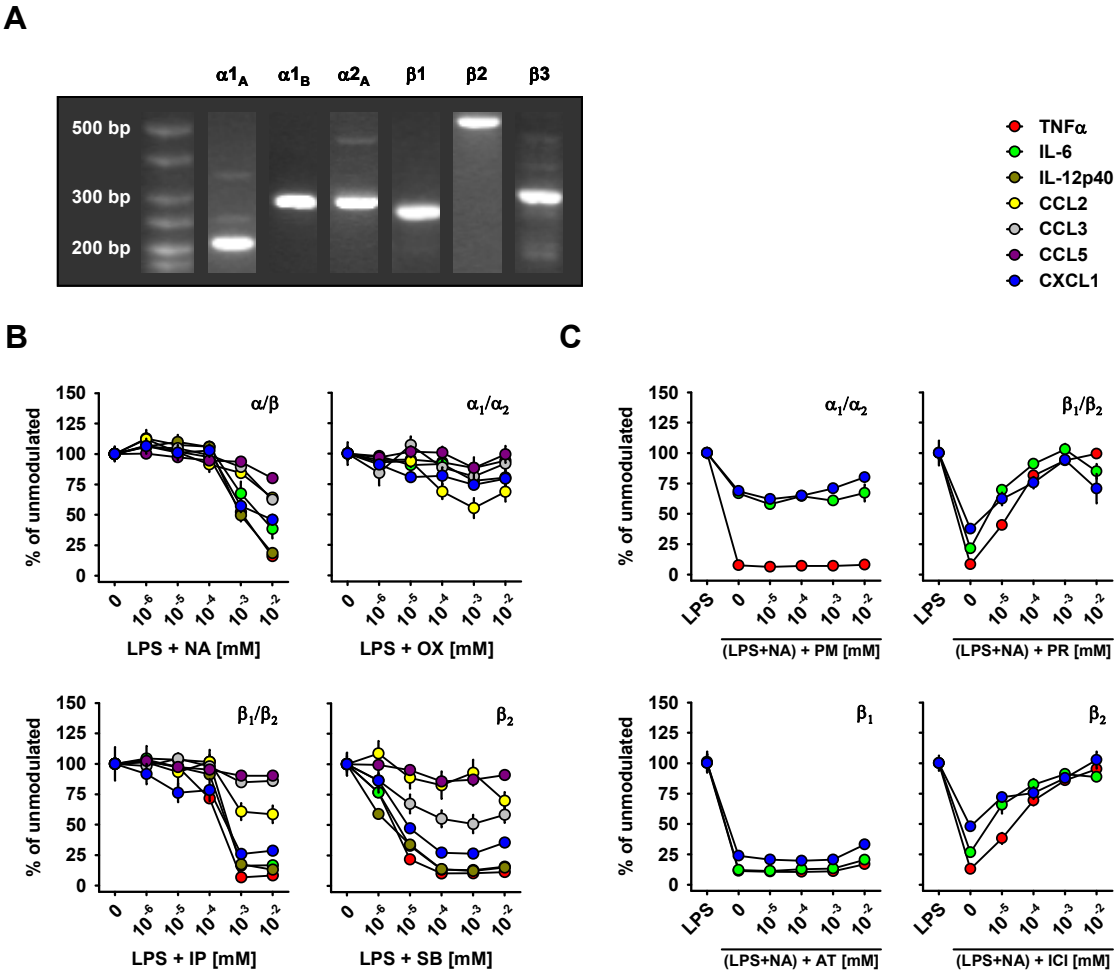


Fig. 4.20: Microglial responses to TLR4 stimulation are modulated by noradrenalin through a β2-adrenergic receptor-dependent mechanism. (A) PCR products representative for the detection of the indicated adrenergic receptor subtypes expressed in mouse microglia (P0, C57Bl/6). **(B)** Mouse microglia (P0, C57Bl/6) were stimulated with a saturating concentration of Re-LPS (10 ng/ml) in the presence of increasing concentrations of noradrenaline (NA), oxymetazoline (OX), isoproterenol (IP) or salbutamol (SB), respectively, for 18 h. **(C)** Cells were stimulated with Re-LPS (as in B) in the presence of 10 µM NA and increasing concentrations of phentolamine methansulfonate (PM), propranolol (PR), atenolol (AT) or ICI118,551 (ICI), respectively, for 18 h. Cyto-and chemokines were determined in the supernatants and the absolute amounts were normalized to the release obtained by control cells stimulated with Re-LPS only (B) or Re-LPS + NA (C, unmodulated cells set to 100 %). Data are mean ± SEM with n=18 from 3 individual experiments for NA, n=12 from 2 individual experiments for SB and ICI and n=6 for OX, IP, PM, PR and AT, respectively.

It is important to note that none of the tested agonists/antagonists (including NA) had by themselves an effect on microglia in terms of the induction of cyto/chemokine release as far as they were considered here.

The β–AR signaling is well-known for the activation of adenylate cyclase (AC) accompanied by a subsequent increase in cAMP levels. The addition of NA to microglial cells led to a rapid increase in the intracellular cAMP concentration within 1 min (Fig. 4.21A). The level reached a plateau at 15 min and then declined over a time course of about 2 h. There was also a cAMP increase in the extracellular compartment, following the intracellular rise and staying elevated up to the end of the experiment (probably also due to the lack of cAMP-degrading enzymes in the culture supernatant). To further substantiate a critical role for cAMP in transmitting the NA effect microglial cells were co-stimulated with LPS and forskolin, a direct AC activator (Fig. 4.21B). High doses of forskolin were capable of mimicking the effect of NA on the LPS response, thereby proving the critical involvement of cAMP as part of the signaling.

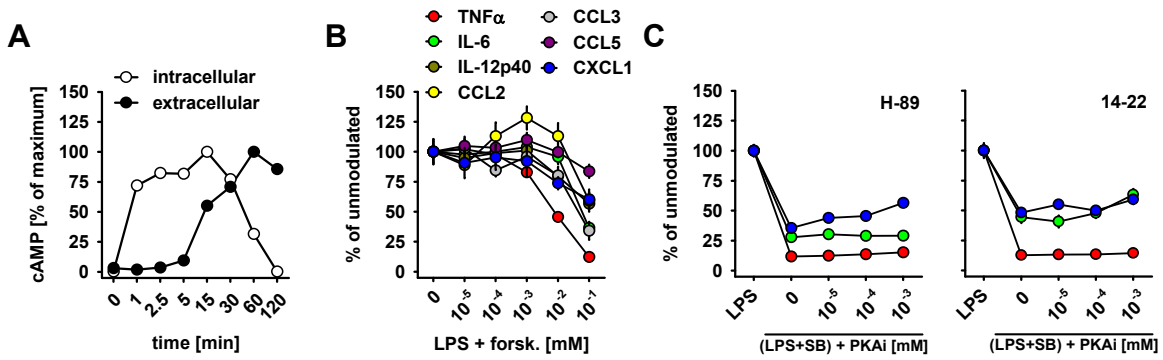


Fig. 4.21: cAMP but not PKA is critically involved in the effect of NA on the microglial TLR4 response. (A) Mouse microglia (P0, C57Bl/6) were stimulated with 10 µM NA for the indicated periods of time. Afterwards, cell culture supernatants were isolated and cells were lysed. In both, the lysates (intracellular) and the supernatants (extracellular) cAMP levels were determined. Absolute values were normalized to the respective experimental maximum (set to 100 %). **(B)** Cells were stimulated with a saturating dose of Re-LPS (10 ng/ml) in the presence of increasing doses of forskolin for 18 h. **(C)** Cells were stimulated with a saturating dose of Re-LPS (as in B) in the presence of 1 µM SB and increasing doses of the PKA inhibitors H-89 and 14-22, respectively, for 18 h. Cyto-and chemokines were determined in the supernatants and the absolute amounts were normalized to the release obtained by control cells stimulated with Re-LPS only (B) or Re-LPS + SB (C) (unmodulated cells set to 100 %). Data are mean ± SEM with n=2 for (A), n=8 for (B) and n=6 for H-89 and n=12 from 2 individual experiments for 14-22, respectively, in (C).

Cyclic AMP is known to activate protein kinase A (PKA). To test a possible involvement of PKA in the NA effect-mediating signaling microglial cells were co-stimulated with

LPS+SB in the presence of a PKA inhibitor (Fig. 4.21C). Surprisingly, neither the potent PKA inhibitor H-89 nor the myristoylated – and therefore better cell-permeable – PKA inhibitor 14-22 could reverse the NA/SB effect on the microglial LPS response. These findings led to the conclusion that cAMP was crucial, but PKA would not represent the main target. PKA mechanisms could therefore not be part of the signaling allowing NA to regulate microglial TLR responses.

Notably, the NA effect was not limited to the inhibition of a TLR4 response but could also be shown for microglial responses to Pam_3CSK_4 (TLR1/2) and MALP-2 (TLR6/2), respectively. Moreover, all TLR responses were sensitive to NA as well as SB modulation, suggesting the usage of a uniform receptor and signaling mechanism (Fig. 4.22). However, the selective modulation in terms of a suppressive *versus* permissive control pattern proved to be unique for the TLR4 response (Fig. 4.22 and 4.24A).

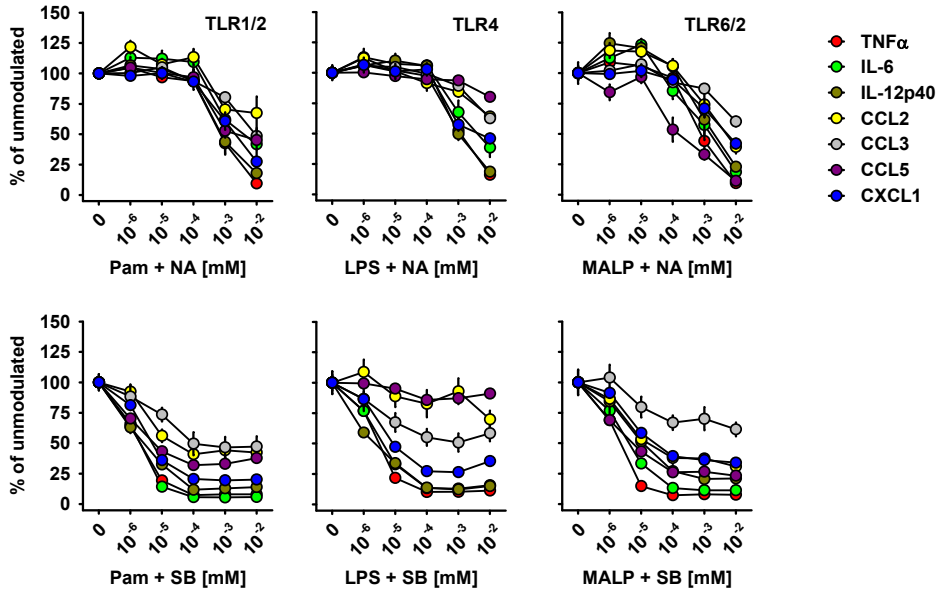


Fig. 4.22: NA can modulate microglial responses to various TLR stimulations. Mouse microglia (P0, C57Bl/6) were stimulated with saturating doses of TLR1/2, TLR4 and TLR6/2 agonists (10 ng/ml, each), respectively, in the presence of increasing concentrations of NA or SB, respectively, for 18 h. Cyto- and chemokines were determined in the supernatants and the absolute amounts were normalized to the release obtained by control cells stimulated with the respective TLR agonist only (unmodulated cells set to 100 %). Data are mean ± SEM with n=18 from 3 individual experiments for NA and n=12 from 2 individual experiments for SB, respectively.

As it was shown in Part B of the Results section, certain genes preferentially 'use' one and/or the other TLR signaling adaptor molecule (MyD88 and/or TRIF) when induced

through TLR4. In order to translate this phenomenon also to the NA modulatory effect co-stimulation experiments were performed with microglia deficient in MyD88 or TRIF, respectively. In the TRIF$^{-/-}$ situation, the microglial response to LPS could be modulated by the addition of either NA or SB, this time unselectively down-regulating all factors (Fig. 4.23A). In the opposite scenario, namely the MyD88$^{-/-}$ situation, most of the factors were potently inhibited, except for CCL5, where the down-regulation was still less potent. Summarizing the data shown in Figs. 4.22 and 4.23, the NA effect seemed to be most potent on genes that were induced through a MyD88-dependent signaling mechanism. However, NA is (in part) also capable to modulate genes induced through TRIF, when microglial TLR4 was stimulated. This was especially apparent for the modulation of IFNβ. As shown in Part A of the Results section, LPS – regardless of its chemotype – is capable of inducing IFNβ release when signaling through TLR4. Indeed, this induction of IFNβ could also be potently down-regulated by the addition of NA (data not shown) indicating that the association of a given gene with TRIF signaling alone is insufficient to 'escape' noradrenergic control.

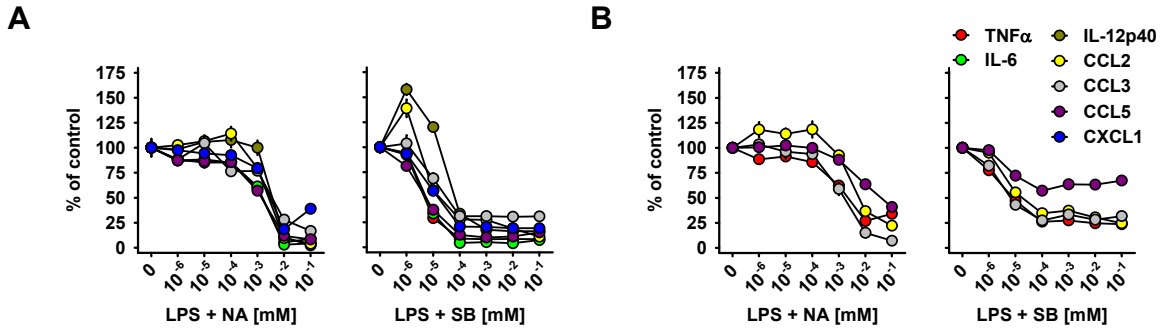


Fig. 4.23: NA strongly regulates genes induced through MyD88. Mouse microglia (P0) with a deficiency in TRIF **(A)** or MyD88 **(B)**, respectively, were stimulated with a saturating dose of Re-LPS (10 ng/ml) in the presence of increasing concentrations of NA and SB, respectively, for 18 h. Cyto- and chemokines were determined in the supernatants and the absolute amounts were normalized to the release obtained by control cells stimulated with Re-LPS only (set to 100 %). Data are mean ± SEM with n=10 from 2 individual experiments for TRIF$^{-/-}$ (A) and n=9 from 2 individual experiments for MyD88$^{-/-}$ (B).

The selective modulation of TLR4-triggered cyto- and chemokines by NA led to their classification as 'permissive', i.e. not or only mildly down-modulated, *versus* 'non-permissive', i.e. suppressed by NA, genes (Fig. 4.24A). Based on this observation, the question evolved whether either of the two groups would also be more or less sensitive to a certain timing of the NA addition. Indeed, in experiments with transient TLR stimulations

(induction efficacy studies) we had observed that agonists, such as LPS, were required for extended periods in the culture medium to induce a significant production of factors like TNFα, whereas others, such as CCL5, got relatively quickly organized for effective subsequent induction and release (see Fig. 4.13 in Part B of the Results section). Therefore, microglial cells were stimulated with a saturating dose of Re-LPS (TLR4) and NA was added simultaneously or up to 3 h before (early addition) or after (late addition) the TLR stimulus was applied. Fig. 4.24B shows that there was no such correlation as proposed above. Instead, factors could be assigned to four different groups of sensitivity towards the timing of NA addition. The first group comprised factors (TNFα, CCL3 and CCL5) that were modulated similarly, regardless of the time point of NA addition. This group thus included members of the permissive as well as non-permissive (suppressed) genes. Yet these genes shared the feature of a time-independent regulation. Factors of the second group (IL-12p40 and CCL2) showed an increased sensitivity for modulation the earlier NA was added before the LPS addition and decreasing sensitivity the later NA was added after the onset of the LPS stimulation. The suppressive influence thus faded almost in a monotonous fashion with the shift in relative NA and LPS addition. The induction control of IL-6 showed a distinct behavior, making up a third type, as a modulation was unaltered when NA was added early (similar to TNFα), but sensitivity decreased after late addition of NA (similar to IL-12p40). Finally, CXCL1 felt into a fourth type of timing dependence since it showed to be down-modulated to an extreme upon coincidental NA and LPS addition. Thus simultaneous treatment – with simultaneous signaling onset of the β_2–AR and TLR4 – caused maximal regulation. Interestingly, such 'diving' effect in the time course of CXCL1 regulation upon coinciding addition was also observed in one of our previous studies for the combination of TLR stimulation and the modulation by IFNγ (Häusler et al., 2002). It appears to be a feature of this particular chemokine when it is under the control of TLRs. Interestingly, the inhibitory capacity of NA was the least effective when it was added 2 h after the release-inducing stimulus but gained back part of its inhibitory capacity when added even 3 h after LPS. This phenomenon pointed to a possible second wave of signaling in the release induction where NA could again interfere.

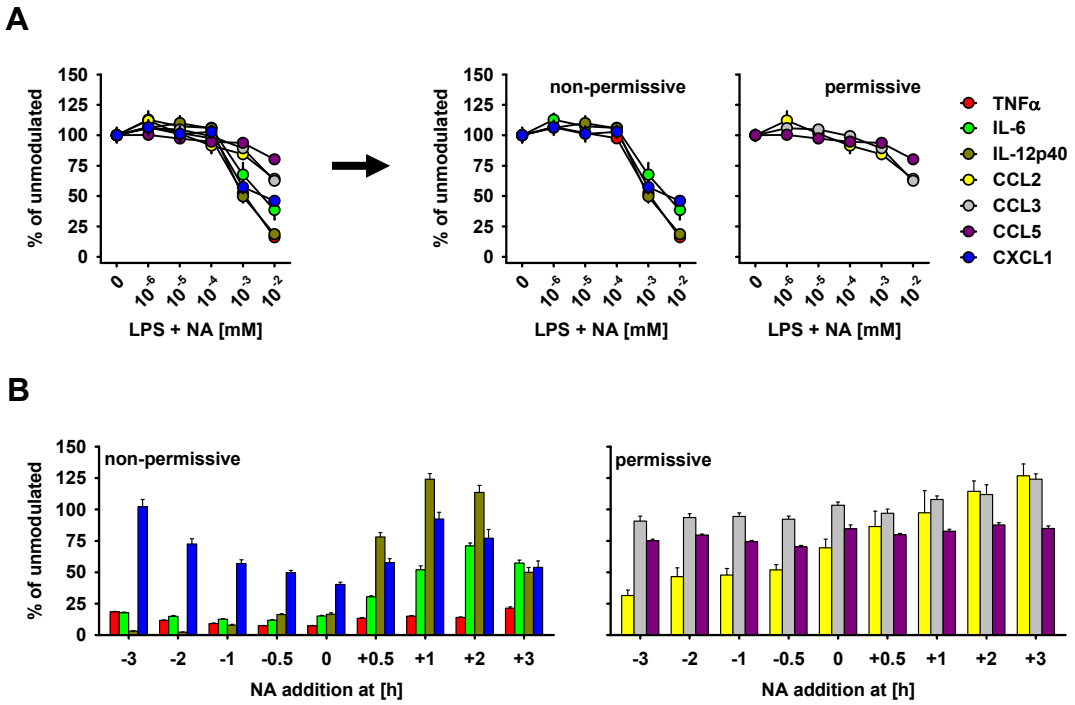


Fig. 4.24: TLR-induced cyto- and chemokines show individual sensitivity to the timing of NA addition. **(A)** Mouse microglia (P0, C57Bl/6) were stimulated with a saturating dose of Re-LPS (10 ng/ml) in the presence of increasing doses of NA for 18 h. Factors largely down-regulated *versus* spared from suppression are grouped as 'non-permissive' and 'permissive', respectively. **(B)** Similarly, cells were stimulated with Re-LPS and NA (10 µM) was added at the indicated time points relative to the onset of Re-LPS stimulation which then lasted for 18 h (time 0 for the addition of Re-LPS). Cyto-and chemokines were determined in the supernatants and the absolute amounts were normalized to the release obtained by control cells stimulated with Re-LPS only for 18 h (unmodulated cells set to 100 %). Data are mean ± SEM with n=18 from 3 individual experiments for (A) and n=8 for (B).

4.16 The interplay of microglia with CNS invading tumor cells

In the previous experiments, microglia were challenged with TLR ligands and the respective responses were modulated by endogenous factors originating from CNS parenchymal cells or from non-CNS immune cells. The experiments described in this section addressed another scenario in which reactive phenotypes of microglia would be either influenced by signals and cells of their environment or influence consequences for the CNS in a pathological context. We addressed the question whether CNS-invading tumor cells would be capable to interact with microglia and whether this potential interaction could also modulate or be modulated by microglial responses to TLR stimulation. Previous experiments could already demonstrate the hypothesized tumor-microglia interaction. In Boyden chamber experiments (collaboration with Prof. Claudia

Binder and Dr. Tobias Pukrop, Department of Oncology and Haematology, University of Göttingen) co-cultures of these two cell types significantly increased the tumors' capability to 'invade' into the microglial compartment. Moreover, this invasion was inhibited when LPS (TLR4) was present. In contrast, Pam_3CSK_4 (TLR1/2) and MALP-2 (TLR6/2), respectively, could not reverse the invasion-supportive effect of microglia (data not shown). In order to test whether the presence of tumor cells, using the mouse breast cancer cell line 410.4, would induce a microglial phenotype both cell types were co-cultured indirectly, i.e. with a physical barrier between tumor and microglia, leaving the opportunity to exchange soluble factors. The conditioned culture medium was then analyzed for the potential release of a broader spectrum of pro- and anti-inflammatory cytokines and chemokines. Control settings with individual microglia or tumor cell cultures were used to assign the respective release to the responsible cell population(s). There was a basal release of TNFα, CCL2 and CCL3 by unstimulated microglia (Fig. 4.25A). When co-cultured together with the tumor cells, this basal release was slightly modulated. CCL2 was substantially up-regulated and CCL3 significantly down-regulated. However, the presence of the tumor did not provoke microglia to release other factors than the ones revealing some basal levels. The 'up-regulation' of TNFα, CCL5, CCL17, CXCL1 as well as CXCL2 in the co-culture could clearly be assigned to the tumor as a responsible source since these factors were produced in similar amounts by the tumor cells alone. This led to the conclusion that, despite the minor adjustments of the basal microglial release pattern, the presence of the tumor did not induce a specific phenotype in microglia, at least as based on the release data. Actually, this was also true for a direct co-culture scenario (no physical barrier between the two cell types) as well as for co-culture experiments with the human breast cancer cell line MCF-7 (data not shown).

A similar picture was seen in co-cultures that were carried out in the presence of (microglia) saturating doses of TLR ligands (Fig. 4.25B). Microglial cells were able to mount a profound proinflammatory response upon stimulation with the TLR4 ligand Re-LPS and the TLR6/2 ligand MALP-2 (also representative for TLR1/2 data), respectively. When co-cultured with the tumor cells, this microglial response was not altered in an apparent manner. The only significant change was a remarkable up-regulation of CXCLl when both cell types were co-cultured. However, this could be explained by a clear contribution of the tumor cells, as they produced high amounts of CXCL1 upon TLR stimulation by themselves ('tumor alone' data not shown in Fig. 4.25B).

