Plasma Factors as Endogenous Agonists and Modulators of TLR4 Signaling in Microglia

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Göttingen, April 2010

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(Signature)

LIST OF PUBLICATIONS

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ABSTRACT

Toll-like receptors (TLRs) comprise a family of pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs) – an assortment of evolutionary conserved structural motifs in microbes. Expressed by innate immune cells, such as CNS microglia, TLRs trigger complex gene activations and functional mechanisms of host defense. TLRs may additionally respond to certain endogenous molecules which are produced, released or structurally modified upon cellular and tissue injury and thereby serve as damage-associated molecular patterns (DAMPs). This study focused on selected plasma proteins associating with the coagulation cascade (thrombin^{HMWM}, fibronectin) or belonging to the humoral limb of the adaptive immune system (immunoglobulins). We found them to be TLR4-agonistic DAMP signals for microglia, analyzed the molecular requirements for their DAMP potential and characterized their dependence on TLR4, accessory receptors and intracellular signaling elements. Importantly, a confounding contamination by bacterial lipopolysaccharide (LPS) as the microbial TLR4 agonist could be ruled out. The recruitment of co-receptors into a TLR4-centered complex seems to contribute to ligand-specific responses, and reactive phenotypes can be further shaped individually by key cytokines. Together, these data demonstrate the versatility of microglial TLR4 mechanisms for sensing and interpreting a variety of distinct ligands. TLR4 could thus function as a decision maker for microglial responses to PAMPs and DAMPs in infectious and non-infectious situations.

ABSTRACT

ZUSAMMENFASSUNG

Toll-like Rezeptoren (TLRs) umfassen eine Familie von Mustererkennungsrezeptoren welche Pathogen-assoziierte molekulare Muster erkennen (PAMPs) – ein Repertoire von evolutionär konservierten strukturellen Motiven in Mikroben. TLRs werden von Zellen des angeborenen Immunsystems, wie ZNS-Mikroglia, exprimiert und lösen komplexe Gen Aktivierungen und funktionelle Mechanismen der Abwehr aus. Zusätzlich können TLRs auf bestimmte endogene Moleküle ansprechen, welche durch Zell- und Gewebsschädigung generiert, freigesetzt oder strukturell modifiziert werden und dadurch als Schädigungs-assoziierte molekulare Muster (DAMPs) wirken können. Diese Studie konzentrierte sich auf ausgewählte Plasmaproteine, die an der Koagulation beteiligt sind (Thrombin^{HMWM}, Fibronektin) oder zum humoralen Arm der erworbenen Immunität gehören (Immunoglobuline). Wir konnten zeigen, dass sie als TLR4-agonistische DAMP Signale wirken, untersuchten die molekularen Voraussetzungen für ihr DAMP Potential und charakterisierten ihre Abhängigkeit von TLR4, assoziierten Rezeptoren und intrazelluären Signalelementen. Eine störende Kontamination durch bakterielles Lipopolysaccharid (LPS), den mikrobiellen TLR4-Agonisten, konnte ausgeschlossen werden. Die Rekrutierung von Ko-Rezeptoren in einem TLR4-zentrierten Komplex scheint zu einer Liganden-spezifischen Antwort beizutragen und die resultierenden reaktiven Phänotypen können individuell durch Schlüsselzytokine moduliert werden. Zusammengefasst zeigen die Daten die Vielseitigkeit des mikroglialen TLR4-Systems, eine Auswahl von verschiedenen Liganden zu erkennen und zu interpretieren. TLR4 könnte deshalb ein Entscheidungsträger für mikrogliale Reaktionen auf PAMPs und DAMPs in infektiösen und nicht-infektiösen Situationen sein.

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ABBREVIATIONS

Antigen-presenting cell	-	APC
Amyloid β	-	Aβ
Alzheimer's disease	-	AD
Blood-brain barrier	-	BBB
Bovine serum albumin	-	BSA
Central nervous system	-	CNS
Cerebrospinal fluid	-	CSF
c-Jun N-terminal kinase	-	JNK
Column volume	-	cv
Cyclooxygenase	-	COX
Damage/danger-associated molecular pattern	-	DAMP
Dendritic cell	-	DC
Dithiothreitol	-	DTT
Dulbecco's modified Eagle's medium	-	DMEM
Endoglycosidase F	-	EndoF
Enhanced chemoluminescence	-	ECL
Enzyme-linked immunosorbent assay	-	ELISA
Extra domain A, B	-	EDA, EDB
Experimental Autoimmune Encephalomyelitis	-	EAE
Extracellular matrix	-	ECM
Fast performance liquid chromatography	-	FPLC
Fc receptor	-	FcR
Fetal calf serum	-	FCS
Fibronectin	-	FN
Fluorescein	-	FITC
Glycosylphosphatidylinositol	-	GPI
Hank's balanced salt solution	-	HBSS
H-D-Phe-Pro-Arg-chloromethylketone	-	PPACK
High mobility group box 1	-	HMGB1
IL-1 receptor-associated kinase	-	IRAK
Immune complex	-	IC
Immunoglobulin	-	Ig

ABBREVIATIONS

Immunoreceptor tyrosine-based activation motif	-	ITAM
Immunoreceptor tyrosine-based inhibitory motif	-	ITIM
Inducible NO synthase	-	iNOS
Interferon	-	IFN
Interferon-regulating factor	-	IRF
Interleukin	-	IL
Intravenous immunoglobulin	-	IVIG
Keyhole limpet hemocyanin	-	KLH
Lipopolysaccharide	-	LPS
LPS binding protein	-	LBP
Macrophage inflammatory protein 1α	-	MIP-1α (CCL3)
Major histocompatibility complex	-	MHC
Mannose receptor	-	ManR
Matrix-Assisted Laser Desorption/Ionization-Time of Flight-Mass Spetrometry	-	MALDI-TOF-MS
Melanoma differentiation-associated gene 5	-	MDA5
Minimal essential medium	-	MEM
Mitogen-activated protein kinase	-	MAPK
Molecular weight cut off	-	MWCO
Monocyte chemoattractant protein 1	-	MCP-1 (CCL2)
MyD88 adaptor-like/ TIR-associated protein	-	MAL/TIRAP
Myeloid differential protein 2	-	MD-2
Myeloid differentiation primary response gene (88)	-	MyD88
Natural killer cell	-	NK cell
Nitric oxide	-	NO
Oligoclonal bands	-	OCB
Organotypic hippocampal slice cultures	-	OHSC
Pathogen-associated molecular pattern	-	PAMP
Pattern recognition receptor	-	PRR
Peptidyl arginine deiminase	-	PAD
Parkinson's diseases	-	PD
Phosphate-buffered saline	-	PBS
Poly-L-lysin	-	PLL
Ployvinylidenfluoride	-	PVDF

ABBREVIATIONS

Protease-activated receptor	-	PAR
Receptor for advanced glycation end products	-	RAGE
Receptor-interacting protein-1	-	RIP1
Regulatory on activation, normal T cell expressed and secreted	-	RANTES
Retinoic acid inducible gene I	-	RIG I
Spleen tyrosine kinase	-	syk
Stroma-derived factor	-	SDF
TAK1 binding protein	-	TAB
T helper	-	Th
Thrombin high molecular weight material	-	thrombin^{HMWM}
TIR-domain-containing adapter-inducing interferon-β/ TIR-domain-containing adaptor molecule 1	-	TRIF/TICAM1
TIR-domain-containing adaptor molecule/ TRIF-related adaptor molecule 2 (TRAM/TICAM2)	-	TRAM/TICAM2
TNF receptor associated factor	-	TRAF
Toll-interleukin 1 receptor	-	TIR
Toll-like receptor	-	TLR
Transforming growth factor β	-	TGFβ
Transforming growth factor-b-activated kinase-1	-	TAK1
Trifluoroacetic acid	-	TFA
Tris(2-carboxyethyl)phosphine	-	TCEP
Tryptophan	-	Trp
Tumor necrosis factor alpha	-	TNFα

1. INTRODUCTION

1.1 Innate and adaptive immunity – the two arms of (and for) defense

The immune system guarantees protection against infection as well as endogenous threats as arising from dysregulated cells. In higher vertebrates, it is based on two major arms, innate and adaptive immunity, which reached in mammals an enormous complexity of specialized cells and molecules. Both (co)operate in the containment and elimination of microbes and parasites, the neutralization of toxins or the fight against tumors. Adaptive (specific, acquired) immunity is carried by T cells with helper, effector and regulatory functions and antibody-producing B cells. T and B cell-mediated cellular and molecular defense mechanisms reveal clonal diversity due to the cell-individual generation of receptors and antibodies for the recognition of a nearly infinite number of antigenic structures. The most effective clones are then selected during an immune response. Moreover, adaptive immunity comes with the feature of increased defense capacity upon reencounter of an antigen, based on the formation of an immunological memory.

The evolutionary older innate (also known as natural or native) immunity is built up on physicochemical barriers of the epithelia, natural killer (NK) cells, proteins with diverse signal and effector functions, namely the complement system, but especially also on phagocytic cells, such as macrophages. It organizes for initial reactions against microbes, employing cytotoxic mechanisms, phagocytic clearance and intracellular killing of pathogens and instructs adaptive immune cells. Presentation of antigens on the surface of antigen-presenting cells (APCs, like macrophages or dendritic cells, DCs) triggers naive T cell activation, clonal expansion as well as differentiation into effector T cells. Innate immune cells are thereby needed for the development of cell-mediated adaptive immunity and the production of antibodies which neutralize toxins or bind microbes. Besides antigen presentation at different steps of an immune response, cells of the innate immunity are also recruited by the adaptive immunity to serve as effector cells. Clearance and killing involve cells, like the macrophages, expressing Fc receptors (FcRs) for the various immunoglobulin (Ig) (sub)classes (isotypes) that can induce a range of effector functions.

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1.2 Microglia as the innate immune cells of the CNS

Microglial cells are resident macrophages of the brain, spinal cord and associated organs (Hanisch and Kettenmann, 2007; van Rossum and Hanisch, 2004). Unlike other glia cells and neurons, they derive from myeloid progenitors in the bone marrow and colonize the CNS in two phases during embryogenesis and early postnatal days (Chan et al., 2007). The replenishment in the adult brain is still under debate (Mildner et al., 2007), but it is likely that they comprise a rather stable, yet not homogenous cell population (Hanisch and Kettenmann, 2007). Estimates in mice calculate 3.5×10^6 cells, with a density ranging between 5% and 13% of parenchymal cells in the cerebral cortex and substantia nigra, respectively (Lawson et al., 1990).

In the healthy adult CNS, the 'resting' microglia present with a small cell body and ramified processes. However, these cells are not inactive. They constantly scan their environment with highly motile processes, each cell covering its territory (Davalos et al., 2005; Nimmerjahn et al., 2005). The behavior completely changes upon, e.g., induction of small lesions. Microglial behavior shifts from apparently random scanning towards a directed migration to the site of injury. This process is formally called microglial 'activation', occurs upon most diverse challenges and is assumed to lead via a stepwise transformation to alerted and finally fully reactive states (Hanisch and Kettenmann, 2007). *In vitro* and *in vivo* studies revealed that reactive microglia can display a plethora of activities, including local proliferation, phagocytosis or antigen presentation with increased expression of various surface structures, such as major histocompatibility complex (MHC) class I and II, cell adhesion molecules or receptors for multiple soluble factors. Furthermore they respond with the release of various neuro- and immunoregulatory cytokines and chemokines (Hanisch, 2002; Prinz et al., 1999; van Rossum and Hanisch, 2004; van Rossum et al., 2008).

1.3 Microglial responses are highly regulated

The general concept of microglial activation was dominated for quite some time by the assumption that cells would respond in a stereotype fashion, exhibiting a rather neurotoxic phenotype with little room for adjustment. The view arose from the observations that microglial activation often correlated with neuropathological changes. *In vitro* and *in vivo* studies proved the toxic potential of microglia for neurons and oligodendrocytes (Hanisch

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and Kettenmann, 2007; Schwartz et al., 2006). However, the assessment of pathological tissue may bias towards cases in which microglia failed to maintain CNS homeostasis. Conceivably, most temporary and focally restricted episodes of microglial activation are never noticed as they do not present with clinical symptoms. On the other hand, isolated experimental use of infection-related stimuli (like the bacterial cell wall component lipopolysaccharide [LPS]) will drive defense-oriented responses, which come with toxic activities. Such experiments do not reflect situations within a tissue where a multitude of influences allows for complex signaling context.

Meanwhile, however, microglial activities are acknowledged for their primarily protective contribution, and the process of microglial 'activation' is seen with more variety in the actual response profiles. It is most likely also not a monophasic event and does not follow a stereotype (Hanisch and Kettenmann, 2007; van Rossum and Hanisch, 2004). Moreover, CNS structures exhibit a high degree of functional specificity, but posses only limited capacities for renewal and regeneration. Thus, microglia and their activities, like inflammatory reactions and immune processes in general, require tight control. Microglia-associated impairment can result from excessive acute (overshooting), chronic (not properly terminated) or maladapted reactions, i.e. when the chosen reactive phenotype is inappropriate (Schwartz et al., 2006).

The concept of phenotype diversity is mainly based on experimental findings on extraneural macrophages, but essentially applies also to microglia. In adopting the nomenclature of T helper (Th) cells, macrophages were initially characterized as M1 (classically activated) or M2 (alternatively activated) according to their functional polarization. Stimulation with the Th1 master cytokine, interferon- (IFN) γ and/or LPS leads to a M1 phenotype with a rather proinflammatory cytokine and chemokine profile, including interleukin-12 (IL-12, especially the p35/p40 heterodimer, p70), IL-6 and tumor necrosis factor alpha (TNFα), production of nitric oxide (NO) and expression of MHC II – but no IL-10. An 'alternative' activation is obtained by stimulation with the Th2 master cytokines IL-4 or IL-13, showing a more anti-inflammatory release profile with a IL-10^{high}/IL-12^{low} signature or transforming growth factor (TGF) β production and expression of characteristic surface receptors, such as mannose receptor (ManR) (Gordon and Taylor, 2005; Mantovani et al., 2004). Macrophages of these distinct polarities often reveal inverse expression of several key molecules, in addition to the IL-10/IL-12 pair. They cover enzymes for alternative metabolic pathways or the generation of mediators (e.g. arginase versus inducible NO synthase [iNOS] cyclooxygenases [COX] 1 versus 2) or

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receptors for soluble factors, cell-cell and cell-matrix interactions (e.g. ManR, CD163). In other cases, the very same factor can be expressed by both M1 and M2 cells, suggesting that the substantially different functional behavior of macrophages – promotion of pro- or antiinflammatory processes, support of either Th1 or Th2 types of immune responses, orientation for defense or tissue repair – is rather built on the ensemble of simultaneously expressed genes. Indeed, the M1/M2 paradigm soon turned out to be insufficient to describe the complex, partially reciprocal, partially overlapping inductions of genes and functional features. Further subvariants got introduced (Gordon and Taylor, 2005), but macrophages may rather come in major global orientations, such as for homeostasis, defense, repair and immune regulation, on which adapted functional profiles are established by tailored assembly of expressed genes (Mosser and Edwards, 2008). Although little is known for microglia, increasing evidence points to a similarly versatile response organization (Hanisch and Kettenmann, 2007; van Rossum and Hanisch, 2004; van Rossum et al., 2008). Their functional phenotypes would thus depend on the stimulus and its context. Tissue architecture (proximity to myelinated and blood-brain barrier [BBB] structures, extracellular matrix [ECM]), biochemical milieu and the cellular neighborhood (neurons, astrocytes, oligodendrocytes, endothelial cells, infiltrating neutrophils, T and B cells, monocytes/macrophages) would instruct and adjust microglial behavior (Hanisch and Kettenmann, 2007).

Microglia are prepared to recognize a wide range of molecules which indicate a disturbance of the homeostasis. The activation process thereby follows two distinct signaling principles (Biber et al., 2007; Hanisch and Kettenmann, 2007; van Rossum and Hanisch, 2004). Certain constitutive signals, e.g. expressed by neurons, provide a calming influence to keep the surveillance state – such as CD200, CX_3CL1 (fraktalkine) or SIRPα, which have complementary receptors on microglia, i.e. CD200R, CX_3CR1 and CD47 (Barclay et al., 2002; Matozaki et al., 2009). Such factors may serve in an 'off' signaling. Disruption of their signaling would lead to activation. Microglia could thus sense a dangerous situation with neuronal impairment without having particular receptors for the danger molecule itself. Furthermore, microglia express several neurotransmitter receptors to sense neuronal activity (Färber and Kettenmann, 2006; Pocock and Kettenmann, 2007). In contrast, 'on' signals are factors that are usually not present or 'visible' for microglia (Hanisch and Kettenmann, 2007). Their sudden appearance, change in concentration or molecular format would then drive an activation through already

expressed receptors. By 'on' and 'off' signaling, microglia could readily respond to known as well as unknown signs of homeostatic disturbance.

1.4 Toll-like receptors (TLRs) for pattern recognition

Microglia serve in the first line to defend the CNS against viral or bacterial invasion. Much of their sensor and effector functions depend on receptors that had evolved to recognize and fight infection. Pattern recognition receptors (PRRs) in general are sensing pathogen-associated molecular patterns (PAMPs). They allow the innate immune system to detect invading germs. Furthermore, they are important for the control of the adaptive immunity and to govern the cooperative activities of effector cells (Hanisch et al., 2008; Padovan et al., 2007; Sutmuller et al., 2006). PRRs are also increasingly understood to organize for responses to tissue damage, to serve neuroprotective functions beyond antimicrobial activities and to have implications in neurogenesis (Hanisch et al., 2008).

PRRs come in major families with surface or intracellular expression. First, there are the lectin-type (ManR, ß-glucan receptors) receptors. The second family comprises nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs, with more than 20 members of NOD receptors and NALPS). Third, receptors identifying by RNA helicase and caspase-recruitment domains (CARD), such as retinoic acid inducible gene (RIG) I and melanoma differentiation-associated gene 5 (MDA5), constitute the RIG-I-like receptors (RLR). Finally, there are the Toll-like receptors (TLR) (Creagh and O'Neill, 2006; Kumar et al., 2009).

The feature of recognizing a 'pattern', i.e. a structural motif which is common to many bacterial strains or viruses, fungi and protozoans, appears to be an innovative achievement for two reasons. First, the recognized motifs often relate to molecules which cannot be varied easily by the microbes as they are involved in essential cell structures and functions, such as cell walls, envelopes or genomic DNA/RNA. Only at the expense of impaired vitality or functionality these core elements could be mutated to escape detection by PRRs. Second, by 'extracting' these essential keys, the innate immune cell can afford to express a still limited number of PRRs while covering a wide range of common pathogens. It is thus important to bear in mind that the promiscuous 'pattern' recognition is not misinterpreted as 'non-specific' interaction.

TLRs are evolutionary conserved molecules, and the original Toll receptor was discovered in *Drosophila melanogaster* for its importance in the establishment of the

dorsoventral polarity. Subsequently, mutations in Toll signaling were found to increase the susceptibility to fungal infections, thus linking Toll to immune responses (Akira and Takeda, 2004; Lemaitre et al., 1996). Until today, more than a dozen homologues proteins were discovered in man and mice. They are expressed on the cell surface or in the endosomal compartment of various cell types, including neural cells (Tab. 1.4.1.). TLRs recognize an assortment of evolutionary conserved motifs found in lipids, glycolipids, lipopeptides, proteins, RNA and DNA of microbial origin. TLR signaling triggers production of inflammatory cytokines and chemokines that help to recruit, activate or instruct cells of the innate and adaptive immune system. Thus, deficiency in TLRs comes with an increased susceptibility to infection.

TLR signaling involves a complex network of several sorting and signaling adapter proteins, protein kinases and transcription factors, which are more and more understood – also in terms of cross-interactions among TLR and non-TLR pathways (Kumar et al., 2009). Signaling is mediated by a set of Toll-interleukin 1 receptor- (TIR) domain-containing adapter molecules i.e. myeloid differentiation primary response gene 88 (MyD88), TIR-containing adaptor protein/MyD88-adaptor-like (TIRAP/MAL), TIR-containing adaptor inducing interferon-β (IFNβ)/TIR-domain-containing adaptor molecule 1 (TRIF/TICAM1) and TIR-domain-containing adaptor molecule/TRIF-related adaptor molecule 2 (TRAM/TICAM2) (Fig. 1.4.1.). Upon TLR activation, they are recruited in a specific combination to organize further downstream events. Two major routes of TLR signaling are described – the MyD88-dependent and the MyD88-independent (TRIF-dependent) signaling pathway. TLRs signal via the MyD88-dependent route, except TLR3 that solely depends on TRIF, whereas TLR4 is the only family member enjoying use of both adapters (Yamamoto et al., 2003). MyD88 signaling from membrane-associated TLRs leads to activation of members of the IRAK (IL-1 receptor-associated kinase) family of protein kinases i.e. IRAK1 and IRAK4 (Akira and Takeda, 2004). The phosphorylated complex of IRAK1/IRAK4 interacts with TNF receptor associated factor (TRAF) 6, that recruits a protein kinase complex involving TAK1 (transforming growth factor-b-activated kinase-1) and TABs (TAK1 binding proteins). The TAK1 complex then sorts the signaling into various pathways leading to the activation of the NFκB system or mitogen-activated protein kinase (MAPK) pathways, involving JNKs (c-Jun N-terminal kinases), p38^{MAPK} and p42/44^{MAPK}, to induce genes encoding for various proinflammatory factors. The NFκB signaling via the MyD88-dependent route is assumed to be an early event in the TLR signaling (regarded early phase).

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Tab. 1.4.1.: TLRs and ligands. TLRs recognize an assortment of evolutionary conserved motifs found on pathogens of the microbial origin (PAMPs). Some may also recognize endogenously derived molecules (DAMPs). Several TLRs are expressed by/in neural cells. Only major elements and features are listed.

TLR	expression in neural cells	compartment	exogenous ligands (PAMPs)	endogenous ligands (DAMPs)
TLR1/2	microglia astrocytes	plasma membrane	lipoproteins triacylated lipopeptides zymosan	unknown
TLR2*	oligodendrocytes	plasma membrane	bacterial lipoproteins lipoarabinomannan glycolipids	heat shock proteins HMGB1 GPI-anchored proteins
TLR6/2	microglia astrocytes	plasma membrane	MALP-2 (mycoplasmal macrophage-activating lipopeptide) diacylated lipopeptides zymosan	unknown
TLR3	microglia astrocytes oligodendrocytes neurons	endosomes	double-stranded RNA poly(I:C) mRNA/tRNA	unknown
TLR4	microglia astrocytes (neurons)	plasma membrane	lipopolysaccharide (LPS), lipid A, flavolipin monophosphoryl lipid A glycoinositolphospholipids	hyaluronate HMGB1, heparan EDA fibronectin, fibrinogen heat shock proteins
TLR5	microglia astrocytes	plasma membrane	flagellin	unknown
TLR7	microglia	endosomes	imiquimod, gardiquimod single-stranded RNA polyuridylic acid	unknown
TLR8	microglia	endosomes	single-stranded RNA polyuridylic acid	unknown
TLR9	microglia astrocytes	endosomes	unmethylated CpG DNA CpG ODNs	IgG-chromatin complexes
TLR10	unknown	plasma membrane	unknown	unknown
TLR11	unknown	plasma membrane	profilin	unknown
TLR12	unknown	plasma membrane	unknown	unknown
TLR13	unknown	plasma membrane	unknown	unknown

Abbreviations: GPI – Glycosylphosphatidylinositol; HMGB1 - high mobility group box 1
* usually forms heterodimers with TLR1 or TLR6, homodimers are suspected,
  association for recognition of endogenous ligands is unknown

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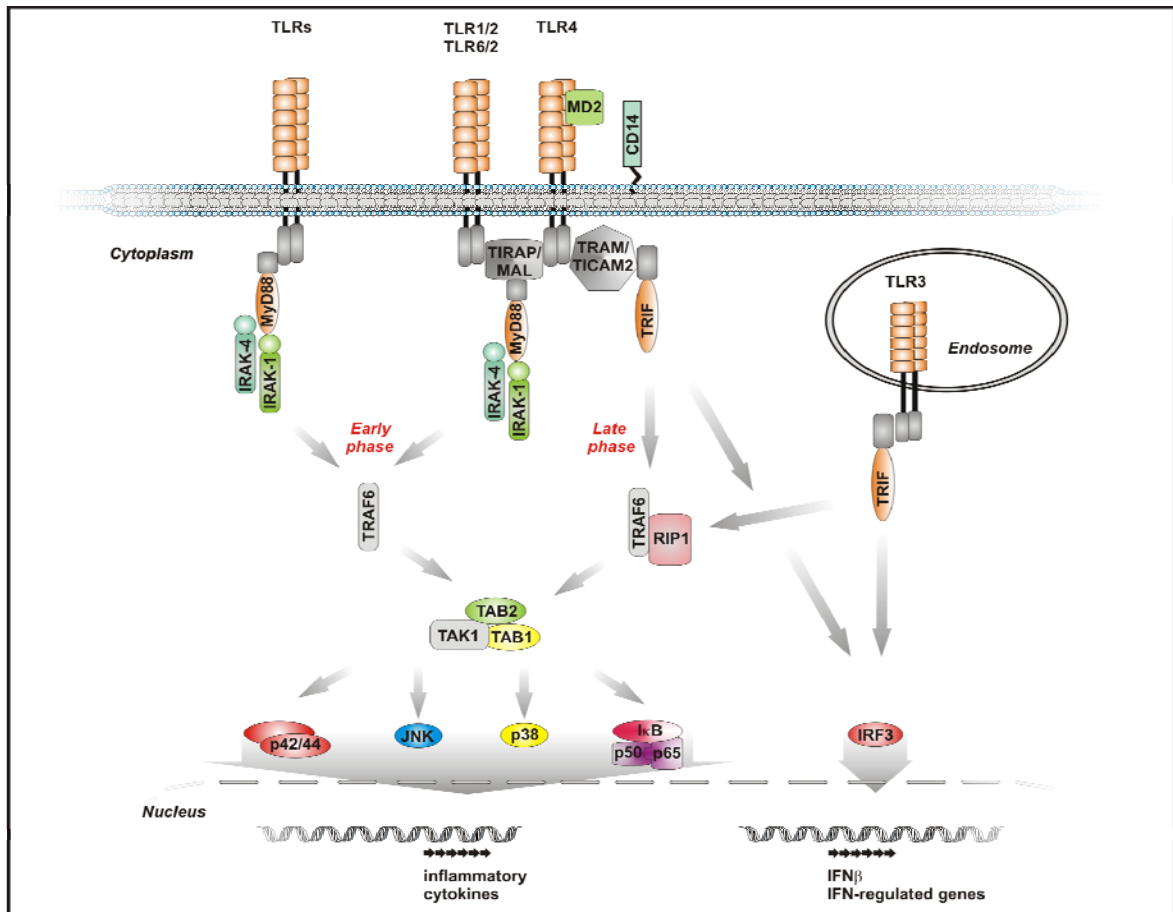
Fig. 1.4.1.: TLR signaling pathways. TLR signaling is mediated by the TIR-domain-containing adapter molecules TIRAP/MAL, TRAM/TICAM2, MyD88 and TRIF. They are recruited in a specific combination upon TLR activation. TLRs signal via MyD88 except for TLR3 which depends on TRIF. TLR4 is the only TLR which utilizes both, the MyD88- and the TRIF-dependent pathways, involving TIRAP/MAL and TRIF/TICAM2, respectively. MyD88 activation recruits IRAK1 and IRAK4 to phosphorylate TRAF6 and to activate the TAK1 complex, which then sorts the signaling into various pathways, involving NFκB, JNKs, p38^{MAPK} and p42/44^{MAPK} to control inflammatory responses by the induction of proinflammatory factors. MyD88-induced activation of NFκB is assumed to be an early event in TLR4 signaling. The TRIF-dependent pathway primarily induces an IFNβ response via IRF3. However, TRIF also interacts with TRAF6 and RIP1 to mediate the late phase activation of NFκB. This simplified scheme shows only major elements of TLR signaling.

The MyD88-independent TRIF pathway is basically known to induce IFNβ via the interferon-regulating factor (IRF) 3. However, the TRIF-mediated signaling also activates NFκB (Akira and Takeda, 2004) by recruitment of TRAF6 and RIP1 (receptor-interacting protein-1). NFκB activation via this pathway is assumed to be slower (regarded late phase) (Yamamoto et al., 2003). MyD88 and TRIF signaling would thus occur sequentially – the former at the plasma membrane, the latter upon receptor internalization (Kagan et al., 2008). However, MyD88 and TRIF pathways cooperate more tightly than previously assumed. For example, depending on the LPS chemotype (structural variant), TLR4 is

capable to vary the quality of signaling, preferring either TRIF or MyD88 or both (Jiang et al., 2005).

1.5 TLRs are sensors of damage signals

In the classical view, TLRs are sensors of microbial structures. They initiate and promote innate immune reactions and organize further adaptive immune responses (Creagh and O'Neill, 2006). However, TLRs do not only help to mount responses against PAMPs but also sense certain host-derived molecules that are collectively described as damage or danger-associated molecular patterns (DAMPs). The idea that endogenous molecules can induce immune responses is based on the 'danger model' proposed by Matzinger (Matzinger, 1994). Accordingly, an immune response is initiated when cells of the innate immune system would sense molecules that are produced and/or released into the extracellular space upon tissue damage and cell injury. Damage could arise even in the absence of an infectious scenario, as a result of cellular stress that finally leads to necrosis. Interestingly, responses to infection and damage are quite similar. A reason for this similarity might be that infection often follows a trauma. Microbes easily penetrate wounds. Thus, it would be efficient to respond to damage and infection in a similar way. DAMPs may amplify or prepare for stronger responses to PAMPs, while triggering emergency programs also in their absence. However, both, infection and trauma can lead to further tissue damage as well as cell death. Yet the actual outcome may vary with the particular DAMP and situation, ranging from support of repair mechanisms to aggravated inflammation.

There is increasing evidence for the recognition of endogenous ligands by TLRs, especially TLR2 and TLR4 (Beg, 2002; Rifkin et al., 2005; Seong and Matzinger, 2004) (Tab. 1.4.1). For example, members of the heat-shock protein family, i.e. HSP60, HSP70 and GP96, were shown to be strong activators of inflammatory responses in APCs via TLR4 and TLR2 (Basu and Srivastava, 2000; Ohashi et al., 2000; Roelofs et al., 2006). DAMPs are not only generated upon non-apoptotic cell death i.e. necrosis. Some can be actively released by cells of the innate immune system, like macrophages or microglia. High-mobility group box 1 (HMGB1) is a chromatin-associated protein and can be released by either necrotic cells or actively from hematopoetic cells via a lysosome-mediated pathway in response to cellular stress (Rubartelli and Lotze, 2007). It interacts with the receptor for advanced glycation end products (RAGE), but is also suggested to

be a potent TLR4/TLR2 ligand (Apetoh et al., 2007). Similar DAMP properties are suggested for S100A8 and S100A9 (Mrp8/Mrp14) (Vogl et al., 2007). In addition to intracellular factors which are released under unphysiological conditions, quite a range of molecules with diverse functions can qualify as a DAMP to indicate tissue injury. Heparin sulfate, soluble hyaluronate or fibronectin (fragments), which are usually bound in the ECM, are suggested to induce inflammatory responses via TLR4. ECM degradation by tissue disruption or cell migration may thus provide an extracellular source of DAMPs.

Factors circulating in the blood could represent another pool of (latent) DAMPs. Especially for the CNS as being shielded by the BBB, their appearance in the parenchyma would indicate vascular leakage. Rapid microglial responses to even small blood vessel lesions have been demonstrated (Nimmerjahn et al., 2005), and inundating blood content has multiple consequences for neurons and glial cells (Gingrich and Traynelis, 2000). Yet little is known about the nature of plasma factors exerting microglial activity, although coagulation-associated proteins can exhibit such a capacity (Hanisch et al., 2004). Fibrinogen (coagulation factor I) was thereby already linked to a TLR4-dependent effect in macrophages (Hodgkinson et al., 2008; Smiley et al., 2001). The present study thus aimed to identify plasma factors with a TLR(4)-agonistic DAMP character for microglia.

It should be emphasized that studies on endogenous TLR ligands always bear the risk (or raise the suspicion) of confounding contaminations by microbial agents. Bacterial LPS is a frequent impurity in recombinant proteins and commercial preparations and may account for cellular effects intentionally assigned to DAMPs (Weinstein et al., 2008b). Thus, the use of appropriate controls is indispensable for distinguishing cellular responses to DAMPs from responses to PAMPs.

1.6 TLRs in the CNS – a double-edged sword

TLRs are expressed in various CNS cells (Hanisch et al., 2008) (Tab. 1.4.1.), and their activation has been frequently associated with detrimental effects for the tissue, as in infectious situations. TLR-driven responses, especially by microglia and to lesser extent astrocytes, were directly shown to be neurotoxic *in vitro* and *in vivo*. For instance, microglia stimulated with LPS *in vitro* is toxic to neurons and oligodendrocytes in a TLR4-dependent manner (Lehnardt et al., 2003). Local administration of LPS into the CNS triggers a TLR4-mediated activation of micro- and astroglia and results in a severe loss of neurons in the hippocampus and the substantia nigra (Carpentier et al., 2008). Similar

observations were made for TLR2 (Hoffmann et al., 2007). Toxicity appears to be mediated via NO. Both microglia and astrocytes upregulate iNOS in response to TLR stimulation, and pharmacological interference is able to prevent neuronal death in the presence of activated glial cells (Lehnardt et al., 2006). TLR9 activation is associated with the induction of meningitis. Patients suffering from bacterial meningitis showed increased levels of fibronectin (FN) in the cerebrospinal fluid (CSF). Costimulation of TLR2, TLR4 and TLR9 with FN, in the presence of IFNγ, resulted in an enhanced inflammatory profile of the cells, suggesting synergistic contribution to the neuronal damage in meningitis (Goos et al., 2007). Furthermore, TLRs might be involved in several neurodegenerative diseases, such as in Alzheimer's and Parkinson's diseases (AD, PD), as well as in autoimmune diseases, like multiple sclerosis. Increased expression of TLRs was described in brains of AD as well as in the brains and spinal cord of multiple sclerosis patients. In AD, the uptake of amyloid β (Aβ) has been demonstrated to be modulated and partially dependent on TLRs. TLR binding of Aβ may promote inflammatory cascades and the deposition of Aβ fibrils (Tahara et al., 2006). HMGB1, by itself a potential TLR4 and TLR2 ligand, is elevated in AD and negatively influences Aβ uptake of microglial cells (Takata et al., 2003). In experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, MyD88 was shown to be important for disease induction, while signaling of TLR3, TLR4 and TLR9 contributed to innate immune cell activation, promotion of differentiation of Th17 cells and thus pronounced inflammation (Marta et al., 2009).

However, TLRs may also participate in mechanisms of neuroprotection as well as neurogenesis. Considering the vulnerability to inflammation and the limited regenerative potential of the CNS, immune responses need a tight control to limit self damage. Regarding TLRs, the simplest way to contain their excessive activity is the proper termination of signaling. This can be achieved by elimination or degradation of the agonistic signal, e.g. by phagocytosis. TLR stimulation promotes phagocytic activity and autophagy in macrophages (Sanjuan et al., 2007). Microglia revealed enhanced phagocytic activity for bacteria when co-stimulated with high doses of FN (Ribes et al., 2010). TLRs might have a substantial impact on the removal of Aβ plaques in AD (Tahara et al., 2006). Thus, clearance of germs as part of the host defense could simultaneously organize for the disappearance of the pro-inflammatory signals, a principle likely applying also to non-microbial, i.e. endogenous material. On the other hand, TLR signaling can also be eliminated even in the presence of the stimulus, e.g. by rapid internalization of the receptor. Prolonged or repeated stimulation with LPS leads to a desensitization of

microglia or macrophages, a phenomenon called endotoxin tolerance (Biswas and Lopez-Collazo, 2009; Mantovani et al., 2007). However, receptor internalization does not only lead to a general desensitization but also to modifications of the intracellular signaling (Kagan et al., 2008). Down-regulation of inflammatory signals may synergize with a delayed induction of anti-inflammatory mediators. Microglial cells exert a late IL-10 release when stimulated for a prolonged period with TLR agonists (unpublished data). Several proteins and principles were recently identified to organize for self limitation and control over TLR signaling (An et al., 2008; Choi et al., 2006; O'Neill, 2008).

Conceivably, in addition to the clearance of infectious agents TLRs may also contribute to neuroprotection. Their signaling appears to be effective and essential in supporting CNS cell survival, tissue preservation, CNS functioning and even neurogenesis (Glezer et al., 2007; Hanisch et al., 2008; Kigerl et al., 2007; Simard and Rivest, 2007). TLR engagement is thus not per se detrimental or mainly driving bystander damage in host defense. After all, the original Toll in Drosophila was discovered for its morphogenetic functions first. In summary, TLR engagement can have a detrimental as well as a beneficial outcome for the CNS depending on the source of the TLR agonists, the affected tissue and the situational context.

1.7 Toll-like receptor 4 – a versatile sensor of PAMPs and DAMPs

Among the TLR family, TLR4 has a unique position for various reasons. First of all, TLR4 is assumed to interact with various co-receptors and adapter molecules. For instance, LPS binding to TLR4 requires the association of LPS with the serum factor LPS binding protein (LBP). LBP conveys the association with – membrane-bound or soluble – CD14 (Schumann et al., 1990). CD14 may even help in the differentiation between chemotypes of LPS, i.e. structural variants of the strains (Jiang et al., 2005). Furthermore, TLR4 essentially requires the adapter molecule myeloid differential protein 2 (MD-2) for its ligand-induced dimerization (Kobayashi et al., 2006). Additional co-receptors were postulated for heterogeneous supramolecular complexes – including FcγRs (CD16, CD32, CD64), integrins (CD11b/18) and others (Pfeiffer et al., 2001). The composition of such activation clusters would vary with the type and activation state of the cells (Triantafilou and Triantafilou, 2002). Conceivably, these flexible interactions serve as an explanation for the exceptional diversity of accepted ligands (Tab. 1.4.1). Moreover, TLR4 organizes cytosolic events via all four TIR-domain-containing adapter molecules (chapter 1.4, Fig.

1.4.1.). While other TLRs signal through either the MyD88 or the TRIF route and interact with them directly, or in the case of TLR2 via the sorting adaptor TIRAP/MAL, TLR4 uses a TIRAP/MAL as well as a TRAM bridge to indirectly recruit MyD88 and TRIF, respectively. This probably provides more options for differential and tuned signaling already at an upstream stage, as in the case of LPS chemotype distinction. TLR4 can also trigger IFNβ release, while other TLRs known to induce the type I IFNs signal from the intracellular compartment as sensors of nucleic acids (Akira and Takeda, 2004; Kagan et al., 2008). TLR4 thus provides access to this important immunoregulator in response to extracellular agonists as well. Furthermore, TLR4 involves non-classical TLR signaling components. The spleen tyrosine kinase (syk) linked to the FcR-ITAM (immunoreceptor tyrosine-based activation motif) signaling constitutively connects with TLR4 to modulate responses in monocytic cells (Chaudhary et al., 2007). Thus, syk could facilitate the assumed crosstalk between FcRs and TLR4. Intriguingly, co-stimulation of FcRs with immune complexes (ICs) and TLR4 with LPS, which alone induce inflammatory macrophage phenotypes, results in a rather M2-like (i.e. M2b) anti-inflammatory orientation (Anderson et al., 2002; Gerber and Mosser, 2001). Moreover, it instructs monocyte-derived dendritic cells to promote the development of regulatory T cells (Treg), while inhibiting T cell proliferation and inducing IL-13 production by T cells (Wenink et al., 2009).

These findings illustrate a broad ligand acceptance as well as distinction, an extraordinary flexibility of signaling and thus an exceptional status of TLT4 among the TLRs/PRRs. Receptor clustering on the one hand and the selective recruitment/use of intracellular signaling components on the other hand may enable the TLR4 not only to recognize a broad spectrum of danger signals but to translate their binding into appropriate cellular responses depending on the nature and context of the stimulus.

2. AIM OF THE STUDY

Microglial cells and the TLR system are considered to be important components of the innate immunity in the CNS. They are essential for tissue homeostasis and anti-microbial protection. They can, however, also contribute to tissue damage caused by excessive inflammatory reactions. TLR4-mediated microglia activation may not exclusively depend on bacterial LPS. Certain endogenous DAMP factors have TLR4-agonistic capacity. We focused on selected plasma-derived proteins and their DAMP signaling potential for microglia upon BBB impairment or tissue destruction. Two of the candidates associate with the coagulation cascade, whereas others belong to the adaptive immune system. Assuming critical roles of TLR4, the following specific questions were addressed:

(1) Is TLR4 responsible for the microglial (pro)inflammatory responses as assigned to thrombin and as actually carried by a thrombin^{HMWM} coagulation factor complex? Which factor(s) could account for the activity and how is the receptor/signaling mechanism organized?

(2) Could plasma FN as a coagulation factor and putative thrombin^{HMWM} constituent serve as a TLR4-agonistic DAMP to drive microglial responses?

(3) Do certain IgG isotypes directly activate microglia responses in antigen absence, i.e. by a monomeric (non-immune complex) presentation? Which receptors, namely non-FcR structures and especially TLR4, would serve in the cellular activation? What are the conditions and molecular requirements that would allow IgGs to gain such a function?

(4) Can TLR4 differentiate between exogenous PAMP and endogenous DAMP signals to install distinct microglial consequences?

Considering the diversity of the microglial reactive phenotypes, this work aimed at demonstrating how versatile a single receptor system could sense and interpret agonists of different origin, structure and (patho)physiological context. TLR4 could thereby prove as a decision maker in microglial responses to PAMPs and DAMPs.

3. MATERIAL AND METHODS

3.1 Animals

NMRI, C57BL/6J and C3H/HeJ wildtype mice were routinely obtained from the central animal facility of the University of Göttingen. Mutant strains, being deficient for CD14 ($CD14^{-/-}$), TLR4 ($TLR4^{-/-}$), MyD88 ($MyD88^{+/-}$ and $MyD88^{-/-}$) and TRIF ($TRIF^{-/-}$) were from our own breeding. Mice being deficient for FcγRI ($CD64^{-/-}$) and Fcγ signaling chain ($Fc\gamma^{-/-}$) were obtained from Prof. Sjef Verbeek (University Medical Center, Leiden, Netherlands). All animals were housed and treated according to the guidelines for animal care at the central animal facility (Universitätsmedizin Göttingen, Germany).

3.2 Primary microglial cultures

All experiments were carried out in 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 (all Biochrom, Berlin, Germany).

Primary microglial cell cultures were prepared from whole brains of newborn mice from the different strains and cultured in DMEM as previously described (Häusler et al. 2001). In brief, brains were freed from meninges and blood vessels, washed with Hank's balanced salt solution (HBSS, Biochrom) and incubated with trypsin (2.5 %, Biochrom) for 10 min at 37°C. Trypsination was stopped by addition of DMEM supplemented with DNAse (0.4 mg/ml, CellSystem, St. Katherine, Switzerland) followed by an incubation at 37°C for 5 min. Cells were mechanically separated and centrifuged at 200 x g at 4°C for 10 min. The supernatant was removed and fresh medium was added. Cells were bottled in a 75 cm^2, poly-L-lysin- (PLL, Invitrogen/Gibco) coated culture flask and cultured in a humid atmosphere with 5 % CO_2 at 37°C. After 1 day, primary mixed-glia cultures were washed 3 times with phosphate-buffered saline (PBS, Invitrogen/Gibco) and fresh medium was added. After 5 days of primary cultivation, microglial proliferation was stimulated by changing the culture medium to DMEM supplemented with 30 % of L929 cell culture supernatant. After 3-5 days, microglial cells were separated from other cell types by shaking, using the different adhesion properties of the cells. Cells were harvested, washed with medium and counted. The purity of the preparations was determined to be

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routinely >98 %, based on nuclear and cell type-specific immuno- and lectin-cytochemistry (data not shown).

For experiments, cells were plated in 96-well plates with a density of 15,000/well, on PLL-coated coverslips with a density of 50,000/coverslip or in petri dishes (diameter 60 mm) with a density of 1×10^6/dish. For the gene array analysis, microglial cells were plated on a biomembrane surface (0.4 µm, Millicell-CM, Millipore, Schwalbach, Germany) with a density of 200,000 cells/insert.

Coverslips and culture flasks were coated with PLL immediately before being used for microglial or astrocytic cell cultures by incubation with PLL (100 µg/ml) for 30 min at room temperature. Excess of PLL was washed off by rinsing coverslips and flasks with sterile distilled H_2O.

3.3 Organotypic hippocampal slice cultures (OHSC)

Slice cultures were prepared in cooperation with Dr. Oliver Kann (Charité, Berlin, Germany) as previously described (Kann et al., 2003). In brief, hippocampal slices (300 µm) were prepared from 7- to 9 day old C57Bl/6 mice under sterile conditions in ice-cold minimal essential medium (MEM, Invitrogen/Gibco). Slices were maintained on a biomembrane surface (0.4 µm, Millicell-CM, Millipore) between 1 ml of culture medium (50% MEM, 25% HBSS, 25% FCS, and 30 mM glucose) in a humid atmosphere with 5 % CO_2 at 37°C. After 3 days, the medium was changed (93 % Neurobasal medium, 2 % B27 supplement, 5 % FCS [all Invitrogen/Gibco]). The culture medium was changed twice a week. Slice cultures were used for experiments after 10 days *in vitro*. For stimulation, 500 µl of the respective solution of bovine thrombin (20 U/ml, Sigma, Taufkirchen, Germany), IgG$_3$ (200 µg/ml, Bethyl Laboratories, Montgomery, USA), IgG$_3$-FITC (200 µg/ml, refer to chapter 3.15) or LPS (20 ng/ml, Sigma) were added to the well in exchange of 500 µl of medium. Aliquots of the supernatants were taken after 24 h and 48 h of stimulation and the amounts of cytokines and chemokines were determined by ELISA (refer to chapter 3.9). OHSC which received IgG$_3$-FITC were further analyzed after 48 h of stimulation on a Noran OZ confocal laserscanning microscope system (Prairie Technologies, Middleton, USA) using the 488 nm line Krypton-argon lasers (Omnichrom 643, Melles Griot, Bensheim, Deutschland) and a 60x water-immersion objective.

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3.4 L929 mouse fibroblast cultures

L929 mouse fibroblasts were cultured in DMEM (10% FCS) and splitted (1:5) every 2 weeks. After 14 days of cultivation, the culture supernatant was taken and stored at -20°C until being used for the stimulation of microglial proliferation as described above.

3.5 Generation and cultivation of mouse hybridoma cell cultures

Mouse hybridoma cell lines were generated for the production of anti-DsRed monoclonal IgGs in cooperation with BioGenes GmbH (Berlin, Germany). In brief, animals (8 weeks old female NMRI mice) were immunized by intra peritoneal injection of purified recombinant DsRed (refer to chapter 3.25) in complete Freund's adjuvants over a time period of 39 days with 4 injections. Splenocytes were fused with SP2/0-Arg14 myeloma cells (ratio 2:1) and plated in 96-well plates containing peritoneal feeder cells. Hybridoma cells were cultured for 10 days in HAT-medium (DMEM supplemented with 20% FCS and Aminopterin). Culture medium was changed every 5 days. Supernatants were tested for the presence of anti-DsRed IgGs by ELISA (refer to chapter 3.9). IgG-positive primary cultures were subcloned by dilution to a density of 1-3 cells/well and plated in 96-well plates containing peritoneal feeder cells. Cells were grown for 10 days and again tested for anti-DsRed IgG presence as above. The cloning procedure resulted in two positive clones secreting IgG_1 and IgG_{2A} antibodies. The clones were transferred to serum free ISF-1 medium containing 100 U/ml penicillin and 100 µg/ml streptomycin (all Biochrom).

Hybridoma cell lines were cultured in ISF-1 medium in the incubator with 5 % CO_2 at 37°C. Every 3 days, cells were harvested by centrifugation at 200 x g for 10 min at 4°C. Cells were counted and bottled in fresh medium with a density of 3.0×10^6 cells/ml. The IgG-containing supernatant was stored at 4°C until being further processed by protein G affinity chromatography (refer to chapter 3.22).

3.6 Microglial stimulation

For stimulation experiments, we used thrombin preparations of bovine and mouse origin (T-3399 and T-8397, Sigma). Human γ-thrombin was a gift from Dr. Thomas Möller (University of Washington, Seattle, USA). Bovine, rat and human FN were from Sigma, mouse FN was from Innovative Research (Novi, Michigan, USA). Immunoglobulin

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preparations were from R&D Systems (supplier A, Wiesbaden, Germany), Arcis (supplier B, Herford, Germany), Bethyl Laboratories (supplier C, Montgomery, USA), Sigma (supplier D), Fitzgerald Industries (supplier E, North Acton, USA) or purified from hybridoma culture supernatants by protein G affinity chromatography (refer to 3.22 and Tab. 4.15.1). Highly purified Toll-like receptor agonists PAM_3CSK_4 ([(S)-[2,3-Bis-(palmitoyloxy)-2(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4-OH·3HCL], TLR1/2 ligand), Poly (I:C) (polyinosinic-polycytidylic acid, TLR3 ligand), lipopolysaccharide (LPS, *E. coli*, serotype R515, TLR4 ligand), MALP-2 (S-(2,3-bisAcyloxypropyl)-cysteine-GNNDESNISFKEK, TLR6/2 ligand) and CPG oligo-desoxynucleotides (OND) 2395 (TCG TCG TTT TCG GCG CGC GCC G, TLR9 ligand) were obtained from Axxora (Lörrach, Germany). Lower hinge region peptide (pIgG1 [CPAPELLGGPSV], 99.05 % purity) was from PANATecs GmbH (Tübingen, Germany). Syk inhibitor 3-(1-Methyl-1H-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfon-amide was from Calbiochem (Merck KGaA, Darmstadt, Germany). Monoclonal rat-anti-mouse CD14 (clone biG53) was from Cell Sciences (Canton, USA), monoclonal rat-anti-mouse CD14 (clone 4C1) and mouse-anti-mouse CD14 (clone rmC5-3) were from BD Pharmingen (San Diego, USA,). Monoclonal rat-anti-mouse TLR4/MD2 antibody was purchased from Axxora, rat anti-mouse CD11b (αM integrin, clone M1/70.15), rat anti-mouse CD18 (β2 integrin, clone M18/2), rat anti-mouse CD51 (αV integrin, clone RMV-7) rat anti-mouse CD439d (α4 integrin, clone R1-2) and rat-anti-mouse CD16/32 (FcγRII/III) were from AbD Serotec (Düsseldorf Germany). Rat-anti-mouse FcγRI (CD64) and recombinant cytokines IFNγ, IFNβ, IL-4 and IL-10 (carrier free solutions) were purchased from R&D Systems. All working solutions were freshly prepared from frozen (-20°C) or refrigerated (4°C) stocks by dilution in DMEM.

Antibodies, containing NaN_3 as preservative were filtered prior to use with a MICROCON YM-50 filter (Millipore) with a 50-kDa MWCO (molecular weight cut off). Antibodies were loaded onto the filter and washed 3 times with PBS (Invitrogen/Gibco) by centrifugation at 13,000 x g for 15 min. Finally, the filter was put upside down, 100 µl of PBS were added on the backside and the antibody was collected by centrifugation at 4000 x g for 5 min.

All experiments concerning ELISA methods were routinely performed in 96-well plates (Greiner, Frickenhausen, Germany). For the detection of modulated FN-, LPS-, or IgG-induced release 0.1, 1 and 10 ng/ml of the respective cytokines were mixed with the stimulation agent and given at the same time to the cells. For the detection of modulated

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TLR agonist-induced release by thrombin[HMWM] or FN the respective TLR agonist was mixed with 10 U bovine thrombin or FN at various concentrations before stimulation and given at the same time to the cells. In other experiments, thrombin (10 U/ml) was added at various time points of the ongoing LPS (1 ng/ml) stimulation (0, 1, 2, 3, 4 and 5 h). In 'kiss and run' studies, cells received a combination of LPS (1 ng/ml) and thrombin (10 U/ml) or just thrombin for various time periods (15, 30, 60, 120 and 180 min). Subsequently, cells were washed and incubated with LPS (1 ng/ml) or just medium, respectively, for additional 18 h. When working with blocking antibodies, cells received a pre-incubation with the respective antibody for 30 min. Antibodies were previously tested for their own release induction capacity and were found to be inactive on microglial cells. In studies using the lower hinge region peptide pIgG, cells received a pre-incubation with the peptide for 15 min. Cells for combined treatments with the syk inhibitor received a pre-incubation at the respective concentration for a 1h. Then, cells received fresh medium or medium containing IgGs (100 µg/ml, supplier A; 25 µg/ml supplier C) or LPS (1 ng/ml) either alone or in combination with the inhibitor, the blocking antibody or pIgG. For thermosensitivity profiles, LPS (1 µg/ml), bovine thrombin (1000 U/ml) or bovine FN (1 mg/ml) were incubated at various temperatures for 10 min or at 70°C for varying time periods. Subsequently, samples were diluted in medium to standard concentrations (see below) and cells were stimulated for 18 h. Furthermore, heat treated thrombin was mixed with untreated LPS and used for microglial stimulation as described above. For gene array analysis, microglial cells were incubated with LPS (1 ng/ml), FN (bovine, 100 µg/ml) or medium as a control for 24 h. Supernatants analyzed by ELISA (refer to chapter 3.9) to confirm the response. Cells were washed with PBS and used for Microarray studies (refer to chapter 3.21).

Cells were stimulated in groups of 12, 8 or 4 well, respectively, with 100 µl of respective substances in medium or just medium as controls. Cells were kept in an incubator for 18 hours in a humid atmosphere with 5 % CO_2. Supernatants were taken and stored at -20°C until being assed (refer to chapter 3.9).. A WST-1 cell viability assay was routinely performed after stimulation as described below. The applied concentration of thrombin (mouse or bovine) or thrombin-PPACK was routinely 10 U/ml (enzymatic activity units served for quantification understanding, however, certain biological effects of the thrombin preparation were independent of the catalytic property), FN 100 µg/ml (mouse or bovine), IgGs 100 µg/ml (supplier A, B, D, E and anti-DsRed) or 25 µg/ml

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(supplier C), PAM$_3$CSK$_4$ (10 ng/ml), Poly (I:C) (50 µg/ml), LPS (1 ng/ml), MALP-2 (10 ng/ml) or CPG (10 µg/ml) (if not otherwise stated).

3.7 WST-1 cell viability assay

Microglial cells were plated on a 96-well plate and stimulated as described above. After 18 hours, cells received fresh medium (100 µl/well) and 10 µl of WST-1 reagent and were incubated at 37°C for 3h. The color reaction was measured using a microplate reader (BioRad, München, Germany) at 450 nm with 750 nm as reference.

3.8 Immunocytochemistry

Cultured microglial cells were stained with FITC-conjugated isolectin B4 (ILB4, *Griffonia simplicifolia*, Vector Laboratories, Burlingame, Canada). FN was stained with a cross reactive rabbit anti-human FN (Dako, Glostrup, Denmark) and nuclei were labeled with 4, 6-diamidino-2-phenylindol (DAPI, Roche, Mannheim, Germany). As the secondary antibody anti rabbit-Cy3 (Sigma) was used.