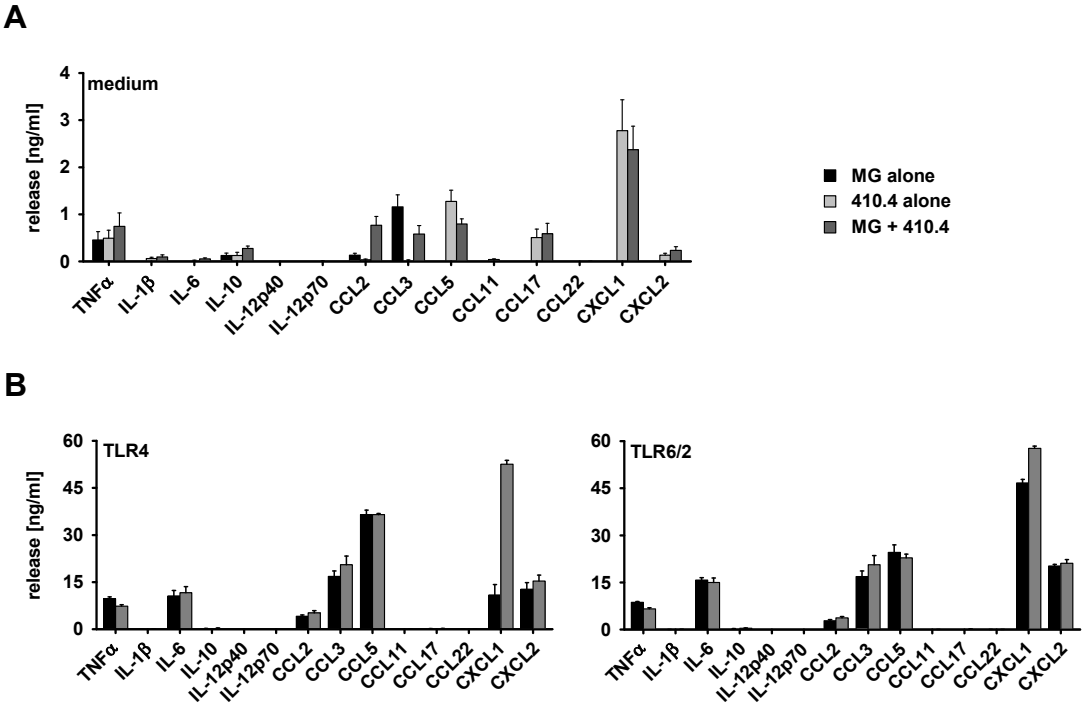


Fig. 4.25: Co-cultures with 410.4 tumor cells do not provoke a microglial phenotype and have no influence on the microglial responses to TLR stimulation. Mouse microglia (P0, NMRI) were indirectly co-cultured with cells of the mouse breast cancer cell line 410.4 for 18 h. As controls, microglia and tumor cells, respectively, were cultured alone within the same experimental settings. **(A)** The cultures were left unstimulated or **(B)** were supplemented with the TLR4 or TLR6/2 ligands Re-LPS and MALP-2 (10 ng/ml each), respectively. Cyto- and chemokines were determined in the supernatants. Data are mean ± SEM with n=8 from 2 individual experiments. Note the different scaling of y axes in (A) and (B). The basal release activity of microglia in (A) was most likely due to a reaction of these cells to the culture insert material (polycarbonate).

Confirming the observations from the unstimulated co-culture, the microglial response to TLR stimulation was also not altered when co-cultured directly with 410.4 tumor cells or indirectly in co-culture with human MCF-7 cells, respectively (data not shown). In conclusion from the Boyden chamber experiments the tumor cells communicated with microglia with the help of one or several (unknown) factor(s) in order to invade the microglial compartment. On the other hand, interpreting the data shown in Fig. 4.25, the simple presence of the tumor neither provoked a microglial phenotype nor did it alter the microglial response to TLR stimulation, leading to the hypothesis that the tumor 'uses' microglia for invasion but, at the same time, keeps these cells 'silent' in terms of a proinflammatory (potentially anti-tumor or tumoricidal) microglial response.

Since the mouse tumor cell line 410.4 is characterized by a rather mild metastasizing and subsequent growth behavior it was of great interest to test whether a

more 'aggressive' cell line would behave different in the above described co-culture scenario. The mouse breast cancer cell line 4T1 was used to answer this question.

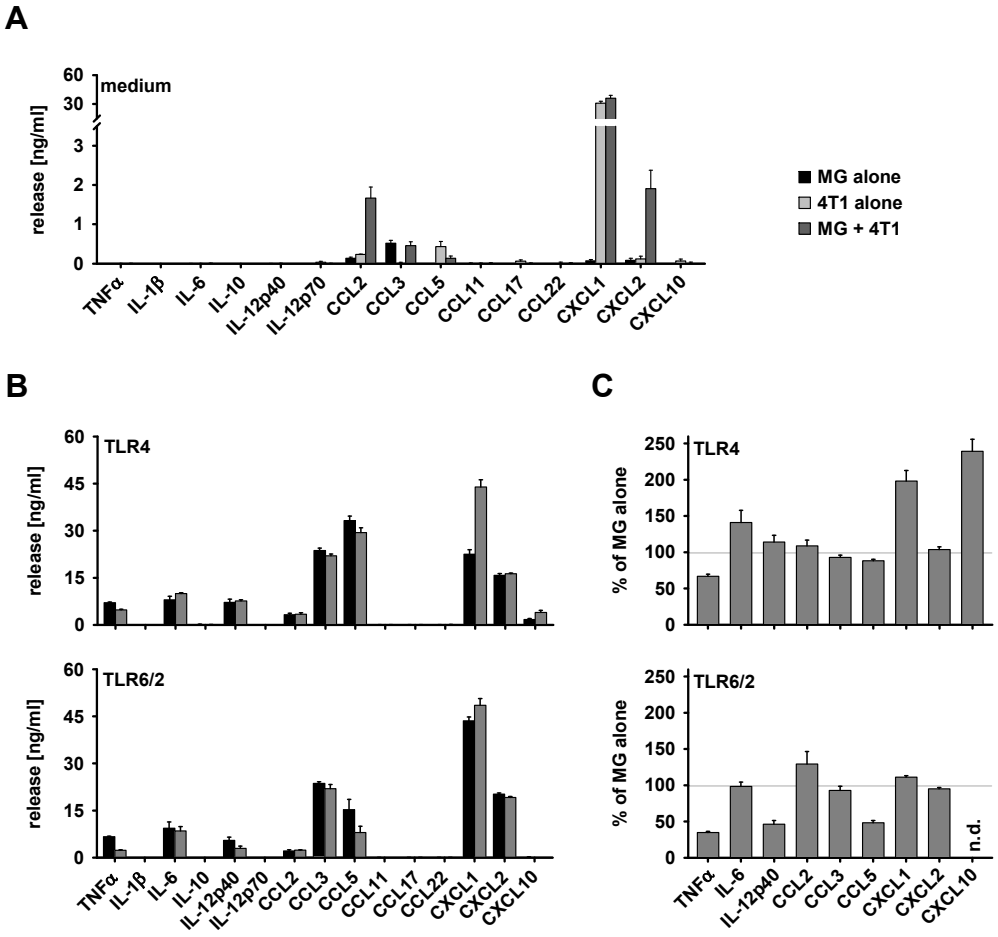


Fig. 4.26: 4T1 tumor cells induce a microglial phenotype and alter microglial responses to TLR stimulation. Mouse microglia (P0, NMRI) were indirectly co-cultured with cells of the mouse breast cancer cell line 4T1 for 18 h either **(A)** in the absence or **(B)** presence of TLR agonists as it was described in Fig. 4.25. Cyto-and chemokines were determined in the supernatants. Data are mean ± SEM with n=8 from 2 individual experiments. **(C)** Absolute release data from the co-culture in (B) were normalized to the release obtained by control microglia stimulated with the respective TLR ligands in the absence of tumor cells (set to 100 %). n.d. = not detected.

As demonstrated in Fig. 4.26A, the tumor did not induce a profound microglial reactive phenotype. However, the co-culture provoked a remarkable up-regulation of CCL2 and CXCL2, chemokines responsible for the attraction of monocytes/macrophages and neutrophils, respectively. Moreover, the more aggressive 4T1 tumor cells also modulated the microglial response to TLR stimulation (Fig. 4.26B/C). Thereby, modulation of the microglial response to TLR4 was characterized by a down-regulation of TNFα and

substantial up-regulation of CXCL1 and CXCL10 as well as by a considerable up-regulation of IL-6. In contrast, the microglial response to TLR6/2 stimulation (again also representative for TLR1/2 stimulation) was generally rather dampened, as TNFα, IL-12p40 and CCL5 were significantly down-regulated while the other factors remained at and around control levels. These findings contrasted the previous observations made with 410.4 cells as 4T1 tumor cells were able to induce a microglial phenotype as well as to alter the microglial response to TLR stimulation. As it was not tested so far, the data also encourages testing the behavior of 4T1 cells in terms of invasion and migration toward microglia as it was shown for 410.4 cells.

Part D – Functional *ex vivo* analysis of mouse microglia responses to TLR stimulation reveals ontogenetic changes from birth to adulthood, regional differences and intrapopulational heterogeneity

4.17 Microglial developmental stages correlate with distinctive TLR responses

In order to investigate whether the response to TLR stimulation would undergo changes during the ontogenetic development, wild type microglial cells isolated from animals of various ages were stimulated *ex vivo* (*in vitro)* with saturating doses of the TLR1/2 agonist Pam_3CSK_4, the TLR4 agonist Re-LPS and the TLR6/2 agonist MALP-2, respectively. The resulting responses were determined by the representative release of the pluripotent and proinflammatory cytokine TNFα and the neutrophil-attracting chemokine CXCL1. Their release from cells at the various postnatal stages was normalized to the amounts as produced by strain-matched neonatal (P0) microglial cells, which got stimulated in parallel with the TLR agonists under otherwise identical conditions.

It is important to stress that the protocol was established by developing a strategy of microglial isolation which would enable to harvest sufficient amounts of vital cells especially from animals of postnatal and adult stages. Even more importantly, these cells could survive in culture for weeks without obvious alterations in their physiological features and response properties. On the other hand, and as outlined below, microglia taken out from the CNS into the *ex vivo* setting presented with some differences in their response behavior depending on the animal age reached at isolation. Although the *ex vivo* approach does not claim to reflect the actual *in vivo* situation, it allowed for experimental manipulations and monitoring of – especially – functional reactions which are otherwise not easily accessible. Importantly, the direct comparison of some physiological, namely electrophysiological, properties of the P0 (standard cultures) and postnatal cells *ex vivo* with those characterized previously in slices of living mouse brain tissue (Boucsein et al., 2003) suggested that, indeed, microglia mature postnatally as well as keep characteristics *ex vivo* as they would present with *in situ* (based on a collaboration with Prof. Helmut Kettenmann, Cellular Neurosciences, Max Delbrück Center for Molecular Medicine, Berlin).

The results obtained with C57Bl/6 wild type microglia are shown in Fig. 4.27A. As it was seen before in other experimental setups the microglial responses after stimulation

of TLR1/2 and TLR6/2 appeared very similar, because of which they could be summarized as TLRX/2 responses. The ontogenetic profiles for TLRX/2-stimulated cells revealed a critical dropdown of about 50 % of the response by microglia isolated from 21 days old animals. This effect could be observed for both cytokines, and the inducible release of both of them came back to P0 control levels at postnatal day 35. At later time points in the developmental profile, the TNFα response remained relatively stable at control levels, whereas the CXCL1 response showed another dramatic dropdown at ontogenetic day 42 before reaching again control levels by day 56. The TLR4 response revealed a developmental profile very close to that seen for TLRX/2 responses. As a major difference, however, we noticed an extreme 'oscillation' of the CXCL1 response which also dropped at ontogenetic days 21 and 42, respectively. Instead of returning to P0 control levels at days 35 and 56, the response was rather shooting over the control-defined level before entering the next drop-down. In contrast, the TNFα response, after dropping at day 21, recovered and remained very stable at control levels throughout the experimental time frame with microglia isolated from animals of up to 63 days of age.

Surprisingly, the ontogenetic profiles as obtained with NMRI wild type microglia revealed more or less a distinct course, contrasting that being observed with C57Bl/6 wild type cells (Fig. 4.27B). Instead of a drop, the TLRX/2 responses (again being very similar for TLR1/2 and TLR6/2) showed an augmentation of the response at postnatal day 21. Again, this was seen for both cytokines chosen as representatives in this study. After this peak, the release levels went back to P0 control levels before experiencing a second and even more dramatic augmentation. This time, the TNFα levels seemed to be more affected as they did not reach a peak or plateau level within the experimental time frame. In great contrast, the time course for the NMRI wild type microglia stimulated with Re-LPS (TLR4) presented as relatively stable. Despite minor variations, the release of both cytokines remained close to the control levels for cells isolated from animals up to postnatal day 49. Only after that point, a clear augmentation of the release was observed, however, to a lesser extent as it was seen, for example, in the case of a TLR6/2 response. As a general theme throughout strains and TLR challenges, the TNFα levels inducible at later developmental stages were more or less higher than those elicited in P0 cells, whereas CXCL1 productions were either less elevated (compared to TNFα) or even lower in comparison to their own P0 values.

Taken together, the developmental profiles demonstrate that, in terms of the responses to TLR stimulation, microglial cells undergo changes from birth to adulthood. Although both of wild type nature, the two mouse strains (C57Bl/6 and NMRI) revealed major differences in their respective profiles, supporting the idea of an ontogenetic development of microglia at the one hand, but excluding at the same time a simple 'common pattern' that could be easily applied to any given mouse strain. On the other hand, staying with a certain receptor system, i.e. comparing responses of the two distinct yet closely related TLR1/2 and TLR6/2, which share much of the signal transduction and intracellular pathways, the profiles prove each other to support the reliability of the *ex vivo* analysis approach. Moreover, cells which left the *in vivo* context at a given postnatal day could thereafter – by prolonged cultivation *ex vivo* – not develop the same response properties they would have acquired during this period within the tissue. It suggests that isolation of microglia kind of terminates their maturation, a finding already indicated by an earlier study of our group (Draheim et al., 1999).

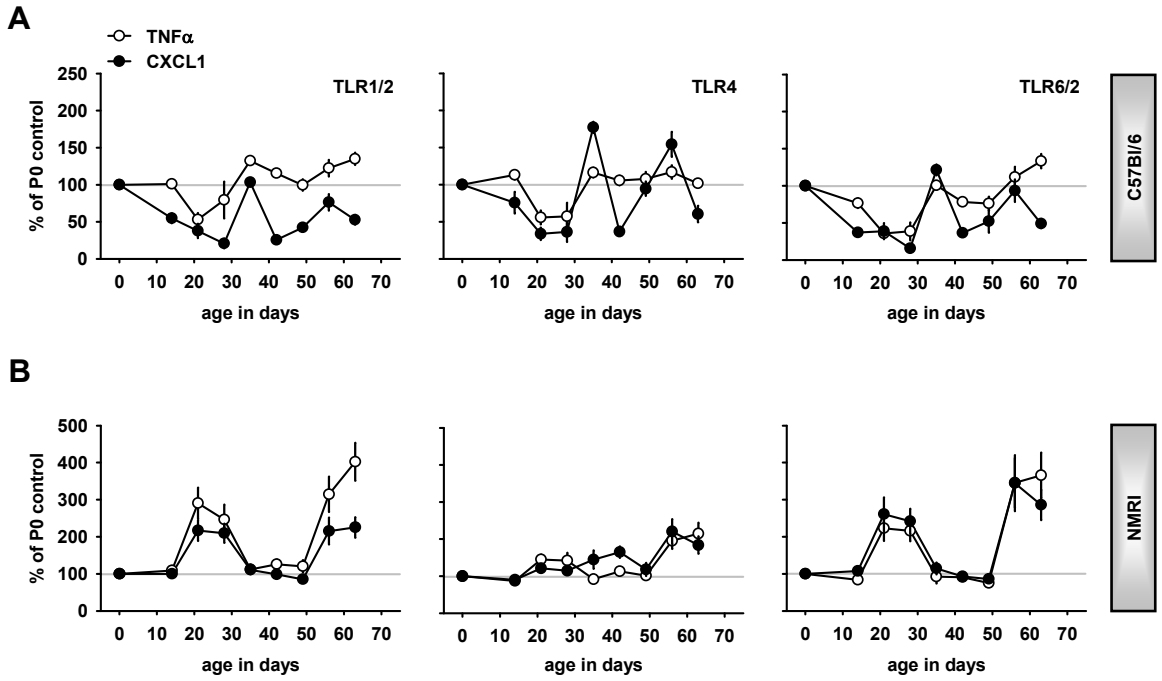


Fig. 4.27: TLR stimulations of microglial cells reveal a developmental profile from birth to adulthood. Mouse microglia were prepared from C57Bl/6 and NMRI wild type mice, respectively, of various ages (x-axis values correspond to the respective animal age at the time of preparation). Cells were harvested up to 4 times from one preparation (1 mouse) and stimulated with saturating doses of the TLR1/2 agonist Pam_3CSK_4, the TLR4 agonist Re-LPS and the TLR6/2 agonist MALP-2 (10, 1 and 10 ng/ml, respectively) for 18h. Cyto-and chemokines were determined in the supernatants and the absolute amounts were normalized to the release obtained by strain-matched neonatal cells that were cultured and stimulated in parallel to each adult preparation (P0 control set to 100 %). Data are mean ± SEM with an average of n=18-24 from 2 individual animals (corresponding to up to 8 harvests).

One of the major findings with respect to the developmental profiles of C57Bl/6 wild type microglia relates to the substantial drop of the TLR(4) response by cells isolated from animals with an age of 21 days. The next experiment was performed to answer the question whether this altered response would also be seen when microglia of this particular age (P21) would be challenged with different LPS chemotypes (see also Part A of the Results section). P21 microglia responded with similar profiles of released proinflammatory cyto- and chemokines to stimulations with the various LPS chemotypes (Fig. 4.28). In addition to the profiles for TNFα and CXCL1 (Fig. 4.27), P21 microglia also significantly down-regulated the production of IL-6 and IL-12p40, as well as CCL2, CCL3 and CCL5, always in a direct comparison to the response of neonatal control cells. Despite minor variations for the individual factors, the overall release pattern could be reproduced for all LPS structural variants tested. The response of P21 microglia to stimulation with Lipid A represents the only exception. A general down-regulation was also apparent but to a markedly lesser extent than seen for the other compounds. Moreover, the release of TNFα even exceeded control levels, pointing to a special importance of Lipid A in the context of a postnatal reorganization of agonist recognition and interpretation by the TLR4 system.

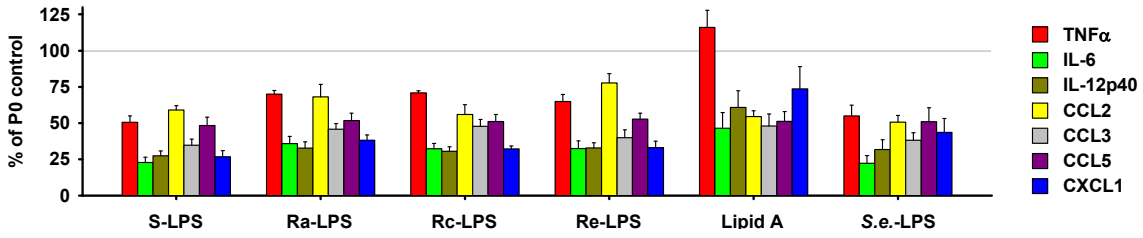


Fig. 4.28: P21 microglia respond with similar cyto/chemokine induction profiles to stimulations with various LPS chemotypes. Mouse microglia were prepared from 21 days old C57Bl/6 wild type mice and stimulated with saturating concentrations of various LPS chemotypes (1 ng/ml each) for 18h. Cyto-and chemokines were determined in the supernatants and the absolute amounts were normalized to the release obtained by strain-matched neonatal cells that were cultured and stimulated in parallel to each adult preparation (P0 control set to 100 %). Data are mean ± SEM with n=9 from 3 individual experiments.

One should keep in mind that the differential response behavior of microglia by postnatal age of the animals reveals an altered TLR signaling as it would only play a role when these receptors are challenged. The assumed reorganization of the receptor/effector systems would take place 'silently' to mature from birth to adulthood. They would thus only be functionally relevant and translate into CNS consequences when TLR agonists would challenge microglia, such as by infection. On the other hand, considering the growing list

of DAMPs, some of the endogenous agonists may appear and play a role in the process of tissue maturation. A time course of signaling and response features of a given TLR could thus cover potential (latent) features as well as actual impacts.

As another key feature of microglial TLR signaling we observed a varying induction efficacy of selected factors/genes (see Part B of the Results section). The developmental profiles obtained from TLR-stimulated C57Bl/6 microglia raised the question whether this feature would also be adapted during development, especially at the critical age of 21 days. Fig. 4.29 demonstrates that there were only minor changes in the induction efficacy of the individual cyto/chemokines when comparing Re-LPS-stimulated P0, P21 and P49 microglia. Thereby, the induction efficacy of TNFα presented with the most obvious adjustment. Whereas in the neonatal microglia this particular cytokine needed long-lasting receptor stimulation (agonist presence) in order to be efficiently induced, already a short-term stimulation of 15 min resulted in the induction of substantial amounts of TNFα in the adult situation, when compared to permanently stimulated control cells. This phenomenon was similarly observed for P21 as well as P49 microglia. In the same direction, minor adjustments could be seen for the induction efficacy of IL-6 and CXCL1. The induction efficacies of other factors, including CCL2, CCL3 and CCL5, remained unchanged in terms of postnatal adjustments. Of course, the kind of illustration in Fig. 4.29 solely represents the qualitative nature of the particular responses. As already indicated in the developmental profiles (Fig. 4.27), the absolute amounts of cyto- and chemokines that were produced by neonatal *versus* adult microglia differed significantly (data not shown).

Taken together, during their postnatal development, microglia undergo major changes with respect to their ability to respond to TLR stimulations with the production of critical cyto- and chemokines. Nevertheless, despite minor adjustments, key features, like the cells ability to specifically react to different LPS chemotypes or to induce particular proinflammatory cyto- and chemokines with varying efficacy, are preserved during these developmental processes.

The period covered by this analysis revealed some marked variations in TLR effects at critical time windows, the data from P21-derived microglia being an example. This period is critical for several reasons. First, it marks the late settlement of microglia postnatally entering the brain. Second, it associates with the maturation of myelin structures. Third, it coincides with the separation of the pups from their mother. All these

events represent developmental steps involving or affecting either the innate immunity or the CNS or both. After all, Toll as the name-giving homologue in *Drosophila* got originally discovered for its morphogenetic functions and only subsequently for contributions to host protection against infection. TLRs may thus play also roles in mammalian development. The marked alterations in CXCL1 with the age of the mice may relate to a cellular activity noticed in addition to neutrophil attraction – the chemokine serves as a stop signal for the migration of oligodendrocyte precursors (Tsai et al., 2002), a feature intriguingly related to myelination.

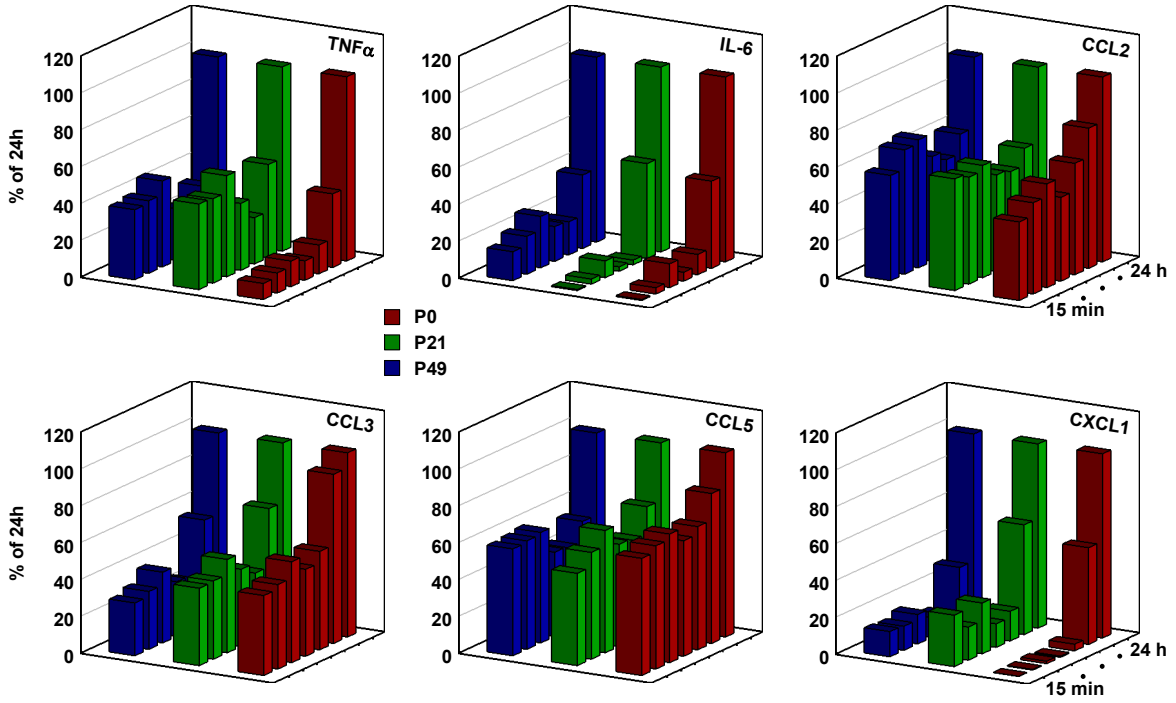


Fig. 4.29: Induction efficacies of cyto/chemokines upon TLR4 stimulation undergo minor adjustments during postnatal development. Mouse microglia isolated from animals at the indicated ages were stimulated with a saturating dose of Re-LPS (1 ng/ml) for the indicated time periods from 15 to 180 min, replacing the stimulus thereafter with fresh medium for incubation up to 180 min. Subsequently, all cells received a medium change and continued in culture for additional 21h (see Fig. 4.13B and Material and Methods for experimental details). Cyto-and chemokines were determined in the supernatants and the absolute amounts were normalized to the release obtained by control cells stimulated throughout the complete 24 h period (set to 100 %). Data are mean with n=12 from 2 individual experiments for P0 and n=7 from 2 experiments for P21 and P49 microglia, respectively.