Mouse microglial cells were plated on PLL-coated cover slips (50.000 cells) and cultured in DMEM for 18 h under serum free conditions. Cells were washed twice with PBS (Biochrom) and fixed for 15 min in 4 % paraformaldehyde at 4°C. All the following incubations were performed at room temperature. Cells were rinsed 5 min in PBS-T (PBS, 0.1% BSA, 0.1% Triton X-100) and blocked for 1 hour in Block-T (PBS, 5% BSA, 0.5% Triton X-100). Then, cells were washed twice with PBS-T and incubated with ILB4-FITC (microglia, 1:200 in PBS-T) for 1 h. Cells were rinsed three times with PBS-T. FN was stained with anti-hFN antibody (1:400 in PBS-T, 1 h) followed by incubation with an anti rabbit-Cy3 (1:100 in PBS-T) for 1 hour and DAPI (1:1000 in PBS-T) for 15 min, including thorough rinsing (3 x 5 min with PBS-T) after each incubation step. Finally, cover slips were rinsed in distilled H$_2$O and mounted with fluorescence mounting medium (Dako) on slides. Slides were kept in the dark at 4°C until photographed. For microscopic observations, we used an Olympus BX51 microscope (Olympus, Hamburg, Germany) with a 40x objective. Pictures were taken with the Color-View soft imaging system (CellF, Olympus).

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3.9 Cyto-/chemokine quantification (ELISA)

Following microglial stimulation, culture supernatants were collected and the release of cyto-/chemokines was quantified by an enzyme-linked immunosorbent assay (ELISA) based on mouse- and factor-specific antibody pairs and standards as described in the manufacturer's instructions. ELISA kits for tumor necrosis factor α (TNFα) and total IL-12p40 were from BioLegend (San Diego USA), KC (CXCL1), RANTES (regulatory on activation, normal T cell expressed and secreted, CCL5), MCP-1 (monocyte chemoattractant protein 1 (CCL2), MIP-1α (macrophage inflammatory protein 1α, CCL3), MIP-2 (CXCL2) and IL-6 were from R&D Systems. The color reaction was measured with a microplate reader (Model 680, BioRad) at 450 nm with 540 nm as reference. Release was calculated as pg/ml, or expressed as percentage relative to the LPS-induced release or to the respective control. The release quantification on volume basis (instead of pg/mg total cell protein) was verified to be suitable.

3.10 Measurement of anti-DsRed IgGs in hybridoma culture supernatants

MaxiSorb ELISA plates (Nunc, Roskilde, Denmark) were incubated with purified DsRed protein (100 µg/ml, refer to chapter 3.25) overnight at room temperature with shaking. Plates were rinsed 3 times with PBS (Biochrom) including 5% Tween 20 and blocked with 5% bovine serum albumin (BSA, Serva, Heidelberg, Germany) for 1 h. Plates were washed as before and incubated for 2 h with hybridoma culture supernatants. Anti-DsRed IgGs were detected with an HRP-conjugated anti-mouse IgG Fc-specific antibody (Sigma). The color reaction was measured as described above.

3.11 Measurement of phosphorylated NFκB p65 and p38^{MAPK}

Microglial cells were plated in petri dishes and stimulated with LPS (1 ng/ml) or FN (bovine, 100 µg/ml) for 0, 5, 15, 30, 60 and 240 min. Then, cells were washed with PBS (Biochrom) and lysed in cell lysis buffer (Cell Signaling Technology, Danvers, USA). The lysates were stored at -80 °C until being assayed. ELISA kits (Phospho-NFκB p65 and p38^{MAPK} Sandwich ELISA, Cell Signaling), were used to quantify the phosphorylation of the transcription factor NFκB p65 and the p38^{MAPK}. The assays are based on mouse- and factor-specific antibodies that bind the phosphorylated as well as the non-phosphorylated

versions of either factor. Subsequently, only the phosphorylated proteins were detected. The ELISAs were carried out according to the manufacturer's instructions.

3.12 Citrullination of FN

Original solutions of mouse or bovine FN (1 mg/ml, Sigma) were supplemented with $CaCl_2$ (10 mM) and DTT (Dithiothreitol, 5 mM). The samples were incubated with peptidyl arginine deiminase (PAD, 7 U/mg of protein, Sigma) for 24 h at 37°C with shaking. The reaction was stopped by the addition of EDTA (10 mM). In order to remove the PAD from the samples, immobilized trypsin inhibitor (Thermo Scientific, Bonn, Germany) was used (10 µl/U of PAD of a 50% slurry). Beads were washed twice with PAD buffer (50 mM Tris, 0.5 M NaCl, 10 mM $CaCl_2$, 5 mM DTT, pH 7.4), added to the samples and incubated for 30 min at 4°C with shaking. Samples were centrifuged at 5,000 x g for 1 min at 4°C. Processed and unprocessed solutions (treatment with or without PAD incubation) were diluted in medium to final concentrations of FN (100 µg/ml) and used for microglial stimulation as above.

3.13 Protease digestion of TLR4 agonists

Trypsin digestion used immobilized trypsin (50 U/mg of protein, Thermo Scientific). Beads were washed twice with PBS (Biochrom) and added to the respective samples of LPS (100 ng/ml), IgGs (supplier C or supplier A, 1 mg/ml in original solution), or PBS as a control. Samples were incubated for 24 h at 37°C with shaking. Trypsin was removed by centrifugation at 5,000 x g for 1 min. Processed and unprocessed solutions (treatment with or without trypsin incubation) were analyzed by SDS-PAGE or diluted in medium to final concentrations of LPS (1 ng/ml) or IgGs (100 µg/ml) and used for microglial stimulation.

Proteinase K digestion used soluble proteinase K (0.5 U/mg of protein, Roche, Mannheim, Germany). The protease was added to the respective sample of LPS (100 ng/ml), IgGs (supplier C or anti DsRed IgG, 1 mg/ml in original solution), FN (bovine, 1 mg/ml in original solution) or PBS as a control. Samples were incubated for 24 h at 37°C with shaking and analyzed by SDS-PAGE. The protease was removed by filtration using Microcon YM50 filter devices (Millipore). Samples were loaded on the filter, and washed twice with PBS by centrifugation at 13,000 x g for 15 min. Subsequently, the filter was put

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upside down and PBS was added to the backside. Proteins retaining on the filter were eluted by centrifugation at 3,000 x g for 5 min. In LPS and control (PBS) samples the enzyme was inactivated by an incubation at 100°C for 10 min. Processed and unprocessed solutions (treatment with or without proteinase K incubation) were diluted in medium to final concentrations of LPS (1 ng/ml), IgGs (100 µg/ml) or FN (100 µg/ml) and used for microglial stimulation.

3.14 Enzymatic deglycosylation

Enzmatic deglycosylation used endoglycosidase F (EndoF, 20 U/mg of protein, Roche) or (2→3,6,8,9) neuraminidase (20 U/mg of protein, Sigma). The glycosidase was added to the respective sample of LPS (100 ng/ml), IgGs (1 mg/ml in original solution) or PBS (Invitrogen/Gibco) as a control. Samples were incubated for 16 h at 37°C with shaking and analyzed by SDS-PAGE. The protease was removed by filtration using Microcon YM50 filter devices as described above. In LPS and control (PBS) samples the enzymes were inactivated by treatment at 100°C for 10 min. Processed and unprocessed solutions (treatment with or without glycosidase incubation) were diluted in medium to final concentrations of LPS (1 ng/ml) and IgGs (100 µg/ml) and used for microglial stimulation.

3.15 Conjugation of IgG to N-Hydroxysuccinimide-Fluorescein

IgG_3 (supplier C) was fluorescent labeled with N-Hydroxysuccinimide-Fluorescein (NHS-FITC, Thermo Scientific) according to the manufacturers instructions. In brief, IgG_3 was dialyzed against 50 mM borate, pH 8.5 using Slide-A-Lyzer dialysis cassettes (Thermo Scientific) with a MWCO of 3,500 Da at 4°C. The buffer was changed three times after every 4th hour of dialysis. The IgG was incubated with a 20 fold molar excess of NHS-FITC (reconstituted in DMSO) for 1 h at room temperature. Excess of non-reacted FITC was removed by dialysis against PBS (Biochrom) as described. IgG_3-FITC were stored in the dark at 4°C and used for stimulation of OHSC as described above.

3.16 IgG refolding

Assessment of diverse protein refolding strategies on an IgG_3 (supplier A), which spontaneous lost its activity on microglia, used the iFOLD® Protein Refolding System 1

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(Novagen, Darmstadt, Germany) according to the manufacturers instructions. A few of them (15 out of 92) were found to restore the activity on microglial cells (data not shown). Further experiments on IgG refolding were performed as follows. IgGs from original solution were transferred into denaturation buffer (50 mM Tris-HCl, 50 mM NaCl, 0.5 mM EDTA, 5% glycerol, 5% laurylsarcosine pH 8.0) or denaturation buffer containing 2 mM tris(2-carboxyethyl)phosphine (TCEP) by filtration using MICROCON YM-50 filter (Millipore) as described. The protein concentration was adjusted 2 mg/ml with denaturation buffer followed by incubation for 12 h at 37°C with shaking. Laurylsarcosine was removed by dialysis against dialysis buffer (10 mM Tris-HCl, 0.05 mM EDTA, pH 8.0) using the Slide-A-Lyzer dialysis cassette (Thermo Scientific) as described. Refolding was achieved by rapid dilution (1:100) in refolding buffer (500mM L-arginine, 12.5mM β-cyclodextrin, 50 mM Tris-HCl pH 8.0) followed by incubation over night at room temperature with rotation. Refolded IgGs were dialyzed against PBS (Biochrom) as before and concentrated with an AMICON Ultra-15 filter device (Millipore) by centrifugation at 4,000 x g to a concentration of 1 mg/ml. Refolded IgGs were used for microglial stimulation, size exclusion chromatography (refer to chapter 3.22) and tryptophan fluorescence spectroscopy. For the latter, a Safire^2 UV-VIS spectrophotometer (Tecan, Crailsheim, Germany) was used. The emission spectrum was recorded from 300-400 nm using excitation at 295 nm.

3.17 Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry (MS)

Thrombin^{HMWM} was separated from a bovine thrombin preparation by size exclusion or thrombin-affinity chromatography (refer to chapter 3.22). Samples were precipitated as previously described (Wessel and Flugge, 1984). In brief, to one part of sample, 4 parts methanol and one part chloroform were added. The mixture was vortexed, centrifuged at 9,000 x g for 10 seconds and 3 parts of distilled water were added. For phase separation the samples were vortexed and centrifuged at 9,000 x g for 1 min. The upper phase was carefully removed and further three parts of methanol were added. The samples were mixed and centrifuged again for 2 min at 9,000 x g to form a protein pellet. The supernatant was removed and the protein pellet was dried under vacuum. Pellets were reconstituted with 40 µl of trypsin solution (10 ng/µl in 100 mM NH$_4$HCO$_3$) and incubated at 37 °C overnight.

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MS analysis was performed in cooperation with Dr. Hassan Dihazi (University of Göttingen, Germany) as previously described (Dihazi et al., 2005). In brief, peptide samples were co-crystallized with matrix (α-cyano-4-hydroxycinnamic acid) on a stainless steel target using 1 µl of matrix and 1 µl of sample. A Voyager-DE STR Time of Flight mass spectrometer (Applied Biosystems, Foster City, USA) operating in delayed reflector mode with an accelerated voltage of 20 kV, was used to generate peptide mass maps. Mass spectra were obtained by averaging 50 individual laser shots. A peptide mixture of des-Arg-bradykinin ($[M + H]^+$ 904.46), angiotensin I ($[M + H]^+$ 1296.68), Glu-fibrinopeptide B ($[M + H]^+$ 1570.67), ACTH-(1–17) ($[M + H]^+$ 2093.08), and ACTH-(18–39) ($[M + H]^+$ 2465.19) was used for external calibration. Resulting mass spectra were internally calibrated with trypsin autolysis products (m/z 842.50 and m/z 2211.10). Monoisotopic peptide masses were assigned and then used in Mascot database searches against the Mass Spectrometry Protein Sequence Database (MSDB) or the National Center for Biotechnology non-redundant (NCBInr) database.

3.18 SDS-PAGE and Imidazol–SDS–Zinc staining

Proteins were analyzed by SDS-PAGE under reducing and non-reducing conditions. Depending on the size of the analyzed protein, resolving gels with 7% to 12% and stacking gels with 4% were used. Samples were mixed 1:4 with 4x sample buffer (Laemmli, 1970) with (reducing) or without (non reducing) 2-mercaptoethanol and heated to 95°C for 5 min. Electrophoresis was performed by 200 V for 50 min using 25 mM Tris, 190 mM Glycine, 0.1% SDS as running buffer.

In gel protein staining was performed using the Imidazol–SDS–Zinc method as previously described (Hardy et al., 1996). In brief, following SDS-PAGE the gel was rinsed in distilled water for 1 min, incubated for 10 min in 0.2 M imidazol solution containing 0.1 % SDS. Subsequently the gel was immersed in 0.2 M zinc sulfate solution until the background became white leaving the protein bands transparent. The reaction was stopped by rinsing with water. Photographs were taken with a Canon EOS 450D camera. For immunoblotting, gels were destained by incubation with 100 mM EDTA solution for 10 min followed by a short rinse in distilled water for 5 min.

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3.19 Immunoblot and Dot blot analyses

Experiments used the following antibodies/reagents for protein detection: rabbit ant-human FN (Dako, Hamburg, Germany), goat anti-human thrombin-biotin (R&D), goat anti-mouse IgG-HRP (Fc-specific), rabbit anti goat IgG-HRP, goat anti rabbit IgG-HRP, streptavidin-biotin (all Sigma), D-Phe-Pro-Arg-chloromethylketone-biotin (PPACK-biotin; Calbiochem).

Following SDS-PAGE, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was moistened with methanol and transfer buffer (25 mM Tris, 190 mM Glycine, 10 % methanol) and a semi-dry transfer blot was performed at 140 mA for 30 min using a Trans Blot SD system (BioRad). Following the blotting procedure, the membrane was blocked with 5% skim milk in PBS (Biochrom) for 1 h. Immunodetection was based on protein-specific antibodies followed by incubation with the respective HRP-conjugated antibody or streptavidin-HRP conjugate. Thrombin was also detected by active site-labeling using PPACK-biotin and streptavidin-HRP conjugate. The visualization was based on enhanced chemoluminescence (ECL) reaction, using an ECL reagent kit (Sigma) and film (Roche, Mannheim, Germany).

Dot blotting was performed using a Bio-Dot apparatus (BioRad). Following size exclusion chromatography, proteins were directly transferred to a nitrocellulose membrane by vacuum and visualized as described above.

3.20 Thrombin activity assay

Thrombin (bovine, 1 mg/ml in original solution, Sigma) was enzymatically inactivated by incubation with various concentrations of PPACK (Calbiochem) for 15 min at RT. Excess of PPACK was removed by filtration using a MICROCON YM-10 filter (Millipore) as described. Thrombin-PPACK was also used for microglial stimulation (refer to chapter 3.6). H-D Phenylalnyl-L-pipecolyl-L-arginine-p-nitroaniline (S-2238, Chromogenix, Milano, Italy) was used as a chromogenic substrate for thrombin and diluted to 100 µM in assay buffer (50 mM Tris/HCL, pH. 7.4, 140 mM NaCL). Samples of thrombin or thrombin-PPACK were diluted in assay buffer to final concentrations of 0.1 U. Subsequently, 20 µl of the sample were incubated with 200 µl of substrate for 15 min. Absorbance was measured with a microplate reader (BioRad) at 405 nm wavelength. All tests were run in triplicate with the substrate solution as blank value.

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3.21 Gene expression analysis (4x44K Agilent microarray)

Microarray studies were performed at the Transkriptomanalyselabor (University of Göttingen, Germany). In brief, following stimulation of microglial cells for 24h with LPS (1ng/ml) or FN (bovine, 100 µg/ml) cells were washed with PBS (Invitrogen/Gibco) and total RNA was isolated using the TRIzol Reagent (Invitrogen, Karlsruhe, Germany) including a DNase I digestion. RNA integrity and quantity was analyzed with the Agilent 2100 bioanalyzer and the NanoDrop DD-1000 UV-VIS spectrophotometer (PecLab, Erlangen, Germany) . For global gene expression analysis we used the murine 4x44K Design (Agilent, Böblingen, Germany) and the "Low RNA Input linear Amplification Kit Plus, One Colour" protocol (Agilent) as well as the "RNA Spike-In Kit for One Colour" (Agilent). 700 ng of total RNA were used for cDNA synthesis. Labeling efficiency was determined using the NanoDrop spectrophotometer (>10 pmol/µl). 1.6 µg were fragmented and hybridized on the 4x44K Agilent microarray for 17 h at 10 rpm and 65°C in a hybridization oven (Agilent). Washing and staining of the arrays were done according to the manufacturer's instructions. Cy3 intensities were detected on an Agilent DNA microarray scanner at 5 micron resolution. The significance of the differences between groups was tested with the Kruskal-Wallis test and the Student`s t-test. P-values <0.05 were considered significant.

3.22 HPLC and FPLC analyses

Protein analysis was performed on a computer-assisted ÄEKTA purifier 10 HPLC system with an A950 fraction collector (GE-Healthcare, München, Germany) and a RF-10AXL fluorescence detector (Shimadzu, Duisburg, Germany). All buffers were filtered and degassed prior to use.

Size exclusion chromatography used a Superose 12 10/300 GL or a Superose 6 10/300 GL column (GE-Healthcare) with separation ranges of 3,500-300,000 Da and 4,000-5,000,000 Da, respectively. All runs were performed with PBS (Biochrom) as elution buffer at 4°C with a constant flow rate of 0.5 ml/min. Before loaded onto the column, protein samples were centrifuged at 13.000 x g for 10 min. Subsequently, the respective samples of thrombin (bovine or mouse, 200 U, Sigma), IgG$_3$ (100 µg, supplier A), normal mouse serum (50 µg, Sigma) or IgG-DsRed IC (25 µg) were diluted in PBS to a final volume of 300 µl and manually injected. The protein was monitored by absorbance at 280

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nm, DsRed fluorescence was recorded by excitation at 482 nm and emission at 608 nm wavelength. Fractions of 300 µl were collected and further analyzed by microglial stimulation, thrombin activity assay, mass spectrometry analysis or were directly reapplied to the column to analyze the reformation of IgG_3 associates. A mixture of thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa) was used for calibration (high and low molecular weight calibration kit, GE- Healthcare).

Thrombin-affinity chromatography was performed with a HighTrap BenzamidineFF 1ml column (GE Healthcare). Runs were performed at 4°C with a constant flow rate of 1 ml/min. The column was equilibrated with 10 column volumes (cv) of buffer A (50 mM Tris, 1 M NaCL pH 6.0). Thrombin (bovine, 100 U, Sigma) was applied to the column and washed with 20 cv of buffer A. Elution of the bound protein from the column was achieved by switching to buffer B (10 mM glycine pH 3.0) for 8 cv. Protein was monitored by absorbance at 280 nm and fractions of 300 µl were collected. Proper fractions were pooled and further analyzed by mass spectrometry analysis.

Ni^{2+}-affinity chromatography was performed with a self made column (5 ml) of ProCatch His resign (Miltenyi, Bergisch Gladbach, Germany). All runs were performed at RT with a constant flow of 1 ml/min. The column was equilibrated with 10 cv of PBS (Biochrom). Before loaded onto the column, the protein solution was centrifuged at 13.000 x g for 10 min. DsRed (tag-containing or tag-free [after factor Xa treatment, refer to chapter 3.25]) was applied to the column followed by washing with 5 cv of PBS. Elution of the bound protein from the column was achieved by switching to buffer C (50 mM CH_2COONa, 300 mM NaCl, pH 5.0) for 10 cv followed by equilibration with PBS for 10 cv. Note that tag-free DsRed does not bind to the column and comes in the flow through while tag-containing DsRed comes during elution.

Protein G-affinity chromatography was performed with HighTrap protein G HP 5 ml columns (GE-Healthcare). Before applied to the column, hybridoma culture supernatants containing anti DsRed-IgGs were filtered with a SteriTop™ filter unit (0.22 µm, Millipore). IgGs were loaded onto the column followed by washing with 5 cv of PBS (Biochrom) or buffer E (50 mM Tris, 150 mM NaCl pH 7.4). Elution of the bound IgGs from the column was achieved by switching to buffer F (50 mM glycine pH 2.5), buffer G (50 mM glycine pH 2.7), buffer H (50 mM glycine pH 2.9) or gentle elution buffer (pH 6.6, Thermo Scientific) for 10 cv followed by equilibration of the column with PBS of buffer E for 10 cv. Note that the use of the gentle elution buffer essentially requires washing with buffer E.

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The eluate was immediately neutralized with 1M Tris pH 9.5. Subsequently, the buffer was changed to PBS using VIVASPIN 4 filter devices (Millipore) with an MWCO of 50,000 Da and centrifugation at 4,000 x g at RT for 15 min. The protein concentration was determined using the MicroBCA protein assay (Thermo scientific) according to the manufacturer's instructions. IgG concentrations were adjusted to 1 mg/ml with PBS.

LPS-affinity chromatography was performed with EndoTrap® LPS removal columns (Profos AG, Regensburg, Germany) according to the manufacturer's instructions and under sterile conditions. Columns were washed twice with regeneration buffer (RB), drained out completely, washed twice with elution (EB) and drained out again. The respective samples of LPS (100 ng/ml), IgG_1 (1 mg/ml, supplier C), IgG_{2A} (1 mg/ml, anti-DsRed), IgG_{2A}+LPS (*IgG_{2A}, 1 mg/ml IgG_{2a} and 100 ng/ml LPS) or PBS (Biochrom) were loaded onto the columns and washed with 1ml EB. Each eluate was collected immediately, including the void volume, until the column was drained. The dilution factor was determined for each sample and the respective control sample (unfiltered) was then diluted with EB according to the dilution factor. Processed and original solutions were diluted in medium to final concentrations as follows: LPS (4 ng/ml), IgG_1 (10 µg/ml), IgG_{2A} (20 µg/ml) and *IgG_{2A} (20 µg/ml IgG_{2a} and 4 ng/ml LPS).

High performance anion exchange chromatography with pulsed amperometric detection was performed in collaboration with Dr. Nicole Poulsen (Georgia Institute of Technology, Atlanta, USA) on a computer-assisted DX-500 system (Dionex, Bannockburn, USA) with autosampler. Monosaccharides were released from IgGs (supplier C or anti-DsRed IgG, 100 µg) by acidic hydrolysis in 2 M TFA for 4h at 100°C. The volume was reduced to dryness under vacuum and samples were resolved in 15 mM NaOH (210 µl). 24 µl were loaded onto a CarboPac PA20 column (Dionex). Separation was performed at a constant flow of 0.5 ml/min by isocratic elution for 15 min with 15 mM NaOH followed by 20 min gradient to 100 mM NaOH, 100 mM NaOAc. Monosaccharides were detected with pulsed amperometric detection and were identified by their retention times established for standards of fucose, galactose and mannose.

3.23 Preparation of chemically competent cells

Experiments used LB medium (5 g/l yeast extract, 10 g/l tryptone 10 g/l NaCl), SOB medium (5 g/l yeast extract, 20 g/l tryptone, 0.5 g/l NaCl, 2.5 mM KCL), SOC-medium (SOB supplemented with 20 mM glucose) and PBS (Biochrom).

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Bacterial overnight cultures were grown in LB at 37°C with shaking at 150 rpm. The next day, cultures were diluted in LB and grown until OD_{600} reached 0.6. Bacteria were harvested by centrifugation at 4°C for 10 min at 1000 x g. The supernatant was discarded and cells were resuspended in ice cold 100 mM $MgCl_2$, incubated on ice for 30 min and harvested by centrifugation at 4°C for 10 min at 2000 x g. Cells were resuspended in ice cold 100 mM $MgCl_2$, 15% glycerol followed by centrifugation as before. The pellet was resuspended in $MgCl_2$/glycerol and split into small aliquots of 100 µl on ice. Bacteria were immediately frozen in liquid nitrogen and stored at -80°C until used for transformation.

3.24 Cloning of DsRed cDNA

The DsRed-Monomer vector was purchased from Clonetech (Saint-Germain-en-Laye, France), the pET42a (+) vector was from Novagene (Darmstadt; Germany). Competent *E.coli* (JM109) were from Promega (Mannheim, Germany) and BL21 cells were from our own glycerin stock. Endonucleases *BamHI* and *HindIII* were from Fermentas (St. Leon-Rot, Germany). Taq polymerase, T4 ligase and IPTG (isopropyl-β-D-thiogalacto-pyranosid) were from Promega, Kanamycin was from Roth (Karlsruhe, Germany). Experiments used the following primer pairs:

(I)	T7_for	5'- TAA TAC GAC TCA CTA TAG GG - 3'
(II)	Dsred_rev	5'- TTG AAG TCG CAG GTG TAG - 3'
(III)	42a_Bam_Dred_for	5'- CGA ACG GGA TCC GAC AAC ACC -3'
(IV)	Dred_Hind3_rev	5'- TAT CCC AAG CTT GGG CTA CTG GGA -3'

BamHI and *HindIII* restriction sites were inserted to the 5' or 3' end of the *DsRed* respectively by PCR using the primer pair (III; IV) as shown in Tab. 3.24.1.

The PCR protocol was as follows: A hold at 95°C for 5 min, 35 cycles of 95°C for 1 min, 65°C for 1 min, 72°C for 1 min, a hold at 72°C for 5 min. The amplified PCR product was separated by agarose gel electrophoresis (1% gel) and extracted from the gel using the DNA extraction kit (Promega) according to the manufacturer's instructions. DNA was treated with *BamHI* and *HindIII* for 2 h at 37°C, purified from an agarose gel and ligated into the *BamHI* and *HindIII*-cleaved pET42a (+) vector by incubation at 4°C over night with T4-DNA-ligase (1 U/µg of DNA). The construct was transformed into competent JM109 bacteria via heat shock. Cells were grown for 1 h in SOC medium and plated on LB-agar

plates supplemented with 50 µg/ml kanamycin. Colonies were analyzed for the pET42a-*DsRed* construct by PCR as described before using the primer pair (I; II) followed by agarose gel electrophoresis on a 1 % gel.

Tab. 3.24.1. : Reaction mixture for amplification of DsRed from the original vector.

solution	stock concentration	final concentration/amount
H_2O	-	add to 25 µl
Taq-buffer	5 x	1 x
$MgCl_2$	25 mM	1.5 mM
primer (III)	100 pmol/µl	1 pmol/µl
primer (IV)	100 pmol/µl	1 pmol/µl
DsRed-monomer vector	1 µg/µl	10 ng
Taq polymerase	10 U/ml	0.1 U

3.25 Expression and purification of recombinant DsRed protein

For DsRed protein expression the construct was transformed into BL21 chemically competent cells as described above. Cultures were grown in LB medium supplemented with kanamycin (50 µg/ml) until OD_{600} reached 0.5. Protein expression was induced by addition of IPTG (1 mM). Bacteria were grown for 10 h at 30°C with vigorous shaking at 200 rpm (adequate oxygen is important for proper protein folding). Bacteria were harvested by centrifugation at 3,500 x g at 4°C for 15 min, washed in ice cold PBS (Biochrom) and centrifuged as before. Cells were lysed by sonification on ice. The DsRed protein was purified by Ni^{2+}-affinity chromatography as described above. The N-terminal Tag was removed by treatment with factor Xa (10 U/mg of protein) over night at RT followed by Ni^{2+}-affinity chromatography as described above. Eluates were collected immediately, including the void volume. The product was analyzed for its purity by SDS-PAGE (12 % gel) as well as for its fluorescent properties using a Safire[2] UV-VIS spectro-photometer (Tecan).

4. RESULTS (PART A)

4.1 Thrombin and fibronectin as DAMP signals for microglial cells

TLRs comprise one of the families of PRRs that recognize an assortment of motifs in RNA/DNA, lipids, lipo- and glyoproteins from viruses, bacteria, fungi and protozoans, collectively known as PAMPs. Some TLRs also trigger responses in non-infectious scenarios by sensing host-derived molecules that are generated, released or modified upon impairment of cells, the vascularity or ECM. These molecules are formally known as DAMPs. Usually, most circulating proteins are excluded from the CNS parenchyma by the BBB. Upon BBB disruption and vascular damage, however, even larger molecules will gain access to the CNS parenchyma, including microglia. While it is well documented that microglia respond to blood vessel lesions, little is still known about the nature of plasma factors with microglial activation potential, the involved receptor/signaling mechanisms and the resulting consequences for neurons and glial cells. Based on previous studies of our group, this part of the thesis focused on plasma/coagulation factors and their potential to function as DAMP signals for microglia. The first chapters deal with the role of the central blood clotting factor thrombin (factor IIa). Besides its well understood function in the coagulation cascade, thrombin also exerts a multitude of cellular effects by limited proteolysis of protease-activated receptors (PARs). This mechanism has also been considered for the induction and release of proinflammatory mediators by microglia. Although microglia express functional PARs (Balcaitis et al., 2003) and another thrombin-relevant receptor (CD42), previous work from our group found them to be dispensable for the induction of cyto- and chemokines (Hanisch et al., 2004). In fact, several thrombin preparations contain a minor fraction of thrombin-associated high molecular weight material (thrombin^{HMWM}) as the sole and potent carrier of this activity. Now we demonstrate that microglia respond to this material via a TLR4-centered receptor/signaling complex. Mass spectrometry of the thrombin^{HMWM} identified thrombin, fibronectin (FN) and other coagulation factors – known to be recruited upon a thrombin-catalyzed coagulation process – among a presumably complex protein aggregate. FN is known to stabilize the growing thrombus, an explanation for its association with thrombin^{HMWM}. We show that FN alone can induce a similar response profile in microglia and that it thereby requires TLR4 signaling similar to thrombin^{HMWM}. FN thus appears as the TLR4-agonistic molecular component in the thrombin^{HMWM} complex. Moreover, FN can

functionally displace the classical TLR4 agonist LPS. However, both agonists present also with individual response features, indicating differences in the signaling and contributions of additional co-receptors. Importantly, FN is only recognized in the soluble format, whereas the immobilized protein – as present in the ECM – is not active on microglia.

In summary, data presented in this study underline the outstanding position of TLR4 in sensing tissue damage and homeostatic disturbance by innate immune cells of the CNS. The versatility and flexibility of the receptor system thereby allows for individual cyto- and chemokine responses to diverse PAMPs and DAMPs – and the instruction of distinct reactive phenotypes of microglia.

4.2 Microglial release responses to thrombin preparations depend on thrombin[HMWM]

Thrombin is a multifunctional serine protease with distinct substrate specificity. The proteolytically active Arg-specific enzyme of about 39 kDa derives from its 72-kDa zymogen (prothrombin, factor II) via cleavage by the factor Xa-containing prothrombinase complex. It is best known for the cleavage of fibrinogen to fibrin, which leads to the formation of the fibrin clot (Davie and Fujikawa, 1975). Besides this important function, thrombin tightly controls the coagulation cascade by positive and negative feedback mechanisms and is thereby the most important enzyme to maintain hemostasis (Bukys et al., 2006; Davie et al., 1991). Interestingly, thrombin's activity spectrum does not only cover cleavage of soluble peptide substrates but it can control several functions of various cell types by limited proteolysis of PARs. It has been shown to act as a mitogen on lymphocytes, monocytes, platelets (Coughlin, 2000), the endothelium (Lum et al., 1994; Lum and Malik, 1996), smooth muscle and mesenchymal cells (O'Brien et al., 2000). Effects of thrombin on microglial cells were first reported in 2000 independently by two groups (including ours). It could be demonstrated that cells respond to thrombin exposure with proliferation, a profound release of nitric oxide (NO) and several cyto- and chemokines, including TNFα, IL-6, IL-12 and CXCL1. Furthermore, microglia showed transient Ca^{2+} signals in response to thrombin stimulation, with properties clearly indicating dependence on PARs (Möller et al., 2000; Ryu et al., 2000). More recent studies demonstrate enhanced CD40 and CD95 expression upon exposure to thrombin (Weinstein et al., 2008a; Weinstein et al., 2009). *In vivo*, microglial activation by thrombin has been linked to a neurotoxic phenotype of these cells, promoting neurodegeneration (Fujimoto et al., 2007; Lee da et al., 2006; Ohnishi et al., 2007). While some of the

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reported activities were shown to be PAR-mediated – by use of agonists, inhibitors and pharmaceutical-grade recombinant thrombin preparations – others rather assumed PAR mediation – or concluded on their involvement from parallel experiments. The release-triggering activity of thrombin, however, was subsequently considered to be independent of its protease activity (Hanisch et al., 2004; Möller et al., 2006).

In previous work of our group, the release induction potentials of bovine and mouse α-thrombin as well as human γ-thrombin were tested on primary microglial cells. While the α-thrombins were mature and fully functional enzymes, γ-thrombin represents a version with a cleavage in the substrate binding site. γ-Thrombin is per se enzymatically active, but reveals a markedly decreased ability to bind and process larger substrates, including PARs (Bezeaud and Guillin, 1988; Fenton and Bing, 1986). All of the preparations were commonly used in the literature to describe thrombin-related/mediated effects *in vitro* as well as *in vivo* (Hanisch et al., 2004).
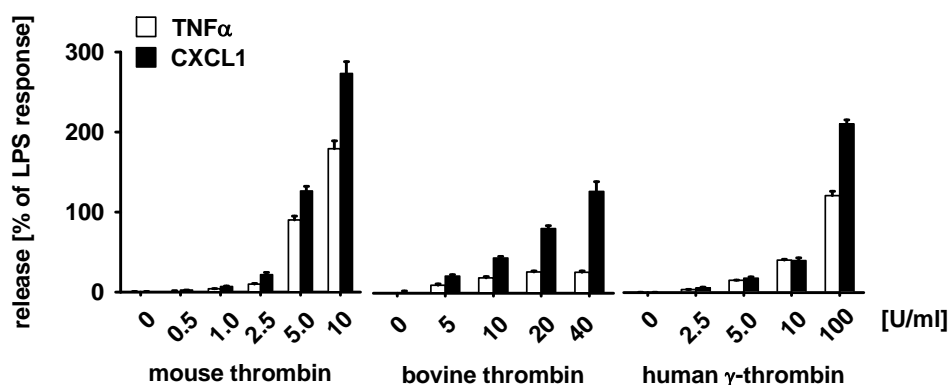
Fig. 4.2.1.: Induction of cytokine and chemokine release in microglial cells by thrombin. Primary mouse microglial cells were stimulated for 18 h with various thrombin preparations at indicated concentrations. The enzymatic activity (U/ml) thereby served as a basis to compare equivalent amounts of thrombin. As representative examples, release of TNFα and CXCL1 was measured in the supernatant and is shown as percentage of LPS-induced amounts. Data are mean ± SEM from n=38 (left), n=20 (middle) and n=36 (right) taken from 5, 2 or 4 experiments.

Cells were treated with thrombin for 18 h at concentrations as indicated. Release of TNFα and CXCL1 was determined in the supernatant as representative examples of a broader profile. Indeed, all thrombin preparations were capable of inducing TNFα and CXCL1 in mouse microglia (Fig. 4.2.1.), the strongest activity being found for the mouse preparation. Surprisingly, even γ-thrombin induced microglial responses, suggesting a PAR-independent mechanism. Consistent with previous observations from our lab, the small and very potent serine protease inhibitor PPACK was not able to interfere with the

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release-inducing activity of α- and γ-thrombins, even at a high dose of 100 µM, while their capacity to process the chromogenic substrate H-D-Phe-Pip-Arg-pNA•2HCl (S-2238) was completely blocked at ~50 nM PPACK (Fig. 4.2.2.B, C). The inhibitor binds irreversible to the active site of the enzyme. It was, therefore, used to visualize also truncated versions and fragments, usually not detected by antibodies, but with an assumed potential to be proteolytically active (Fig. 4.2.2.A).
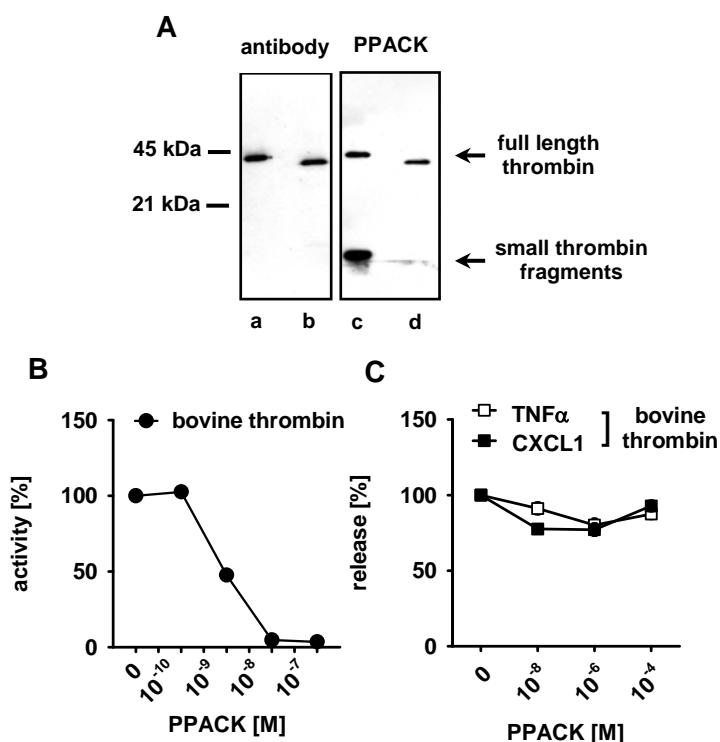
Fig. 4.2.2.: Relation between the protein, the enzymatic and cellular thrombin activities. (A) Thrombin (bovine, 0.1 U) was separated by SDS-PAGE (12% gel) under both non-reducing (a, c) and reducing (b, d) conditions. It was then visualized with an anti-thrombin-biotin antibody (a, b) or by active site-labeling using PPACK-biotin (c, d), followed by incubation with a streptavidin-HRP conjugate. While immunoblotting revealed single proteins within the expected size range, labeling by the enzyme inhibitor also detected a smaller fragment containing the active site of the protease. (B, C) Thrombin (bovine, 20 U) was incubated with PPACK at indicated concentrations for 15 min at RT. Excess of unbound PPACK was removed by filtration. (B) Enzyme activity was analyzed by conversion of the chromogenic substrate H-D-Phe-Pip-Arg-pNA·2 HCl. (C) Microglia were stimulated with thrombin or thrombin-PPACK (10 U/ml) for 18 h and the release of TNFα and CXCL1 was measured in the supernatant. Data are mean ± SEM with n=36 from 3 experiments.

Obviously, the release-triggering activity of thrombin was independent of its proteolytic capacity. Moreover, several further lines of evidence ruled out that microglial responses were dependent on proteolysis or known receptor interactions. First, interference with hirudin (two versions), an inhibitor of substrate binding, did not block the release induction. Second, a panel of PAR-agonistic peptides (considering PAR1, 3, 4 as well as the trypsin-activated PAR2 and species-specific sequences) could not mimic thrombin effects. Third, two thrombin-derived protein domains (TP508, B loop) reported in the past for some cellular thrombin effects failed to act as agonists or antagonists in driving the microglial reactions to thrombin. Finally, we had indentified microglia to express the GPIbα/Ibβ/V/IX

(CD42) complex, a receptor for von-Willebrandt factor and thrombin. Yet functional data, i.e. interference with low molecular weight (LMW) forms of heparin, argued also against this complex as being relevant. All these lines thus rendered more and more unlikely that proteolysis or any known receptor would play a role in the proinflammatory responses of microglia (data not shown).

As a consequence, we addressed two questions: (i) Is another component in the thrombin preparations responsible for the thrombin-assigned microglial activity? (ii) Which receptor and signaling mechanism would convey the responses? In a series of fast performance liquid chromatography (FPLC)-based studies, a minor fraction of high molecular weight protein was observed in the bovine as well as the mouse thrombin preparation as the sole carrier of activity – expanding former findings (Hanisch et al., 2004). Calibration-assisted size exclusion chromatography revealed three mayor peaks for bovine thrombin: A LMW protein peak at fraction 41 (13.1 ml elution volume, matching the size of thrombin proper), a large protein peak between fractions 27 and 35 (8.8 to 12.6 ml elution volume) and a small protein peak eluting with fraction 22 (7.9 ml) in the void volume of the column, representing protein of more than 300 kDa (Fig. 4.2.3.A). Even on a column with 5,000 kDa size exclusion, this material eluted in the void volume (data not shown), suggesting aggregated/associated rather than individual proteins. Because of its size, this material was termed thrombin^{HMWM}. Fractions (300 µl) were collected during the whole elution period and tested for proteolytic and release-triggering capacity. As expected, fractions matching thrombin proper by size contained most of the enzymatic activity (Fig. 4.2.3., solid line). Interestingly, these fractions had no release-inducing activity (dashed line). In contrast, microglial activity was found in the void volume fractions of thrombin^{HMWM}. The mouse thrombin preparation, being by far more homogeneous (purer) in its protein profile, presented with virtually the same thrombin^{HMWM} regarding microglial activity (Fig. 4.2.3.B). This was even more surprising as the potent fractions were not accompanied by a prominent protein peak. The chromatogram had only a small-sized peak – presumably degraded thrombin – at fraction 55 (16.7 ml of elution) and a major protein peak at fraction 42 (13.6 ml), i.e. proteolytic active thrombin. Thus, very small amounts of thrombin^{HMWM} protein carried the tremendous impact on microglia.
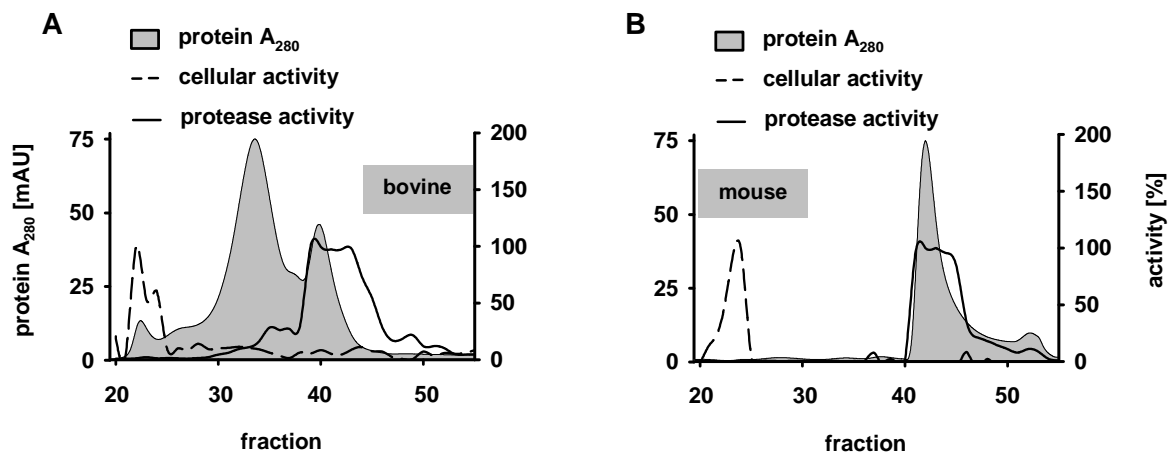
Fig. 4.2.3.: Separation of cellular and protease activity of thrombin preparations by size exclusion chromatography. (A) Thrombin (bovine, 200 U) or **(B)** thrombin (mouse, 200 U) were analyzed with a Superpose 12 10/300 gelfiltration column, using a constant flow of 0.5 ml/min of PBS at RT. Absorbance was measured at 280 nm. Fractions of 300 µl were collected. Each fraction was analyzed for protease and microglial activity as in Fig. 4.2.2., showing the release of CXCL1 as a representative example. Data are given as percentage of the maximum response from 1 representative experiment. The release-driving material is referred to below as thrombin[HMWM].

However, microglial reactions are specific and triggering them not simply a feature of any bigger protein complex or aggregate. For example, cells did not respond with measurable release to incubation with the Keyhole Limpet Hemocyanin (KLH), a very large protein complex in the hemolymph of *Megathura crenulata* (data not shown). It is comprised of 350 kDa subunits forming complexes of 4,500 up to 13,000 kDa. Since thrombin[HMWM] did not show any proteolytic activity, the release-triggering activity could be clearly separated from the protease nature – and was thus PAR-independent. It was likely not a feature of thrombin proper, but of some thrombin-associated, frequently co-purified factor(s). Consistent with these observations, several mouse, bovine or even human thrombin preparations of high specific enzyme activity (units/mg protein) and apparent homogeneity stimulated the cells, while others, including those of pharmaceutical grade, did not. The species independence and potency of thrombin[HMWM] indicated a conserved recognition and effective signaling mechanism, e.g. as it is known from PRRs.

4.3 Thrombin[HMWM] critically depends on TLR4 and can interfere with LPS signaling

TLRs are known to accept an assortment of evolutionary conserved structural motifs in commonly termed PAMPs (refer to chapter 1.4). Especially TLR4, the LPS-sensing

receptor, has been reported for accepting also several DAMPs as generated, released or modified upon cell and tissue impairment.
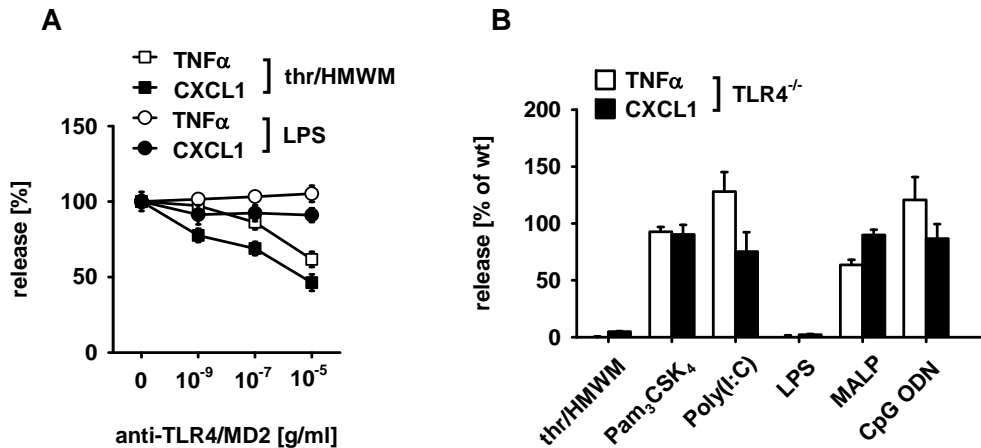
Fig. 4.3.1.: Effect of TLR4 blockade or deficiency on the release induction in microglial cells. **(A)** Wildtype cells were stimulated with thrombin[HMWM] (bovine, 10 U/ml) or LPS (1 ng/ml) in the presence or absence of a TLR4/MD2 blocking antibody for 18 h. Cells received a preincubation with the antibody for 30 min. **(B)** Microglia from mice being deficient for TLR4 (TLR4^{-/-}) were stimulated with specific agonists for TLR1/2 (Pam$_3$CSK$_4$, 10 ng/ml), TLR3 (Poly(I:C), 100 µg/ml), TLR4 (LPS, 1 ng/m), TLR6/2 (MALP, 10 ng/ml), TLR9 (CpG ODN, 5 µg/ml) or thrombin[HMWM] (bovine, 10 U/ml) for 18 h and release of cyto/chemokines was measured. The concentrations of TLR agonists were found to be sufficient to drive already maximal responses in previous experiments (not shown). Data are shown as percentage of the response without the blocking antibody or of wildtype cells and are mean ± SEM with an average of n=8 (A) or n=18 (B) from 3 experiments. Poly(I:C) has been recently found to further signal through an additional dsRNA sensor molecule, referred as melanoma differentiation-associated protein-5 (MDA5).

In an effort to identify the microglial receptor for thrombin[HMWM], we found the TLR4 system to have mandatory importance for its recognition and signal transduction. Use of an anti-TLR4/MD2 blocking antibody – at concentrations that had no effect on their own – revealed an inhibition of microglia stimulation with thrombin[HMWM] (Fig. 4.3.1.A). Responses to LPS were not affected by this clone, pointing to sterical hindrance as preventing the large thrombin[HMWM] but not the small LPS to bind to the receptor. As a more robust approach, we tested for thrombin[HMWM] effects on microglia prepared from a mouse strain being deficient for TLR4 (TLR4^{-/-}, Fig. 4.3.1.B) or expressing a mutant TLR4 version (C3H/HeJ) with a loss-of-function point mutation in the TLR4 gene (data not shown). In these situations, thrombin[HMWM] completely failed to induce release responses. In contrast, responses to TLR1/2 (Pam$_3$CSK$_4$), TLR3 (Poly (I:C), TLR6/2 (MALP) or TLR9 (CpG ODN) agonists were not affected (Fig. 4.3.1.B).

Fig. 4.3.2.: Modulation of TLR agonist-induced microglial release activity by thrombin[HMWM].
(A) Cells were incubated for 18 h with Pam$_3$CSK$_4$ (10 ng/ml), LPS (1 ng/ml) or MALP (10 ng/ml) in the absence or presence of thrombin (bovine, 10 U/ml). Release of TNFα and CXCL1 was determined and expressed as percentage of the response to the respective TLR agonists alone. Data are mean ± SEM with an average of n=8. **(B)** Cells were incubated with mouse thrombin alone at various concentrations or in combination with LPS (1 ng/ml) for 18h. Release of TNFα and CXCL1 was determined by ELISA and is shown as percentage of the response to LPS. Arrows indicate the critical threshold concentration, where both agonists were equally potent. Data are mean ± SEM from n=38 from 5 experiments.

Use of the TLR4 by thrombin[HMWM] thereby came with an additional interesting feature. As shown in Fig. 4.2.1., some responses to bovine thrombin[HMWM] especially at a standard concentration of 10 U/ml (U still being taken as a dose unit even though enzymatic activity is not required) were weak when compared to the release induced by LPS (1 ng/ml). Conceivably, LPS could bind more efficiently to TLR4 than thrombin[HMWM]. This is not the case. When paired with LPS in a co-stimulation, thrombin[HMWM] was able to compete for TLR4 and to displace LPS, indicated by a reduced response. Both agonists together caused an intermediate release, because the potent signaling of LPS got lowered by the less effective contribution of thrombin[HMWM] (Fig. 4.3.2.A). This effect was TLR4-specific since co-stimulations of thrombin[HMWM] with other TLR agonists rather resulted in an enhanced response. Further proof derives from the combination of LPS with a per se more potent mouse thrombin preparation (Fig. 4.3.2.B). At a critical threshold concentration, where both agonists are equally potent (red arrow), the combined release was not different from each single agonist-triggered response. Above this threshold, the stronger release is dominated by thrombin[HMWM]. Moreover, a correlation of cyto- and chemokine amounts induced by thrombin[HMWM] alone versus a combination with LPS

reveals that the pattern is more and more dictated with increasing thrombin[HMWM] concentrations (Fig. 4.3.3).
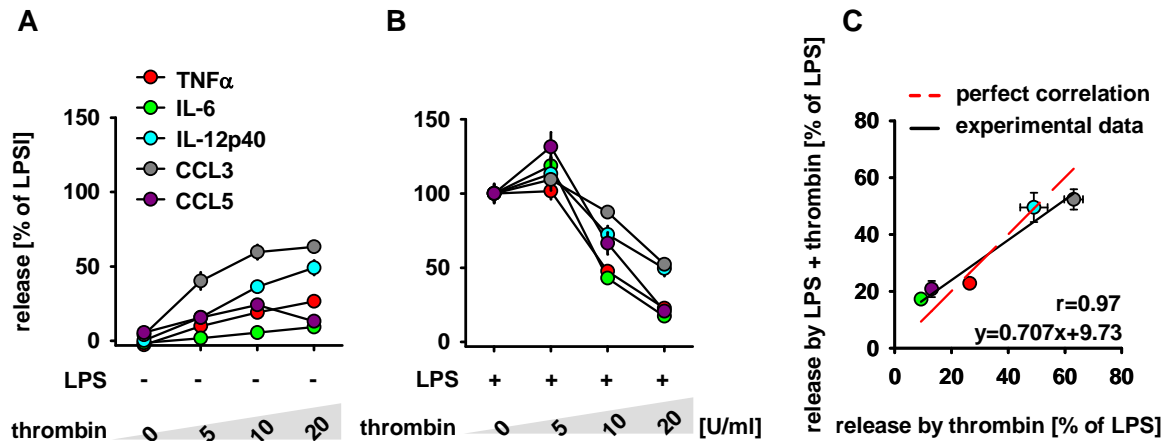
Fig. 4.3.3.: Induction, co-induction and correlation analysis of cytokine and chemokine release by thrombin[HMWM] and LPS. Cells were treated with **(A)** bovine thrombin alone or **(B)** together with LPS (1 ng/ml) at various concentrations for 18 h. Release of cyto/chemokines was determined and **(C)** correlated for both conditions for 20 U/ml thrombin. In (B), thrombin altered the LPS-induced amounts as it triggered itself less TNFα, IL-6, IL-12p40, CCL3 and CCL5. The higher the concentration of thrombin, the more the combined stimulation resembled in its profile the release as triggered by thrombin alone. At a thrombin concentration of 20 U/ml, it dominated the release profile, reflected by the direct correlation in (C). Presence of LPS in this situation had no more influence on the release pattern, suggesting a full functional replacement, most likely based on physical competition for TLR4. Data are mean ± SEM, average of n=24 from 5 experiments.

Thrombin[HMWM] could also interfere with an ongoing LPS stimulation (Fig. 4.3.4.A). Even at 5 h of delayed addition, thrombin[HMWM] still reduced the LPS-evoked release. Effective release induction requires continuous presence of LPS (Hanisch et al., 2001). The same holds true for thrombin[HMWM], as shown by 'kiss and run' studies (Fig. 4.3.4.B). Here, microglia were stimulated for short periods to compare subsequently released amounts. Even 3 h of thrombin[HMWM] exposure resulted in only partial responses. In contrast, the occupation of TLR4 seemed to be a very fast process (Fig. 4.3.4.C). Microglia were stimulated with LPS and thrombin[HMWM] in combination for short periods, followed by an exposure to LPS alone. Already 15 min of thrombin[HMWM] presence during a co-stimulation were sufficient to affect the subsequent, i.e. predominantly LPS-evoked release. The findings suggest (i) that LPS and thrombin[HMWM] require significant time for organizing full responses, (ii) that TLR4 occupation by thrombin[HMWM] is a rapid and then (functionally) lasting process and (iii) that binding and signaling are two kinetically separated events. Support for the latter notion also derived from experiments on the thermosensitivity of thrombin[HMWM], intending to rule out LPS contamination as the cause of microglial TLR4

activation. Along these studies, we found that defined thermal stress abolished the own agonistic activity of the thrombin[HMWM], while maintaining binding capacity. This regime thus created a stable antagonist (Fig. 4.4.1.).