4.18 Microglia from different CNS regions show diversity in TLR responses

The isolation, *in vitro* cultivation and *ex vivo* analysis of microglia from variable postnatal stages already gave some insights into developmental changes these cells seem to undergo. In addition, this new protocol allows for investigations of microglial features in distinct CNS compartments – studies not being as possible when using neonatally derived cells. With the maturation of the CNS, not only the neuronal circuits become fully established but also synaptic contacts shaped – a process that likely involves microglial contributions. Functional features of these cells may thus change when they acquire the adult status, formerly described as 'resting' state (which is now known to be rather active by tissue surveillance). In addition, myelination proceeds after birth. Again, microglia might affect this process as well, even though in a still largely unknown way. The white matter environment, however, has an impact on microglia, as shown by the different expression of surface molecules (Anderson et al., 2007). It is thus important to rely on postnatal cell preparations when addressing features of microglia as they could vary by regions.

Taking advantage of the *ex vivo* isolation protocol, microglial cells were isolated from 48 days old NMRI wild type mice concentrating on three different CNS compartments: the cortex (liberated from any visible myelin) as representative for grey matter, the cerebellum and the (complete) spinal cord as anatomical entities with a higher white matter index, representing tissues with grey and a substantial amount of white matter. As with the whole brain, adult microglia from the region-specific isolates were cultured *in vitro* and could be harvested up to four times from one preparation. Fig. 4.30 (upper panel) shows representative photomicrographs of the respective cells *in vitro*. All harvested cells were found positive in the ILB4 staining, confirming the purity and identity of microglia. The preparations from cortex and cerebellum, respectively, had a similar appearance with a predominance of elongated and bipolar shaped cells. In slight contrast, microglial cells isolated from spinal cord presented in a variety of shapes, including a substantial amount of ramified cells.

To test for functional differences, microglial cells isolated from the various CNS regions were stimulated with TLR agonists as described for the whole brain postnatal microglia (see above). Fig. 4.30 (lower panel) summarizes these responses by means of TNFα and CXCL1 release. As it was already seen for the morphology, the responses to TLR stimulation by cells originating from either the cortex or the cerebellum were found to

be very similar, with the release of CXCL1 being only slightly above or comparable to P0 control levels. The TNFα release from cortical microglia was also close to control levels for TLRX/2 stimulations, but lower in the case of a TLR4 challenge. Cells originating from the cerebellum revealed in general a lower TNFα production. Moreover, spinal cord microglia showed in general a stronger response to all TLR stimuli, as TNFα was released in higher amounts under the TLR1/2 activation and CXCL1 levels were higher than the P0 reference throughout. These results indicated regional variation in the TLR-inducible reactions, suggesting that whole CNS preparations constitute a blend of (adult) microglia. In addition, the data further support the notion that microglia once isolated from the tissue may maintain features in the *ex vivo* setting.

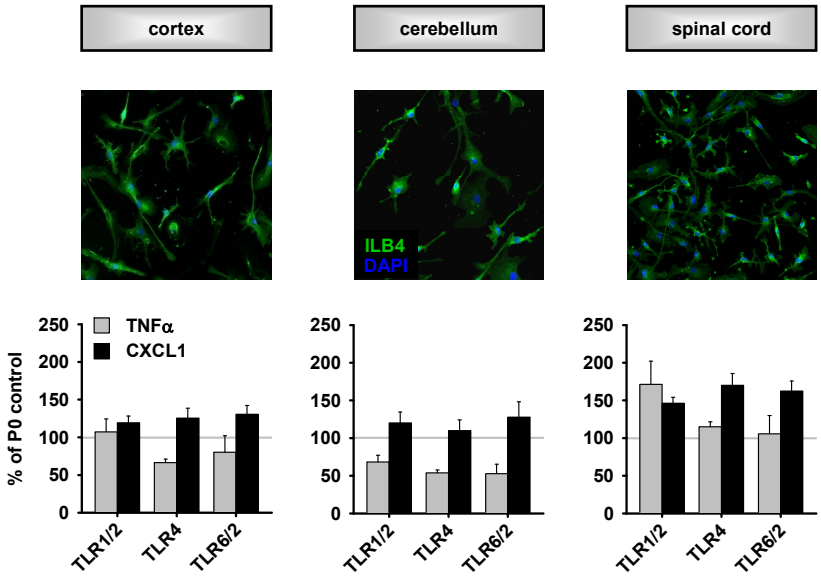


Fig. 4.30: Region-specific microglial cells show diversity in responses to TLR stimulations. Mouse microglia were prepared from cortex, cerebellum and spinal cord, respectively, of 48 days old NMRI wild type mice. Cells were harvested 4 times from this preparation and each time stimulated with saturating concentrations of TLR1/2, TLR4 and TLR6/2 agonists (as in Fig. 4.27), respectively, for 18h. Cyto- and chemokines were determined in the supernatants and the absolute amounts were normalized to the release obtained by strain-matched neonatal cells (whole brain) that were cultured and stimulated in parallel to each adult preparation (P0 control set to 100 %). Data are mean ± SEM with n=16 from 4 individual experiments. The upper panel shows representative photomicrographs of unstimulated cells derived from the individual cultures.

4.19 Patterns of intracellular proteins indicate intrapopulational heterogeneity

While microglia may adapt to the environmental conditions of different CNS regions as they come with distinct tissue architecture, vascular properties or neuronal signaling, very

little is known about the specialization of these cells within a rather circumscribed community. The understanding of microglia thus far had either ignored their heterogeneity or considered an instruction of reactive phenotypes upon a challenge. Conceivably, microglia would be able to undergo 'provincial' adjustments by tissues, but at the same time fall into individual subtypes even within a tissue. Such 'predestination' could provide a tissue with cells of diverse response features, varying by proliferative potential or inducible genes upon a challenge. Constitutive heterogeneity by and within CNS regions could thus be a principle of microglia overlaying with the diversity of reactive phenotypes. In addition, the composition of 'the' microglia may also undergo changes throughout ontogenetic development.

As a first approach to the study of inhomogeneous properties among microglia, cells of different postnatal ages were stimulated with a saturating concentration of Re-LPS (TLR4) and stained for a variety of surface and intracellular proteins to be analyzed by flow cytometry (and immunocytochemistry) (Fig. 4.31). Cells of all ages were capable of up-regulating the expression of MHC I molecules on their surface. About 90 % revealed such a response, regardless of their ontogenetic background. A more differentiated picture was seen with the expression of COX2. Adult microglia (P49) showed a higher number of COX2-positive cells upon LPS challenge, when compared to P0 control cells. Moreover, when comparing the two postnatal preparations, the portion of COX2-positive P21 microglia exceeded the one seen for P49 cells, representing another evidence for functional adjustments during microglial development after birth. In addition, although not quantified, immunocytochemical analysis of COX2 expression already revealed the inhomogeneous distribution of this protein when compared for microglia with varying ontogenetic backgrounds (Fig. 4.31).

The characterization of potential subpopulations was expanded to an intracellular staining of proinflammatory cyto- and chemokines. TNFα and CCL3 were chosen as representatives to determine whether all microglia would contribute to their production or whether the induction could be confined to individual, i.e. specialized subpopulations only. Since releasable factors may not necessarily reveal increased intracellular levels, the induction was triggered by TLR stimulation, but the release was subsequently blocked. We evaluated the most appropriate time window, based on the time courses of release (see Fig. 4.9) to interfere with the protein export before the synthesis got terminated. Moreover, we optimized the staining protocols, namely also the sequence of antibody-mediated

detections, to avoid interference or underestimation of the accumulated protein amounts. Single and double staining approaches were thus compared to confirm reliable measurement.

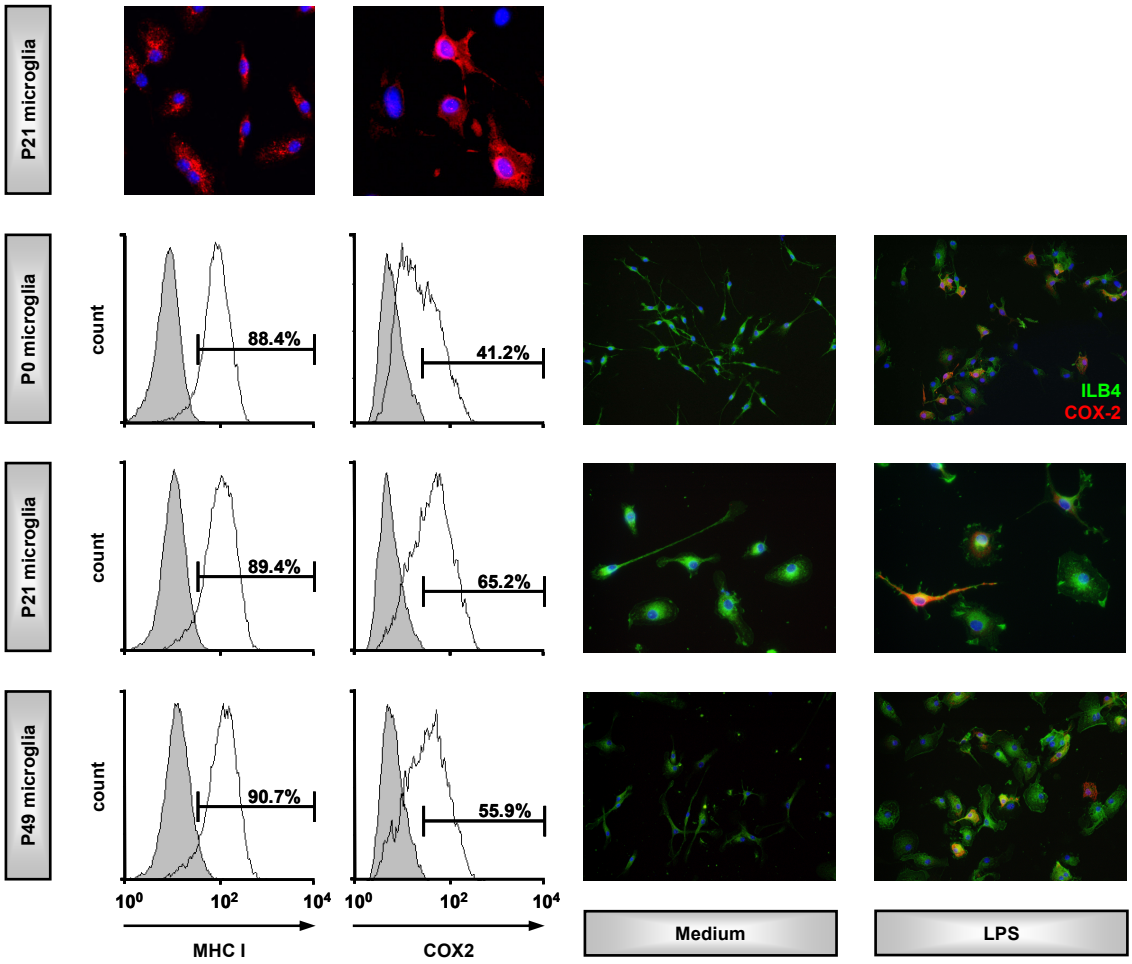


Fig. 4.31: Expression pattern of surface/intracellular proteins upon LPS challenge reveals ontogenetic adjustments. Mouse microglia were prepared from C57Bl/6 wild type mice of the indicated ages and stimulated with a saturating concentration of Re-LPS (10 ng/ml). After the incubation cells were removed from the culture dish and stained with antibodies against MHC I and COX2, respectively, for acquisition by flow cytometry. Representative histograms are depicted, comparing unstimulated (grey areas under grey curve, always in the back plane) *versus* Re-LPS-stimulated cells (transparent areas under black curve, always in the front plane). Numbers indicate percentages of positive events for the respective staining. The upper panel shows representative photomicrographs of the respective staining (red) with a nuclear counterstaining (DAPI, blue) exemplarily for Re-LPS-stimulated P21 microglia. On the right representative photomicrographs are shown to illustrate the (LPS-inducible) inhomogeneous distribution of intracellular COX2 expression. Note that the histograms (middle) do not reflect the quantification of the staining (right).

As Fig. 4.32 shows for neonatal microglia, only a small portion of cells produced CCL3 upon LPS challenge, and about one third of the cells accounted for the production of

TNFα. When looking at the respective double-staining, the cyto/chemokine-producing microglia could further be divided into two major groups, one producing only TNFα and another one being able of producing both TNFα and CCL3. In addition, there was a tiny subpopulation of cells which apparently produced only CCL3, but no TNFα. These findings could be reproduced for the postnatal situations as well. However, correlating with the increasing age of the animals at the time of microglia isolation, the percentage of

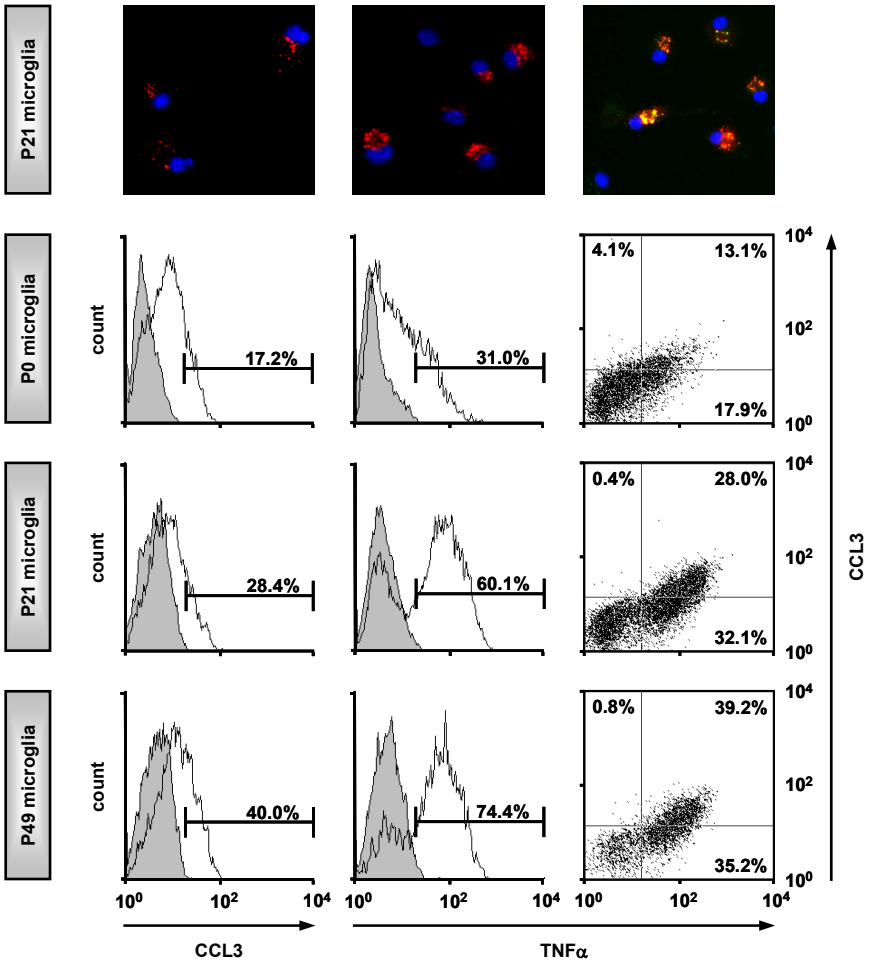


Fig. 4.32: Specialized subpopulations of microglia produce proinflammatory cytokines upon LPS challenge. Mouse microglia were prepared from C57Bl/6 wild type mice of the indicated ages and were stimulated with a saturating concentration of Re-LPS (10 ng/ml). As of 5 h, Monensin was added in order to prevent the release of the cyto/chemokines and to thus enforce their intracellular accumulation (for experimental details, see Materials and Methods). After additional 3 h of incubation, the cells were removed from the culture dish and stained with antibodies against intracellular TNFα and CCL3 for acquisition by flow cytometry. Representative histograms are depicted, comparing unstimulated (grey areas under grey curve, always in the back plane) *versus* Re-LPS-stimulated cells (transparent areas under black curve, always in the front plane). Corresponding dot plots of Re-LPS-stimulated cells (right column) show the double staining. The panels on top are representative photomicrographs of a respective staining for TNFα (in red) and CCL3 (in red for the single and green for the double staining), with a nuclear counterstaining (DAPI, blue) exemplarily for Re-LPS-stimulated P21 microglia.

cyto/chemokine-producing cells increased as well. Notably, the ratio of cells producing either one or both cyto/chemokines was slightly shifted when comparing the two adult preparations. Whereas for P21 microglia, the fractions of cells producing TNFα alone *versus* TNFα and CCL3 were similar, this ratio was shifted in favor of TNFα/CCL3-producing cells in P49 microglia. At both postnatal stages, the subpopulation of only CCL3-producing cells almost disappeared. Interestingly, for P21 and P49 microglia, production of TNFα revealed two distinct populations by itself. Histograms showed two peaks for cells with no (or very low) and clearly induced TNFα levels, respectively. The separation of these two populations was also visible in the dot plots from the double staining approach (Fig. 4.32, panels on the right). As a kind of 'zoom in' study to substantiate the findings with whole brain preparations we expanded the intracellular cytokine approach to P49 microglia isolated from distinct CNS regions as described in section 4.18. Indeed, we could find the 'specialized' sub-populations of cytokine producing microglia also in preparations originating from the cortex, cerebellum and spinal cord, respectively (Fig. 4.33). As it was seen before for the released protein amounts (Fig. 4.30) also the intracellular staining revealed an apparently higher production of cyto/chemokines by spinal cord-derived microglia. However, global differences between the overall microglial populations – as derived from different CNS compartments – were only of minor character. Still, the intrapopulational heterogeneity proofed to be a feature also of more defined sub-populations of microglia. Considering other TLR and non-TLR challenges as well as other gene products and cellular properties, the variety of microglia by their expression or combination could be even much bigger. Only two factors gave here rise to three subtypes already.

Taken together, in addition to adjustments during the ontogenetic development, cells could further be divided into sub-populations with specific 'duties' when challenged through their TLRs. The experiments support the assumption that individual cells occur within a given microglial pool. The findings also point again to the ability of microglia in an *ex vivo* setting to keep (individual) features as they were likely instructed *in vivo*. With the special emphasis on TLR4, finally, the study encourages research on its organizational variability not only in different cell types, such as DCs, mast cells, macrophages or microglia. TLR4 may (re)organize its complex arrangements on the cell surface and its connection to the signaling events also in a given cell type and its populational varieties as they present with individuality during development, by and within tissue regions.

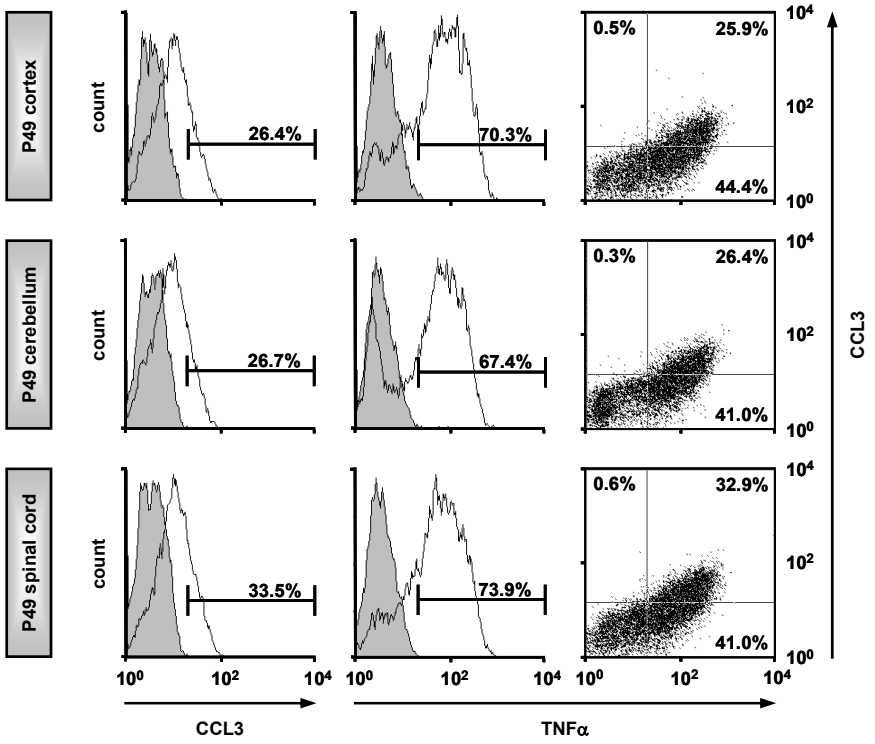


Fig. 4.33: Specialized subpopulations of microglia can be found in distinct CNS regions. Mouse microglia were prepared from cortex, cerebellum and spinal cord, respectively, of 49 days old C57Bl/6 wild type mice and were stimulated with a saturating concentration of Re-LPS (10 ng/ml). Afterwards, cells were treated, stained and acquired as described in Fig. 4.32. Representative histograms are depicted, comparing unstimulated (grey areas under grey curve, always in the back plane) *versus* Re-LPS-stimulated cells (transparent areas under black curve, always in the front plane). Corresponding dot plots of Re-LPS-stimulated cells (right column) show the double staining.

5. Discussion

5.1 Mouse microglia respond to LPS of various chemotypes

Concerning the responses of the innate immune system, the structural variants of LPS, also referred to as LPS chemotypes, represent essential features of the actual Gram-negative bacterial strains by which they are produced – and TLR4 is their major receptor. Of course, other PAMPs add to the full spectrum of structural indicators for such an infection. Bacterial DNA plays a critical role by itself, and cooperativity between the various TLRs is being more and more understood to shape the host response. Yet much of Gram-negative infection can be mimicked just by LPS, suggesting its great impact.

Discrimination between 'smooth' (S-LPS) and 'rough' (R-LPS) variants is thereby not reduced to a property of the molecule itself, but represents once more a correlation with the underlying strain, its physiology and pathogenetic importance. When cultured on agar plates, bacteria incorporating S-LPS into their outer membrane form colonies that have a 'smooth' appearance, whereas the other group grows in 'rough' looking colonies. S-LPS is thereby widely accepted as the 'wild type' LPS molecule, with the R-forms representing various mutations that have evolved with time. At the molecular level, rough mutants are characterized by a lack of the O-polysaccharide (also known as O-antigen) as well as by a more or less truncated core polysaccharide. This core carbohydrate structure is relatively conserved among all known LPS variants. In turn, it allows for further classification of the rough family members, ranging from Ra-LPS with a complete core polysaccharide to Re-LPS (in many studies referred to as 'deep rough' LPS) as carrying only the minimal and essential carbohydrate residues. Common to all known LPS structures, the Lipid A portion of the molecule is a (usually) bi-phosphorylated and up to hepta–acylated disaccharide which is covalently bound to the core polysaccharide. Lipid A anchors the molecule within the outer bacterial membrane and is the general carrier of LPS pathogenicity. *E.coli* Lipid A represents the 'gold standard', with the highest known endotoxic activity. Variations of this model structure always come with lowered pathogenic potential (Rietschel et al., 1994). However, Lipid A is not the sole determinant of the level of endotoxic activity as structural variants with an identical Lipid A backbone – but diverse carbohydrate assemblies – have been shown to exert varying effects on a number of cells, including mast cells and macrophages (Gangloff et al., 2005; Jiang et al., 2005b; Huber et al., 2006). In

other words, LPS-carried innate immunological properties do not solely reside in the Lipid A domain and a more refined approach to the understanding of cellular and systemic responses must pay attention also to the previously neglected carbohydrate moieties.