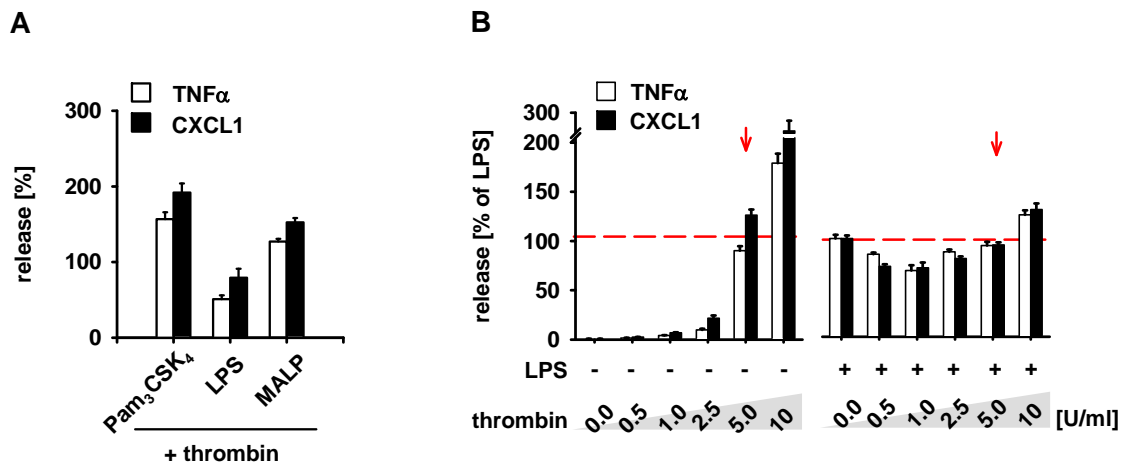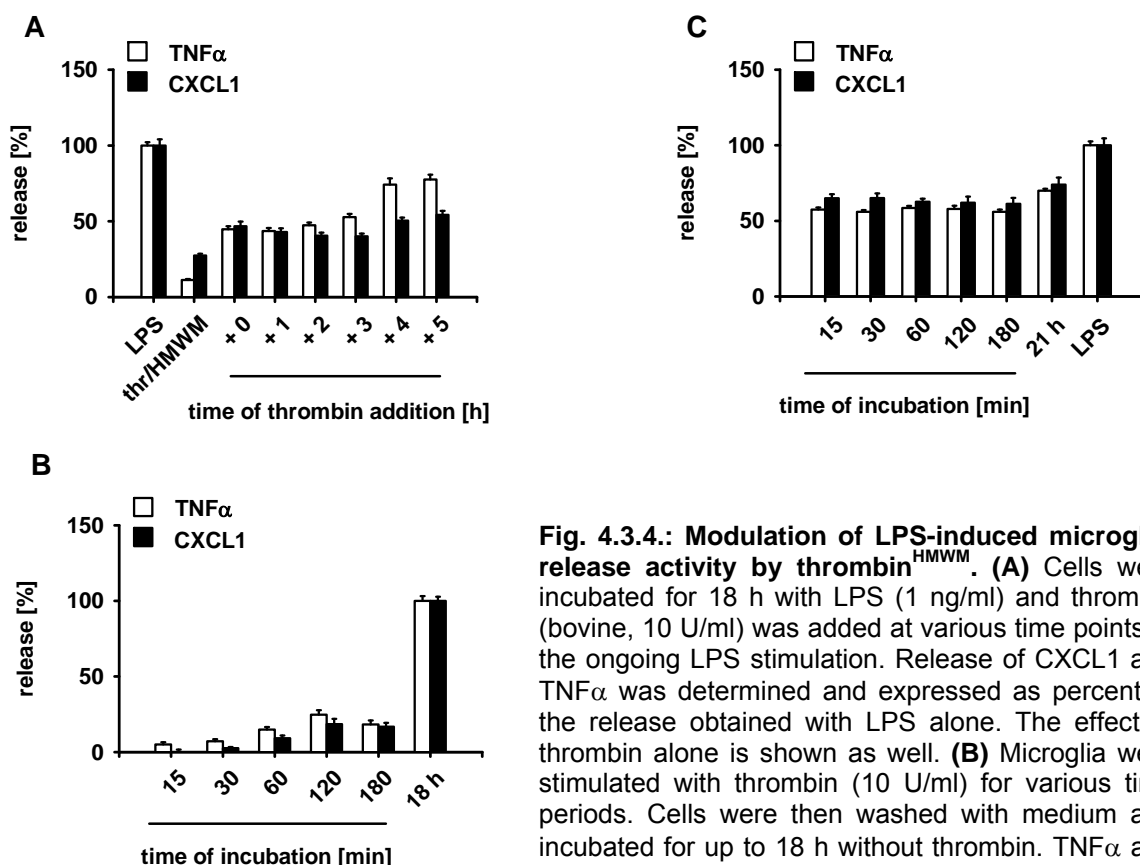**Fig. 4.3.4.: Modulation of LPS-induced microglial release activity by thrombin[HMWM]. (A)** Cells were incubated for 18 h with LPS (1 ng/ml) and thrombin (bovine, 10 U/ml) was added at various time points of the ongoing LPS stimulation. Release of CXCL1 and TNFα was determined and expressed as percent of the release obtained with LPS alone. The effect of thrombin alone is shown as well. **(B)** Microglia were stimulated with thrombin (10 U/ml) for various time periods. Cells were then washed with medium and incubated for up to 18 h without thrombin. TNFα and CXCL1 were collected in the time window between 3 and 18 h to allow for a direct comparison. Release was expressed as percent of release obtained with continuous thrombin stimulation (18 h, the first 3 h not affecting much the total amount). **(C)** Microglia were co-stimulated with LPS (1 ng/ml) and thrombin (10 U/ml) for various time periods, washed with medium and further incubated for 18h with LPS alone. Another group received the combination for the whole period of 21 h. CXCL1 and TNFα release was determined in comparable time windows and expressed as percentage of the amounts induced by LPS alone. Data are mean ± SEM from 3 experiments with an average of n=24 (A, C) or n=36 (B).

4.4 Thrombin[HMWM] activity for TLR4 does not derive from LPS contamination

Studies on endogenous TLR(4) agonists are always suspected to suffer from confounding impurities by microbial ligands, especially LPS (Bausinger et al., 2002; Tsan and Gao, 2007; Weinstein et al., 2008b). Competition for microglial TLR4 with the experimentally applied LPS did not rule out that contamination with LPS chemotypes from e.g. other bacterial strains (with lower potency) was responsible for the cellular activity assigned to

thrombin[HMWM]. Therefore, the effect of transient heat exposure was analyzed (Fig. 4.4.1.). Heat treatment did not influence the cellular activity of LPS, as tested over a wide concentration range (from 0.01 to 100 ng/ml, data not shown). However, gradual exposure of thrombin showed that incubation for 4 min at 70°C was sufficient to completely abrogate both, the cellular and protease activities, whereas LPS again remained unaffected (Fig. 4.4.1.A, B; red arrows).
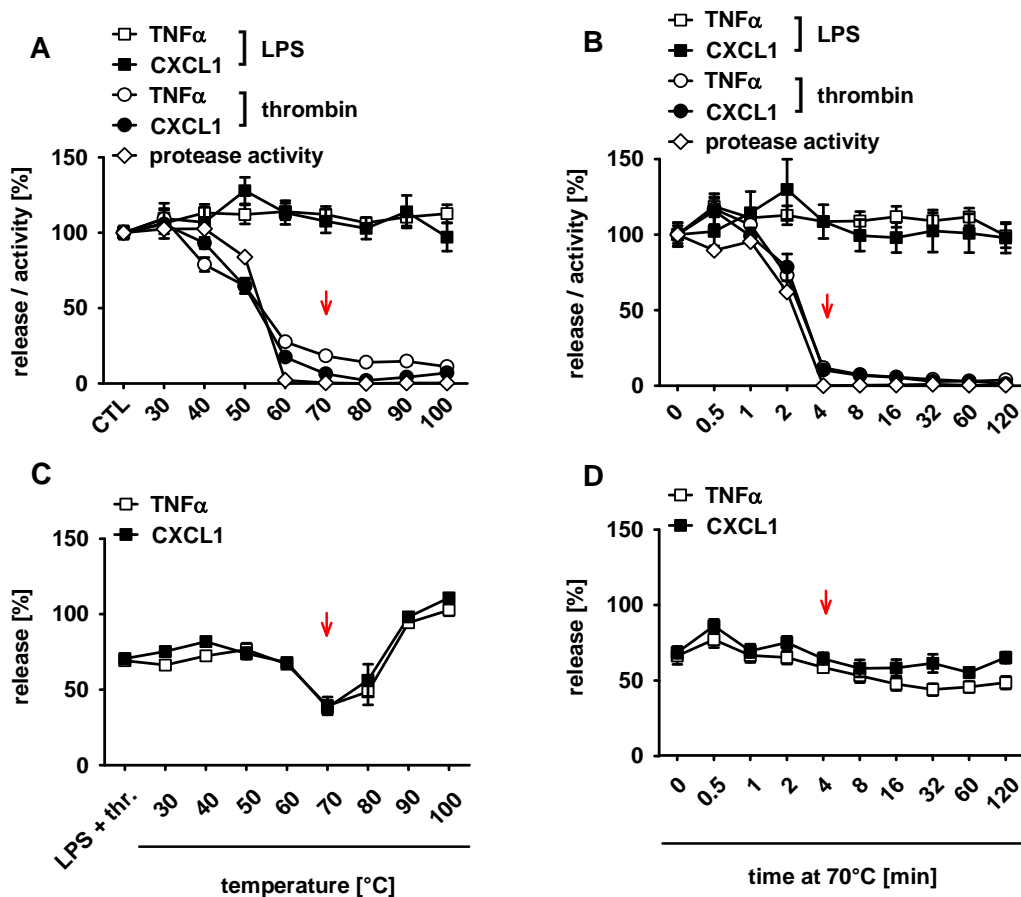
Fig. 4.4.1.: Dissociation of thrombin- and LPS-induced microglial release activity as well as of thrombic enzyme activity by thermosensitivity profiling. Solutions of bovine thrombin or LPS were incubated at **(A)** different temperatures for 10 min or **(B)** at 70 °C for various time periods, then diluted with medium to final concentrations of 10 U/ml and 1 ng/ml, respectively, and applied to cells for 18 h. TNFα and CXCL1 release was determined in the supernatants and is shown as percent of the respective release obtained with untreated thrombin or LPS. Proteolytic activity of thrombin was determined as in Fig. 4.2.2. Data are mean ± SEM with an average n=27 (A) and n=17 (B) from 3 experiments. **(C)** Bovine thrombin was incubated at different temperatures for 10 min or **(D)** at 70 °C for various time periods, subsequently mixed with untreated LPS, diluted with medium to final concentrations of 10 U/ml and 1 ng/ml, respectively, and applied to cells for 18 h. Release was determined as described above. Data are mean ± SEM with an average n=27 (A) and n=17 (B) from 3 experiments.

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Interestingly, the ability to compete with LPS was more stable, indicated by the reduced release upon co-stimulation as compared to the LPS effect alone. At 70°C, the 'inhibition' of LPS-induced release by thrombin^{HMWM} seemed to be even stronger (Fig. 4.4.1.C), because thrombin^{HMWM} lost at this step its own induction capacity and turned into a pure inhibitor (antagonist). However, exposure to more than 90°C finally destroyed the ability of thrombin^{HMWM} to bind TLR4. Signaling was now solely dependent on LPS and the release intensity 'recovered' to the control levels of LPS alone. A T of 70°C thus seems to be the critical threshold where TLR4 binding and signaling of thrombin^{HMWM} can be separated. Even longer incubation at 70°C did not cause a loss in the competitive, i.e. TLR4 binding function. Defined thermal stress thus led to the formation of a stable TLR4 antagonist (Fig. 4.4.1.D).

These data demonstrate that the microglia-activating potential of thrombin^{HMWM} is not simply a consequence of LPS contamination. Experiments employing the endotoxin binding antibiotic polymyxin B support this finding (not shown). Based on digestion with proteinase K, the protein nature of the activity carrier was subsequently also more directly confirmed (data not shown). Together, one could conclude that thrombin^{HMWM} critically depends on TLR4 to induce microglial responses and that it is capable to compete with LPS for the receptor.

4.5 Thrombin^{HMWM} signaling depends on a CD14/TLR4/MyD88/TRIF complex

As already described, TLR4 is known to associate with additional adapter and co-receptor molecules in order to facilitate appropriate responses to LPS and other agonists. The GPI-anchored CD14 is one of the key elements in the formation of a TLR4 receptor complex. It assists together with LBP in the transfer of LPS to the MD-2/TLR4 formation and its further dimerization. Involvement of CD14 in thrombin^{HMWM}-induced microglial responses was analyzed using CD14 blocking antibodies as well as cells from CD14^{-/-} mice (Fig. 4.5.1.). Blockade by the anti-CD14 clones 4C1 (Fig. 4.5.1.A) and big53 (data not shown) abrogated responses to LPS and thrombin^{HMWM}, whereas another clone (rmC5-3) failed to block both (data not shown). A more effective sterical hindrance could easily explain the stronger block for thrombin^{HMWM}, than for LPS. Experiments with cells being deficient for CD14 independently proved its mandatory function for thrombin^{HMWM} effects (Fig. 4.5.1.C).

Unmatched by other family members, TLR4 connects to both signaling adapters, MyD88 and TRIF, which control downstream kinases and transcription factors for TLR-

induced responses. MyD88 is thought to primarily control expression of proinflammatory mediators, whereas TRIF may largely control interferon-β (IFNβ) and interferon-regulated genes. For links to MyD88 and TRIF, TLR4 recruits the sorting adapters TIRAP/MAL or TRAM/TICAM-2, respectively. The dual and bridged access to MyD88 and TRIF provides TLR4 with a flexibility to convert binding of diverse agonists into adapted gene induction patterns.
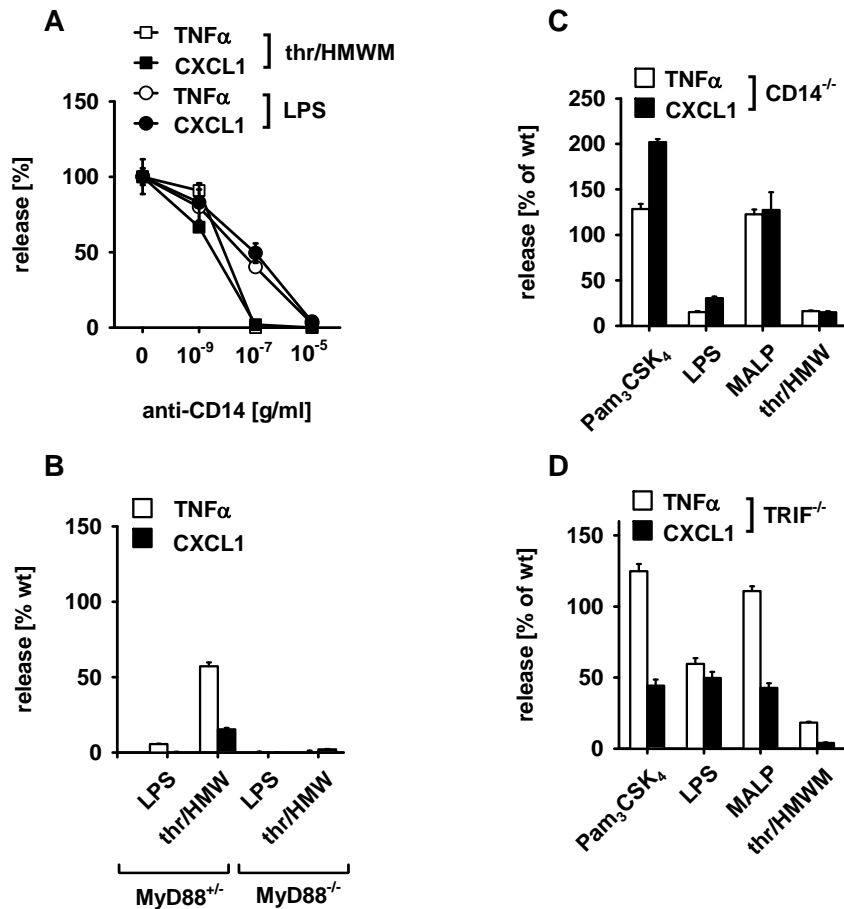
Fig. 4.5.1.: Effect of CD14 blockade as well as CD14, MyD88 and TRIF deficiencies on microglial cytokine and chemokine induction. Wildtype cells were stimulated with **(A)** LPS (1 ng/ml) or thrombin (bovine, 10 U/ml) in the presence or absence of a CD14 blocking antibody (clone 4C1) for 18 h, including a preincubation for 30 min. Another clone (big53) proved also to be inhibitory, while a third clone (rmC5-3) failed to block reactions to both thrombin[HMWM] and LPS (data not shown). Cells were also prepared from mice with **(B)** heterozygous (MyD88^{+/-}) or homozygous (MyD88^{-/-}) MyD88 deficiency or lacking **(C)** CD14 (CD14^{-/-}) or **(D)** TRIF (TRIF^{-/-}). Cells were stimulated with Pam$_3$CSK$_4$ (10 ng/ml), LPS (1 ng/ml), MALP (10 ng/ml) or thrombin (as above) for 18 h. Release of TNFα and CXCL1 was measured and is shown as percentage of the response of unblocked or wildtype cells. Data are mean ± SEM from 6 (A) or 3 (B, C, D) experiments with an average of n=24 or n=15, respectively.

To test the involvement of these adapter molecules for thrombin[HMWM]-induced responses, microglia from MyD88^{+/-}, MyD88^{-/-} and TRIF^{-/-} mice were used for stimulation experiments (Fig. 4.5.1.B, D). Absence of MyD88 led to an abrogation of thrombin[HMWM] and LPS effects, whereas cells expressing one functional allele (MyD88^{+/-}) remained partially responsive. Interestingly, TRIF deficiency had also a strong impact on thrombin[HMWM], whereas signaling of LPS and other TLR agonists was still allowed. The data also reveal some cooperativity between the pathways and even for TLR1/2 and TLR6/2. The dependence of thrombin[HMWM] on functional TRIF was surprising, as microglia did not show any INFβ release, a typical function governed by TRIF, for example upon LPS challenges (data not shown). As shown in chapter 4.18, similar results were obtained for IgGs. Thus, dependence on TRIF signaling despite lack of IFNβ induction was a common phenomenon of the DAMPs which were analyzed in this study.

4.6 Thrombin[HMWM] is a complex of coagulation-associated proteins

After demonstrating TLR4 as the essential receptor, the nature of the active thrombin[HMWM] constituents needed to be identified. Assumed to be co-purified with thrombin proper, thrombin[HMWM] was suspected to consist of some coagulation-associated plasma proteins.
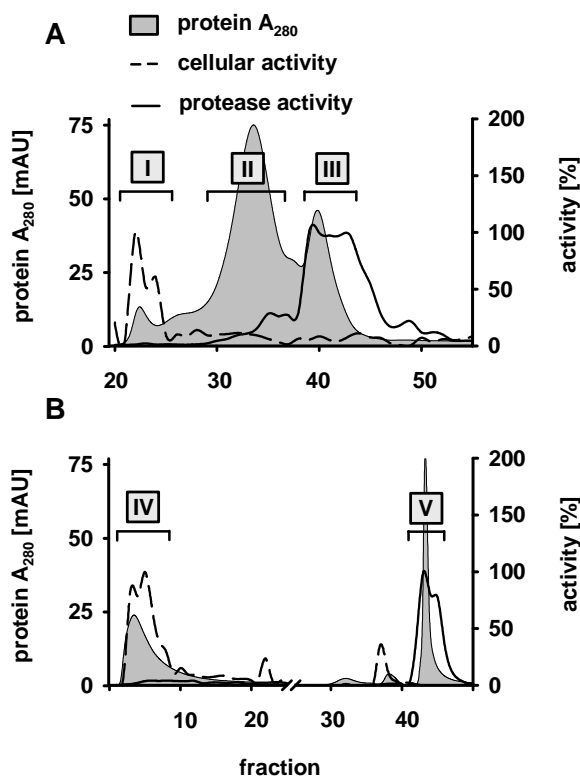
Fig. 4.6.1.: Separation of cellular and protease activities in a preparation of thrombin by size exclusion and affinity chromatography. Thrombin (bovine, 200 U) was analyzed with a **(A)** Superpose 12 10/300 gelfiltration column with a constant flow of 0.5 ml/min of PBS at RT or **(B)** with a HighTrap BenzamidineFF column, using a constant flow of 1 ml/min of loading buffer for 40 min, followed by elution buffer for 5 min at RT (see Material and Methods for details). Absorbance was measured at 280 nm. Fractions of 300 µl were collected. Each fraction was analyzed for protease and microglial activity as in Fig. 4.2.2. Fractions were pooled as indicated, incubated with trypsin (10 ng/µl) overnight at 37°C and the peptides were extracted with ACN/TFA, and analyzed by MALDI-TOF-MS as described elsewhere (Dihazi et al., 2005).

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Tab. 4.6.1.: Mascot search results of peptide fingerprints obtained from MALDI-TOF-MS. The table summarizes significant matches with a score* >28 and shows corresponding protein identities and accession numbers (SwissProt database). Fractions are indicated in which the proteins were found.

Fraction	Protein identity	Accession number	Protein mass (Da)	Queries matched	Score*
I	Thrombospondin-1**	Q28178	129,330	9	359
I, IV	Factor V**	Q28107	251,514	4	165 (I)
				2	86 (IV)
I	Tubulin-beta 2B chain	Q6B856	49,639	2	89
I	Alpha actinin-4	A5D7D1	104,788	2	73
I	Actin, cytoplasmic 1	P60712	41,710	2	68
I	Apolipoprotein A-I	P15497	30,759	1	67
I, II	TCP-zeta-2	Q3T084	57,597	2	31 (I)
				1	29 (II)
I, II	Complement C4	P01030	192,650	4	228 (I)
				2	48 (II)
I, II, IV	Fibronectin**	P07589	262,442	1	54 (I)
				1	54 (II)
				1	54 (IV)
I, II, III, IV	Inter-alpha-trypsin inhibitor H2**	P56652	106,370	7	287 (I)
				7	252 (II)
				4	252 (III)
				7	264 (IV)
II, IV	Actin, aortic smooth muscle	P62739	41,982	2	122 (II)
				2	72 (IV)
II	Lumican	Q05443	38,405	2	77
II	CD44	Q29423	81,503	1	65
II, III, IV	Inter-alpha-trypsin inhibitor H1**	Q0VCM5	101,326	2	46 (II)
				1	77 (III)
				3	108 (IV)
II, III, IV	Factor X**	P00743	54,697	3	162 (II)
				1	53 (III)
				3	139 (IV)
III, IV, V	Prothrombin**	P00735	69,992	3	108 (III)
				1	66 (IV)
				1	67 (V)

* Score is -10*Log(P), where P is the probability that the event is random, calculated as sum over all significant matches; scores >28 indicate identity or extensive homology (p<0.05).

** Proteins that are assumed to have a function in coagulation processes

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To further separate and enrich microglia-active components, a series of density gradient centrifugation (data not shown) and FPLC-based separation approaches were performed, proving size exclusion and affinity chromatography to be most suitable for unraveling the carrier(s) of activity (Fig. 4.6.1.A, B). Accordingly, bovine thrombin fractions from these FPLC procedures were analyzed for microglial and protease activity, pooled by activity, subjected to trypsin digestion and analyzed by MALDI-TOF-MS (in cooperation with Dr. Hassan Dihazi, Department of Nephrology and Rheumatology, University of Göttingen). Resulting spectra were used as peptide mass fingerprints to search for corresponding proteins in the Mascot database. Overall, 17 proteins were identified, 7 entries matching coagulation-associated proteins (Table 4.6.1., entries being marked by asterisks) and several others indicating cytosceletal proteins. As expected, thrombin (assigned as its precursor prothrombin) was found in fraction III (Fig. 4.6.1.), which contained the protease activity. Interestingly, one fragment was also found in fraction IV, although being devoid of enzymatic activity. However, it matched with our previous studies (Hanisch et al., 2004), suggesting that enzymatically inactive thrombin is incorporated into the HMW material. Most important for the potential to activate microglia via TLR4, fibronectin (FN) – and most likely the plasma-derived version, i.e. pFN – among fractions I, II and V represented a promising candidate (Tab. 4.6.1.). FN fragments were previously found to stimulate TNFα secretion by monocytes (Beezhold and Personius, 1992) and the extra domain A (EDA) of cellular fibronectin (cFN) was suggested to exhibit TLR4 activation potential (Okamura et al., 2001).
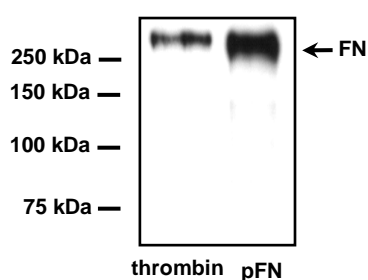
Fig. 4.6.2.: Immunoblot detection of FN in thrombin and FN preparations. Thrombin (bovine, 0.1 U) or pFN (bovine, 10 ng) were separated by SDS-PAGE (7% gel). FN was visualized with a cross-reactive anti-human FN antibody and an anti-rabbit HRP-conjugated secondary antibody.

Immunoblot analysis confirmed the presence of FN in thrombin[HMWM] (Fig. 4.6.2.). As shown below, FN does, indeed, share most of its microglial activation features. Yet it also differs in some aspects, suggesting that it contributes but does not account for all of the activity. The amount of pure FN needed to induce similar responses of microglia would be too high to explain the potency of thrombin[HMWM] (approximately 100fold as estimated from FPLC analysis). The complex nature or additional constituents – alone or in their combination – are probably of importance. Since MS analyses revealed several

coagulation-associated molecules in thrombin[HMWM], we assumed that the complex is generated during coagulation, i.e. as a consequence of thrombin action. As this is work still in progress, it was not included into the thesis.

4.7 Microglia responses to pFN depend on CD14/TLR4/MyD88 signaling

FNs are high molecular weight glycoproteins with complex structure and functions. They are found in extracellular fluids and represent a major component of the ECM. FNs are known to play important roles in cell adhesion and migration processes, hemostasis, inflammation and wound healing as well as tissue maintenance through interaction with other ECM, plasma and cell surface molecules (Moretti et al., 2007). In humans, more than 20 different isoforms are known so far, whereas in mice only 12 variants are described. The variations are a consequence of alternative splicing events of a single primary transcript and posttranslational modifications. The processing mainly occurs at three sites, i.e. at the extra domain A (EDA also known as EIII-A or EDI), extra domain B (EDB, EII-B or EDII) and the type III homologies connecting segment (IIICS). Splicing depends on the species and is regulated by the developmental stage, age and cell type. FN occurs in two major forms. pFN is a soluble form in the plasma and is comprised of two disulfide bond-linked subunits of 220 and 240 kDa lacking both EDA and EDB. It is produced by hepatocytes and secreted into the blood. cFN is produced by fibroblasts, epithelial and other cells and exists in the format of crosslinked, multimeric fibrils in the ECM material. In contrast to pFN, it contains both the EDA and EDB (Astrof et al., 2004).