Here, we sought to elucidate potentially varying effects of structural LPS variants on microglia as the resident innate immune cells of the CNS. We employed a set of ultra-pure preparations of various LPS chemotypes as well as naturally occurring and mutated (monophosphorylated, MPLA) forms of Lipid A, all preparations originating from the highly pathogenic bacterial strains *E.coli* and *Salmonella* species (Tab. 4.1). We found microglia to respond to all tested preparations by means of the release of proinflammatory cyto/chemokines. Actually, these responses were dose-dependent as microglia were most sensitive to stimulations with either of the rough LPS mutants. S-LPS as well as Lipid A needed a 10-fold higher concentration to elicit a measurable response. However, both preparations could saturate the microglial response at a dose around 1 ng/ml, similar to what was observed for rough LPS. Lipid A lacking one phosphate moiety (MPLA) as well as its synthetic counterpart (sMPLA) showed a substantially lower microglial activation potential as they needed a 100-fold and 1000-fold, respectively, higher concentration to elicit a cellular response, compared to rough LPS. Patterns of varying sensitivity towards specific structural variants of LPS were also demonstrated by others for a number of different cell types, including murine peritoneal macrophages (Gangloff et al., 2005; Jiang et al., 2005b; Mata-Haro et al., 2007), bone marrow-derived macrophages (Mata-Haro et al., 2007) and mast cells (Huber et al., 2006). However, individual cell types revealed distinct patterns concerning the recognition of LPS chemotypes, thus favoring the idea of a cell-specific organization of the TLR4 complex and its associated intracellular signaling. In this context, microglia proved to have a discrete pattern as well, especially by 'favoring' the rough chemotypes over S-LPS and Lipid A. It must be noted that the dose-response relations of the various structural LPS variants were compared as based on weight-volume, rather than on molar concentrations. While differences in the molecular weight have only minor effects on the comparisons among the various rough chemotypes it might well be that they influence the interpretation of differences between S-LPS and R-LPS. Differences in the activities could be overestimated when performed on the weight-per-volume basis. However, molar concentrations are still hard to compare. Due to incomplete biosynthesis, S-LPS actually represents a highly heterogeneous mixture of molecules with varying lengths of the O-polysaccharide and even containing molecules of some rough

chemotypic nature (Huber et al., 2006). It is, therefore, nearly impossible to determine the precise molecular weight of a given S-LPS preparation, which led us to operate with the weight-volume approach. Nevertheless, we tried to roughly estimate the molecular weights of the LPS preparations used here – based on findings concerning specific carbohydrate compositions and sugars used (Kenne et al., 1983; Stenutz et al., 2006). Accordingly, the theoretical molecular weight of S-LPS would exceed the one of Ra-LPS by 3.4 to 7 times. Due to the above mentioned generally extreme heterogeneity of S-LPS preparations we assume that this difference in our case will be in the lower range, i.e. S-LPS will be maximally 5 times bigger than Ra-LPS. In turn, this (rough) estimation would support our data of microglia being slightly less sensitive to stimulation with S-LPS – needing a 10-fold higher concentration to elicit a response – compared to challenges with rough LPS preparations.

LPS signaling through TLR4 critically depends on a number of co-receptors. There is consensus about the indispensable role of MD2 to recognize LPS in association with TLR4 (Medzhitov et al., 1997; Shimazu et al., 1999; Nagai et al., 2002). Recently, this complex interaction has also been resolved by crystal structure analysis (Park et al., 2009). Furthermore, LPS binding protein (LBP) seems to be instrumental for the delivery and presentation of LPS to the receptor complex (Hailman et al., 1994; Hamann et al., 2005). Much more controversy evolved about the role of another co-receptor – CD14 – since it was first shown to participate in LPS recognition and signaling by Wright and colleagues (1990). The molecule thereby facilitates the delivery of the LPS-LBP complex to the TLR4-MD2 heterodimer (Vasselon et al., 1999; da Silva Correia et al., 2001). However, the participation of CD14 was then later revealed to be no mandatory event in general, but rather to aid the discrimination between structural LPS variants. CD14 would thus represent a 'tool' for interpreting the LPS type – and thus to identify the strain. Conceivably, TLR4/CD14-coexpressing cells could adjust their responses as some strains may require more resolute reactions than others. This way, CD14 proved to be most essential for the recognition of S-LPS in monocytes/macrophages (Perera et al., 1997; Gangloff et al., 1999; Moore et al., 2000; Gangloff et al., 2005; Jiang et al., 2005b) as well as in mast cells (Huber et al., 2006).

Since microglia showed in our studies a discrete way of discriminating between structural variants of LPS we sought to decipher the role of CD14 in this regard. Using CD14-deficient microglia we observed a similar pattern of CD14 dependence as it was

shown before for extra-neural macrophage populations. $CD14^{-/-}$ microglia showed an approximately 100-fold lower sensitivity to stimulation with S-LPS compared to wild type cells. Importantly, the lack of CD14 affected microglial sensitivity to all members of the rough LPS group in a similar manner, however, with Rc- and Re-LPS being gradually less affected. In addition, the recognition of free Lipid A was affected in the same direction with a drop in sensitivity by a factor of 10. These findings contrast the view of CD14 being solely associated with the recognition of S-LPS. They rather go along with similar observations for peritoneal macrophages made by Gangloff and colleagues (2005). Interestingly, (s)MPLA was recognized by $CD14^{-/-}$ microglia at similar concentrations as by wild type cells, implicating a CD14-independent mechanism of recognition by the TLR4/MD2 complex for this particular compound. A striking exception from the above mentioned observation was the production of CCL3. For this particular chemokine, dose-response relations of all tested compounds appeared to be unaltered in $CD14^{-/-}$ cells, implying that the production of CCL3 by microglia upon stimulation with X-LPS is generally CD14-independent. Such a phenomenon could find a first explanation by some individual dependence of the various inducible genes on the rather complex signaling routes organized by TLR4. MyD88 and/or TRIF contribute with varying importance to individual genes, while CD14 is of different importance for the two pathways controlled by the adaptor proteins (see further below).

Another intriguing observation within this set of experiments regards the altered pattern of released cyto/chemokines by $CD14^{-/-}$ cells in terms of their absolute amounts. Regardless of the actual stimulus (LPS chemotype), these cells produced significantly more TNFα, IL-6 and IL-12p40 as well as substantially more CXCL1 as compared to their wild type counterparts. At least for TNFα, this phenomenon was also observed by others (Gangloff et al., 2005). However, as absolute levels of CCL3 and CCL5 were comparable between $CD14^{-/-}$ and wild type situation and levels of CCL2 were even lower in $CD14^{-/-}$ cells, an explanation for this observation remains to be found. One might speculate that animals with a constitutive lack of CD14 undergo a slightly different development than their wild type littermates, with possible consequences for the architecture and strength of the TLR(4) signaling cascade. This hypothesis may find confirmation in an experiment where $CD14^{-/-}$ cells would react with similarly altered release profiles upon TLR stimulation with ligands known to signal CD14-independently. Alternatively, CD14 involvement may exert a 'negative' control over certain gene inductions, and lack of such

containment unleashes the full capacity. CD14 involvement could thus shape the TLR4 response pattern.

To confirm the differential involvement of CD14, we further tested the possibility to neutralize CD14 function in wild type cells by the use of a blocking antibody. This anti-CD14 antibody (clone 4C1) had proven to be very effective in other studies investigating DAMP signaling through the TLR4 receptor complex. In a pilot experiment with LPS, however, we made an unexpected observation. While this antibody was able to dose-dependently block the response of wild type microglia to Re-LPS at close-to-saturation doses (0.1 and 1 ng/ml Re-LPS), this blocking effect was reversed when cells were stimulated with a saturating dose (10 ng/ml) of Re-LPS, meaning that application of the antibody dose-dependently potentiated the Re-LPS-induced release response. While the effect is striking, its interpretation is far from trivial. Lifting of a control, as suggested as an option for the $CD14^{-/-}$ cells above does not easily fit with the blocking outcome at lower LPS concentrations. Another possible explanation for this phenomenon might be the existence of low-affinity binding sites of TLR4, which might have been exposed as a consequence of rearrangements of the receptor environment due to the presence of the antibody. This might also explain why the observed effect depends on the actual LPS dose, as the low affinity binding sites would conceivably need higher ligand concentrations for effective signaling than their high-affinity counterparts. The nature and location of 'low affinity' sites would still be obscure. In the light of more and more findings on the complex arrangement of TLR4, however, it may turn out that the individual TLR4 molecules are not completely identical in terms of their functional features – due to differences in their integration in larger receptor arrangements. Identification of such inhomogeneous – in the easiest scenario two – binding sites would require a classical binding assay with direct detection of the bound ligand. Yet the extremely low expression levels of TLR4 in our cells render such an approach difficult. Indeed, we found microglia to express TLR4 only at low (yet functionally significant) levels, using confocal microscopy, flow cytometry as well as an ELISA (data not shown).

At the end, we took advantage of this unexpected phenomenon and applied the various structural LPS variants at a common saturating dose of 10 ng/ml together with increasing doses of the CD14-blocking antibody. As a result, all LPS chemotypes induced discrete patterns of released cyto/chemokines when CD14 was neutralized by the blocking antibody. The individuality of the responses came either by the magnitude of antibody-

mediated response augmentation or by the pattern of genes which were modulated or not. However, a clear gradient of CD14-dependence from S-LPS to Lipid A, as it was seen in the $CD14^{-/-}$ situation, could not be observed this time. Still, all structural LPS variants responded with unique signaling consequences when CD14 was blocked, underlining once more the capacity of microglial TLR4 to discriminate between discrete agonists. Moreover, the finding of exacerbated release of CXCL1 when CD14 is neutralized and LPS comes at high concentrations confirms findings made in the $CD14^{-/-}$ situation. Again, this favors the idea of CD14 as a regulatory element within the TLR4 receptor complex, thereby preventing overshooting microglial (CXCL1) responses to LPS challenges with (extremely) high agonist concentrations.

TLR4 signaling is unique among the members of the TLR family as this particular receptor employs both known routes of TLR signaling, i.e. the MyD88-dependent and the TRIF-dependent signaling. These two routes with partially overlapping but also discrete signaling consequences were proposed to engage sequentially rather than simultaneous (Kagan et al., 2008). Especially TRIF-dependent signaling was shown to critically depend on the involvement of CD14 in conveying cellular consequences to LPS challenges (Jiang et al., 2005b). As we could show a differential dependence on CD14 among the various structural LPS variants in microglial TLR4 signaling, this in turn implies a likewise differential usage of the TRIF-dependent signaling route. However, based on microglia deficient in either of the two signaling adaptors we could show that, in both scenarios, the cells responded with comparable patterns of released cyto/chemokines regardless of the LPS chemotype used for stimulation. From this finding we conclude that the microglial discrimination of structural variants is not associated with a differential usage of either of the TLR signaling adaptors *per se*. It seems more likely that this discrimination already happens at the level of ligand recognition (critically involving CD14).

Despite the various LPS preparations with high endotoxic potential there are also naturally occurring antagonists of endotoxins. LPS (Rs-LPS) and Lipid A (RSLA) isolated from the photosynthetic bacterium *Rhodobacter sphaeroides* have been shown to be potent antagonists in human monocytes and neutrophiles (Golenbock et al., 1991; Aida et al., 1995) as well as in murine monocytes/macrophages (Strittmatter et al., 1983; Qureshi et al., 1991). However, this feature is restricted to certain species as the same Rs-LPS serves as TLR4 agonist in hamster and equine cells, respectively (Delude et al., 1995; Lohmann et al., 2007). We aimed to confirm the antagonistic properties of Rs-LPS for our murine

microglia. Surprisingly, Rs-LPS was not able to antagonize the release of proinflammatory cyto- and chemokines induced by microglial stimulation with highly endotoxic Re-LPS, regardless of the concentration of the endotoxin. In contrast, Rs-LPS itself proved to have agonistic properties on microglia, as it strongly induced proinflammatory cyto/chemokines.

This agonistic property was, however, partially TLR4-independent. The Lipid A portion of Rs-LPS strikingly resembles the structure of *Porphyromonas gingivalis* LPS with its penta-acylated and mono-phosphorylated Lipid A. As *P.gingivalis* LPS was shown to be recognized by TLR2, rather than TLR4, in murine macrophages (Hirschfeld et al., 2001) it is likely that also Rs-LPS is able to signal through TLR2 (in microglia), which partially might explain its agonistic properties. Differential receptor usage of Rs-LPS might also indirectly be supported by the findings of Thieblemont and colleagues (1998), where agonistic LPS (like Re-LPS) was transported into the intracellular space (probably in a complex with TLR4), whereas antagonistic Rs-LPS remained at the plasma membrane (possibly bound to TLR2). As many experimental designs rely on transfection or an overexpression paradigm, such cells might lack a functional TLR2 component – thereby missing out on a potential agonism mediated through this receptor, while concentrating on the antagonism mediated through TLR4. In turn, we can at the same time still not totally exclude the possibility that the antagonistic features of Rs-LPS in microglia are simply 'overwritten' by its agonistic properties. However, in murine BMDM we could show Rs-LPS to be at least partially antagonistic on Re-LPS-induced responses, despite being an agonist at high doses in these cells as well. We conclude from these findings that structural and functional properties of TLRs in general – and of TLR4 in particular – are not only differentially organized among different species, but that they come with cell type-specific features even within a given species. Again, these data and the conclusion drawn from them are in support of a heterogeneous and possibly adaptive organization of TLR4.

Microglia respond to LPS stimulation not only with the induction of a range of proinflammatory cyto- and chemokines but also with the up-regulation of surface MHC I as well as the inhibition of myelin phagocytosis (unpublished own observations). These regulatory activities got partially already assigned to the signaling pathways. For example, up-regulation of MHC I obviously depends on TRIF. It is seen with TLR4 and TLR3, but not with TLR1/2 or TLR6/2. The first two TLR members have links to TRIF, the latter two supposedly not. We wanted to know whether these features would be differentially affected by the various LPS chemotypes. Indeed, the capacity to up-regulate MHC I was

comparable for S- and R-LPS preparations, but was significantly lower for Lipid A and almost absent for sMPLA. This pattern was specifically changed in the $CD14^{-/-}$ situation. Here, the ability of S-LPS to induce MHC I expression was severely impaired (>50 %) whereas for the rough LPS preparations this feature was only slightly diminished. Bearing in mind the suggested discriminating role of CD14 and its connection to TRIF, the various LPS chemotypes would differ by CD14 engagement, but would still depend on TRIF. For the various Lipid A preparations, the effect was even reversed as they gained MHC I up-regulating potential in the absence of CD14. This pattern of CD14 dependence closely resembled the one we had seen earlier for the production of cyto/chemokines. Indeed, lack of TRIF completely abolished the capability of all LPS preparations to up-regulate microglial MHC I, indicating that this feature is organized through TRIF and in a MyD88-independent manner. The 'enhancer' effect, on the other hand, would then again argue for some regulatory control of CD14 for certain TLR4 ligands. Together, these results again favor the idea that CD14 is strongly involved in TRIF-dependent signaling events induced through TLR4 – yet with an impact varying with the agonist.

Importantly, this time Rs-LPS had no agonistic but strong antagonistic properties with respect to MHC I up-regulation, contrasting the results for cyto/chemokine release. This antagonism by Rs-LPS over Re-LPS was apparently CD14-independent, suggesting that the antagonism is based on a competition for the receptor (TLR4) rather than resulting from functional interference at the signaling level. Moreover, the combined data on Rs-LPS agonism/antagonism imply that the induction of cyto- and chemokines and the up-regulation of MHC I are discrete signaling events as they are differentially sensitive to an antagonism.

The ability of microglia to clear myelin debris by means of phagocytosis is of great importance for the maintenance of CNS tissue homeostasis and even more in the context of demyelinating disorders. This microglial function can also be investigated *in vitro* by incubation of the cells with fluorescence-labeled mouse myelin which then can be tracked inside the cells at a given time point after the initiation of phagocytosis (van Rossum et al., 2008). From our studies we also know that the ability of otherwise unstimulated microglia to take up myelin is strongly suppressed by the presence of LPS. We therefore asked the question whether different LPS chemotypes would also differentially alter the myelin uptake by microglia. Briefly, despite minor variations, all tested structural variants of LPS (except for sMPLA) were able to suppress myelin phagocytosis to a comparable degree.

This was also not changed in a $CD14^{-/-}$ situation, and the lack of TRIF only had minor consequences in reducing the suppressive effect (slightly for Lipid A and significantly for MPLA). Moreover, Rs-LPS failed to develop any agonistic and antagonistic properties in this context. The data clearly indicate that the TLR4 signaling consequences that interfere with the induction/control of phagocytosis are insensitive to discrimination between LPS chemotypes. Following the idea that LPS discrimination is a feature mainly associated with CD14/TRIF, one might speculate that the effect of suppressing myelin phagocytosis is mediated through MyD88. Indeed, suppression of myelin phagocytosis was seen also with a stimulation of TLR1/2 and TLR6/2, both of which are thought to signal through MyD88 only. However, the $TRIF^{-/-}$ situation should have changed the pattern more obviously as the lack of TRIF also renders MyD88-dependent signaling to discriminate between LPS chemotypes (Jiang et al., 2005b). Nevertheless, and as stressed above, TLR4 organization reveals cell type individuality and it thus may not surprise that the cooperativity among the adaptor protein pathways is among the most prominent aspects that differ accordingly.

Taken together, we could show that microglia, like other extra-neural macrophage populations, are able to discriminate between structural variants of pathogenic LPS in the context of TLR4 signaling. This differentially affects the release of proinflammatory cyto- and chemokines as well as the LPS-induced up-regulation of MHC I, but apparently spares the ability of LPS to suppress microglial myelin phagocytosis. CD14 plays a crucial role in the discrimination between LPS chemotypes with consequences being biased towards TRIF-dependent rather than MyD88-dependent signaling events. The actual outcome of a TLR4 activation is a function of the cell type (and probably status) and allows for some discrimination between agonists. While the flexibility of TLR4 to differentiate between PAMPs and DAMPs may be important to organize microglial reactive phenotypes upon infection *versus* damage differently, the individual interpretation of microbial structures could be important for adapted responses to different Gram-negative strains. TLR4 as a special PRR and the most complex TLR family member could thus prove as a decision maker for microglial challenges in diverse CNS-endangering situations.

5.2 Microglial organization of TLR signaling

TLRs are broadly expressed by the cells of the CNS (Bsibsi et al., 2002; Hanisch et al., 2008) and their role, especially in the context of microglial TLR signaling, in fueling

neurodegenerative processes is widely accepted (reviewed by Lehnardt, 2010). However, there is also growing consensus about the neuroprotective and repair-favoring properties of activated microglia (van Noort, 2007; Hanisch et al., 2008). In this context it becomes more and more evident that there is a subtle balance between beneficial and detrimental microglial contributions (van Noort and Bsibsi, 2009). For example, in mouse experimental cerebral ischemia, both TLR2 and TLR4 have been shown to contribute to tissue damage, as in the respective receptor ko animals CNS damage and, in particular neuronal loss, were markedly reduced (Lehnardt et al., 2007; Ziegler et al., 2007; Caso et al., 2008; Kilic et al., 2008). In contrast, when animals were exposed to the specific TLR agonists before disease induction, their resistance to ischemia-caused injury was significantly increased (Hua et al., 2008). Such an apparent discrepancy gets even more pronounced when certain *in vitro* findings cannot be reproduced or even present with opposite outcomes upon translation into the experimental setting *in vivo*. Owens aimed to summarize such phenomena by proposing the 'strength of signal' hypothesis (Owens, 2009). On the basis of a number of different *in vitro* scenarios, we tried to unmask the relationship between 'strength of signal' and the resulting microglial phenotype in response to TLR stimulation. Thereby, the actual 'strength' of the signal was determined by its duration, the sensitivity of the response to be modulated or the possibility of repeated receptor ligations with varying ligand concentrations.

Timing matters

A considerable shortcoming of many *in vitro* studies of microglial activation (upon TLR stimulation) is the actual stimulation time frame looked at. The majority of studies concentrate on stimulation periods of 24 h or less which may not reflect the *in vivo* situation in its entirety. Given the experimental circumstances of the static *in vitro* stimulation paradigm we were able to monitor the microglial response to TLR stimulation for a maximal period of 72 h. And indeed, some of the released factors constantly accumulated over time periods that clearly exceeded the 24 h mark. In general, the 'kinetics' of cyto- and chemokine release was quite similar between microglial cells stimulated with TLR1/2, TLR4 and TLR6/2 agonists, respectively, although the response of TLR6/2-stimulated cells appeared a bit 'decelerated' compared to TLR1/2 or TLR4 responses. In terms of the released factors, the immediate response of all stimulation paradigms involved the release of TNFα, the T_H1 cell attractant CCL3 and the neutrophil

attractant CXCL1, all of which being produced in substantial amounts within the first 12 h and reaching a plateau within 24 h of stimulation. The second 'wave' of cytokines included IL-6, the monocyte/macrophage attractant CCL2 as well as the general T_H cell-attractant CCL5. These cytokines constantly accumulated over longer time periods, in the case of CCL2 even over the complete 72 h.

Considering the temporal induction of releasable factors – which are by far not yet all known – autocrine loops may play also an important role in triggering, enforcing or stabilizing signaling activity. For example, induction of TNFα by TLR stimulation will require MAPK contributions, such as by p38 members. In turn, TNFα would drive p38 in a second wave when binding to TNF receptors. In other words, release products may act on signaling events again that were used for their induction and would thus affect also factors depending on them.

The fine-orchestrated release of individual cyto- and chemokines points to well-defined defense programs that are organized by microglia depending on specific (PAMP) signals. The most striking observation from experiments with the prolonged time course, however, was the relative late induction of the prototypic anti-inflammatory cytokine IL-10, which was released the earliest after 36 h of stimulation with either of the above mentioned TLR agonists. Usually a characteristic feature of M2-like orientations, macrophages are also known to produce IL-10 upon TLR(4) challenge both *in vitro* (Boonstra et al., 2006; Chang et al., 2007) and *in vivo* (Siewe et al., 2006), and also microglia have been proposed to be a source of IL-10 (Hanisch, 2002; Jack et al., 2005). However, as discussed earlier, also all of these studies were based on the 24 h stimulation paradigm and therefore missed the time points that proofed to be critical in our studies. The discrepancy between the 'early' IL-10 in human microglia (Jack et al., 2005) and our observations of 'late' IL-10 induction might well find an explanation in species-specific differences in TLR signaling organization, which was discussed earlier for the species-dependent antagonistic/agonistic properties of the TLR4 ligand Rs-LPS (section 5.1), and which was further shown for TLRs in general and, for example, for TLR3 in particular (Rehli, 2002; Heinz et al., 2003). The timing of IL-10 induction is absolutely critical and determines whether secreted IL-10 can act as an autocrine negative feedback mechanism (Hu et al., 2008) or rather regulates neighboring effector cells in a paracrine fashion (Katakura et al., 2004). Our data clearly favor the paracrine mechanism as only simultaneously present IL-10 (in a physiologically relevant dose of 10 ng/ml) could

effectively suppress/modulate the TLR-induced production of proinflammatory cyto- and chemokines by microglia. When the anti-inflammatory cytokine was added at later time points (12 or 24 h post TLR signaling induction), the immuno-suppressive effect was negligible. These results go in line with *in vivo* findings where pre-treatment with recombinant human IL-10 reduced endotoxin-induced inflammatory reactions whereas the equivalent post-treatment (1 h after endotoxin) failed to do so (Pajkrt et al., 1997). For the special situation of the CNS, where sustained microbial challenge is not acceptable, it appears conceivable that any potential infection is given the chance to be cleared effectively before the defense-oriented response is redeemed by resolution and repair mechanisms. In other words, the microglial pro-inflammatory response is given a 'head start' before anti-inflammatory IL-10 (among others) comes into play to reduce potential tissue damage.

Selective versus cooperative use of adaptor protein routes

Signaling downstream of TLR critically depends on adaptor molecules and their associated signaling pathways (O'Neill and Bowie, 2007). In this context, two distinct signaling routes are known to be associated with TLRs, the MyD88-dependent signaling route being associated with all TLRs except for TLR3, and the TRIF-dependent (also known as MyD88-independent) signaling route, which is exclusively used by TLR3 and in optional combination with MyD88 by TLR4. To confirm this pattern of differential adaptor and signaling route usage for microglia, we applied the above mentioned 72 h stimulation protocol on microglia deficient in either of the two signaling adaptors, MyD88 or TRIF, respectively. As seen and discussed before for TLR4 stimulation with Re-LPS (see section 5.1), this specific Toll-like receptor has an exceptional position as it is the only family member using both adaptor molecules. Accordingly, genes are differentially induced with respect to the signaling routes. In our studies, the majority of proinflammatory cyto- and chemokines was induced in a MyD88-dependent manner. Other genes, like IFNβ, are exclusively induced through the TRIF-dependent route. On top of that, there are genes – the best example being *CCL5* – which can be induced through either of the two routes. However, when TLR4 signaling was 'forced' through either of the two signaling pathways, non of the LPS-induced factors could get produced in amounts comparable to the wild type situation, except for CCL5 when induced through TRIF (but also not when induced through MyD88). In other words, TLR4 signaling critically depends on the simultaneous

signaling through both signaling routes, in order to induce a response with full potential. Although this mandatory interaction is part of the state-of-the-art knowledge about TLR signaling, an explanation for this phenomenon has yet to be discovered (Kawai and Akira, 2010). The concerted (successive) activation of the NFκB system could be one option.