Although the molecular mechanisms involved in FN processing are well known since two decades, the resulting functional consequences remain elusive. However, the EDA has been in the focus of investigations because it is elevated upon tissue damage and wound healing, pathologies, like rheumatoid arthritis, as well as during inflammation (Okamura et al., 2001). A link between EDA-containing cFN or its fragments and TLR4-induced inflammation was made for the first time by a study showing that recombinant EDA could activate THP-1 monocyte/macrophage cells and TLR4-transfected HEK 293 cells (Okamura et al., 2001). However, the EDA was produced in *E.coli*. Studies on DAMPs and TLRs, in general, arouse suspicion as to contaminations with microbial agonists, in this case LPS (Tsan and Gao., 2007). Moreover, it remained unclear whether EDA would be, indeed, the correctly assigned (true and only) interacting domain, especially since pFN would be devoid of it. To determine the microglia activation potential,

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we systematically analyzed preparations of pFN from various species as to the release induction capacity (Fig. 4.7.1.). Cells were stimulated at concentrations of ≤150 µg/ml, which are below plasma levels in mice (~600 µg/ml) and humans (~300 µg/ml) (Moretti et al., 2007). We found mouse, bovine and human pFN to trigger microglial release, whereas responses to rat pFN were negligible. This was surprising, because mouse and rat FN are most closely related, showing 97 % sequence identity, while bovine and human FN share only about 91 % with the mouse structure (clustalW sequence alignment).
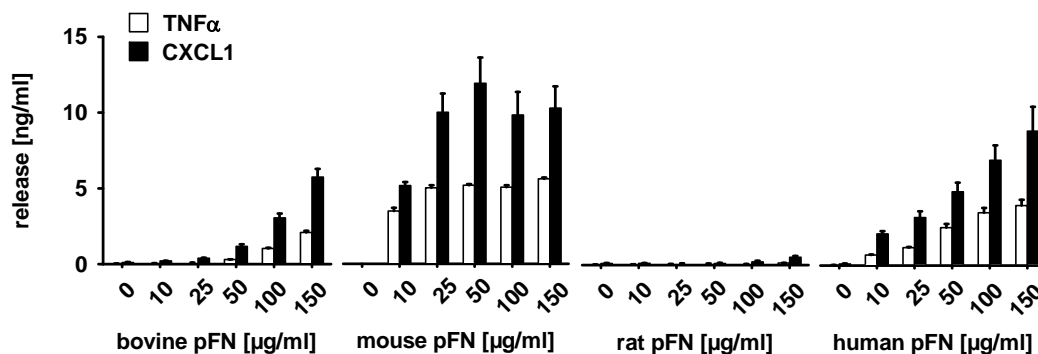
Fig. 4.7.1.: Induction of cytokine and chemokine release in microglia by plasma (p)FN. Cells were stimulated for 18 h with pFN from various species at different concentrations. TNFα and CXCL1 release was measured in the supernatants. Data are mean ± SEM from 2 experiments with an average of n=8.

As shown for thrombin[HMWM] (Fig. 4.2.1.), mouse pFN showed the highest activity for the mouse microglia, agreeing with the finding that it is an important constituent of this material. Importantly, microglial cultures would not be exposed to critical FN levels in their standard culture medium (10% FCS-supplemented), since the FN levels were in the subthreshold range (estimated from immunoblot analysis, data not shown).
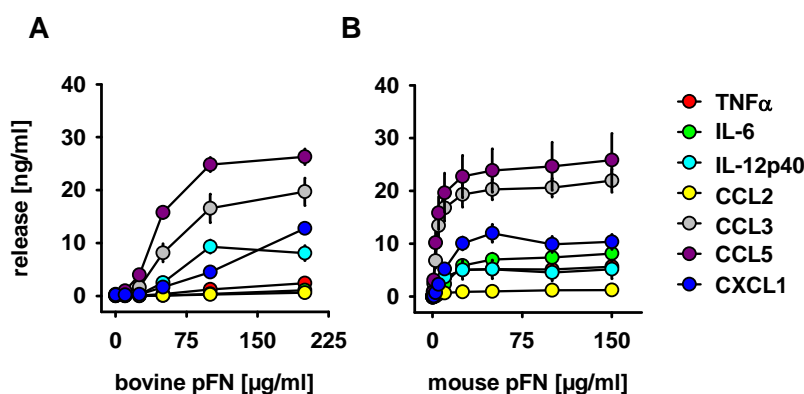
Fig. 4.7.2.: Microglial cyto- and chemokine induction pattern of pFN. Cells were stimulated for 18 h with plasma-derived **(A)** bovine or **(B)** mouse pFN and the release of cyto/chemokines was determined in the supernatants. Data are mean ± SEM from 2 experiments, with an average of n=8.

The induced pattern included the pluripotent immunoregulatory factors TNFα, IL-6 and IL12p40 as well as chemoattractants for T cells (CCL3/MIP-1α and CCL5/RANTES),

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neutrophils (CXCL1/KC, equivalent of human GROα) and monocytes/macrophages (CCL2/MCP-1, at very low levels) (Fig. 4.7.2.). We also measured NO production (Goos et al. 2007). The patterns of released factors were thereby identical for mouse and bovine pFN, although with a shift in their dose-response curves. A comparison of the responses from cells from the C57/Bl6 and NMRI strain also revealed virtually identical profiles (data not shown). Overall, the release pattern suggested a bias towards a proinflammatory (classical or M1-like) phenotype in microglia, as it appears in the context of an infectious challenge. Of course, the actual M1 or M2 orientations, or their respective facets, cannot be easily defined on a limited set of genes (see below). Meanwhile, however, release induction of representative cyto- and chemokines served as a reliable measure of cellular responses.
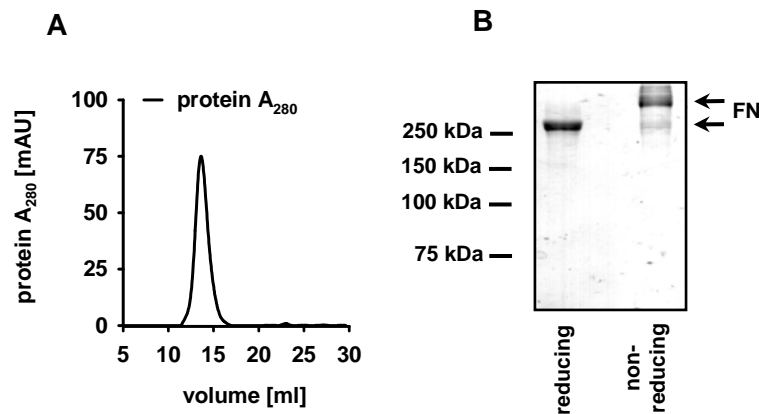
Fig. 4.7.3.: Analysis of pFN by size exclusion chromatography and SDS-PAGE. (A) pFN (bovine, 50 µg) was analyzed with a Superpose 6 10/300 gelfiltration column, using a constant flow of 0.5 ml/min of PBS at RT. Absorbance was measured at 280 nm. The single peak (at 13.7 ml elution volume) represented heterodimeric FN (~460 kDa). No components with higher (polymers, fibrillary protein) or lower molecular mass (fragments) were detected. **(B)** Separation of pFN (bovine, 10 ng) by SDS-PAGE on a 10% gel under reducing and non-reducing conditions. No further protein impurities were detected.

Calibration-assisted size exclusion chromatography of the bovine pFN revealed the heterodimer (~ 460 kDa) as the predominant form. No compounds with a higher molecular mass, i.e. oligo- or polymers, or fibrillary protein, or lower molecular mass, i.e. fragments, were detected (Fig. 4.7.3.A). The preparation was also free of other protein contaminations as shown by SDS-PAGE (Fig. 4.7.3.B).

As for thrombin[HMWM], microglial responses to pFN were strictly depended on the complex of CD14/TLR4/MyD88 (Fig. 4.7.4.; Goos et al., 2007). In contrast to thrombin[HMWM], however, TRIF signaling had only a minor contribution. There was just a

slight shift in the dose-response relation for TRIF$^{-/-}$ cells (Fig. 4.7.4.D). Accordingly, pFN also failed to induce IFNβ (data not shown).
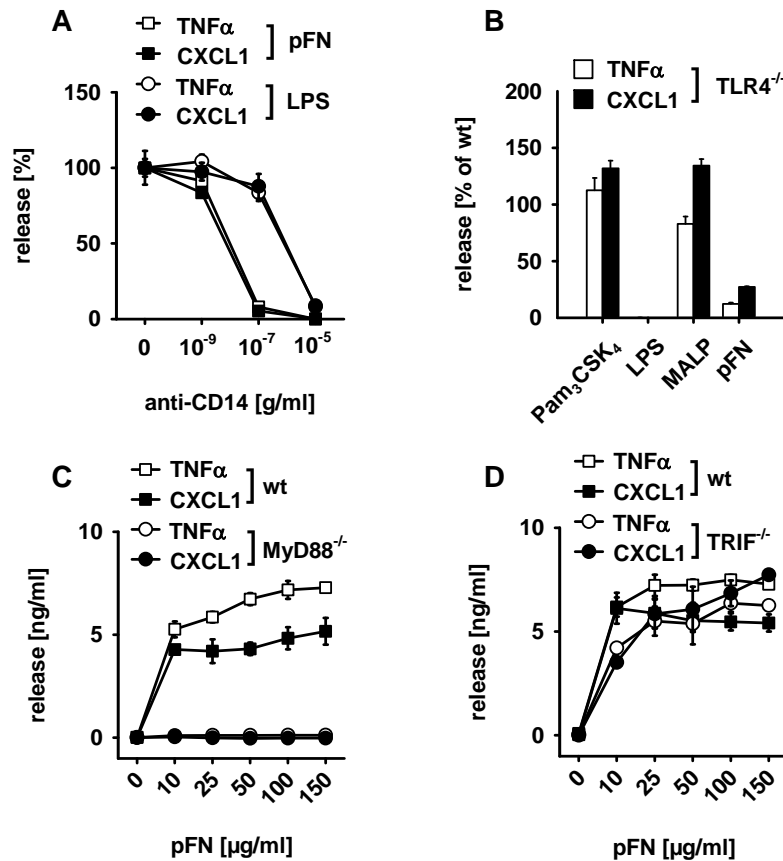
Fig. 4.7.4.: Effect of CD14 blockade or TLR4, MyD88 and TRIF deficiencies on the cytokine and chemokines induction in microglia by pFN. Wildtype cells were stimulated with **(A)** LPS (1 ng/ml) or pFN (bovine, 100 µg/ml) in the presence or absence of a CD14 blocking antibody (clone 4C1) for 18 h. Cells received a preincubation with the antibody for 30 min. Note that another clone (big53) proved to be inhibitory, whereas a third clone (rmC5-3) failed to block reactions to pFN and LPS. Cells were prepared from mice being deficient for **(B)** TLR4 (TLR4$^{-/-}$), **(C)** MyD88 (MyD88$^{-/-}$) or **(D)** TRIF (TRIF$^{-/-}$). Cells were stimulated with Pam$_3$CSK$_4$ (10 ng/ml), LPS (1 ng/ml), MALP (10 ng/ml) or pFN (100 µg/ml), or with various concentrations of pFN for 18 h. Release of TNFα and CXCL1 is shown as the percentage of the response of unblocked or wildtype cells, or in terms of a dose response as absolute values. Data are mean ± SEM with n=8 (A) and n=4 (B to D) per group.

Thrombin^{HMWM} and pFN also differed in their thermosensitivity profiles. Thrombin^{HMWM} lost the ability to stimulate microglia by a 10 min exposure to 70°C, whereas FN still induced a full-blown responses (Fig. 4.7.5.A), as further supported by the kinetics of inactivation at 70°C (Fig.4.7.5.B). The dissociated curves for TNFα and CXCL1 (between 1 and 10 min) suggest a different impact of stepwise FN denaturation on the TLR4 receptor/signaling mechanism for their induction. Exposure to 100°C or incubation at 70°C for 120 min finally abrogated microglial responses to FN.
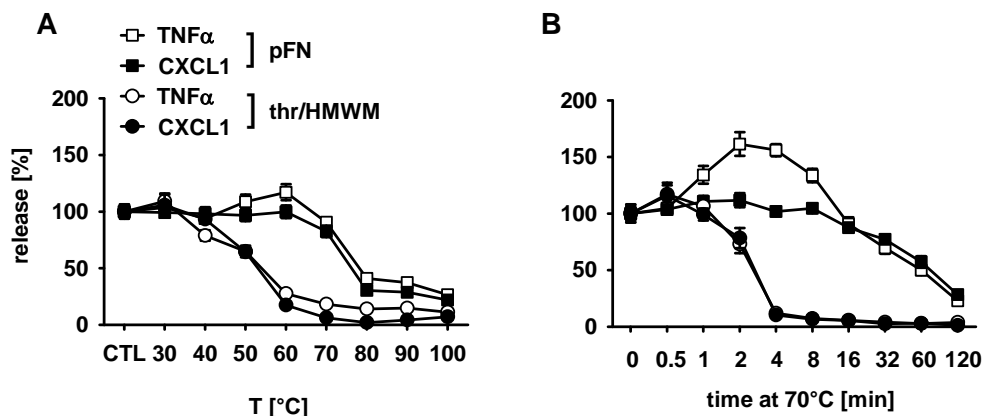
Fig. 4.7.5.: Thermosensitivity of thrombin^{HMWM} and pFN. Preparations of the bovine proteins were incubated **(A)** at various temperatures for 10 min or **(B)** at 70 °C for various time periods, subsequently diluted with medium to final concentrations of 10 U/ml and 100 µg/ml, respectively, and applied to the cells for 18 h. Release of TNFα and CXCL1 was measured in the supernatants and is shown as percent of release obtained with untreated proteins. Data are mean ± SEM from 2 experiments with an average of n=16.

The thermosensitivity of FN already indicated that microglial responses are not due to entrapped endotoxin. Proteinase K digestion confirmed the strict protein dependence by a complete loss of induction capacity (Fig. 4.7.6.). Since FN fragments containing EDA were postulated to activate TLR4 (Okamura et al., 2001), we sought to detect (or rule out) its presence within our full-length pFN by immunoblot analysis as well as surface enhanced laser desorption ionization time-of-flight-mass spectrometry. However, neither approach delivered evidence for EDA in the pFN (data not shown), suggesting that this domain is dispensable for the TLR4-mediated activation of microglia (see Discussion).
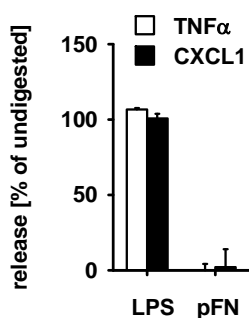
Fig. 4.7.6.: Effect of FN proteolysis on the induction of cytokines and chemokines in microglia. Solutions of LPS or FN were subjected to proteolysis by soluble proteinase K for 24 h at 37°C. The protease was removed, samples were diluted in medium to final concentrations of 1 ng/ml and 100 µg/ml, respectively, and applied to cells for 18 h. The release of TNFα and CXCL1 was measured in the supernatants and is shown as percentage of untreated controls. Data are mean ± SEM with an average of n=4.

TLR4 stimulation leads to the activation of the transcription factor NFκB and – especially via the MyD88 route – several downstream kinases, e.g. p38^{MAPK}, p42/44^{MAPK} or JNK (see Introduction). While NFκB would also be activated by TRIF (late response), its rapid activation and engagement of p38^{MAPK} would indicate a MyD88-driven response (Kawai

and Akira, 2007). Thus, the phosphorylation of p38^{MAPK} and NFκB p65 upon LPS and FN challenges was measured. The NFκB pathway responded within 5 min of LPS stimulation, with a maximal phosphorylation of the p65 subunit after 30 min (Fig. 4.7.8.A). Thereafter, the signal slowly declined. In response to pFN, the signaling was slower, showing the first p65 phosphorylation after 15 min. Maximal phosphorylation was also reached after 30 min, followed by a decline as seen for LPS.
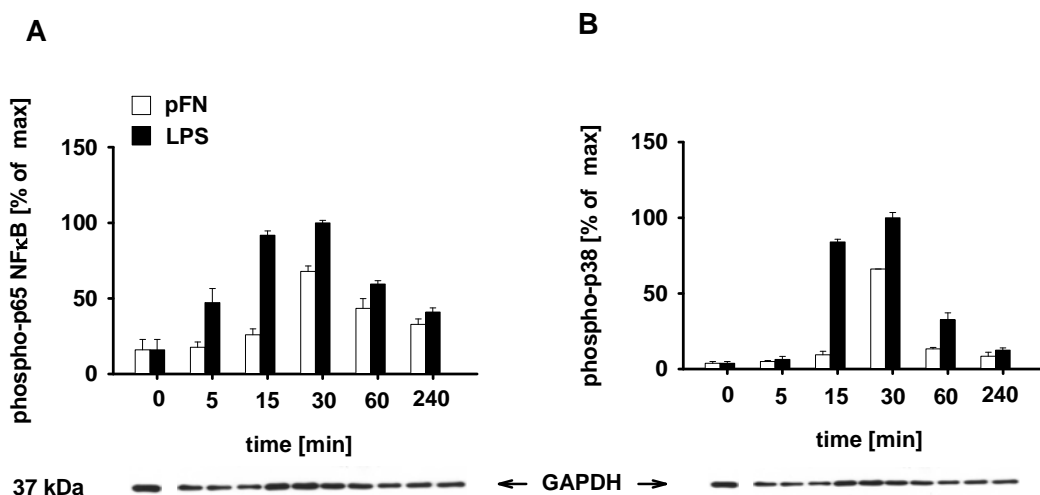
Fig. 4.7.8.: Activation of microglial NFκB p65 and p38^{MAPK} by LPS and pFN. Cells were incubated with LPS (1 ng/ml) or pFN (bovine, 100 µg/ml) for various time periods. Following cell lysis, proteins were harvested and the phosphorylation of **(A)** NFκB p65 or **(B)** p38^{MAPK} was measured by ELISA. Samples were also analyzed by GAPDH immunoblots to verify equal sample load. Data are mean ± SEM from 2 experiments with n=4.

Analysis of p38 phosphorylation revealed similar profiles as seen for NFκB, yet with distinct kinetics. Again, LPS triggered a phosphorylation more rapidly than seen with FN, but taking 15 min for a first increase, followed by a sharp decline of the signal (Fig. 4.7.8.B). The response to pFN basically followed the LPS profile, however, the time window of efficient p38^{MAPK} phosphorylation was even smaller, showing only a signal at the 30 min time point. Together the data indicate that both, LPS and pFN, signal via a MyD88-dependent route, however, with distinct kinetics (and intensities).

4.8 pFN can functionally displace LPS from its receptor

The data presented thus far suggest that the microglial responses to pFN require the CD14/TLR4/MyD88 pathway. Nevertheless, we sought to prove a TLR4 dependency by another method. TLR4 expression levels on microglial cells are very low, as we found by FACS analysis and immunostaining (data not shown), making it impossible to show the

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direct interaction of pFN with TLR4 by optical methods. We thus investigated, whether pFN would interfere with LPS signaling and modulate LPS-induced release responses as thrombin[HMWM] did (chapter 4.3). In a series of experiments, pFN was thus combined at increasing concentrations with LPS, used at its saturating concentration of 1 ng/ml.
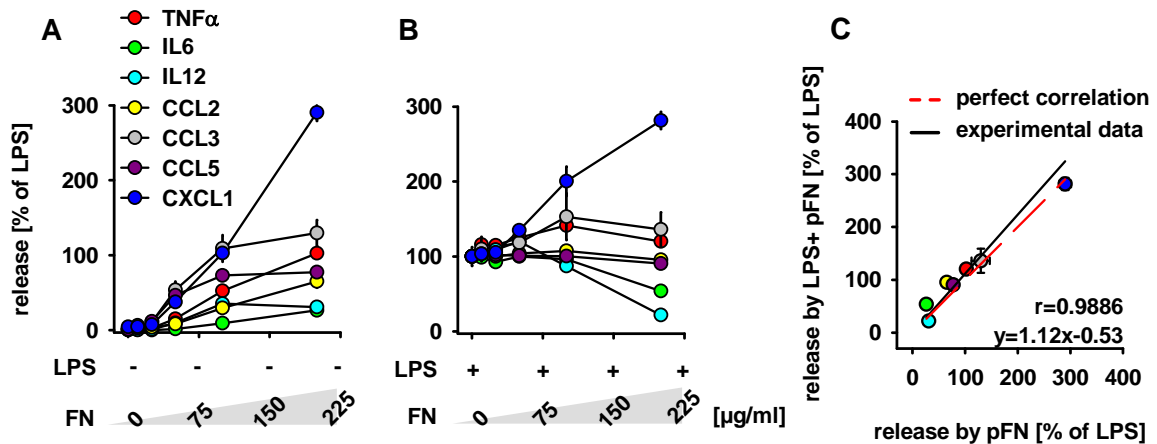
Fig. 4.8.1.: Induction, co-induction and correlation analysis of cytokine and chemokine release by FN and LPS. Cells were treated with **(A)** bovine pFN alone or **(B)** together with LPS (1 ng/ml) at various concentrations for 18 h. Release of cyto/chemokines was determined and then **(C)** correlated for both conditions under the highest FN concentration used (200 µg/ml). In (B), FN altered the LPS-induced amounts as itself triggered more release of CXCL1, less of IL-12p40 or IL-6 and about the same for, e.g. CCL2 and CCL5. The higher the concentration of pFN, the more the combined stimulation resembled in its profile the release as triggered by pFN alone. At a pFN concentration of 200 µg/ml, it dominated the release profile, reflected by the direct correlation in (C). Presence of LPS in this situation had no more influence on the release pattern, suggesting full functional replacement, most likely based on physical competition for TLR4. Note the perfect match of the regression analysis and also the parameters of slope and y axis intercept. Data are mean ± SEM with an average of n=8.

pFN alone produced a specific release pattern, with first detectable amounts of cyto/chemokines at about 50 µg/ml and reaching almost maximal induction at 200 µg/ml. This pattern was distinct from that of LPS (Fig. 4.8.1.A) and that of thrombin[HMWM] as well (Fig. 4.3.3.). Factors, like IL-6 or IL-12p40, were less, others, i.e. CXCL1, were stronger induced, and again others were almost equally triggered (TNFα, CCL5). FN added at increasing concentrations to the combined stimulation then altered the release induced by LPS (Fig. 4.8.1.B). IL-6 and IL-12p40 were reduced, CXCL1 was increased, and others remained more or less unaffected. At 200 µg/ml, the release profile resulting from the combined stimulation with LPS was nearly identical to that generated by FN alone, as reflected by an almost perfect correlation (Fig. 4.8.1.C). In other words, FN and LPS did not synergize. At a sufficiently high concentration, FN dominated the response pattern. FN

had functionally replaced LPS, because it succeeded to displace the microbial agonist from the joint receptor, TLR4.

4.9 Thrombin[HMWM]-, pFN- and LPS-induced microglial responses are differently modulated by immunoregulatory cytokines

The data presented thus far revealed that thrombin[HMWM] as well as pFN – as a HMWM constituent – can induce microglial responses by utilization of TLR4. Thereby, these molecules would function as DAMPs. A major question was how the system would facilitate the recognition and binding of molecules having not much structural similarities – such as LPS and pFN. The fact that TLR4 recruits co-receptor and binding molecules, such as CD14 and LBP, for appropriate and differentiated LPS recognition suggests that TLR4 may also associate with accessory surface receptors for DAMP binding. This would not only provide the required binding specificity to the various ligands but also allow for further intracellular signaling options – as indicated by the differential use of TRIF (see chapters 4.5 and 4.7) and IFNβ induction. We thus aimed at a demonstration of functional discrimination between LPS as a PAMP versus FN and thrombin[HMWM] as DAMPs. Thrombin[HMWM], pFN and LPS were paired with certain key immunoregulatory cytokines in co-stimulatory experiments to determine the influence on the resulting cyto/chemokine profile (Fig. 4.9.1.). In these settings, any contribution of a given cytokine would apply in the very same way to the three agonists, provided that their TLR4 signaling is completely identical. In contrast, if the joint stimulation outcome with the cytokine would differ for the TLR4 agonists, their signaling has to be differently organized in first place. The former assumption considers LPS, thrombin[HMWM] and FN as non-differentiating receptor agonists. The latter concept would allow for individual consequences.

IFNβ had not much influence on the release profile of LPS, it basically suppressed the chemoattractive signals for neutrophils (CXCL1). In contrast, it enhanced chemo-attractants for macrophages and monocytes (CCL2) or IL-6, while sparing TNFα and signals for T cells (CCL3, CCL5) in the case of thrombin[HMWM] and pFN (Fig. 4.9.1.). Although being similar, the profile modulation also revealed differences among the DAMPs, such as by the regulation of CCL5 and IL-6. These findings are of particular interest, because DAMPs did not induce IFNβ – in contrast to LPS. In an infectious situation, LPS could thereby modulate responses to DAMP signals by auto/paracrine loops while not being influenced itself.
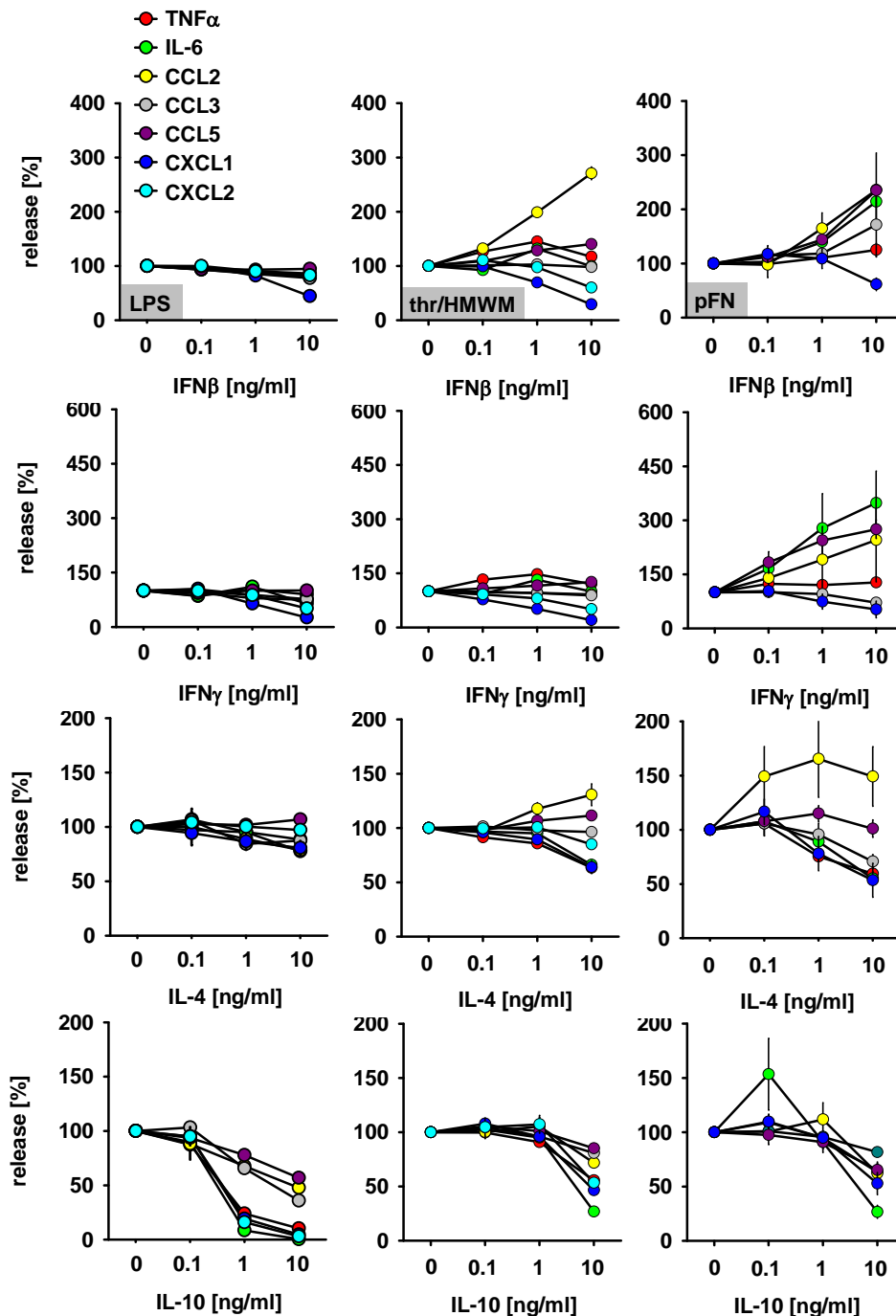
Fig. 4.9.1.: Modulation of LPS-, pFN- and thrombin[HMWM]-induced microglial release profiles by immunoregulatory key cytokines. Cells were stimulated with LPS (1 ng/ml), thrombin (bovine, 10 U/ml) or pFN (bovine, 100 µg/ml) for 18 h in the presence or absence of IFNβ, IFNγ, IL-4 or IL-10 at various concentrations. The TLR4 agonists and the cytokines were given at the same time. Release of cyto/chemokines was measured and expressed as percentage of controls (stimulation in the absence of the modulatory influences). Data are mean ± SEM with n=12 from 3 experiments. In this study, cytokines by themselves were found to not trigger relevant release.

Moreover, IFNβ is used as a therapeutic agent in multiple sclerosis (Goodkin D, 1994, Prinz et al., 2008), with a recently revealed involvement of innate immune cells, namely microglia. The Th1 cytokine IFNγ, which is released by activated Th1 and NK cells, could influence the profiles of all three TLR4 agonists. Microglial responses to thrombin[HMWM] and LPS revealed an almost identical modulation, basically suppression of attractants for neutrophils. In the context of a pFN stimulation, however, the cells further responded with upregulation of IL-6, CCL2 and CCL5. The influence of the Th2 master cytokine IL-4 would then reflect situations as they may occur in Th2 type immune responses or during later phases of microglial activation and termination of inflammatory processes. IL-4 did not alter much the LPS-induced release, but affected the responses to thrombin[HMWM] and pFN – again in a similar yet individual fashion. Only under IL-10, the immunosuppressive cytokine produced e.g. by alternatively activated macrophages or B cells, all of the TLR4 agonist-induced reactions underwent a downregulation.

These experiments allow for three conclusions. First, TLR4 accepts microbial LPS and endogenously derived agonists but then transduces distinct signaling. Differentiating consequences are even more prominent when TLR4 stimulation is embedded in a more complex signaling context. Second, TLR4-triggered response programs in an infectious emergency situation are less variable under certain cytokine and interferon influences, than the responses to endogenous ligands as occurring upon damage or in sterile inflammation. While thrombin[HMWM] and FN could efficiently compete with LPS for TLR4 usage, a predominant LPS presence could exert a self-sparing release control of DAMPs via IFNβ. Third, the ability to distinguish even between closely related DAMPs (see also chapter 4.20) may, indeed, require some co-receptors conferring binding and signaling specificity.

4.10 Microglial responses to pFN involve integrin assistance

Integrins belong to the large family of cell adhesion receptors which mediate cellular attachment to the ECM as well as cell-cell interactions. They are obligate heterodimeric molecules. 18 α and 8 β subunits – known so far in mammalia – assemble to a total of 24 integrin molecules with specific functions. Integrins play key roles in development and the regulation of diverse functions, like hemostasis, tumor growth and metastasis, leukocyte trafficking and inflammation (Hynes, 2002). Intracellularly, integrins are connected to actin-based filaments and provide mechanical links from the extracellular space to the

cytoskeleton. However, integrin engagement also triggers several signaling events to control and modulate cellular behavior (Clark and Brugge, 1995; Hynes, 2002).
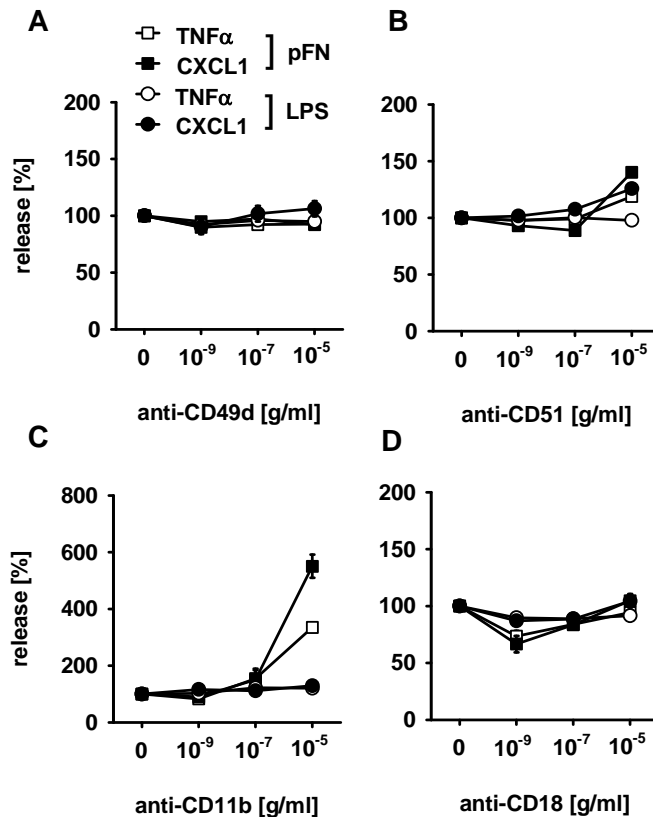
Fig. 4.10.1.: Effects of interference with integrin functions on the microglial cytokine and chemokine release induction by pFN. Cells were stimulated with pFN (bovine, 100 µg/ml) or LPS (1 ng/ml) in the presence or absence of **(A)** anti-CD49d (α4), **(B)** anti-CD51 (αV), **(C)** anti-CD11b (αM) or **(D)** anti-CD18 (β2) blocking antibodies for 18 h. Cells received a 30 min pre-incubation with antibodies. Note that anti-CD51 induced a release response by itself at 10^{-5} g/ml, a phenomenon probably relating to the work in chapters 4.13-4.24, whereas other antibodies used here were inactive (data not shown). TNFα and CXCL1 were measured and are shown as percent of release in the absence of blocking antibodies. Data are mean ± SEM with an average of n=4 per group.

FN contains several copies of the tripeptide sequence RGD that is recognized by a set of integrins and several integrins are known for FN interaction. Conceivably, integrins could facilitate binding and/or signaling events in complex with TLR4. We determined potential integrin contributions to pFN-triggered responses of microglia. The studies were based on blocking antibodies against certain integrin subunits since knockout mice show severe phenotypes ranging from early lethality (embryonic and perinatal) to major defects in cell functions (Hynes, 2002). Experiments included antibodies against the α4 (CD49d), αV (CD51), αM (CD11b) and β2 (CD18) subunits (Fig. 4.10.1.). Antibodies were tested for their own release induction capacity (data not shown) and proved to be inactive on microglia, except for the αV antibody, that showed profound release induction at the highest concentration (10^{-5} g/ml). Potential underlying mechanisms are actually addressed in chapters 4.13-4.24.

Block of the integrin subunit αM with an anti-CD11b antibody resulted in a strong enhancement of the pFN-driven release response, while a LPS-induced release was not

affected. This subunit joins β2 (CD18) to assemble the heterodimeric integrin αMβ2 (Mac-1, complement receptor 3, CR3), that is specifically found on cells of the hematopoetic linage, including microglia. Blockade of CD18, however, did not affect the pFN-induced release, suggesting different sterical hindrance and/or roles for both subunits. Indeed, while the αM subunit would facilitate binding to various αMβ2 ligands, the β2 subunit has a more limited repertoire of ligands and basically supports cellular migration (Solovjov et al., 2005). It is noteworthy, that CD11b/CD18 was already reported for the binding of plasma proteins, such as fibrinogen and fibronectin. Interestingly, we could not observe responses to (mouse) fibrinogen, nor did we see consequences of a CD11b block with thrombin[HMWM] (data not shown), implying selectivity for the FN-TLR4 signaling in microglia.

The enhanced response under anti-CD11b blockade could be interpreted as a negative control by the integrin to reduce FN-driven TLR4 consequences. We sought for another method to prove this hypothesis. Citrullination is a post-translational modification. Specific enzymes convert arginine residues into citrulline by deimination, a process also intimately linked with rheumatoid arthritis (Alivernini et al., 2008). Conversion of the positively charged arginine into citrulline alters the protein net charge and can result in dramatic structural changes (Tarcsa et al., 1996). Most importantly, it alters FN-integrin interactions (Chang et al., 2005). Thus, pFN was citrullinated *in vitro* by peptidyl arginine deiminase (PAD) and used for microglial stimulation.
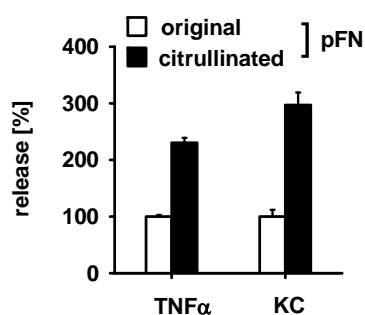
Fig. 4.10.2.: Effect of pFN citrullination on its cytokine and chemokine release induction in microglia. Mouse pFN was citrullinated using PAD (7 U/mg protein, at 37°C, 24 h). The enzyme was removed by affinity chromatography using immobilized soybean trypsin inhibitor. The cells were stimulated with citrullinated or unprocessed pFN (50 µg/ml) for 18 h. Release of TNFα and CXCL1 was measured and is shown as percentage of the release of the unprocessed pFN. Data are mean ± SEM with an average of n=4 per group. Experiments on bovine pFN gave the same results (data not shown).

Agreeing with the findings on CD11b blockade, citrullination of pFN did enhance microglial release responses. The αMβ2 integrin might thus be recruited to TLR4 upon FN binding, dampening part of the responses, while unleashing full signaling when being blocked or otherwise disabled. Its contribution to a TLR4 complex would thus not be mandatory for binding, but rather regulatory for signaling.

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4.11 The microglial activation by pFN requires conformational flexibility

While pFN is produced and secreted into the blood stream by hepatocytes, cFN is locally produced by various cell types (Astrof et al., 2004). As CNS cells, astrocytes and microglia are also capable of producing substantial amounts of cFN (Liesi et al., 1986); Fig. 4.11.1.). It is cross-linked with other ECM proteins, such as collagen and laminin, and deposited as insoluble fibrils to form tissue ECM (Pankov and Yamada, 2002). The concept of DAMP signaling suggests that endogenous molecules (only) gain such a character when being produced, released or modified upon cell and tissue injury. While pFN would be usually denied access to the CNS parenchyma by the BBB, cFN as part of the ECM should thus not be recognized as a DAMP by microglia under normal conditions. We, therefore, raised the question whether microglia would respond to immobilized (p)FN as they do to the soluble form.
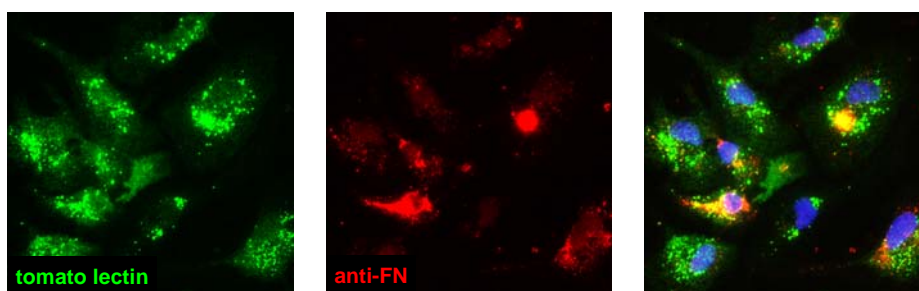
Fig. 4.11.1.: Expression of FN by microglia. (A) Microglial cells were plated on PLL-coated coverslips, cultured under serum-free conditions for 18 h and fixed with 4% PFA. Microglia were stained with tomato lectin-FITC (green). FN was visualized with a cross-reactive anti-hFN-Cy3 antibody (red). Nuclei were stained with DAPI (blue). Pictures were taken at a magnification of 400x.

Microglia did not execute any release when being placed on plates which were coated with pFN (Fig. 4.11.2.A, B), even to a saturated level to maximize the contact surface (Fig. 4.11.2.C). Moreover, neither were the responses to TLR1/2 and TLR6/2 ligands or LPS and thrombin[HMWM] – and soluble pFN itself – affected. Although not totally mimicking the ECM complexity, these data indicate that FN needs some conformational flexibility to be functionally recognized by TLR4. ECM-incorporated cFN would miss this important feature. ECM breakdown upon injury or proteolysis by infiltrating cells would liberate soluble FN (fragments) to alert microglia. Similarly, pFN inundating the microglial environment from the circulation would spell danger as associated with vascular leakage.

A

B

C
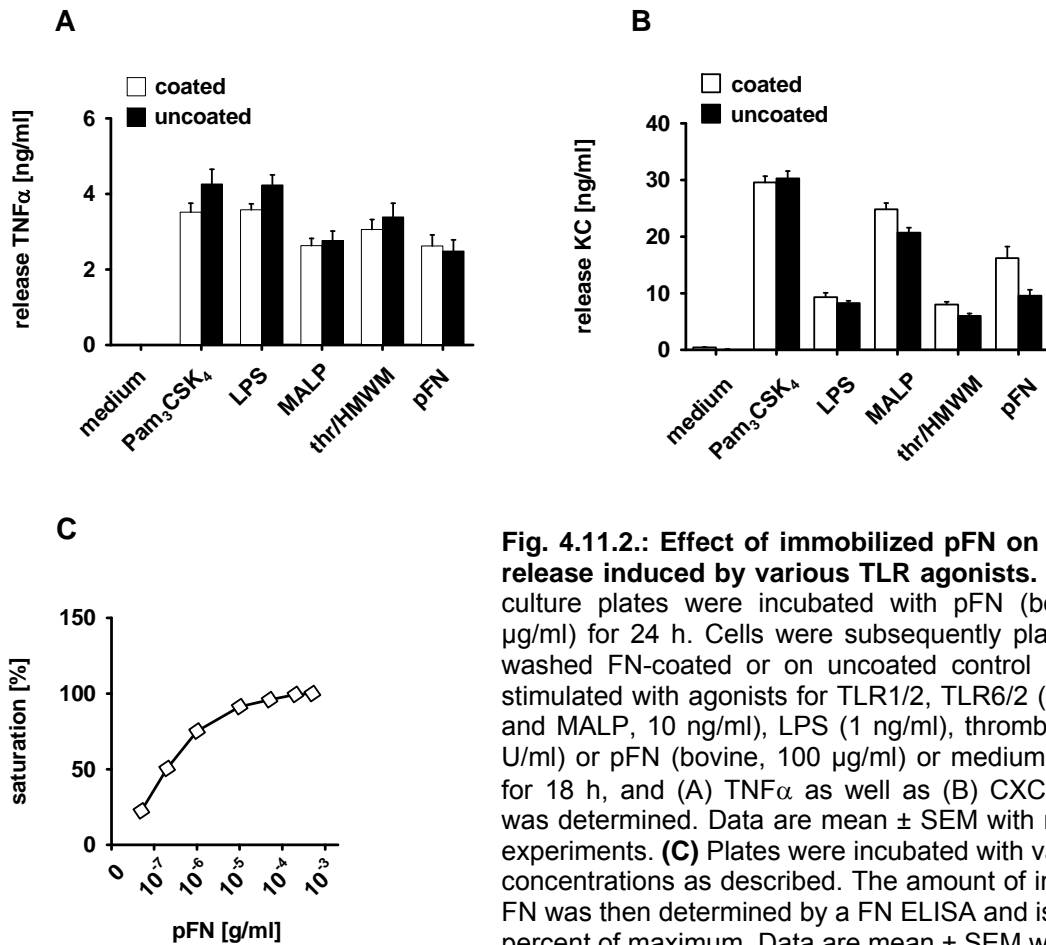
Fig. 4.11.2.: Effect of immobilized pFN on microglial release induced by various TLR agonists. (A, B) Cell culture plates were incubated with pFN (bovine, 100 µg/ml) for 24 h. Cells were subsequently plated on the washed FN-coated or on uncoated control plates and stimulated with agonists for TLR1/2, TLR6/2 (Pam$_3$CSK$_4$ and MALP, 10 ng/ml), LPS (1 ng/ml), thrombin[HMWM] (10 U/ml) or pFN (bovine, 100 µg/ml) or medium as control for 18 h, and (A) TNFα as well as (B) CXCL1 release was determined. Data are mean ± SEM with n=6 from 2 experiments. **(C)** Plates were incubated with various pFN concentrations as described. The amount of immobilized FN was then determined by a FN ELISA and is shown as percent of maximum. Data are mean ± SEM with n=4.

4.12 Microglia respond to LPS or pFN with individual gene expression patterns

The individual cyto- and chemokine patterns induced by LPS versus thrombin[HMWM] and pFN as well as the distinct modulatory influences taken by certain key cytokines could probably stand for more differences in the control of gene expression. To compare such patterns and to identify possible targets serving as markers for individual phenotypes, cells were stimulated with LPS and pFN, or just kept in medium as controls, for analysis by a whole genome gene array (processed in cooperation with Dr. Gabriela Salinas-Riester, Transcriptome Analysis Laboratory, University of Göttingen). The transcriptomes revealed overlapping as well as differential gene regulations by pFN and LPS (Fig. 4.12.1.A). A total number of 3801 genes differed significantly (log$_2$FC≥1 and P≤0.05). Interestingly, among the most downregulated genes under LPS stimulation we found FN and factor XIII – the coagulation factor that cross-links FN into the fibrin clot after thrombin cleaved fibrinogen. This process stabilizes the growing thrombus at the site of injury. In a

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first approach, we classified the regulated genes into categories according to their function in biological processes and to extract any bias towards some 'classical', proinflammatory (M1) or 'alternatively' activated (M2) phenotype. Using the PANTHER (protein analysis through evolutionary relationships) classification tool (Thomas et al., 2003), 2511 genes could be assigned to known gene products. Further analysis revealed 68 entries to have a function in carbohydrate metabolism, 94 to be part of cell adhesion processes, 306 with an impact in immunity and defense and 21 entries that would play a role in coagulation (Fig . 4.12.1.B).
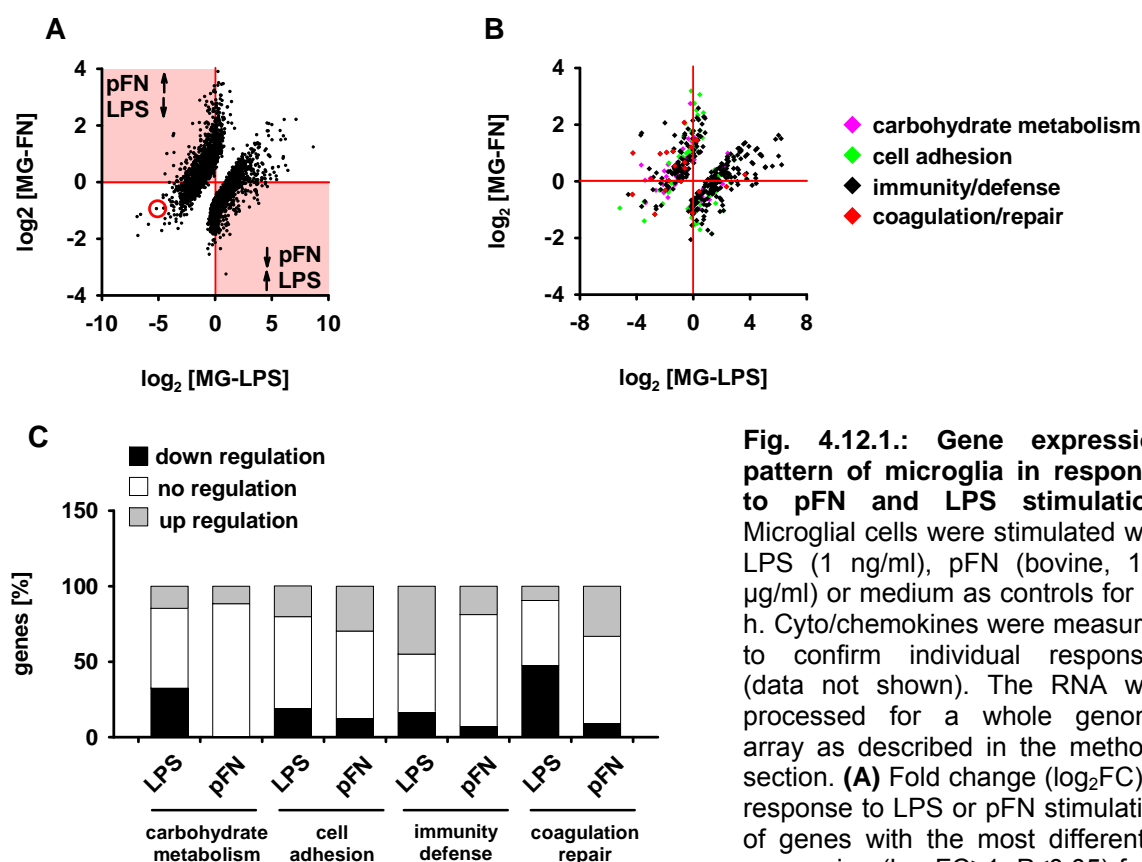
Fig. 4.12.1.: Gene expression pattern of microglia in response to pFN and LPS stimulation. Microglial cells were stimulated with LPS (1 ng/ml), pFN (bovine, 100 µg/ml) or medium as controls for 24 h. Cyto/chemokines were measured to confirm individual responses (data not shown). The RNA was processed for a whole genome array as described in the methods section. **(A)** Fold change ($\log_2$FC) in response to LPS or pFN stimulation of genes with the most differential expression ($\log_2$FC≥1, P≤0.05) from 3 experiments. We found 3801 genes to be differentially expressed. Note that FN was one of the most down-regulated genes by LPS (in red circle). **(B)** Classification of differently regulated genes (as under A) according to their function in carbohydrate metabolism (68), cell adhesion (94), immunity/defense (306) or coagulation (21), identified by using the 'PANTHER' classification tool. **(C)** Classified genes from (B) were grouped according to their regulation (up-regulation by $\log_2$FC≥1 or down-regulation by $\log_2$FC≤1), compared to unstimulated cells. Expression patterns revealed a bias towards up-regulation of immunity/defense-associated genes by LPS, whereas FN up-regulated genes related to coagulation and repair.

The gene expression pattern revealed a bias towards up-regulation of immunity/defense-associated genes by LPS, whereas FN regulated genes related to coagulation and repair

processes. However, as the cyto/chemokine release upon pFN stimulation had already indicated, the gene pattern did not point clearly to either a M1 or M2 decision. The lack of a simple categorization goes in line well with observations on macrophages, describing numerous 'non-classical' phenotypes by transcriptional and non-transcriptional features in different tissues or disease situations (Gordon and Taylor, 2005). Thus, the FN-instructed phenotype comes with elements that are part of inflammatory reactions while others are linked to wound healing and repair. While a detailed analysis of the induced expression patterns and its correlation with functional consequences will be addressed elsewhere, a first view already supported the previous observations that microglial cells do not respond to the individual TLR4 agonists in a stereotyped fashion. Their TLR4 acts as a versatile antenna for diverse exogenous and endogenous signals which can interpret the nature of the agonist. Forming complexes with additional surface molecules seems to be a mean to achieve this capacity. The following part of the thesis will provide evidence that some microglial responses to immunoglobulins can occur independently of FcRs – by involving a TLR4-centered signaling complex.

RESULTS (PART B)

4.13 Immunoglobulins as DAMP signals for microglial cells

This part of the work focuses on the cellular and molecular mechanisms by which IgGs can recruit and instruct microglial cells for functional responses by y non-FcR mechanism. In the following chapters we show that IgGs that are not in complex with an antigen (i.e., monomeric IgGs) can activate microglia to produce characteristic cyto- and chemokine patterns. The microglial activity of the IgGs is thereby controlled by the isotype, environmental influences, like the pH, as well as posttranslational modifications, such as glycosylation. Microglia recognize the IgGs via a receptor mechanism involving a TLR4-centered signaling complex with only partial contribution of functional Fc receptors (FcRs, in particular FcγRs). Analogous to the findings on thrombin[HMWM] and FN, this interaction would provide the ligand specificity of microglial responses. These findings underscore once more the importance of TLR4 in recognizing not only exogenous PAMPs but also endogenous DAMPS. Furthermore, our observations go in line with the hypothesis of microglial phenotype diversity, assuming that these cells react with distinct – assumed to be appropriate – programs depending on the nature of a stimulus and its situational context. Our findings are largely based on mouse IgGs and primary preparations of P0 microglia from mouse strains. Experiments employing human IgGs on mouse cells were performed to prove, at least partially, species independence of the principle of TLR4-Ig binding and signaling.

4.14 Microglia respond to IgG stimulation with release of cytokines and chemokines

Based on the initial finding that microglia can respond with the release of cyto- and chemokines upon exposure to IgGs that are not bound to their corresponding antigen, i.e. not presented as an immune complex (IC), we systematically analyzed IgG preparations of various sources and suppliers for their induction capacity (Tab. 4.15.). We stimulated cells for 18 h with IgGs at doses of up to 250 µg/ml. These concentrations are at least one order of magnitude below the normal isotype plasma levels and still in the range of CSF levels (as determined in samples from patients with relapsing-remitting MS, [Innsbruck cohort of CSF and serum], data not shown). Although preparations from the various suppliers differed in their activity (Tab. 4.15.1.), we found in general IgG$_3$, IgG$_{2B}$ and IgG$_1$

to trigger microglial release (induction capacity this order, i.e. $IgG_3 \geq IgG_{2B} \geq IgG_1$), whereas responses to the IgG_{2A} isotype were usually negligible (Fig. 4.14.1., Tab. 4.15.1.).
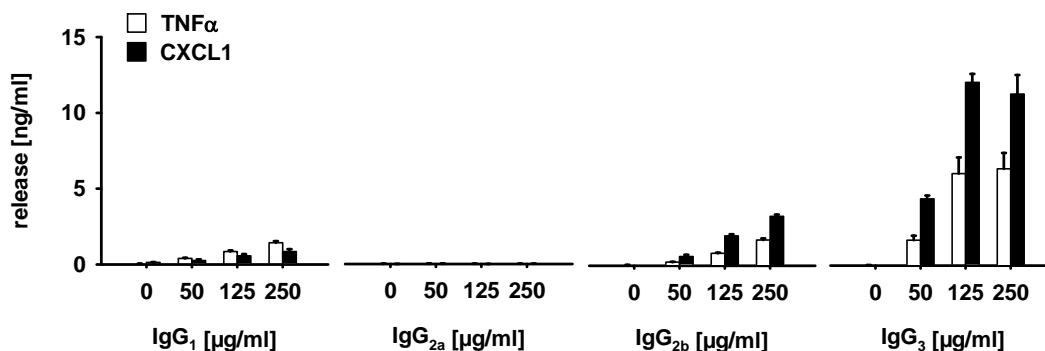
Fig. 4.14.1.: Induction of cytokine and chemokine release in microglial cells by IgG isotypes. Primary cultures of mouse microglia were stimulated for 18 h with mouse IgG isotypes (supplier A, Tab. 4.15.1) at indicated concentrations. The release of $TNF\alpha$ and CXCL1 was measured in the supernatants. Data are mean ± SEM from 2 experiments with an average of n=24.

The induced pattern included the pluripotent immunoregulatory factors $TNF\alpha$ and IL-6, chemoattractants for T cells (CCL3 and CCL5), neutrophils (CXCL1 and CXCL2) as well as low levels of the monocyte/macrophage-attracting CCL2 (Fig. 4.14.2.). Overall, the release profile suggests a bias towards a proinflammatory (classical or M1-like) phenotype in microglia as it appears in the context of tissue damage (see also chapters 4.3 and 4.7), infection or LPS stimulation.
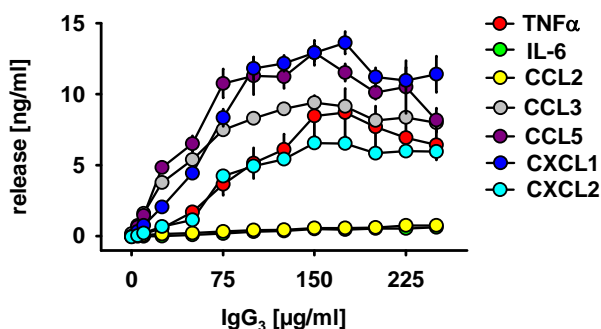
Fig. 4.14.2.: Induction of microglial cytokine and chemokine release by mouse IgG_3. Cells were stimulated for 18 h with IgG_3 (supplier A, Tab. 4.15.1) at various concentrations. Release of cyto- and chemokines was measured in the supernatant. Data are mean ± SEM from two independent experiments with an average of n=14.