One might speculate about a role in controlling and probably limiting responses to bacterial endotoxin, as especially LPS-producing *Enterobacteriaceae* are an essential part of the commensal flora. Keeping this symbiotic 'partnership' in balance, macrophages in the periphery might contain themselves – not responding to minute amounts of LPS as 'spilling' from the gut. In contrast, microglia in the shielded CNS compartment could be less tolerant. Differentiation between LPS forms and the responses elicited by them could be a cell type-dependent feature. Controlled interaction of MyD88 and TRIF downstream of TLR4 might be one of the mechanisms to achieve this goal. Interestingly, in our studies employing endogenous agonists, the respective TLR4 responses appeared to be largely MyD88-dependent, yet potently inducing cyto/chemokine release comparable to that of LPS-induced responses. As in these studies other co-receptors are essentially involved in the TLR4 receptor complex, it might be interesting whether the co-receptor signaling is able to lift or circumvent the 'TRIF-control' in these cases. The assumption that TRIF is less important for DAMPs derives from the lack of measurable IFNβ production. Yet it has to be added that we still found some TRIF dependence for a coagulation-associated protein complex with remarkable TLR4 activity. DAMPs may thus come with some atypical TRIF involvement.

With respect to adaptor protein usage, the situation for TLRX/2 responses presents somewhat 'easier' as both TLR1/2 and TLR6/2 heterodimeric receptor complexes are believed to rely exclusively on the MyD88-dependent signaling route (O'Neill and Bowie, 2007; Kawai and Akira, 2010). Indeed, in microglia deficient for MyD88, the production of proinflammatory cyto- and chemokines in response to TLR1/2 and TLR6/2 stimulation, respectively, was completely abolished. Surprisingly, when microglia with a deficiency in TRIF were stimulated with TLRX/2 agonists, responses were also significantly altered, thereby questioning the theory of solely MyD88-dependent signaling downstream of TLRX/2. In fact, the absence of TRIF resulted in diverse regulation of different genes as the production of factors was either inhibited (CXCL1), left more or less unaltered (TNFα and CCL3) or even got significantly enhanced (CCL5). This pattern of differential influences clearly points to a regulatory role for a TRIF-based or TRIF-related mechanism.

It is important to mention that a possible TRIF component in TLRX/2 signaling is not simply due to a contamination, such as with TLR4-activating LPS. $TLR4^{-/-}$ microglia responded unaltered to respective TLR1/2 and TLR6/2 agonists, compared to wild type cells (data not shown). In an attempt to find an explanation for this unexpected TRIF involvement several ideas can be considered. First of all, there is some evidence already for an interaction of TLR2 and CD14 (Dziarski et al., 1998; Schwandner et al., 1999; Hermann et al., 2002; Parker et al., 2008). Given the still not completely resolved relation between CD14 and TRIF signaling (especially in TLR4 signaling) one might speculate about a similar relation of TRIF and a potential TLR2/CD14 receptor complex. Similarly to TLR4, TLR2 and its co-receptors are believed to be organized in cholesterol-rich membrane compartments, called lipid rafts (Triantafilou et al., 2006). In such rafts, signaling partners establish a spatial proximity to each other, thereby ensuring optimal conditions for ligand binding, receptor complex building and subsequent signaling initiation. Moreover, TRAM, the adaptor responsible for TRIF recruitment in TLR4 signaling, was shown to translocate to the plasma membrane (Rowe et al., 2006), presumably also targeting lipid rafts. Together it is imaginable that, within the lipid raft assembly, TRIF comes in close enough proximity to the TLRX/2 receptor complex to interfere by an unknown mechanism. Importantly, in this scenario, TRIF would not induce any signaling consequences by itself but would rather regulate signaling induced through TLRX/2 and MyD88. Apart from these assumptions, a number of TLR2 responses have been shown to be (at least in part) MyD88-independent (Santos-Sierra et al., 2009; Yang et al., 2009; Burns et al., 2010). Moreover, TRIF has also been demonstrated to interfere with TLR5 signaling, representing another TLR exclusively signaling through MyD88 (Choi et al., 2010). Taken together, there is (so far) no clear evidence for TRIF-involvement in TLRX/2 signaling, although our data and other reports strongly support such an idea. A most recent publication could yet twist the conventional notion of TLR4 homodimers (Stewart et al., 2010). TLR4/TLR6 heterodimers were found to mediate responses to oxidized low-density lipoprotein and Aβ, involving recruitment of CD36. It would be worth considering a bridging by TLR6 between classical TLR4 and TLRX/2 signaling options. Bearing in mind the enormous complexity of TLR signaling and assuming that we are still at the beginning to fully understand this signaling network with all its associated control mechanisms, it appears worthwhile to follow these and our findings and to shed more light on the potential cross-signaling and control mechanism.

The concept of second wave signaling

Coming back again to the 'strength of signal' hypothesis, we were further interested in the question of how long a TLR stimulus would need to be present/available for its respective receptor in order to effectively induce a proinflammatory cytokine response. For this purpose, we stimulated microglia with a saturating dose of Re-LPS for only a short period of time, ranging from 15 to 180 min. Afterwards, the stimulus was replaced by plain medium and the cells were monitored for their ability to produce proinflammatory cyto- and chemokines after such transiently stimulation (for experimental details refer to section 3.6 in Material and Methods). Concerning the efficacy with which individual factors were induced by short-term stimulation we observed substantial differences depending on the particular gene. Broadly, genes could be classified in three groups: (i) factors that needed long-term continues stimulation (more than 180 min), including TNFα, IL-6 and CXCL1; (ii) factors that were clearly induced by short-term stimulation but with absolute amounts reaching only 20-40 % of those obtained with continuously (24 h) stimulated control cells, including IFNβ, CCL2 and CCL3; (iii) factors which were potently induced already by a transient stimulation of 15 min and in absolute amounts close to control levels, as for CCL5. Efficient phosphorylation/activation of MAPKs and NFκB, thereby covering major signaling elements responsible for the induction (transcription) of proinflammatory cyto- and chemokines, only required a LPS pulse of 5 min. This surprisingly indicates that the initial signaling wave must be followed by additional signals to efficiently induce genes from groups (i) and (ii). Importantly, the second signal wave cannot be explained by some autocrine loop, as mentioned above, since it came from the receptor/ligand complex. The TLR agonist had to be available for prolonged time periods.

Of note, the feature of differentially 'fast' induced genes upon TLR4 stimulation required the cooperative effort of both signaling routes. When limited to either of the two (MyD88$^{-/-}$ *versus* TRIF$^{-/-}$ cells), the formerly fast genes turned into intermediate induction efficacy and, accordingly, the formerly intermediate ones lost most of their property to be induced upon short-term stimulation. Once again, the findings underpinned the necessity for cross-talk between MyD88 and TRIF signaling upon TLR4 stimulation. Interestingly, MyD88-dependent signaling alone was generally insufficient for the fast induction of genes, as transiently TLRX/2-stimulated microglia – limited to MyD88 signaling – induced any gene with slow to intermediate efficacy only. Thus, despite mandatorily depending on

signaling cooperation, TLR4 seems to be equipped with a unique feature, compared to other TLR family members, underlining the importance of this particular TLR.

The relationship between signal (stimulus) duration and differential signaling consequences is already known for other cell types. For example, in T cells, termination of the signal by stimulus removal has a profound impact on various responses, including proliferation, fitness and differentiation (Lanzavecchia and Sallusto, 2002). Moreover, in human monocyte-derived DCs, continuous triggering of TLR4 was shown to be needed for LPS-induced cytokine production. In contrast, the LPS-induced up-regulation of MHC, co-stimulatory molecules and CCR7 only required a transient receptor triggering of less than 15 min (Macagno et al., 2006).

Run-down of TLR-triggered responses

With a variation from the latter experiments we sought to examine the induction stability of microglial TLR stimulations. For this purpose we designed an experiment where the stimulus was removed after 24 h of an ongoing TLR response. After this time period the proinflammatory cytokine responses were already established, however, depending on the individual factors, not to the full extent. Independent of the stimulus (TLR1/2, TLR4 or TLR6/2 agonists, respectively), microglia stopped their reactive program within 12 to 24 h after removal of the stimulus. In other words, upon constant presence of the stimulus, some of the factors would have been produced and accumulated for another 36 to 48 h, as we have seen it in the 72 h stimulation paradigm. However, when the situation changes (as indicated here by the sudden disappearance of the stimulus) microglia immediately adapt to the new situation. This way, also long-term reactive programs still require the continuous presence of the stimulus that initiated the reaction in the first place. These observations integrate in the findings of Stout and colleagues (2005), describing sequential changes of functional phenotypes upon changes in the macrophage environment. By various pre-conditioning paradigms, the studies showed that macrophages would repeatedly adapt their reactive phenotype depending on the actual trigger/condition. Quite similarly, our approach showed that microglia immediately adapt to changes in their environment. Thereby, reactive programs will not be executed until the maximal response potential is reached. Such programs will rather be terminated if changes in the environment require it.

Desensitization of TLR-triggered responses

In line with the former sequential changes in functional phenotypes we finally sought to elucidate whether microglial responses to TLR stimulation could be desensitized by pre-conditioning of the cells with sub-threshold concentrations of the same TLR ligands. The idea behind this question addressed, for example, a situation where a chronic pathogenic challenge – which by itself does not elicit a 'measurable' response – would render cells more or less insensitive to a full-blown challenge with the very same pathogen.

To mimic such a scenario *in vitro*, microglia were challenged with various sub-threshold concentrations of TLR agonists for 24 h before the stimulus was replaced with a saturating dose of the same TLR agonist for a second 24 h incubation period. For TLR1/2-stimulated microglia (also representing TLR6/2-stimulated cells), we observed a certain desensitization phenomenon. However, this was restricted to individual factors. Whereas the production of TNFα, CCL3 and CXCL1, respectively, was not altered, other factors, like IL-6 and CCL5, were produced to a substantially lower degree when cells were pre-conditioned by a sub-threshold concentration of the TLRX/2 agonists. Representing an extreme, the CCL2 production was severely diminished, regardless of the pre-conditioning dose.

TLR4 responses were characterized by similar yet distinct patterns, depending on the particular factor. Overall, the subsequent response to the saturating dose of LPS appeared to be less affected by desensitization phenomena than it was seen for TLRX/2 responses. For example, the production of CCL5 was stably close to control levels, no matter of the LPS concentration used for pre-incubation. Once more, the fact underlines the obviously essential role of this particular chemokine in the microglial response to LPS.

Overall, it appears that microglial TLRs can desensitize when being exposed to sub-threshold concentrations of their ligands, a phenomenon known as homotolerance. Importantly, despite differential outcomes for individual TLRs, also the induced genes are differentially prone to this phenomenon. Thus, subsequent responses differ in quality and quantity compared to otherwise unaltered responses. It should be added that (experimental) pre-treatment mimicking (natural) pre-challenges can also enhance subsequent responses, a phenomenon known as priming and as, for example, functional for IFNγ and TLR agonists (Häusler et al., 2002) or DAMPs and PAMPs (Ribes et al., 2010a , 2010b).

Summarizing the findings regarding the signaling organization of microglial TLR stimulation, we could show that these responses are fine orchestrated and highly sensitive

to changes in the (extracellular) environment. Moreover, certain phenomena described here cannot be generalized throughout TLRs or inducible genes, but rather come with distinct consequences for individual responses. Especially TLR4 proved to have unique features, which in part seem to be based on the special situation of TLR signaling adaptor usage. In future studies, it will be interesting to extend these findings beyond the level of cyto- and chemokine induction. Nevertheless, and at even more general level, the actual observations add some more pieces to the puzzle of the microglial response versatility.

5.3 Microglial TLR responses are modulated by secondary signaling events

Macrophage activation represents a potent mechanism of innate immunity in order to elicit first-line responses against invading pathogens as well as to initiate and orchestrate subsequent actions of the adaptive arm of immunity. However, extensive or overshooting defense-oriented (proinflammatory) programs exerted by macrophages (and of course other immune cells) always carry the risk of 'collateral damage', including local tissue injury and spreading of the inflammatory reaction throughout whole tissues and organs up to a systemic dimension. In the worst case, such uncontrolled/uncontrollable immune reactions can lead to chronic inflammation and autoimmunity. Conceivably, all cells of the immune system are equipped with a battery of intrinsic control mechanisms, including negative feedback gene regulation, multi-step activation processes (especially for T and B lymphocytes) and apoptosis of (over)activated cells, thereby representing means of limiting, controlling and terminating activation of individual cells. Additionally, there is the complex network of cyto- and chemokines, representing another level of control which can have booth, autocrine and paracrine consequences.

In peripheral tissues, two important mechanisms have evolved that promote the resolution of innate immune responses: (i) blood-borne monocytic cells infiltrate into injury sites and phagocytose dead or dying cells (van Rossum et al., 2008); (ii) these and other proinflammatory cells exit the tissue via the lymphatic system, thereby allowing to shift reactive programs of residing cells from 'defense' to 'repair'. In the special situation of the CNS, however, the above mentioned mechanisms are unlikely to be of great importance. Under physiological conditions, the blood-brain barrier potently restricts the movement of cells from the vasculature into the CNS parenchyma (Abbott et al., 2006), a function that might be altered only in the context of injury and disease (Soulet and Rivest,

2008). Importantly, studies on this topic have been suspected to suffer from a technical pitfall, which renders interpretation of the respective data difficult (Mildner et al., 2007). On the other hand, the CNS is devoid of a defined lymphatic system, and although the exchange of fluids and soluble proteins between CNS and the peripheral lymphatic system has been shown, the passage of CNS-resident cells through this route is still under debate (Carare et al., 2008). Based on these 'limitations', other control mechanisms of macrophage activation gain relevancy in the context of microglia as resident innate cells of the CNS.

Cytokines are potent mediators of various 'instructions', including activation of cells, modulation or shifting of reactive programs as exerted by already activated cells, or attraction of cells along chemotactic gradients (e.g. established by complement factors or chemokines) to the sites of action. As our studies are concentrating on microglial reactive phenotypes that result from TLR stimulation we asked the question to which extent such reactions would be susceptible to be modulated when secondary immune signals come into play. Among the numerous immunomodulatory cytokines, we focused on four representatives: (i) IFNγ served as a prototypic proinflammatory cytokine that originates mainly from activated T_H1 cells. Although macrophages and microglia have been shown to produce IFNγ under specific circumstances (Suzuki et al., 2005), there are still debates whether these cells significantly contribute to the production of this cytokine. Assuming that microglia are no major source, the presence of IFNγ would mimic an advanced phase of inflammatory reaction where T_H1 cells (among others) already have entered the site of injury/infection. (ii) IL-4 represents a 'master' cytokine secreted by T_H2 cells, which has a clear tendency to exert anti-inflammatory properties. (iii) In the same line, IL-10 can be seen as the prototypic anti-inflammatory cytokine, which is produced by a number of immune as well as non-immune cells throughout the organism, including alternatively activated macrophages and B cells. The anti-inflammatory properties of IL-10 on TLR-stimulated microglia were already discussed in section 5.2. As IL-10 is known to be produced by macrophages (Saraiva and O'Garra, 2010), including microglia (Hanisch, 2002; Jack et al., 2005), the main source of IL-4 seems to reside in the adaptive immune compartment. Importantly, although both cytokines are characterized as anti-inflammatory, they induce distinct phenotypes when acting on macrophages. Whereas IL-4 (like IL-13) induces the 'M2' phenotype of alternative activation, stimulation of macrophages with IL-10 (and/or TGFβ) will result in the phenotype of 'acquired deactivation' (Gordon, 2003; Gordon and Taylor, 2005). Therefore, it appeared reasonable to incorporate both anti-

inflammatory cytokines in our study. In addition to the above-mentioned three classical immunomodulatory cytokines we also tested the response of TLR-stimulated microglia in co-incubation with IFNβ. This cytokine is of special interest as its administration proved to be beneficial in patients suffering from the neurodegenerative disease MS (Rudick et al., 1997; Li and Paty, 1999; Weinstock-Guttman et al., 2008). Due to the compromised integrity of the BBB in MS patients it is very likely that the systemically administered cytokine also reaches the CNS parenchyma, where it then might act on (activated) microglia. Indeed, the neuroprotective potential of IFNβ on microglia was already demonstrated *in vitro* (Jin et al., 2007) and *in vivo* (Prinz et al., 2008). Moreover, macrophages/microglia are also potent producers of IFNβ in response to viral infection. The fact that the underlying signaling of this response is TLR-dependent makes it even more interesting to check for the regulatory potential of (autocrine) IFNβ on microglial TLR signaling.

Within this set of experiments, we tested microglial responses to saturating doses of TLR1/2, TLR4 and TLR6/2 agonists, respectively, in the presence of one of the above mentioned immunomodulatory cytokines. Generally, all observed effects seen with either TLR1/2 or TLR6/2 stimulation were very similar, despite minor variations. Due to the shared TLR2 component, this finding was not unexpected. In contrast, the modulation of TLR4-induced signaling showed marked differences in almost any of the co-stimulation paradigms. IFNγ modulated the TLRX/2-induced response by means of substantial up-regulation of a T_H cell attracting signal (CCL5) and simultaneous suppression of the attraction of neutrophils (CXCL1/CXCL2). This pattern confirms earlier findings made with pneumococcal cell walls (PCW) as the primary stimulus (Häusler et al., 2002). PCW thereby represent a mixture of various molecules, including proteoglycans and lipoteichoic acid, two agents that in the meantime were identified as agonists for TLR1/2 and TLR6/2. Clearly, this modulated pattern indicates an advanced inflammatory response where early effectors, like neutrophils, are no longer required, but where the T cell response is in full operation. Similar to the TLRX/2 situation, IFNγ also suppressed neutrophil attraction in response to TLR4 stimulation. However, here the general call for T cells was unaltered whereas chemoattraction of specifically T_H1 cells was down-modulated, indicating a bias towards T_H2 cells. Also in agreement with the findings of Häusler et al. (2002) – and their principle as confirmed by Feuerer et al. (2006) – this IFNγ-induced shift of the microglial TLR4 response appears to represent a 'self-limiting' control mechanism as T_H1 cells would

be the major source of (more) IFNγ at the site of inflammation. Collectively, the data on TLR/IFNγ co-stimulations demonstrate that a secondary (modulatory) signal can substantially influence the microglial response, thereby shifting reactive phenotypes with respect to the actual situation and the involved effector cells.

IL-4 had the expected suppressive effect on the TLRX/2-induced proinflammatory cyto- and chemokines. However, T cell-attracting signals were in part spared from this modulation, also favoring the presence of T_H2 over T_H1 cells. As IL-4 is a typical T_H2 cytokine, this argues for a continuation of a T_H2-biased response, probably already in the resolution phase of the inflammatory reaction. In addition, IL-4 instructed for an increased attraction of monocytes/macrophages (CCL2). Following the idea of an already resolving immune reaction, these additional macrophages (microglia) would probably be needed for clearance of debris or apoptotic/dead cells as well as for the organization of subsequent tissue repair processes. Interestingly, the microglial response to TLR4 stimulation was basically unaltered when IL-4 was added. The fact that microglial TLR stimulations are differentially sensitive to IL-4 modulation may correlate with the 'importance', i.e. the potential threat of a given PAMP and its parent pathogen. This way, in a given context involving the presence of IL-4, microglia would allow for resolving the proinflammatory response to Gram-positive invaders (via TLRX/2), whereas Gram-negative threats (TLR4) would require a continuously strong defense response. Yet the special TLR4 conditions could also be relevant for the various endogenous agonists (see below). In contrast, the presence of IL-10 would not allow for such a distinct decision. In its presence, as expected, all TLR-induced responses were dose-dependently down-modulated, with the effect being less potent for signals attracting T cells and monocytes/macrophages. As we could show microglia to be a source of IL-10 in (late) response to TLR stimulation, this cytokine would also represent a 'self-limiting' factor, acting in a paracrine fashion.

Modulation of microglial TLRX/2 responses by IFNβ appeared quite different from the observations made with the 'classical' immunomodulatory cytokines. There was no marked suppression of proinflammatory cytokines. Instead, a general call for T cells was apparent by the up-regulation of CCL5. In contrast, there was no obvious modulation of microglial TLR4 responses, despite a partial down-regulation of monocyte/macrophage attractants. This latter finding appears to be somehow contradictory. On the one hand, it is known that TLR4 stimulation with LPS results in the production of IFNβ. On the other hand, however, this intrinsic IFNβ would have no modulatory effect except to contain the

recruitment of (more) macrophages/microglia. One might speculate whether this phenomenon represents a mechanism of Gram-negative invaders aiming to escape innate immune processes.

In our studies comparing TLR4 signaling in response to LPS (as a representative PAMP) *versus* various DAMPs we could show that these responses were differentially modulated by the set of modulatory cytokines described above (unpublished data). This way, we could demonstrate that different agonists, which bind to and signal through the same receptor (TLR4), all employ specialized signaling routes downstream of the receptor, as the identical second signal (for example IFNγ) had distinct impacts on TLR4 signaling, depending on the individual PAMP/DAMP agonist. Bringing this concept one step further, we asked whether a second signal would also have differential influences on the different structural LPS variants as they signal through TLR4 (see also section 5.1). As expected, all LPS chemotypes were modulated in a similar fashion. However, we also observed subtle differences, especially under the IL-4 and IFNβ co-stimulation paradigms, pointing to at least some degree of individuality with respect to the TLR4 signaling induced by structural LPS variants. It must be stressed, however, that the observed differences did not correlate with the composition or length of the carbohydrate chains as associated with the LPS chemotypes. Once more, our results argue against a generalized correlation of the degree of glycosylation of a given LPS molecule and the underlying TLR4 signaling organization.

Notably, however, in terms of modulation of their inducible release profiles, TLR4-active PAMPs and DAMPs seemed to differ as classes of agonists. As mentioned, IFNβ did not affect much the LPS-driven responses. In sharp contrast, we found IFNβ to change the cyto/chemokine output drastically as it was primarily induced by DAMPs. This feature applied to several endogenous proteins indentified in our laboratory as true TLR4 agonists (not shown). On the other hand, LPS could induce IFNβ production, whereas all DAMPs failed to do so. In other words, a PAMP→TLR4-driven microglia could provide IFNβ to reorganize a (simultaneous) response to DAMPs, while the response to the very PAMP signal itself remains unaltered. DAMPs cannot reciprocally use IFNβ to act on the PAMP pattern, since they are unable to induce it and since the PAMP reaction would ignore it. IFNβ could thus be quite instrumental in a unidirectional PAMP-DAMP cross-regulation.

Summarizing the experiments of this section, we could confirm that microglial reactive phenotypes in response to TLR stimulation are prone to shifts upon appearance of

a modulatory (second) signal. These fine-tuned modifications probably gain complexity the more signaling consequences (factors and functions) are considered. Depending on the nature of the microglial activation (stimulus), the outcome of the respective modulation may differ significantly. This level of complexity should be kept in mind when interpreting data from experiments involving microglial activation with more than 'just' one defined stimulus.

5.4 Noradrenaline modulates the microglial response to TLR stimulation

As discussed earlier, the CNS represents a specialized tissue concerning basic immune surveillance and the absence of a broad repertoire of immune effector cells, as compared to peripheral organs and structures. Conceivably, 'non-classical' mechanisms had to evolve in order to efficiently organize for the induction, control and/or termination of activities dealing with 'dangers' and 'strangers'. In this context, neurotransmitters have proven not to be 'limited' to their role in synaptic signal transmission but to also exert extra-synaptic activities, thereby modulating additional neuronal and glial processes, including those of microglia (Agnati et al., 1995). Indeed, microglia express receptors for a number of neurotransmitters, including GABA (Charles et al., 2003), glutamate (Noda et al., 2000) and (nor-) adrenaline (Mori et al., 2002). Moreover, microglia already have been shown to be subject to efficient neurotransmitter-related modulations (Pocock and Kettenmann, 2007). Concentrating on noradrenaline (NA), this neurotransmitter is a well-known immunosuppressor (Sternberg, 2006). In the periphery, where NA also acts as a hormone, it was shown to suppress the production of proinflammatory cytokines produced by macrophages (Hu et al., 1991; Hasko and Szabo, 1998) and DCs (Maestroni and Mazzola, 2003). In the CNS, a similar anti-inflammatory potential of NA was shown for glial cells in general (Feinstein et al., 1993, 2002) and for microglia in particular (Prinz et al., 2001; Heneka et al., 2002). In a recent study, Heneka and colleagues showed that NA exerts control over microglial phagocytosis and migration in response to Aβ peptide. We contributed to this study by showing that the microglial proinflammatory cytokine response to Aβ stimulation can be suppressed by NA *in vitro* (Heneka et al., 2010). As the link between Aβ-mediated microglial activation and the involvement of TLRs in this process already found acceptance (Landreth and Reed-Geaghan, 2009; Stewart et al., 2010), we hypothesized that NA strategically targets the microglial TLR signaling to be

modulated in response to DAMP/PAMP stimulation. We could show that *in vitro* cultivated primary microglia are equipped with all known adrenergic receptor (AR) subtypes, as we found the respective mRNA transcripts in unstimulated cells, thereby confirming and expanding the earlier findings of Mori et al. (2002). When subjected to stimulation with the TLR4 agonist Re-LPS, the microglial proinflammatory cyto- and chemokine response was efficiently inhibited by NA in a dose-dependent manner. Of note, the NA-mediated modulation of the microglial response was most effective at a dose of 10 μM, representing a physiologically relevant concentration that might even be exceeded in areas proximal to projection terminals of NA-producing neurons. Strikingly, this inhibitory effect was of a selective manner as particularly the expression of the chemokines CCL2 (attracting monocytes/macrophages), CCL3 and CCL5 (both attracting T_H cells) was almost spared from being inhibited by NA. This modulatory rather than solely suppressive potential of NA will be discussed in more detail below.

In order to identify the responsible AR subtype(s), we employed a set of specific agonists and antagonists to examine their potential in mimicking (agonists) or reversing (antagonists) the NA-induced effect, respectively, focusing on the microglial response to TLR4 stimulation. Based on this receptor pharmacology, we could rule out an involvement of α_x-AR. Instead, the β-AR subclass, and specifically β_2-AR, could be identified as the critical receptor, as the β_2-AR-specific agonist salbutamol (SB) potently resembled the NA effect. Moreover, the β_2-AR-specific antagonist ICI118,551 (ICI) could completely reverse the inhibition mediated by NA. Interestingly, β_1-AR also appeared to be largely dispensable for the NA-mediated effect, despite having a considerably higher affinity for the neurotransmitter, compared to β_2-AR (Hoffmann et al., 2004). This phenomenon may find an explanation in the fact that shared downstream signaling elements are differentially organized, resulting in distinct signaling consequences (Chen-Izu et al., 2000). Actually, the low affinity of NA towards the β_2-AR may also explain the rather high dose needed to elicit the described effect, compared to the β_2-specific SB, which is already fully effective at a 100-fold lower dose. Notably, we did not specifically test the potential involvement of the β_3-AR. Although there is already substantial and complementary evidence in favor of the β_2-AR, for completeness, the potential role of the β_3-AR has still to be investigated in more detail. Moreover, work on other microglial functions indicated that the additionally expressed AR subtypes have their own implications. They may contribute to some extent

also to the pattern of cyto/chemokine regulation as outlined below. Indirect evidence for this assumption derives from the 'cleaner' separation of the permissive and suppressive effects when comparing the profiles in the order NA to β-AR agonist to β_2-AR agonist.

Agonist binding of β_2-AR leads to the Gs protein-dependent activation of adenylate cyclase (AC), which then generates cyclic AMP (cAMP) (Rosenbaum et al., 2009). Indeed, when NA was added to microglia, we could detect substantial amounts of intracellular cAMP as early as of 1 min after the NA treatment, which declined only after 30 min. After its intracellular accumulation, the second messenger was translocated to the extracellular compartment, where the levels remained stable, probably owing to the lack of degrading enzymes. The critical involvement of cAMP in NA-mediated effects was substantiated by the effects of forskolin. The nonspecific activator of AC could closely resemble the NA-mediated modulation of the microglial response. The still observed subtle differences are likely due to the compartmentalized AC recruitment via the natural receptor pathway, whereas forskolin would stimulate AC activities throughout the cell.

We then sought to decipher the potential role of protein kinase A (PKA), the prime target of cAMP (Rosenbaum et al., 2009). Surprisingly, PKA could not be shown to participate in the NA-mediated signaling cascade. Both the potent 'classical' PKA inhibitor H-89 as well as the myristoylated (and therefore better cell permeable) PKA inhibitor 14-22 could not interfere with the NA-mediated modulation. Collectively, these results favor a crucial role for cAMP, while at the same time ruling out involvement of PKA. Therefore, other cAMP targets with a potential link to TLR signaling need to be considered in order to dissect the critical signaling events involved in the NA-mediated effects. In another project of our laboratory, the search for such a cAMP-dependent, yet PKA-independent factor was meanwhile successful, as the nucleotide exchanger Epac proved to play a role and its direct activation, indeed, mimicked the pattern of NA and SB modulations of the release profile. Of note, although not involved in the release-regulatory effects of NA as addressed here, PKA still does get activated by cAMP and may thus influence other microglial functions.

As we hypothesized NA to have a general modulatory effect on TLR signaling, we also searched for a potential NA modulation of responses to microglial stimulation with TLR1/2 and TLR6/2 agonists. Indeed, these responses were potently inhibited as well. However, this time we did not observe a 'selective' pattern of modulation as described above for TLR4 responses, where some factors were spared and thus permitted for release.

In contrast, all factors were potently down-regulated with the only exception being CCL3, which was only partially inhibited (similar to what was observed for the modulation of TLR4 responses). As already discussed in section 5.1 regulation of *CCL3* presents with some distinction from all the other genes considered in our studies. Concerning the other induced and differentially modulated factors, we asked whether the different TLR signaling routes are variably sensitive towards interference by NA-induced signaling. As extensively discussed before, TLR4 uniquely uses both TLR signaling routes – depending on MyD88 and TRIF – whereas both TLR1/2 and TLR6/2 employ solely MyD88 (although more and more exceptions from this 'rule' come to light; see for details also section 5.2). Based on this discriminative feature, we hypothesized that the MyD88-dependent signaling would be 'NA-sensitive', with TRIF-induced genes being rather protected from NA regulation.

Indeed, 'forcing' TLR4 signaling to be solely MyD88-dependent, i.e. in a $TRIF^{-/-}$ situation, rendered all genes sensitive to inhibition by NA and SB, respectively. However, creating the opposite scenario in $MyD88^{-/-}$ cells did not result in a 'protection' of the induced genes. In fact, the resulting pattern had a rather 'intermediate' appearance, as all factors were down-modulated by NA/SB, but with reduced inhibitory potential, compared to the wild type situation. Further arguing against a general TRIF-related protection from NA modulation, release of IFNβ, itself being induced solely through the TRIF-dependent signaling route, was also sensitive to be down-modulated by NA. Collectively, these data indicate a certain role for TRIF in mediating the selectivity of NA regulation, however, with the footnote that TRIF dependency alone does not suffice to 'escape' from NA control. We therefore favor the idea that NA selectivity is determined individually for each factor, depending on the respective gene-regulatory elements involved. In other words, genes that solely get induced through MyD88 may get regulated at transcriptional levels or proximal to them. Genes getting activated through MyD88 and TRIF may escape the tight control. Yet being dependent on TRIF is not a guarantee for not having NA-sensitive response elements in the transcription control.

Finally, we asked the question to which extent differential timing of NA addition would influence the modulatory outcome. As we already could demonstrate the importance of 'timing' for the general organization of (microglial) TLR signaling (see section 5.2), we considered a potential importance also for the NA-induced β_2-AR signaling. Consequently, timed addition of NA up to 3 h before or after setting the primary TLR4 stimulus resulted

in a complex rearrangement of the modulated pattern of cyto- and chemokines (see section 4.15 for more details). Several conclusions could be drawn from these results. First of all, basically each individual factor responds with distinction towards a differential NA timing, thereby also disrupting the 'permissive' *versus* 'non-permissive' groups of genes. There is also no correlation between fast induced genes (like CCL3 and CCL5) and their reduced responsiveness to NA modulation, as based on the idea that these genes are already 'on their way' to be translated before NA-induced signaling consequences could interfere. Even a 3 h pre-incubation of microglia with NA did not render these genes NA-sensitive, thus challenging the above mentioned idea. Further, the order of TLR4-induced cyto/chemokine production (e.g. immediate induction of TNFα *versus* later induction of CCL5, see section 4.8) does not correlate with NA sensitivity, as for example TNFα and CXCL1, both being induced more or less simultaneously after TLR4 stimulation, show apparent variations in their response to differentially timed NA addition. Also, based on the inhibition of IL-6, IL-12p40 and CXCL1, NA substantially loses modulatory potential when administered 1 to 2 h after TLR4 stimulation and, moreover, gains back much of its potency when added 3 h post LPS. This phenomenon points to a second wave of NA-induced signaling which needs to be followed in more detail.

All together, the modulatory effect of NA seems to be most potent when TLR(4) stimulus and neurotransmitter signaling are initiated simultaneously. Bearing also in mind the differential NA effects on MyD88- *versus* TRIF-induced genes, the most obvious 'point of interference' seems to be at the level of gene transcription. However, other potential levels of interference, including mRNA stability, feedback signaling loops or neutralization of soluble products by degrading enzymes or decoy receptors must also be considered for future investigations. Fully understanding the NA-induced regulatory mechanism likely will result in a number of options to make use of this potent effect in a therapeutic context. After all, the noradrenergic neurotransmission as one of the major 'diffuse modulating' systems of the CNS proves for another modulatory impact, namely on the resident innate immune cell and its pivotal functions in homeostatic surveillance and protection against endogenous and exogenous threats.

5.5 Microglia support tumor metastasis without acquiring an apparent phenotype

As part of the innate immunity, macrophages play crucial roles in defending the body against 'strangers' and 'dangers' as discussed before. In addition to these various molecules of exogenous and endogenous origin, there is another endogenously derived threat that requires the attention of macrophages, namely the presence of tumors and their spread (metastasis) throughout the organism. Unfortunately, macrophages repeatedly were shown to support rather than counteract tumor development and outgrowth. A specialized subset of these cells, known as tumor-associated macrophages (TAM), proved to play a critical role in cancer cell proliferation, invasion and intravasation as well as in promoting the remodeling and neovascularization of tumor-adjacent tissues (Mantovani et al., 2008). In contrast to classically activated (M1) macrophages, TAM were shown to exert an M2-like phenotype of alternative activation with high levels of IL-10 and low levels of IL-12 secretion (Allavena et al., 2008). Whereas the role of TAM in the context of tumor cell evasion from the primary tumor and subsequent intravasation has already been subject to intense investigation, TAM contributions to the ultimate step of metastasis, i.e. the colonization of the target tissue, remained largely unknown. There are indications for tissue-specific TAM behavior as investigations in distinct body compartments showed differential actions of these cells. In the liver, for example, Kupffer cells (as the resident tissue macrophages) can have both, tumoricidal and prometastatic effects (Gjoen et al., 1989; Heuff et al., 1995; Sturm et al., 2003; Gorden et al., 2007). Similarly, bone marrow-derived monocytes have been shown to support cancer cell colonization in the lung (Hiratsuka et al., 2002, 2008; Kaplan et al., 2005; Kim et al., 2009). Although cerebral metastasis represent the most frequent malignant brain tumors associated with a poor clinical prognosis, the potential role of microglia (as CNS-resident macrophages) in acquiring TAM properties and thereby supporting tumor colonization of the CNS has yet not drawn much attention. The recent report by Pukrop and colleagues (2010) showed for the first time a tumor-supportive role of microglia. Briefly summarized, this study (to which we contributed) could show the presence of microglia in human carcinoma brain metastasis (comprising up to 50 % of all cells) and their co-localization with infiltrating tumor cells. Microglia enhanced *in vitro* invasion rates of human MCF-7 and mouse 410.4 breast cancer cell lines in a Wnt-dependent way. Moreover, the addition of LPS potently inhibited this tumor-supportive microglial phenotype. While TAM may thus ignore a

tumor cell presence or contribute to their immune evasion by serving as M2-instructed sources of IL-10, the tumor-supportive activities can – as now shown for microglia – even reach beyond these levels and exceed to the quality of mediating invasiveness.

This study was complemented by our results as described in chapter 4.16. We made the striking observation that neither indirect nor direct co-cultures of microglia with either of the above mentioned breast cancer cell lines induced a M2-like phenotype of microglia, as it is generally proposed to be the case for TAM (Mantovani et al., 2008). In contrast, otherwise unstimulated microglia kept their 'resting' phenotype irrespective of the absence or presence of the tumor cells, at least with respect to the rather broad pattern of released cyto- and chemokines investigated here. These findings were substantiated by gene arrays (Pukrop et al., 2010). Moreover, the capability of microglia to respond to TLR stimulation with a proinflammatory cyto/chemokine profile remained unaltered when co-cultured with tumor cells.

Additionally, we investigated the same co-culture paradigm using the rather 'aggressive' breast cancer cell line 4T1. Contrasting the previous results, these tumor cells were able to elicit a microglial phenotype, specifically in instructing microglia to attract neutrophils and monocytes/macrophages, thereby also challenging the of microglia acquiring M2-like (TAM-like) properties. Moreover, 4T1 cells could also modulate the microglial response to TLR stimulation. In this context, the tumor-instructed down-regulation of TNFα production by TLR-stimulated microglia was a most intriguing result, as this cytokine is believed to be tumor-promoting (Charles et al., 2009). However, since invasion data for this breast cancer cell line are not available yet, interpretations of the *in vitro* stimulation data are difficult.

Taken together, these findings indicate once again a distinct role for microglia when compared to other (extra-neural) macrophage populations. Obviously, microglia actively support CNS colonization by metastatic tumor cells without acquiring an obvious TAM phenotype. It is tempting to propose that also the definition of TAM with regard to phenotypic characteristics cannot be used universally. More likely, also TAM have to be sub-classified according to their origin (blood-borne or tissue resident), their specific tissue affiliation and the type of tumor they support (or occasionally fight).

5.6 Microglia represent a heterogeneous cell population in diverse ways

In the previous chapters, we discussed the phenotypic diversity of (neonatal) microglia in response to various TLR stimulation paradigms. The term 'diversity', however, should not be limited to acquired and/or shifted phenotypes as induced upon different challenges. This chapter addresses microglial diversity with respect to postnatal developmental processes, which seem to include some maturation of signal interpretation and receptor signaling. The following section also discusses the constitutive heterogeneity of microglial cells by and within sub/populations as organized in defined compartments of the CNS.

Microglial diversity based on developmental changes

It has been recognized for quite some time that microglia of different developmental stages show variations in morphological and functional features. In humans, such studies almost exclusively rely on post-mortem tissue and, therefore, are limited to histological analysis. Only a few laboratories with access to human fetal or biopsy tissue were able to contribute some rare but precious functional *in vitro* data on microglial properties (Jack et al., 2005). On the contrary, using rodents and well-established protocols, microglia can be routinely isolated with high cell yield and purity to allow for diverse functional investigations under controlled experimental conditions. However, the majority of microglial *in vitro* research is still based on neonatal cells, although some defined studies also utilize embryonic cells (Draheim et al., 1999; Gingras et al., 2007) or those from early postnatal (young), adult or aged animals (Carson et al., 1998; Floden and Combs, 2006; de Haas et al., 2007; Sierra et al., 2007; Njie et al., 2010). The majority of studies on adult ages concentrate on aged (senescent) microglia and their contribution to neurodegenerative processes (Streit and Xue, 2009). Yet microglia from adult animals are difficult to bring to culture – as they do not adapt and survive easily. On the other hand, the use of acutely isolated microglia is unavoidably running the risk that cells rapidly respond to the isolation-associated trauma. We could show early preparation-associated activation for rat retinal microglia (Mertsch et al., 2001). If a snap-shot analysis of expressed molecules is performed, rapid isolation and 'freezing' of the status (by fixation) can avoid that tissue damage released factors activate signaling pathways causing functional adjustments or inducing mRNA expression, protein synthesis and/or release/shedding. Otherwise, any functional study on the acutely isolated

microglia will raise doubts as to the cellular responses being critically influenced by the 'injury' experience.

Based on these considerations, we established a protocol for the functional *ex vivo* analysis of microglia, giving access to cells from mice of varying ages. We thereby sought to attempt a systematic approach to the monitoring of changes in the microglial behavior during normal postnatal development from birth (P0) to adulthood (P63 and beyond). For this purpose, we introduced an adaptation to our standard protocol for neonatal microglia, enabling us to culture cells as prepared at P14 to P63 (with 7-day-steps in between) for an extended period of time (up to 1 month; for technical details refer to Material and Methods section). The cultures provided sufficient amounts of vital and obviously quiescent cells for use in functional assays. Importantly, in accordance with the findings of Floden and Combs (2007), our repetitively harvested microglia (up to four times from one original preparation) kept their physiological features and response properties stable throughout the entire *in vitro* cultivation period. On the other hand, microglial responses to the very same stimulus differed in part significantly depending on the age of the animal the cells had been isolated from. Collectively, this means that the cells' status at the time of isolation was (to a certain degree) carried over into the culture dish, without being simply overwritten by *in vitro* conditions. On the other hand, cells could also not further 'mature' *ex vivo*. Microglia kept for four weeks in culture did not match by features of a preparation of cells taken at this time point of the tissue. We are aware of the fact that this *in vitro* phenotype might not to 100 % represent the actual *in vivo* situation. However, as microglia did not further mature outside the CNS environment we could at least rule out an 'artificial' development *in vitro*. The *ex vivo* approach thus relies on some preservation of the *in vivo* state to complement *in vivo* studies by an approach allowing easy manipulation and monitoring of microglia.

We then characterized functional properties of microglia of varying developmental 'history'. We challenged the cells with TLR1/2, TLR4 and TLR6/2 agonists, respectively, and measured the proinflammatory cyto/chemokine release response. Thereby, production of the prototypic proinflammatory cytokine TNFα and the neutrophil attractant CXCL1 served as representative readouts. For a reliable comparison, the results of each experiment with postnatal microglia were normalized to results obtained in parallel from an identical experiment employing strain-matched neonatal microglia.

The 'developmental profile' of responses to TLR stimulation by C57Bl/6 microglia revealed several, in part remarkable, changes. Common to all responses, the time window around P21 proved to be especially critical. Such microglia responded significantly less potent to stimulation. After this 'drop', TNFα production went back to control levels (P0 response) and remained rather stable throughout the further period studied into adulthood. In contrast, CXCL1 levels showed a more 'oscillatory' behavior as they recovered after the drop around P21, but then dropped again, followed by yet another recovery phase. While the profile for TNFα had similarity for the different TLR stimulation paradigms, the CXCL1 induction 'behavior' was more extreme in the case of TLR4 stimulation. Here, the recovery phases even showed an overshooting response, when compared to control levels.

These developmental changes appeared quite intriguing. We sought to substantiate the findings – as they were made with microglia from the inbred strain C57Bl/6 – and to compare them to cells isolated from an outbred strain, namely NMRI. Indeed, these cells (of a more robust, less homogenous wild type background) also showed developmental changes. However, to our surprise, the TLR responses revealed their very own profiles. The NMRI microglia showed a remarkably augmented response to TLR stimulation at and around P21. The responses then went back to control levels but experienced a second dramatic augmentation at P56, the elevated responses than remaining stable until the end of the experimental time frame (P63). This time, developmental changes concerning microglial TLRX/2 responses (once again very similar between TLR1/2 and TLR6/2) appeared much more dramatic as the ones seen for TLR4 responses. However, all went into the rather same direction. Notably, TLR-triggered release responses of C57Bl/6 and NMRI are very similar otherwise – when compared for P0 microglia. Moreover, differences as shown here were based on induced responses to TLR agonists, raising the question as to whether they would be relevant in a non-infected (healthy) animal. On the other hand, the growing set of identified DAMPs may also include factors which access TLRs during normal maturation, making TLR reactions to them a physiologically relevant issue.

In addition to this rather broad examination of TLR response organization during development, we sought to determine also for some key features of the microglial TLR4 response as previously described for neonatal cells (see chapters 4.1 and 4.11). First, we wanted to know whether the altered (Re-)LPS response of C57Bl/6 P21 microglia would also extend to the other LPS chemotypes. Indeed, the responses of P21 cells to all of these

structural variants appeared very similar. Only Lipid A was differentially recognized as this response triggered a TNFα production even exceeding P0 control levels. This outstanding finding points again to the rearrangements in the TLR4 receptor complex and its link to the intracellular effector cascade. It is imaginable that a reorganization of the TLR(4) signaling (as developmental consequence) takes place at the receptor level in terms of co-receptor recruitment or by the interaction with sorting and signaling adaptors, i.e. TRAM, MAL, TRIF and MyD88 – or additional factors not yet understood as to their roles. We also do not know whether this outstanding behavior reflects a special importance of Lipid A *versus* the (lacking) core carbohydrates and other (glycosylated) structures.
Another key feature of TLR4 signaling in neonatal microglia was the efficacy with which genes were differentially induced. To test whether this feature would be altered during microglial development we compared TLR4 stimulations of P0, P21 and P49 microglia. Overall, we observed only minor adaptations with respect to the microglial development. Most obviously, *TNFα* was promoted from a 'slow' (in P0) to a 'fast' (in P21 and P49) induced gene. In the same direction, but less pronounced, the production of IL-6 and CXCL1 was also slightly adapted. Induction efficacies of the other factors (CCL2, CCL3 and CCL5) remained unchanged in postnatal compared to the neonatal microglia.

Collectively, the developmental profiles support the idea that microglia undergo major changes during their development from birth to adulthood. Reorganization was manifested for responses to TLR stimulation, but it conceivably involves other non-TLR systems as well.

The observed differences between the two mouse strains indicate that there is no 'common theme' with respect to the reorganization of TLR responses. Nevertheless, the fact that both cell types independently showed a developmental profile, moreover with in large parts overlapping critical time windows, strongly supports the validity of our findings as reflecting also the actual situation *in vivo*. Of note, profiles determined for TLR1/2 and TLR6/2 revealed striking similarity, likely due to the shared TLR2 signaling. Especially the critical time window around P21 offers a variety of possible interpretations. First, three weeks after birth is usually the time point when the pups are separated from their mother. The discontinuation of maternal supply – especially with respect to immunosupportive factors – may likely provoke major adaptations of the now autonomous immune system of the offspring. Concerning microglia, the phase just before P20 coincides with the second wave of microglia settlement throughout the CNS, which may be followed by a phase of

reorganization in order to adapt to the newly created environment. Also at this time in (mouse) life, myelination will come to an end, thereby also shifting responsibilities for microglia. Most likely, not a single of these developmental 'events' will instruct (or require) the described changes in microglial response reorganization, but more or less the coincidental occurrence of all of them (and presumably others) might be responsible. Actually, it has already been shown that the outcome of an inflammatory – especially LPS – challenge or a bacterial infection can differ significantly depending on the age (developmental stage) of the animal (Wang et al., 2006b; Stolp et al., 2005, 2007). Moreover, long-term consequences of early life exposures to LPS also can vary, again depending on the time window during which the endotoxin challenge occurred. Interestingly, a period between P14 and P21 (in mice and rats) was critical for determining consequences of immune challenges later in adulthood (Spencer et al., 2006; Ellestad et al., 2009). Our observations thus fit these findings and could offer a molecular substrate for understanding sensitive windows in the developing innate immunity, in particular of the CNS.

Microglial heterogeneity

Diversity in inducible reactive phenotypes and the developmental changes already point to versatile response options of microglia. These cells may also constitute an inhomogeneous population by CNS-anatomical regions. Not until recently, the populational heterogeneity has been consciously addressed – and is still a matter of debate as systematic research is still awaited. The issue does not regard differences between parenchymal microglia and other CNS-relevant macrophage populations, such as perivascular, meningeal or ependymal cells, or invading peripheral macrophages/monocytes in the context of a lesion. It rather concerns the existence of distinct populations of microglia proper themselves. Carefully screening the literature, one can find evidence, most often referring to differences of white *versus* grey matter regions (Elkabes et al., 1996; Ren et al., 1999; Schmid et al., 2002; Sriram et al., 2006; Anderson et al., 2007; Hristova et al., 2010). Other reports of location-associated diversity describe, for example, the microglial expression of ManR, which is found primarily in areas close to the vasculature (Linehan et al., 1999; Galea et al., 2005). These cells were also reported to express specifically high levels of the scavenger receptor CD163, which is closely associated with the uptake of hemoglobin (from microhemorrhage events; Fabriek et al., 2005).

We aimed to substantiate the idea of microglial heterogeneity also on the basis of functional data. For this purpose, the development of the above mentioned protocol for *ex vivo* analyses of postnatal and 'adult' microglia was of great advantage as investigations of cells from distinct circumscribed CNS compartments are hardly possible with the neonatal preparations. An investigation at such later stages would also reveal microglial features in the context of the fully matured (e.g. myelinated) tissue. To do so, we isolated microglia from the cortex (liberated of any visible myelin), the cerebellum and the spinal cord from 48 days old NMRI mice and cultured them *in vitro* as described above for the whole brain approach. These regions were chosen based on a differential white matter index, being the highest in cerebellum, about 'even' in spinal cord and much lower in the cortex. Looking at the morphology, we could already observe slight differences as microglia originating from the cortex and cerebellum were predominantly of elongated bipolar shapes, whereas spinal cord microglia showed a higher variability by appearance, with a substantial number of highly ramified cells. We then subjected these cells to our TLR stimulation regimes and observed differences in their responses. Cortical and cerebellar microglia responded with CXCL1 levels comparable to strain-matched (whole brain) P0 controls. However, TNFα production was clearly below control levels in the microglia from cortex and even lower in cells from the cerebellum. In contrast, spinal cord microglia responded with TNFα around control and CXCL1 clearly above control levels. These findings point to morphologically as well as functionally distinct microglia sub/populations, also indicating that whole brain preparations would represent a blend of responses.

We also investigated microglial populations as to their heterogeneous expression of function-associated proteins on the cells surface and in the intracellular compartment. In a first attempt, we compared whole brain preparations from different developmental stages. Upon LPS (TLR4) challenge, we found the majority of cells expressing MHC I molecules, not differing between P0 *versus* P21 *versus* P49 stages. Contrary, COX2 expression was not found in all cells after LPS stimulation. Only about 40 % of P0 microglia stained positive, whereas this number increased in both P21 and P49 microglia to about 60 % of all cells. This enzyme was thereby not only heterogeneously expressed within one pool of cells, but underwent developmental adaptations, adding yet another dimension of heterogeneity. The intracellular flow cytometry approach was then expanded to visualize intracellularly trapped cyto/chemokines upon LPS challenge. In P0 microglia, we observed a surprisingly low number of cells to produce the proinflammatory cytokine TNFα (32 %)

and/or the T_H1 cell-attracting chemokine CCL3 (17 %). These numbers gradually increased correlating with the developmental status of the cells (P21 and P49, respectively). On top of these findings double-staining approaches revealed yet another level of diversity, as we could identify three distinct groups of cyto/chemokine-producing cells. There were cells producing (i) only TNFα, (ii) only CCL3 or (iii) both of them, respectively. Whereas cells producing only CCL3 almost disappeared in postnatal preparations, the number of 'double producers' gradually increased in P21, and even more in P49 microglia. In contrast, the number of cells producing only TNFα was similar in both P21 and P49 cells. While bulk quantifications of releasable factors in the supernatant can reveal levels of production 'averaged' for the whole culture, this trapping approach could identify the individual sources. It is important to stress that the procedure was carefully adapted to the time course of release (not to miss out on producing cells) and to a reliable detection by the antibodies, especially in the double staining. Moreover, the populational diversity was also not due to partially impaired vitality, since a majority of cells expressed, e.g., MHC I. These data thus directly point to the simultaneous presence of several types of microglia.

This pattern of differential cyto/chemokine production in microglia derived from whole brain preparations could then also be confirmed for cells from discrete regions. We characterized, therefore, cultures prepared from the cortex, cerebellum and spinal cord of P49 mice. Despite some minor differences, subpopulations of 'specialized' cyto/chemokine producers were thereby also found, with little differences by anatomical origin. The most intriguing result is the hint to an intrapopulational heterogeneity, even when 'zooming' into a rather circumscribed tissue entity. Microglia may thus not only present themselves with an adaptation to major anatomical areas as differing by myelin content, BBB features or a predominant neurotransmitter milieu. Cells within a local community might be different, i.e. pre-destined to individual functions. As these data collectively already proof intrinsic heterogeneity of a given microglial (sub)population, it will be very interesting to confirm and substantiate the findings in an *in situ* setting. Indeed, preliminary data based on brain slice analysis delivered such confirmation (data not presented).

5.7 Conclusion

All together, we could show that microglia does not present with a static behavior or as a uniform population, but that these cells instead undergo postnatal maturation and later on represent as a variety of subsets by and within CNS regions – with overlapping but also distinct properties. This way, the CNS is surveyed by a (unknown) number of 'specialists' that may cover reactions in all kinds of scenarios, involving 'dangers' and 'strangers'. In addition to their versatile inducible phenotypes, there seems to be also some constitutive diversity to start with. As exemplarily shown for neonatal microglia, when appropriately triggered, these cells do not only respond in a complex and organized manner, but these reactions are also controlled and orchestrated in diverse ways. We showed that microglia can, indeed, respond with the induction of differentiated executive profiles when being exposed to diverse molecular or cellular challenges. We showed that a neurotransmitter, as a CNS-typical messenger, can control microglial functions. Finally, we had shown in the first part that the microglial TLR4 may act as a crucial decision maker. It accepts both PAMP and DAMP signs of danger. We demonstrated how TLR4 manages in combination with CD14 to organize reactions towards structural variants of its major PAMP, namely LPS. CD14 shapes both responsiveness and responses and enables TLR4 for the sensitive recognition of a broad spectrum of agonist versions. It will be indispensable to follow up phenomena of microglial heterogeneity, response diversity and smart response management – also or especially in relation to neuropathological processes. Conceivably, microglia could qualify as a highly relevant target of carefully tailored pharmacological manipulations.

Bibliography

Abbott NJ, Ronnback L, Hansson E. 2006. Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci 7(1):41-53.

Agnati LF, Zoli M, Stromberg I, Fuxe K. 1995. Intercellular communication in the brain: wiring versus volume transmission. Neuroscience 69(3):711-26.

Aida Y, Kusumoto K, Nakatomi K, Takada H, Pabst MJ, Maeda K. 1995. An analogue of lipid A and LPS from Rhodobacter sphaeroides inhibits neutrophil responses to LPS by blocking receptor recognition of LPS and by depleting LPS-binding protein in plasma. J Leukoc Biol 58(6):675-82.

Allavena P, Sica A, Garlanda C, Mantovani A. 2008. The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. Immunol Rev 222:155-61.

Anderson AC, Anderson DE, Bregoli L, Hastings WD, Kassam N, Lei C, Chandwaskar R, Karman J, Su EW, Hirashima M and others. 2007. Promotion of tissue inflammation by the immune receptor Tim-3 expressed on innate immune cells. Science 318(5853):1141-3.

Apetoh L, Ghiringhelli F, Tesniere A, Criollo A, Ortiz C, Lidereau R, Mariette C, Chaput N, Mira JP, Delaloge S and others. 2007. The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy. Immunol Rev 220:47-59.

Beg AA. 2002. Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses. Trends Immunol 23(11):509-12.

Belvin MP, Anderson KV. 1996. A conserved signaling pathway: the Drosophila toll-dorsal pathway. Annu Rev Cell Dev Biol 12:393-416.

Bianchi ME. 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol 81(1):1-5.

Biber K, Neumann H, Inoue K, Boddeke HW. 2007. Neuronal 'On' and 'Off' signals control microglia. Trends Neurosci 30(11):596-602.

Boonstra A, Rajsbaum R, Holman M, Marques R, Asselin-Paturel C, Pereira JP, Bates EE, Akira S, Vieira P, Liu YJ and others. 2006. Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. J Immunol 177(11):7551-8.

Boucsein C, Zacharias R, Farber K, Pavlovic S, Hanisch UK, Kettenmann H. 2003. Purinergic receptors on microglial cells: functional expression in acute brain slices and modulation of microglial activation in vitro. Eur J Neurosci 17(11):2267-76.

Brade H, Brade L, Nano FE. 1987. Chemical and serological investigations on the genus-specific lipopolysaccharide epitope of Chlamydia. Proc Natl Acad Sci U S A 84(8):2508-12.

Bsibsi M, Ravid R, Gveric D, van Noort JM. 2002. Broad expression of Toll-like receptors in the human central nervous system. J Neuropathol Exp Neurol 61(11):1013-21.

Burns E, Eliyahu T, Uematsu S, Akira S, Nussbaum G. 2010. TLR2-dependent inflammatory response to Porphyromonas gingivalis is MyD88 independent, whereas MyD88 is required to clear infection. J Immunol 184(3):1455-62.

Carare RO, Bernardes-Silva M, Newman TA, Page AM, Nicoll JA, Perry VH, Weller RO. 2008. Solutes, but not cells, drain from the brain parenchyma along basement

membranes of capillaries and arteries: significance for cerebral amyloid angiopathy and neuroimmunology. Neuropathol Appl Neurobiol 34(2):131-44.

Cardona AE, Pioro EP, Sasse ME, Kostenko V, Cardona SM, Dijkstra IM, Huang D, Kidd G, Dombrowski S, Dutta R and others. 2006. Control of microglial neurotoxicity by the fractalkine receptor. Nat Neurosci 9(7):917-24.

Carson MJ, Reilly CR, Sutcliffe JG, Lo D. 1998. Mature microglia resemble immature antigen-presenting cells. Glia 22(1):72-85.

Carty M, Goodbody R, Schroder M, Stack J, Moynagh PN, Bowie AG. 2006. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. Nat Immunol 7(10):1074-81.

Caso JR, Pradillo JM, Hurtado O, Leza JC, Moro MA, Lizasoain I. 2008. Toll-like receptor 4 is involved in subacute stress-induced neuroinflammation and in the worsening of experimental stroke. Stroke 39(4):1314-20.

Chan WY, Kohsaka S, Rezaie P. 2007. The origin and cell lineage of microglia: new concepts. Brain Res Rev 53(2):344-54.

Chang EY, Guo B, Doyle SE, Cheng G. 2007. Cutting edge: involvement of the type I IFN production and signaling pathway in lipopolysaccharide-induced IL-10 production. J Immunol 178(11):6705-9.

Charles KA, Kulbe H, Soper R, Escorcio-Correia M, Lawrence T, Schultheis A, Chakravarty P, Thompson RG, Kollias G, Smyth JF and others. 2009. The tumor-promoting actions of TNF-alpha involve TNFR1 and IL-17 in ovarian cancer in mice and humans. J Clin Invest 119(10):3011-23.

Charles KJ, Deuchars J, Davies CH, Pangalos MN. 2003. GABA B receptor subunit expression in glia. Mol Cell Neurosci 24(1):214-23.

Chen-Izu Y, Xiao RP, Izu LT, Cheng H, Kuschel M, Spurgeon H, Lakatta EG. 2000. G(i)-dependent localization of beta(2)-adrenergic receptor signaling to L-type Ca(2+) channels. Biophys J 79(5):2547-56.

Choi YJ, Im E, Pothoulakis C, Rhee SH. 2010. TRIF modulates TLR5-dependent responses by inducing proteolytic degradation of TLR5. J Biol Chem 285(28):21382-90.

Covert MW, Leung TH, Gaston JE, Baltimore D. 2005. Achieving stability of lipopolysaccharide-induced NF-kappaB activation. Science 309(5742):1854-7.

Creagh EM, O'Neill LA. 2006. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. Trends Immunol 27(8):352-7.

da Silva Correia J, Soldau K, Christen U, Tobias PS, Ulevitch RJ. 2001. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. J Biol Chem 276(24):21129-35.

de Haas AH, Boddeke HW, Brouwer N, Biber K. 2007. Optimized isolation enables ex vivo analysis of microglia from various central nervous system regions. Glia 55(13):1374-84.

Delude RL, Savedra R, Jr., Zhao H, Thieringer R, Yamamoto S, Fenton MJ, Golenbock DT. 1995. CD14 enhances cellular responses to endotoxin without imparting ligand-specific recognition. Proc Natl Acad Sci U S A 92(20):9288-92.

Draheim HJ, Prinz M, Weber JR, Weiser T, Kettenmann H, Hanisch UK. 1999. Induction of potassium channels in mouse brain microglia: cells acquire responsiveness to pneumococcal cell wall components during late development. Neuroscience 89(4):1379-90.

Dziarski R, Tapping RI, Tobias PS. 1998. Binding of bacterial peptidoglycan to CD14. J Biol Chem 273(15):8680-90.

Elkabes S, DiCicco-Bloom EM, Black IB. 1996. Brain microglia/macrophages express neurotrophins that selectively regulate microglial proliferation and function. J Neurosci 16(8):2508-21.

Ellestad KK, Tsutsui S, Noorbakhsh F, Warren KG, Yong VW, Pittman QJ, Power C. 2009. Early life exposure to lipopolysaccharide suppresses experimental autoimmune encephalomyelitis by promoting tolerogenic dendritic cells and regulatory T cells. J Immunol 183(1):298-309.

Erridge C, Bennett-Guerrero E, Poxton IR. 2002. Structure and function of lipopolysaccharides. Microbes Infect 4(8):837-51.

Fabriek BO, Van Haastert ES, Galea I, Polfliet MM, Dopp ED, Van Den Heuvel MM, Van Den Berg TK, De Groot CJ, Van Der Valk P, Dijkstra CD. 2005. CD163-positive perivascular macrophages in the human CNS express molecules for antigen recognition and presentation. Glia 51(4):297-305.

Feinstein DL, Galea E, Reis DJ. 1993. Norepinephrine suppresses inducible nitric oxide synthase activity in rat astroglial cultures. J Neurochem 60(5):1945-8.

Feinstein DL, Heneka MT, Gavrilyuk V, Dello Russo C, Weinberg G, Galea E. 2002. Noradrenergic regulation of inflammatory gene expression in brain. Neurochem Int 41(5):357-65.

Feuerer M, Eulenburg K, Loddenkemper C, Hamann A, Huehn J. 2006. Self-limitation of Th1-mediated inflammation by IFN-gamma. J Immunol 176(5):2857-63.

Floden AM, Combs CK. 2006. Beta-amyloid stimulates murine postnatal and adult microglia cultures in a unique manner. J Neurosci 26(17):4644-8.

Floden AM, Combs CK. 2007. Microglia repetitively isolated from in vitro mixed glial cultures retain their initial phenotype. J Neurosci Methods 164(2):218-24.

Galanos C, Luderitz O, Rietschel ET, Westphal O, Brade H, Brade L, Freudenberg M, Schade U, Imoto M, Yoshimura H and others. 1985. Synthetic and natural Escherichia coli free lipid A express identical endotoxic activities. Eur J Biochem 148(1):1-5.

Galea I, Bechmann I, Perry VH. 2007. What is immune privilege (not)? Trends Immunol 28(1):12-8.

Galea I, Palin K, Newman TA, Van Rooijen N, Perry VH, Boche D. 2005. Mannose receptor expression specifically reveals perivascular macrophages in normal, injured, and diseased mouse brain. Glia 49(3):375-84.

Gangloff SC, Hijiya N, Haziot A, Goyert SM. 1999. Lipopolysaccharide structure influences the macrophage response via CD14-independent and CD14-dependent pathways. Clin Infect Dis 28(3):491-6.

Gangloff SC, Zahringer U, Blondin C, Guenounou M, Silver J, Goyert SM. 2005. Influence of CD14 on ligand interactions between lipopolysaccharide and its receptor complex. J Immunol 175(6):3940-5.

Gingras M, Gagnon V, Minotti S, Durham HD, Berthod F. 2007. Optimized protocols for isolation of primary motor neurons, astrocytes and microglia from embryonic mouse spinal cord. J Neurosci Methods 163(1):111-8.

Gjoen T, Seljelid R, Kolset SO. 1989. Binding of metastatic colon carcinoma cells to liver macrophages. J Leukoc Biol 45(4):362-9.

Golenbock DT, Hampton RY, Qureshi N, Takayama K, Raetz CR. 1991. Lipid A-like molecules that antagonize the effects of endotoxins on human monocytes. J Biol Chem 266(29):19490-8.

Gorden DL, Fingleton B, Crawford HC, Jansen DE, Lepage M, Matrisian LM. 2007. Resident stromal cell-derived MMP-9 promotes the growth of colorectal metastases in the liver microenvironment. Int J Cancer 121(3):495-500.

Gordon S. 2003. Alternative activation of macrophages. Nat Rev Immunol 3(1):23-35.

Gordon S, Taylor PR. 2005. Monocyte and macrophage heterogeneity. Nat Rev Immunol 5(12):953-64.

Hailman E, Lichenstein HS, Wurfel MM, Miller DS, Johnson DA, Kelley M, Busse LA, Zukowski MM, Wright SD. 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J Exp Med 179(1):269-77.

Hamann L, Alexander C, Stamme C, Zahringer U, Schumann RR. 2005. Acute-phase concentrations of lipopolysaccharide (LPS)-binding protein inhibit innate immune cell activation by different LPS chemotypes via different mechanisms. Infect Immun 73(1):193-200.

Hanisch UK. 2002. Microglia as a source and target of cytokines. Glia 40(2):140-55.

Hanisch UK, Johnson TV, Kipnis J. 2008. Toll-like receptors: roles in neuroprotection? Trends Neurosci 31(4):176-82.

Hanisch UK, Kettenmann H. 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci 10(11):1387-94.

Hanisch UK, Prinz M, Angstwurm K, Hausler KG, Kann O, Kettenmann H, Weber JR. 2001. The protein tyrosine kinase inhibitor AG126 prevents the massive microglial cytokine induction by pneumococcal cell walls. Eur J Immunol 31(7):2104-15.

Hasko G, Szabo C. 1998. Regulation of cytokine and chemokine production by transmitters and co-transmitters of the autonomic nervous system. Biochem Pharmacol 56(9):1079-87.

Häusler KG, Prinz M, Nolte C, Weber JR, Schumann RR, Kettenmann H, Hanisch UK. 2002. Interferon-gamma differentially modulates the release of cytokines and chemokines in lipopolysaccharide- and pneumococcal cell wall-stimulated mouse microglia and macrophages. Eur J Neurosci 16(11):2113-22.

Heinz S, Haehnel V, Karaghiosoff M, Schwarzfischer L, Muller M, Krause SW, Rehli M. 2003. Species-specific regulation of Toll-like receptor 3 genes in men and mice. J Biol Chem 278(24):21502-9.

Helander IM, Lindner B, Brade H, Altmann K, Lindberg AA, Rietschel ET, Zahringer U. 1988. Chemical structure of the lipopolysaccharide of Haemophilus influenzae strain I-69 Rd-/b+. Description of a novel deep-rough chemotype. Eur J Biochem 177(3):483-92.

Heneka MT, Galea E, Gavriluyk V, Dumitrescu-Ozimek L, Daeschner J, O'Banion MK, Weinberg G, Klockgether T, Feinstein DL. 2002. Noradrenergic depletion potentiates beta -amyloid-induced cortical inflammation: implications for Alzheimer's disease. J Neurosci 22(7):2434-42.

Heneka MT, Nadrigny F, Regen T, Martinez-Hernandez A, Dumitrescu-Ozimek L, Terwel D, Jardanhazi-Kurutz D, Walter J, Kirchhoff F, Hanisch UK and others. 2010. Locus ceruleus controls Alzheimer's disease pathology by modulating microglial functions through norepinephrine. Proc Natl Acad Sci U S A 107(13):6058-63.

Hermann C, Spreitzer I, Schroder NW, Morath S, Lehner MD, Fischer W, Schutt C, Schumann RR, Hartung T. 2002. Cytokine induction by purified lipoteichoic acids from various bacterial species--role of LBP, sCD14, CD14 and failure to induce IL-12 and subsequent IFN-gamma release. Eur J Immunol 32(2):541-51.

Heuff G, van de Loosdrecht AA, Betjes MG, Beelen RH, Meijer S. 1995. Isolation and purification of large quantities of fresh human Kupffer cells, which are cytotoxic against colon carcinoma. Hepatology 21(3):740-5.

Hiratsuka S, Nakamura K, Iwai S, Murakami M, Itoh T, Kijima H, Shipley JM, Senior RM, Shibuya M. 2002. MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. Cancer Cell 2(4):289-300.

Hiratsuka S, Watanabe A, Sakurai Y, Akashi-Takamura S, Ishibashi S, Miyake K, Shibuya M, Akira S, Aburatani H, Maru Y. 2008. The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. Nat Cell Biol 10(11):1349-55.

Hirotani T, Yamamoto M, Kumagai Y, Uematsu S, Kawase I, Takeuchi O, Akira S. 2005. Regulation of lipopolysaccharide-inducible genes by MyD88 and Toll/IL-1 domain containing adaptor inducing IFN-beta. Biochem Biophys Res Commun 328(2):383-92.

Hirschfeld M, Weis JJ, Toshchakov V, Salkowski CA, Cody MJ, Ward DC, Qureshi N, Michalek SM, Vogel SN. 2001. Signaling by toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. Infect Immun 69(3):1477-82.

Hoffmann C, Leitz MR, Oberdorf-Maass S, Lohse MJ, Klotz KN. 2004. Comparative pharmacology of human beta-adrenergic receptor subtypes--characterization of stably transfected receptors in CHO cells. Naunyn Schmiedebergs Arch Pharmacol 369(2):151-9.

Hristova M, Cuthill D, Zbarsky V, Acosta-Saltos A, Wallace A, Blight K, Buckley SM, Peebles D, Heuer H, Waddington SN and others. 2010. Activation and deactivation of periventricular white matter phagocytes during postnatal mouse development. Glia 58(1):11-28.

Hu X, Chakravarty SD, Ivashkiv LB. 2008. Regulation of interferon and Toll-like receptor signaling during macrophage activation by opposing feedforward and feedback inhibition mechanisms. Immunol Rev 226:41-56.

Hu XX, Goldmuntz EA, Brosnan CF. 1991. The effect of norepinephrine on endotoxin-mediated macrophage activation. J Neuroimmunol 31(1):35-42.

Hua F, Ma J, Ha T, Kelley J, Williams DL, Kao RL, Kalbfleisch JH, Browder IW, Li C. 2008. Preconditioning with a TLR2 specific ligand increases resistance to cerebral ischemia/reperfusion injury. J Neuroimmunol 199(1-2):75-82.

Huber M, Kalis C, Keck S, Jiang Z, Georgel P, Du X, Shamel L, Sovath S, Mudd S, Beutler B and others. 2006. R-form LPS, the master key to the activation ofTLR4/MD-2-positive cells. Eur J Immunol 36(3):701-11.

Jack CS, Arbour N, Manusow J, Montgrain V, Blain M, McCrea E, Shapiro A, Antel JP. 2005. TLR signaling tailors innate immune responses in human microglia and astrocytes. J Immunol 175(7):4320-30.

Jiang D, Liang J, Fan J, Yu S, Chen S, Luo Y, Prestwich GD, Mascarenhas MM, Garg HG, Quinn DA and others. 2005a. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. Nat Med 11(11):1173-9.

Jiang Z, Georgel P, Du X, Shamel L, Sovath S, Mudd S, Huber M, Kalis C, Keck S, Galanos C and others. 2005b. CD14 is required for MyD88-independent LPS signaling. Nat Immunol 6(6):565-70.

Jin S, Kawanokuchi J, Mizuno T, Wang J, Sonobe Y, Takeuchi H, Suzumura A. 2007. Interferon-beta is neuroprotective against the toxicity induced by activated microglia. Brain Res 1179:140-6.

Kagan JC, Su T, Horng T, Chow A, Akira S, Medzhitov R. 2008. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. Nat Immunol 9(4):361-8.

Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, MacDonald DD, Jin DK, Shido K, Kerns SA and others. 2005. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 438(7069):820-7.

Katakura T, Miyazaki M, Kobayashi M, Herndon DN, Suzuki F. 2004. CCL17 and IL-10 as effectors that enable alternatively activated macrophages to inhibit the generation of classically activated macrophages. J Immunol 172(3):1407-13.

Kawai T, Akira S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11(5):373-84.

Kenne L, Lindberg B, Soderholm E, Bundle DR, Griffith DW. 1983. Structural studies of the O-antigens from Salmonella greenside and Salmonella adelaide. Carbohydr Res 111(2):289-96.

Kilic U, Kilic E, Matter CM, Bassetti CL, Hermann DM. 2008. TLR-4 deficiency protects against focal cerebral ischemia and axotomy-induced neurodegeneration. Neurobiol Dis 31(1):33-40.

Kim S, Takahashi H, Lin WW, Descargues P, Grivennikov S, Kim Y, Luo JL, Karin M. 2009. Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. Nature 457(7225):102-6.

Kono H, Rock KL. 2008. How dying cells alert the immune system to danger. Nat Rev Immunol 8(4):279-89.

Landreth GE, Reed-Geaghan EG. 2009. Toll-like receptors in Alzheimer's disease. Curr Top Microbiol Immunol 336:137-53.

Lanzavecchia A, Sallusto F. 2002. Progressive differentiation and selection of the fittest in the immune response. Nat Rev Immunol 2(12):982-7.

Lehnardt S. 2010. Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. Glia 58(3):253-63.

Lehnardt S, Lehmann S, Kaul D, Tschimmel K, Hoffmann O, Cho S, Krueger C, Nitsch R, Meisel A, Weber JR. 2007. Toll-like receptor 2 mediates CNS injury in focal cerebral ischemia. J Neuroimmunol 190(1-2):28-33.

Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86(6):973-83.

Li DK, Paty DW. 1999. Magnetic resonance imaging results of the PRISMS trial: a randomized, double-blind, placebo-controlled study of interferon-beta1a in relapsing-remitting multiple sclerosis. Prevention of Relapses and Disability by Interferon-beta1a Subcutaneously in Multiple Sclerosis. Ann Neurol 46(2):197-206.

Linehan SA, Martinez-Pomares L, Stahl PD, Gordon S. 1999. Mannose receptor and its putative ligands in normal murine lymphoid and nonlymphoid organs: In situ expression of mannose receptor by selected macrophages, endothelial cells, perivascular microglia, and mesangial cells, but not dendritic cells. J Exp Med 189(12):1961-72.

Lohmann KL, Vandenplas ML, Barton MH, Bryant CE, Moore JN. 2007. The equine TLR4/MD-2 complex mediates recognition of lipopolysaccharide from Rhodobacter sphaeroides as an agonist. J Endotoxin Res 13(4):235-42.

Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, Washburn NR, Devera ME, Liang X, Tor M, Billiar T. 2007. The grateful dead: damage-associated molecular

pattern molecules and reduction/oxidation regulate immunity. Immunol Rev 220:60-81.

Macagno A, Molteni M, Rinaldi A, Bertoni F, Lanzavecchia A, Rossetti C, Sallusto F. 2006. A cyanobacterial LPS antagonist prevents endotoxin shock and blocks sustained TLR4 stimulation required for cytokine expression. J Exp Med 203(6):1481-92.

Maestroni GJ, Mazzola P. 2003. Langerhans cells beta 2-adrenoceptors: role in migration, cytokine production, Th priming and contact hypersensitivity. J Neuroimmunol 144(1-2):91-9.

Mantovani A, Allavena P, Sica A, Balkwill F. 2008. Cancer-related inflammation. Nature 454(7203):436-44.

Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. 2004. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 25(12):677-86.

Martinez FO, Sica A, Mantovani A, Locati M. 2008. Macrophage activation and polarization. Front Biosci 13:453-61.

Martinon F. 2008. Detection of immune danger signals by NALP3. J Leukoc Biol 83(3):507-11.

Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. 2007. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science 316(5831):1628-32.

Matzinger P. 2002. The danger model: a renewed sense of self. Science 296(5566):301-5.

Matzinger P. 2007. Friendly and dangerous signals: is the tissue in control? Nat Immunol 8(1):11-3.

Medzhitov R, Janeway CA, Jr. 2002. Decoding the patterns of self and nonself by the innate immune system. Science 296(5566):298-300.

Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388(6640):394-7.

Mertsch K, Hanisch UK, Kettenmann H, Schnitzer J. 2001. Characterization of microglial cells and their response to stimulation in an organotypic retinal culture system. J Comp Neurol 431(2):217-27.

Milanski M, Degasperi G, Coope A, Morari J, Denis R, Cintra DE, Tsukumo DM, Anhe G, Amaral ME, Takahashi HK and others. 2009. Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity. J Neurosci 29(2):359-70.

Mildner A, Schmidt H, Nitsche M, Merkler D, Hanisch UK, Mack M, Heikenwalder M, Bruck W, Priller J, Prinz M. 2007. Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions. Nat Neurosci 10(12):1544-53.

Moore KJ, Andersson LP, Ingalls RR, Monks BG, Li R, Arnaout MA, Golenbock DT, Freeman MW. 2000. Divergent response to LPS and bacteria in CD14-deficient murine macrophages. J Immunol 165(8):4272-80.

Mori K, Ozaki E, Zhang B, Yang L, Yokoyama A, Takeda I, Maeda N, Sakanaka M, Tanaka J. 2002. Effects of norepinephrine on rat cultured microglial cells that express alpha1, alpha2, beta1 and beta2 adrenergic receptors. Neuropharmacology 43(6):1026-34.

Mosser DM, Edwards JP. 2008. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8(12):958-69.

Nagai Y, Akashi S, Nagafuku M, Ogata M, Iwakura Y, Akira S, Kitamura T, Kosugi A, Kimoto M, Miyake K. 2002. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. Nat Immunol 3(7):667-72.

Nimmerjahn A, Kirchhoff F, Helmchen F. 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308(5726):1314-8.

Njie EG, Boelen E, Stassen FR, Steinbusch HW, Borchelt DR, Streit WJ. 2010. Ex vivo cultures of microglia from young and aged rodent brain reveal age-related changes in microglial function. Neurobiol Aging.

Noda M, Nakanishi H, Nabekura J, Akaike N. 2000. AMPA-kainate subtypes of glutamate receptor in rat cerebral microglia. J Neurosci 20(1):251-8.

O'Neill LA, Bowie AG. 2007. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol 7(5):353-64.

Okamura Y, Watari M, Jerud ES, Young DW, Ishizaka ST, Rose J, Chow JC, Strauss JF, 3rd. 2001. The extra domain A of fibronectin activates Toll-like receptor 4. J Biol Chem 276(13):10229-33.

Owens T. 2009. Toll-like receptors in neurodegeneration. Curr Top Microbiol Immunol 336:105-20.

Pajkrt D, Camoglio L, Tiel-van Buul MC, de Bruin K, Cutler DL, Affrime MB, Rikken G, van der Poll T, ten Cate JW, van Deventer SJ. 1997. Attenuation of proinflammatory response by recombinant human IL-10 in human endotoxemia: effect of timing of recombinant human IL-10 administration. J Immunol 158(8):3971-7.

Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature 458(7242):1191-5.

Parker LC, Prestwich EC, Ward JR, Smythe E, Berry A, Triantafilou M, Triantafilou K, Sabroe I. 2008. A phosphatidylserine species inhibits a range of TLR- but not IL-1beta-induced inflammatory responses by disruption of membrane microdomains. J Immunol 181(8):5606-17.

Perera PY, Vogel SN, Detore GR, Haziot A, Goyert SM. 1997. CD14-dependent and CD14-independent signaling pathways in murine macrophages from normal and CD14 knockout mice stimulated with lipopolysaccharide or taxol. J Immunol 158(9):4422-9.

Pineau I, Lacroix S. 2009. Endogenous signals initiating inflammation in the injured nervous system. Glia 57(4):351-61.

Pocock JM, Kettenmann H. 2007. Neurotransmitter receptors on microglia. Trends Neurosci 30(10):527-35.

Prinz M, Hausler KG, Kettenmann H, Hanisch U. 2001. beta-adrenergic receptor stimulation selectively inhibits IL-12p40 release in microglia. Brain Res 899(1-2):264-70.

Prinz M, Schmidt H, Mildner A, Knobeloch KP, Hanisch UK, Raasch J, Merkler D, Detje C, Gutcher I, Mages J and others. 2008. Distinct and nonredundant in vivo functions of IFNAR on myeloid cells limit autoimmunity in the central nervous system. Immunity 28(5):675-86.

Pukrop T, Dehghani F, Chuang HN, Lohaus R, Bayanga K, Heermann S, Regen T, Rossum DV, Klemm F, Schulz M and others. 2010. Microglia promote

colonization of brain tissue by breast cancer cells in a Wnt-dependent way. Glia 58(12):1477-89.

Qureshi N, Takayama K, Kurtz R. 1991. Diphosphoryl lipid A obtained from the nontoxic lipopolysaccharide of Rhodopseudomonas sphaeroides is an endotoxin antagonist in mice. Infect Immun 59(1):441-4.

Ransohoff RM, Perry VH. 2009. Microglial physiology: unique stimuli, specialized responses. Annu Rev Immunol 27:119-45.

Rehli M. 2002. Of mice and men: species variations of Toll-like receptor expression. Trends Immunol 23(8):375-8.

Ren L, Lubrich B, Biber K, Gebicke-Haerter PJ. 1999. Differential expression of inflammatory mediators in rat microglia cultured from different brain regions. Brain Res Mol Brain Res 65(2):198-205.

Ribes S, Ebert S, Regen T, Agarwal A, Tauber SC, Czesnik D, Spreer A, Bunkowski S, Eiffert H, Hanisch UK and others. 2010a. Toll-like receptor stimulation enhances phagocytosis and intracellular killing of nonencapsulated and encapsulated Streptococcus pneumoniae by murine microglia. Infect Immun 78(2):865-71.

Ribes S, Ebert S, Regen T, Czesnik D, Scheffel J, Zeug A, Bunkowski S, Eiffert H, Hanisch UK, Hammerschmidt S and others. 2010b. Fibronectin stimulates Escherichia coli phagocytosis by microglial cells. Glia 58(3):367-76.

Rietschel ET, Kirikae T, Schade FU, Mamat U, Schmidt G, Loppnow H, Ulmer AJ, Zahringer U, Seydel U, Di Padova F and others. 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. FASEB J 8(2):217-25.

Rifkin IR, Leadbetter EA, Busconi L, Viglianti G, Marshak-Rothstein A. 2005. Toll-like receptors, endogenous ligands, and systemic autoimmune disease. Immunol Rev 204:27-42.

Rosenbaum DM, Rasmussen SG, Kobilka BK. 2009. The structure and function of G-protein-coupled receptors. Nature 459(7245):356-63.

Rowe DC, McGettrick AF, Latz E, Monks BG, Gay NJ, Yamamoto M, Akira S, O'Neill LA, Fitzgerald KA, Golenbock DT. 2006. The myristoylation of TRIF-related adaptor molecule is essential for Toll-like receptor 4 signal transduction. Proc Natl Acad Sci U S A 103(16):6299-304.

Rudick RA, Goodkin DE, Jacobs LD, Cookfair DL, Herndon RM, Richert JR, Salazar AM, Fischer JS, Granger CV, Simon JH and others. 1997. Impact of interferon beta-1a on neurologic disability in relapsing multiple sclerosis. The Multiple Sclerosis Collaborative Research Group (MSCRG). Neurology 49(2):358-63.

Santos-Sierra S, Deshmukh SD, Kalnitski J, Kuenzi P, Wymann MP, Golenbock DT, Henneke P. 2009. Mal connects TLR2 to PI3Kinase activation and phagocyte polarization. EMBO J 28(14):2018-27.

Saraiva M, O'Garra A. 2010. The regulation of IL-10 production by immune cells. Nat Rev Immunol 10(3):170-81.

Schmid CD, Sautkulis LN, Danielson PE, Cooper J, Hasel KW, Hilbush BS, Sutcliffe JG, Carson MJ. 2002. Heterogeneous expression of the triggering receptor expressed on myeloid cells-2 on adult murine microglia. J Neurochem 83(6):1309-20.

Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J Biol Chem 274(25):17406-9.

Seong SY, Matzinger P. 2004. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. Nat Rev Immunol 4(6):469-78.

Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, Kimoto M. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med 189(11):1777-82.

Sierra A, Gottfried-Blackmore AC, McEwen BS, Bulloch K. 2007. Microglia derived from aging mice exhibit an altered inflammatory profile. Glia 55(4):412-24.

Siewe L, Bollati-Fogolin M, Wickenhauser C, Krieg T, Muller W, Roers A. 2006. Interleukin-10 derived from macrophages and/or neutrophils regulates the inflammatory response to LPS but not the response to CpG DNA. Eur J Immunol 36(12):3248-55.

Soulet D, Rivest S. 2008. Bone-marrow-derived microglia: myth or reality? Curr Opin Pharmacol 8(4):508-18.

Spencer SJ, Martin S, Mouihate A, Pittman QJ. 2006. Early-life immune challenge: defining a critical window for effects on adult responses to immune challenge. Neuropsychopharmacology 31(9):1910-8.

Sriram K, Matheson JM, Benkovic SA, Miller DB, Luster MI, O'Callaghan JP. 2006. Deficiency of TNF receptors suppresses microglial activation and alters the susceptibility of brain regions to MPTP-induced neurotoxicity: role of TNF-alpha. FASEB J 20(6):670-82.

Stenutz R, Weintraub A, Widmalm G. 2006. The structures of Escherichia coli O-polysaccharide antigens. FEMS Microbiol Rev 30(3):382-403.

Sternberg EM. 2006. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. Nat Rev Immunol 6(4):318-28.

Stewart CR, Stuart LM, Wilkinson K, van Gils JM, Deng J, Halle A, Rayner KJ, Boyer L, Zhong R, Frazier WA and others. 2010. CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. Nat Immunol 11(2):155-61.

Stolp HB, Dziegielewska KM, Ek CJ, Habgood MD, Lane MA, Potter AM, Saunders NR. 2005. Breakdown of the blood-brain barrier to proteins in white matter of the developing brain following systemic inflammation. Cell Tissue Res 320(3):369-78.

Stolp HB, Ek CJ, Johansson PA, Dziegielewska KM, Potter AM, Habgood MD, Saunders NR. 2007. Effect of minocycline on inflammation-induced damage to the blood-brain barrier and white matter during development. Eur J Neurosci 26(12):3465-74.

Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK, Suttles J. 2005. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. J Immunol 175(1):342-9.

Streit WJ, Xue QS. 2009. Life and death of microglia. J Neuroimmune Pharmacol 4(4):371-9.

Strittmatter W, Weckesser J, Salimath PV, Galanos C. 1983. Nontoxic lipopolysaccharide from Rhodopseudomonas sphaeroides ATCC 17023. J Bacteriol 155(1):153-8.

Sturm JW, Magdeburg R, Berger K, Petruch B, Samel S, Bonninghoff R, Keese M, Hafner M, Post S. 2003. Influence of TNFA on the formation of liver metastases in a syngenic mouse model. Int J Cancer 107(1):11-21.

Suzuki Y, Claflin J, Wang X, Lengi A, Kikuchi T. 2005. Microglia and macrophages as innate producers of interferon-gamma in the brain following infection with Toxoplasma gondii. Int J Parasitol 35(1):83-90.

Tanamoto K, Zahringer U, McKenzie GR, Galanos C, Rietschel ET, Luderitz O, Kusumoto S, Shiba T. 1984. Biological activities of synthetic lipid A analogs: pyrogenicity, lethal toxicity, anticomplement activity, and induction of gelation of Limulus amoebocyte lysate. Infect Immun 44(2):421-6.

Thieblemont N, Thieringer R, Wright SD. 1998. Innate immune recognition of bacterial lipopolysaccharide: dependence on interactions with membrane lipids and endocytic movement. Immunity 8(6):771-7.

Triantafilou M, Gamper FG, Haston RM, Mouratis MA, Morath S, Hartung T, Triantafilou K. 2006. Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting. J Biol Chem 281(41):31002-11.

Triantafilou M, Lepper PM, Briault CD, Ahmed MA, Dmochowski JM, Schumann C, Triantafilou K. 2008. Chemokine receptor 4 (CXCR4) is part of the lipopolysaccharide "sensing apparatus". Eur J Immunol 38(1):192-203.

Triantafilou M, Triantafilou K. 2002. Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. Trends Immunol 23(6):301-4.

Tsai HH, Frost E, To V, Robinson S, Ffrench-Constant C, Geertman R, Ransohoff RM, Miller RH. 2002. The chemokine receptor CXCR2 controls positioning of oligodendrocyte precursors in developing spinal cord by arresting their migration. Cell 110(3):373-83.

Tsan MF, Gao B. 2009. Heat shock proteins and immune system. J Leukoc Biol 85(6):905-10.

van Noort JM. 2007. Toll-like receptors as targets for inflammation, development and repair in the central nervous system. Curr Opin Investig Drugs 8(1):60-5.

van Noort JM, Bsibsi M. 2009. Toll-like receptors in the CNS: implications for neurodegeneration and repair. Prog Brain Res 175:139-48.

van Rossum D, Hanisch UK. 2004. Microglia. Metab Brain Dis 19(3-4):393-411.

van Rossum D, Hilbert S, Strassenburg S, Hanisch UK, Bruck W. 2008. Myelin-phagocytosing macrophages in isolated sciatic and optic nerves reveal a unique reactive phenotype. Glia 56(3):271-83.

Vasselon T, Hailman E, Thieringer R, Detmers PA. 1999. Internalization of monomeric lipopolysaccharide occurs after transfer out of cell surface CD14. J Exp Med 190(4):509-21.

Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, Nacken W, Foell D, van der Poll T, Sorg C and others. 2007. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med 13(9):1042-9.

Wake H, Moorhouse AJ, Jinno S, Kohsaka S, Nabekura J. 2009. Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. J Neurosci 29(13):3974-80.

Wang J, Shao Y, Bennett TA, Shankar RA, Wightman PD, Reddy LG. 2006a. The functional effects of physical interactions among Toll-like receptors 7, 8, and 9. J Biol Chem 281(49):37427-34.

Wang X, Rousset CI, Hagberg H, Mallard C. 2006b. Lipopolysaccharide-induced inflammation and perinatal brain injury. Semin Fetal Neonatal Med 11(5):343-53.

Weinstock-Guttman B, Ramanathan M, Zivadinov R. 2008. Interferon-beta treatment for relapsing multiple sclerosis. Expert Opin Biol Ther 8(9):1435-47.

Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249(4975):1431-3.

Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K and others. 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 301(5633):640-3.

Yang J, Ryu YH, Yun CH, Han SH. 2009. Impaired osteoclastogenesis by staphylococcal lipoteichoic acid through Toll-like receptor 2 with partial involvement of MyD88. J Leukoc Biol 86(4):823-31.

Ziegler G, Harhausen D, Schepers C, Hoffmann O, Rohr C, Prinz V, Konig J, Lehrach H, Nietfeld W, Trendelenburg G. 2007. TLR2 has a detrimental role in mouse transient focal cerebral ischemia. Biochem Biophys Res Commun 359(3):574-9.

Acknowledgment

I would like to sincerely thank my supervisor (Prof.) Uwe (Hanisch) for his excellent and tireless support. Only his believe in my competence made this present work possible. I know that we had our ups and downs but I will keep the time we had together in best memory!

I would also like to thank Prof. Wolfgang Brück for giving me the opportunity to work in his department, thereby having the chance to fully concentrate on my projects. For their support I also thank the members of my Thesis Committee, Prof. Fred Wouters and Prof. Mikael Simons.

Special thanks go to the members of the 'Hanisch lab', Dr. Denise van Rossum and Christiane Menzfeld for the scientific team work and Elke Pralle, Silke Strassenburg, Susanne Kieke and Arkadiusz Kominowski for excellent technical assistance and beyond. Elke, thank you for all these fantastic cakes!

Very special thanks belong to my dear colleague, cubicle neighbor and friend Jörg Scheffel! Together, we discovered the world of 'Weltforschung', moved once a year and prepared tons of 'Kaiser Glia'. I will miss our shoptalk in and outside the lab. And I will always owe Jörg for the license to use his ELISA macro!!!

Further, I would like to say hello to my former students Marilena, Natalia, Steffi and 'Volti' (also known as Jens). Thank you for teaching me to tech. We had a great time!

I would also like to say thank you to all the other colleagues and friends at the Neuropathology. I hope that some true friendships will persist also after I/we left Göttingen.

Although they were not directly involved during my doctoral fellowship I would like to use the opportunity and say thank you to two special people that influenced and guided my (early) life as a researcher profoundly and with lasting impressions – Dr. Nathalie Arbour from McGill University (now Université de Montréal) in Montreal, Canada and Dr. Volker Heussler from the Bernhard-Nocht-Institute in Hamburg, Germany. Hope to see you again very soon!

The CMPB/GGNB program I would like to thank for administrative and also financial support.

Last but most I have to thank the two most important women in my life – Jutta for supporting me and tolerating the chaos I (still) generate at home, and Heidemarie 'Mutti' Regen for simply being the best mom anyone can have. Thank you both – I love you!

Curriculum Vitae

Tommy Regen, M.Sc.
born December 4, 1979 in Forst/Lausitz, Germany

Current Professional Affiliation

Institute of Neuropathology, University Medicine Göttingen
Phone: +49-551-396622
E-mail: tommy.regen@med.uni-goettingen.de

Education

Doctoral fellow at the Göttingen Graduate School of Neurosciences and Molecular Biosciences (GGNB) within the doctoral program 'Molecular Physiology of the Brain' (CMPB)
02/2007 – present,

Institute for Neuropathology, University Medicine Göttingen
04/2006 – 01/2007

Lausitz University of Applied Sciences in Senftenberg, Germany,
Master of Science (M.Sc.) in Biotechnology, 03/2004 – 03/2006,
Bachelor of Science (B.Sc.) in Biotechnology, 09/2000 – 02/2004

Friedrich-Ludwig-Jahn-Gymnasium (High-School) in Forst, Germany
Received 'Abitur' (A-levels) in 06/1999

Practical Work Experience

Master Thesis at the Montreal Neurological Institute, McGill University, Montreal, QC, Canada, Research Group Dr. Antel, 04/2005 – 12/2005

Practical training period at Institute for Clinical Immunology, University of Leipzig, Germany, Research group Dr. Sack, 09/2004

Bachelor Thesis at the Montreal Neurological Institute, McGill University, Montreal, QC, Canada, Research group Dr. Antel, 08/2003 – 01/2004

Practical training period at Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany, Research Group Dr. Heussler (Malaria), 09/2002 – 02/2003

Scholarships

Bridging Fund for the finalization of doctoral studies, provided by the Göttingen Graduate School for Neurosciences and Molecular Biosciences (GGNB), 02/2010

Short stipend for the realization of the Master Thesis, provided by the German Academic Exchange Council (DAAD), 08/2005

Half stipend for the realization of the Bachelor Thesis, provided by DAAD, 09/2003

Extracurricular Activities

Foundation and Presidency of the departmental student organization for Biotechnology, Chemistry and Process Technology (BCV), 04/2001 – 07/2003

Member of the BCV faculty-council, 07/2001 – 06/2002

Professional Affiliations

Member of DECHEMA (German Society for Chemistry and Biotechnology) and VBIO (Association of German Biologists)

Göttingen, July 2010

Publications

Regen T, van Rossum D, Scheffel J, Kastriti ME, Revelo Nuncira N, Prinz M, Brück W, Hanisch UK (2010) Microglial TLR4 distinguishes between agonists: CD14 shapes responsiveness and responses. (in preparation)

Fitzner D, Schnaars M, van Rossum D, Krishnamoorthy G, Dibaj P, Regen T, Hanisch UK, Simons M (2010) Selective transfer of membrane vesicles containing encephalitogenic antigens from oligodendrocytes to microglia. *J Cell Sci* (submitted)

van Neerven S, Nemes A, Imholz P, Regen T, Denecke B, Johann S, Beyer C, Hanisch UK, Mey J (2010) Inflammatory cytokine release of astrocytes in vitro is reduced by all-trans retinoic acid. *J Neurochem* (submitted)

Orfaniotou F, Nadrigny F, Regen T, Werner HB, Steffens H, Kelm S, Griffiths IR, Mitkovski M, Hanisch UK, Kassmann CM, Nave KA (2010) Myelin-associated glycoprotein is inhibitory to microglial cells. *Proc Natl Acad Sci U S A* (under review)

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. *J Neuroimmunol* (Epub ahead of print)

van Neerven S, Regen T, Wolf D, Nemes A, Johann S, Beyer C, Hanisch UK, Mey J (2010) Inflammatory chemokine release of astrocytes in vitro is reduced by all-trans retinoic acid. *J Neurochem* (Epub ahead of print)

Pukrop T, Dehghani F, Chuang HN, Lohaus R, Bayanga K, Heermann S, Regen T, van Rossum D, Klemm F, Schulz M, Siam L, Hoffmann A, Trümper L, Stadelmann C, Bechmann I, Hanisch U.-K, Binder C (2010) Microglia promote colonization of brain tissue by breast cancer cells in a Wnt-dependent way. *GLIA* 58(12):1477-89

Flatz L, Rieger T, Merkler D, Bergthaler A, Regen T, Schedensack M, Bestmann L, Verschoor A, Kreutzfeldt M, Brück W, Hanisch UK, Günther S, Pinschewer D (2010) T cell-dependence of Lassa fever pathogenesis in humanized mice. *PLoS Pathog* 6(3):e1000836

Heneka MT, Nadrigny F, Regen T, Martinez-Hernandez A, Dumitrescu-Ozimek L, Terwel D, Jardanhazi-Kurutz D, Walter J, Kirchhoff F, Hanisch UK, Kummer MP (2010) Locus ceruleus controls Alzheimer disease pathology by modulating microglial functions through norepinephrine. *Proc Natl Acad Sci U S A* 107(13):6058-63

Ribes S, Ebert S, Regen T, Agarwal A, Tauber S, Czesnik D, Spreer A, Bunkowski S, Eiffert H, Hanisch UK, Hammerschmidt S, Nau R (2010) Toll-like receptor stimulation enhances phagocytosis and intracellular killing of nonencapsulated and encapsulated Streptococcus pneumoniae by murine microglia. *Infect Immun* 78(2):865-71

Ribes S, Ebert S, Regen T, Czesnik D, Scheffel J, Zeug A, Bunkowski S, Eiffert H, Hanisch UK, Hammerschmidt S, Nau R (2010) Fibronectin stimulates Escherichia coli phagocytosis by microglial cells. *GLIA* 58(3):367-76

Ribes S, Ebert S, Czesnik D, Regen T, Zeug A, Bunkowski S, Mildner A, Eiffert H, Hanisch UK, Hammerschmidt S, Nau R (2009) Toll-like receptor prestimulation increases phagocytosis of Escherichia coli DH5alpha and Escherichia coli K1 strains by murine microglial cells. *Infect Immun* 77(1):557-64

Sturm A, Amino R, van de Sand C, Regen T, Retzlaff S, Rennenberg A, Krueger A, Pollok JM, Menard R, Heussler VT (2006) Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science* 313(5791):1287-90

Arbour N, Lapointe R, Saikali P, McCrea E, Regen T, Antel JP (2006) A new clinically relevant approach to expand myelin specific T cells. *J Immunol Methods* 310(1-2):53-61