This view is supported by the lack of typical Th2 cytokines and the absence of the immunosuppressive (M2-associated) IL-10.

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4.15 Microglia respond to IgG isotypes across species borders

Although human IgG isotypes and FcγRs differ from the mouse system – by structural and functional features as well as preferential interactions – we also tested for the effects of human IgG_1, IgG_2, IgG_3 and IgG_4 on cultures of primary mouse microglia.
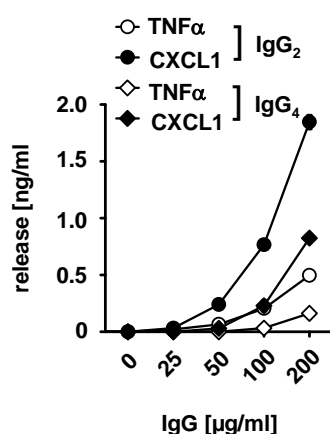
Fig. 4.15.1.: Induction of cytokine and chemokine release in mouse microglia by human IgGs. Cells were stimulated for 18 h with human IgGs (supplier E, Tab. 4.15.1.) at various concentrations. Release of TNFα and CXCL1 (as representative factors) was measured in the supernatant. Data are mean ± SEM with an average of n=8. Note that stimulation with IgG_1 and IgG_3 did not result in a release response.

The cellular response was determined by cyto- and chemokine induction. As shown in Fig. 4.15.1., mouse microglia responded to human IgGs, similarly as they did upon stimulation with the mouse Ig counterparts. The cross-species experiments suggested thereby a general phenomenon of monomeric IgG recognition and subsequent signaling, probably not involving the highly specialized, classical FcγR-IgG interactions. Tab. 4.15.1. summarizes findings obtained with a panel of highly purified mouse and human IgG isotypes. The IgGs were of either commercial origin or were self-generated and purified monoclonal antibodies, as described further below (see also chapter 3.22). To expand findings from purified IgGs to human patient material, CSF samples from MS cases and healthy (control) individuals (kindly provided by Prof. Robert Harris, Karolinska Hospital, Stockholm, and Profs. Dr. Markus Reindl and Thomas Berger, Medical University, Innsbruck) were tested for their release induction potential in microglial cells. However, due to the required sample dilution in medium before addition to the cultures, the samples failed to induce cyto- and chemokine release (data not shown). The limited access to human CSF prohibited a sample concentration before testing.

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Tab. 4.15.1.: Preparations of IgG subclasses tested for microglial release induction capacity. Purified IgGs from various sources are ranked according to the potential for induction of cyto- and chemokines. Ranking as no (-) to very strong (+++) is based on the comparison to LPS-induced release. Resistance to trypsin digestion is indicated as no (-) or partial resistance (+). Experiments focused on IgG isotypes, but also considered some IgA and IgM preparations (data not shown).

Ig structure	Source (purification method)	Supplier (Code)	Induction capacity	trypsin resistance
Mouse IgG_1	hybridoma clone 11711 (AC*)	R&D Systems (A)	++	-
Mouse IgG_1	clone MOPC-21	Acris (B)	+++	n.d.
Mouse IgG_1	clone MI10-102	Bethyl (C)	+++	+
Mouse IgG_1	clone MOPC-31C	Sigma (D)	+	n.d.
Mouse IgG_1	hybridoma clone 7-3-5	self-made	+	-
Mouse IgG_{2A}	hybridoma clone 20102 (AC)	R&D Systems (A)	–	n.d.
Mouse IgG_{2A}	clone MI10-103 (AC)	Bethyl (C)	+++	+
Mouse IgG_{2A}	clone UPC-10 (AC)	Sigma (D)	+	n.d.
Mouse IgG_{2A}	hybridoma clone 53-5-9 (AC)	self-made	+	-
Mouse IgG_{2B}	hybridoma clone 20116 (AC)	R&D Systems (A)	++	n.d.
Mouse IgG_{2B}	clone MI10-104 (AC)	Bethyl (C)	+++	+
Mouse IgG_{2B}	clone MOPC-141 (AC)	Sigma (D)	+++	n.d.
Mouse IgG_3	hybridoma clone 133316 (AC)	R&D Systems (A)	+++	+
Mouse IgG_3	myeloma serum/FLOPC-21	Acris (B)	+++	n.d.
Mouse IgG_3	clone MI10-105 (AC)	Bethyl (C)	++	-
Mouse IgG_3	clone Y5606 (AC)	Sigma (D)	+	n.d.
Human IgG_1	myeloma serum	Fitzgerald (E)	–	n.d.
Human IgG_2	myeloma serum	Fitzgerald (E)	+++	n.d.
Human IgG_3	myeloma serum	Fitzgerald (E)	–	n.d.
Human IgG_4	myeloma serum	Fitzgerald (E)	++	n.d.

n.d., not determined; * affinity chromatography

4.16 Microglia respond to IgGs within a tissue context

The importance as well as relevance of the previous findings got further underlined by experiments in the more complex setting of an organotypic hippocampal slice culture (OHSC, performed in collaboration with Dr. Oliver Kann, Charité Berlin). Slices of the mouse hippocampus were incubated with IgG_3 (supplier C, Tab. 4.15.1.) for 24 h or 48h. The supernatants were analyzed for released cyto- and chemokines as described. Furthermore, slices were incubated for 24 h with fluorescein isothiocyanate-conjugated

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IgG$_3$ (FITC-IgG$_3$) and analyzed by confocal microscopy to reveal the distribution and cellular association of the molecule (Fig. 4.16.1.B).

Fig. 4.16.1.: Induction of cytokine and chemokine release by IgG$_3$ in the organotypic hippocampal slice cultures. Tissue slices (300 µm thickness) were stimulated for 24 h and 48 h with IgG$_3$ (12.5 µg/ml, supplier C, Tab. 4.15.1.). **(A)** Release of cyto- and chemokines was measured in the supernatant. Data are mean ± SEM from 2 experiments with an average of n=6. **(B)** Slices were incubated with FITC-IgG$_3$ for 24 h and analyzed by confocal microscopy. The arrows indicate green fluorescent cells with microglia-like morphology.

As shown in Fig. 4.16.1., the induced release profile was comparable but not identical to the situation in the primary culture (Fig. 4.14.2.). We found high amounts of CXCL1, CCL3 and CCL5 and – in contrast to the primary cultures – IL-6, but only low amounts of TNFα. Differences in the (relative) release pattern could be attributed to the complexity of the slice culture, e.g. presumably to indirect astrocytic contributions. However, by using a fluorescent IgG$_3$ derivative, single cells exhibited a microglia-like morphology when analyzed by confocal microscopy, suggesting them to represent a target and response element of the immunoglobulins in the complex situation of the tissue.

4.17 IgG-dependent activation of microglia reveals partial FcγR contributions

In addition to their role in antigen binding Ig's can modulate immune responses by interacting with FcRs. The family of FcγRs, being responsible for IgG interactions in mice, consists of four members, i.e. FcγRI (CD64), FcγRIIB (CD32), FcγRIII (CD16) and FcγRIV, with orthologues proteins in other mammalian species (Nimmerjahn and Ravetch, 2008). They are categorized according to their affinity for the various IgG isotypes and the intracellular signaling processes they initiate. In the mouse, the FcγRIIB is the only known inhibitory FcγR containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the intracellular domain. With the exception of the human FcγRIIA and FcγRIIC, FcγRs transmit activating signals via a cytosolic immunoreceptor tyrosine-based activating motif

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(ITAM) that is provided by a separate transmembrane signaling unit, i.e. the Fcγ signaling chain. Upon activation, the ITAM is phosphorylated and recruits spleen tyrosine kinase (syk) for downstream signaling events.

Although mouse and human FcγRs share similarities, they differ by their affinity to IgG isotypes, their intracellular signaling domains and cellular expression patterns. These differences have to be taken into account when comparing cellular effects across species. As this work is primarily based on mouse cells and mouse IgGs, only the contribution of mouse FcγRs to IgG-dependent responses of microglial cells will be shown below.
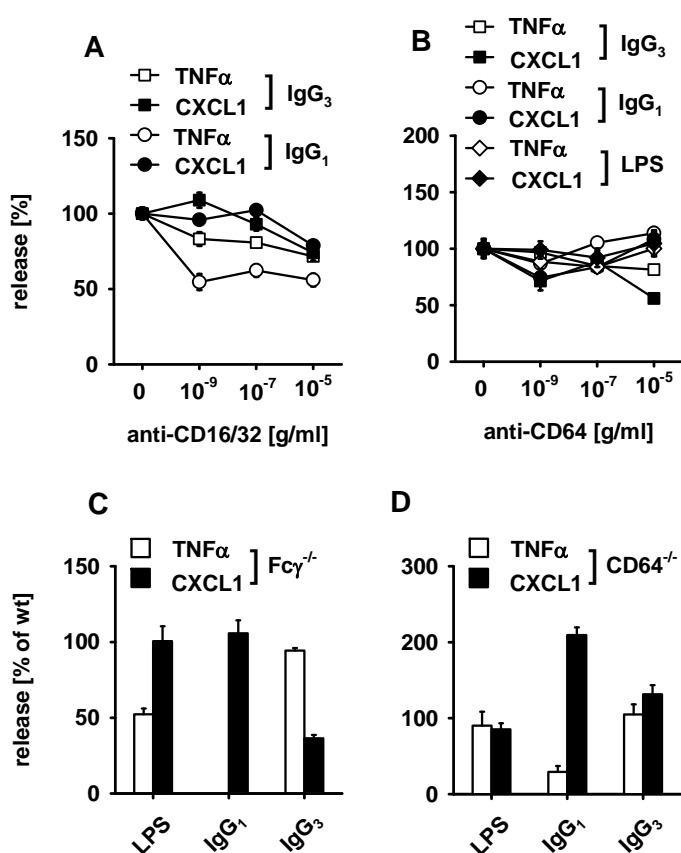
Fig. 4.17.1.: Effects of interference with FcγR functions on the cytokine and chemokine release induction in microglia by IgGs. Wildtype cells were stimulated for 18 h with IgGs (100 µg/ml, supplier A, Tab 4.15.1.) or LPS (1 ng/ml) in the presence or absence of anti-CD16/32 **(A)** or anti-CD64 **(B)** blocking antibody. Cells received a pre-incubation with the blocking antibody for 30 min. Note that the blocking antibodies by themselves did not trigger release. Microglia were prepared from mice deficient for the **(C)** Fcγ signaling chain (Fcγ$^{-/-}$) or **(D)** CD64 (CD64$^{-/-}$) and stimulated with LPS or IgGs. The release of TNFα and CXCL1 was measured in the supernatant and is given as percent of the release in the absence of the blocking antibody or as determined in wildtype cells. Data are mean ± SEM from n=34 (A) n=4 (B, C) n=8 (D) from 3, 1 or 2 experiments.

According to the classical notion, IgGs can only initiate FcγR signaling processes when being bound to their antigen to form ICs. In ICs, the Fc portions of neighboring IgGs thereby mediate the crosslinking of FcγRs. Although monomeric IgGs – in the absence of the antigen as used in this study – may also bind to FcγRs (namely FcγRI), such interactions are not known to efficiently trigger FcγR signaling. A classical FcγR contribution to the observed phenomenon of microglial activation by monomeric IgGs seemed to be unlikely but could not be ignored. Therefore, participation of FcγRs was

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systematically analyzed using blocking antibodies for CD16/32 (FcγRIII/II) and CD64 (FcγRI) as well as cells from mice deficient for CD64 (CD64$^{-/-}$) or the Fcγ signaling (Fcγ$^{-/-}$) chain (Fig. 4.17.1.). Experiments used IgG preparations from supplier A (Tab. 4.15.1.) and, as a control, LPS for a FcR-independent stimulation. Blocking antibodies were tested previously for their own release induction capacity and were found to be inactive on microglial cells at the applied concentrations. From these experiments, partial FcγR contributions to the IgG-induced release activity of microglial cells could be deduced. However, effects of FcR-blocking antibodies and FcR signaling deficiency varied with the tested isotypes and the factors induced by them. For example, the IgG$_1$-induced TNFα release seemed to depend on the Fcγ signaling chain and CD64 (Fig. 4.17.1.C, D), whereas the CXCL1 response was not affected by a Fcγ$^{-/-}$ situation and even increased in CD64$^{-/-}$ cells. In contrast, under IgG$_3$ stimulation, TNFα was not affected in the γ-chain knockout, whereas CXCL1 release was reduced, and CD64 deficiency did not have an impact. Because of these equivocal findings we thought of another method to prove FcR contributions. Based on a study (Radaev and Sun, 2001) in which the authors had designed small peptides for interference with IgG-FcγR interactions we employed the sequence CPAPELLGGPSV (pIgG1) to mimic the lower hinge region of the IgG$_1$ and IgG$_3$ which is thought to contribute to FcγR binding. The peptide, pIgG1, synthesized with high puritiy and used in competition experiments, impeded the IgG-induced responses, with drastic effects at 0.5 mM and 1.0 mM (~20 fold molar excess) (Fig. 4.17.2.).
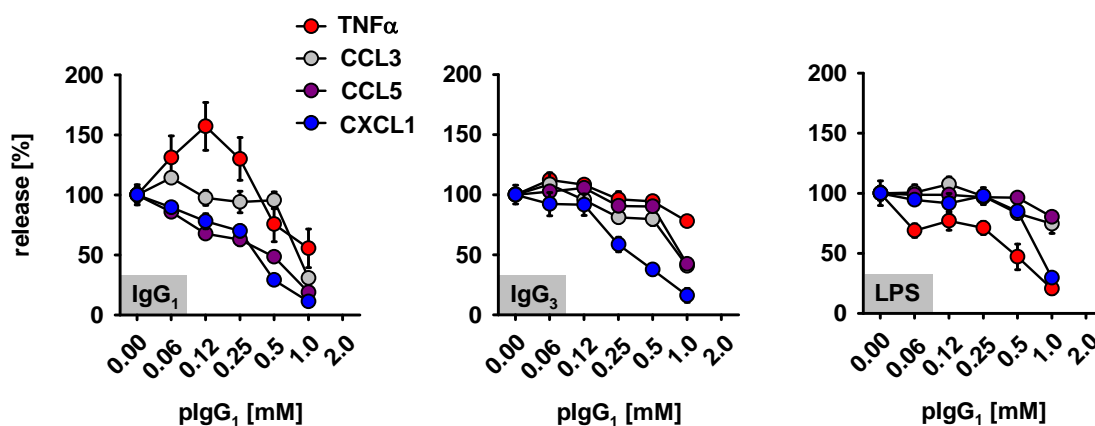
Fig. 4.17.2.: Effect of the lower hinge region peptide pIgG1 on the induction of cytokine and chemokine release in microglial cells by IgGs. Cells were stimulated with IgG$_1$, IgG$_3$ (100 µg/ml, supplier A, Tab. 4.15.1) or LPS (1 ng/ml) in the absence or presence of pIgG$_1$ (CPAPELLGGPSV) at various concentrations for 18 h. Release of cyto/chemokines was measured in the supernatant. Data are shown as percent of release in the absence of the peptide and are mean ± SEM from 3 experiments with an average of n=18.

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Although by themselves not a proof of FcγR contributions these data go in line with the findings of Fig. 4.17.1., suggesting that FcγRs have at least a partial role in the triggering of microglial release activity by monomeric IgGs. Interestingly, also LPS-induced responses were affected by an interference based on the peptide. This fact was surprising at first glance. However, TLR4 responses to LPS were found to be modulated by non-TLR receptors already, as discussed below (Kagan et al., 2008; Pfeiffer et al., 2001).
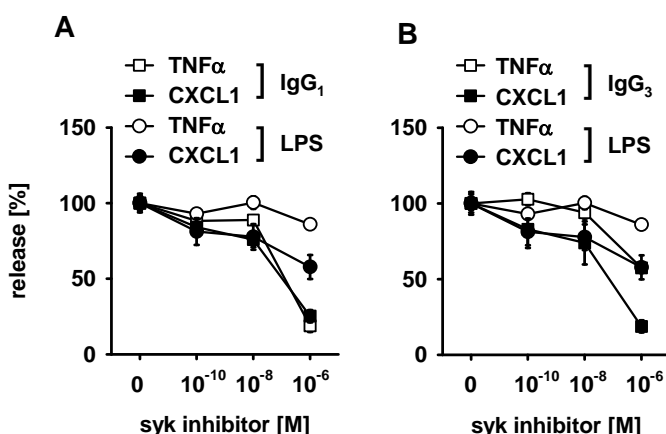
Fig. 4.17.3.: Effect of syk inhibition on cytokine and chemokine release induction in microglia by IgGs. Cells were stimulated with LPS (1 ng/ml) and **(A)** IgG_1 or **(B)** IgG_3 (both 100 µg/ml, supplier E) in the presence or absence of a syk inhibitor (see in the text) at various concentrations. The inhibitor was added 1 h before the stimulation. Release of TNFα and CXCL1 was determined as described above. Data are mean ± SEM from 2 experiments with an average of n=12.

Since the spleen tyrosine kinase (syk) is involved in downstream signaling upon ITAM activation the influence of its inhibitor 3-(1-Methyl-1H-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide on IgG and LPS stimulation was determined (Fig. 4.17.3.). The IgG-induced release was, indeed, quantitatively reduced by the syk inhibitor at 1 µM. The mild effect on LPS-induced release, on the other hand, could well relate to a recent report on the direct interaction of syk with TLR4 in monocytes (Chaudhary et al., 2007). Taken together, FcRs could participate in a signaling of monomeric IgGs. They may facilitate IgG recruitment to the cell surface and confer ligand specificity and selectivity for certain isotypes on an otherwise TLR4-centered receptor complex. The actual involvement of TLR4 is shown in the next chapter.

4.18 IgGs utilize a CD14/TLR4/MyD88/TRIF complex to induce microglial responses

TLR4 was thus far reported to recognize the largest variety of endogenous structures. As already paralleled the species independence of microglial responses to IgGs, we found the TLR4 system to have a mandatory function for their signal transduction (Fig. 4.18.1.). In a first experiment, we tested whether TLR4 receptor blockade could interfere with IgG-induced responses. A TLR4/MD2-directed blocking antibody – at concentrations not

having on their own an effect on the cells – revealed an impact on microglial reactions to mouse IgGs. Influences were even more pronounced for the human Ig counterparts, but not detectable for LPS-triggered responses (Fig. 4.18.1.A). However, experiments with blocking antibodies bear – especially in monocytes/macrophages – the risk of false-positive results, due to FcR expression. Therefore, we took advantage of microglial cells from mouse strains either expressing a mutant TLR4 version (C3H/HeJ) with a loss-of-function point mutation in the TLR4 gene or being completely deficient for TLR4 (TLR4$^{-/-}$). We found that, under both of these conditions, IgGs completely failed to induce release responses in microglia, the same way as LPS did (Fig. 4.18.1.B, C). In contrast, and demonstrating receptor selectivity, responses to specific TLR1/2 (Pam$_3$CSK$_4$) or TLR6/2 (MALP) agonists were virtually not affected. The fact that a partial impairment of MALP-inducible responses was occasionally observed in the physical TLR4 knockout might be explained by an already suggested cooperation between TLR4 and TLR2 (Flo et al., 2002).
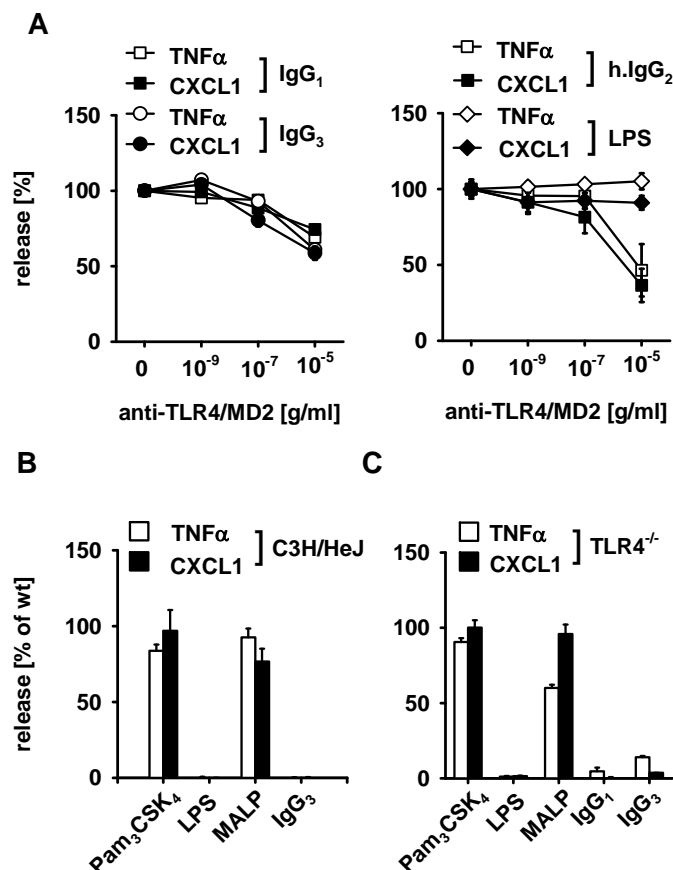
Fig. 4.18.1.: Effects of TLR4 blockade or deficiency on the cytokine and chemokine induction in microglia. (A) Wildtype cells were stimulated for 18 h with mouse IgGs (25 µg/ml, supplier C), human IgG$_2$ (100 µg/ml, supplier E) or LPS (1ng/ml) in the presence or absence of a TLR4/MD2 blocking antibody. Cells received a preincubation with the antibody for 30 min. The blocking antibody by itself did not trigger release. Cells prepared from mice **(B)** carrying a mutated version (C3H/HeJ) or **(C)** lacking TLR4 (TLR4$^{-/-}$) were stimulated with Pam$_3$CSK$_4$ (10 ng/ml), LPS (1 ng/ml), MALP (10 ng/ml) or mouse IgGs (25 µg/ml, supplier C) for 18 h. Release of TNFα and CXCL1 was measured in the supernatant and is shown as percentage of the response without the blocking antibody or of wildtype cells. Data are mean ± SEM from 3 experiments with an average of n=9 (A, B) or n=18 (C).

As described, TLR4 seems to associate with additional co-receptor and adapter molecules to facilitate appropriate responses to LPS and other agonists. CD14 is on of the

key elements in the receptor complex. The GPI-anchored molecule assists not only in the binding of LPS but also participates in the recruitment of MD2 and the TLR4 dimerization. According to experiments in chapter 4.7, involvement of CD14 in TLR4-IgG binding/ signaling was analyzed using three different blocking antibody clones as well as cells from CD14-deficient mice. IgGs (mouse and human) as well as LPS responses were abrogated using the anti-CD14 clones 4C1 (Fig. 4.18.2.A, B) and big53 (data not shown), whereas another clone (rmC5-3) was totally ineffective to interfere with either the IgG or LPS activities (data not shown). Experiments using cells deficient in CD14 proved its essential function in responses to both IgGs and LPS (Fig. 4.18.2.C).
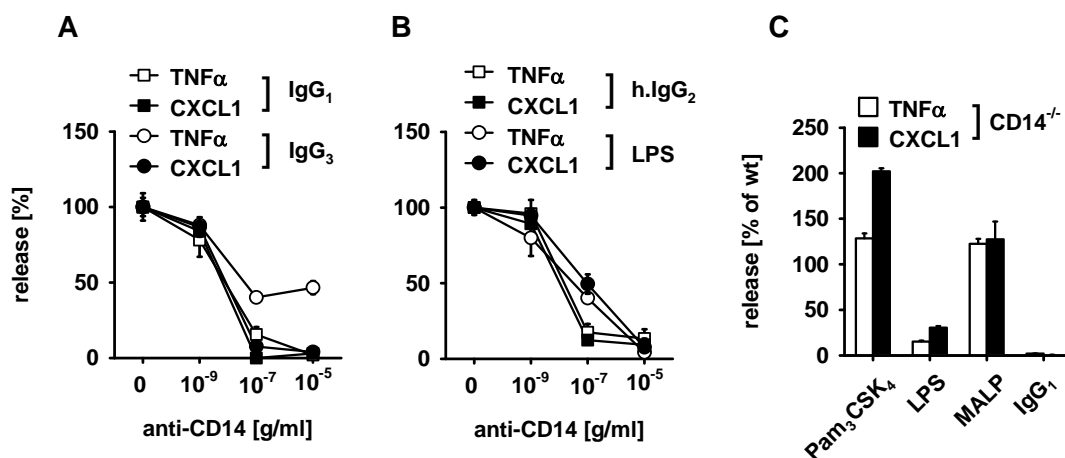
Fig. 4.18.2.: Effects of CD14 blockade or deficiency on cytokine and chemokine induction in microglia. Wildtype cells were stimulated for 18 h with **(A)** mouse IgGs (100 µg/ml, supplier A), **(B)** human IgG$_2$ (100 µg/ml, supplier E) or LPS (1 ng/ml) in the presence or absence of a CD14 blocking antibody (clone 4C1). Cells received a pre-incubation with the antibody for 30 min. The blocking antibody by itself did not trigger release. Note that another clone (big53) proved to be inhibitory, whereas a third clone (rmC5-3) failed to block reactions to IgGs and LPS. **(C)** Microglia prepared from mice lacking CD14 (CD14$^{-/-}$) were stimulated with Pam$_3$CSK$_4$ (10 ng/ml), LPS (1 ng/ml), MALP (10 ng/ml) or mouse IgG$_1$ (100 µg/ml, supplier A) for 18 h. Release of TNFα and CXCL1 was determined in the supernatants and is shown as percentage of the response without the blocking antibody or of wildtype cells. Data are mean ± SEM from 6 experiments with an average of n=24 (A) or 2 experiments with an average of n=8 (B, C).

TLR4 connects to both of the two major intracellular signaling routes depending on the adapter molecules, MyD88 and TRIF. MyD88 is thought to primarily control the expression of proinflammatory mediators, whereas TRIF is suggested to have a major function in the control of IFNβ and, thereby, interferon-regulated genes. Nevertheless, the pathways of the two signaling adapter proteins revealed substantial cooperativity. Overlap in MyD88- and TRIF-controlled signaling elements is most obvious for NFκB and its concerted as well as timed activation upon TLR4 stimulation (see also chapters 4.5 and 4.7). To test for

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the importance of these two signaling elements in IgG-induced responses, microglia of MyD88- and TRIF-deficient animals (MyD88$^{+/-}$, MyD88$^{-/-}$, TRIF$^{-/-}$) were prepared and tested in our cell culture model. Lack of functional MyD88 led to a complete abrogation of IgG- (mouse and human) as well as LPS-triggered responses (Fig. 4.18.3.A, B). Moreover, MyD88$^{-/-}$cells were also unable to mount a release upon TLR1/2 and TLR6/2 activation (data not shown), demonstrating the crucial role in all of these TLR challenges. Experiments also revealed TRIF to have a significant signaling importance as IgG-induced responses were strongly reduced in the knockout cells (Fig. 4.18.3.C). For LPS, as the prototypic microbial TLR4 agonist, the TRIF$^{-/-}$ situation had a less pronounced impact. In this case, MyD88 still compensated for the absence of TRIF. As expected, TLR1/2- and TLR6/2-stimulated TNFα production remained as in the wildtype microglia. Interestingly, however, functional TRIF signaling might be required also in their case, when considering particular responses. CXCL1 levels were clearly lower in TRIF$^{-/-}$ cells, again pointing to MyD88-TRIF cooperativity.
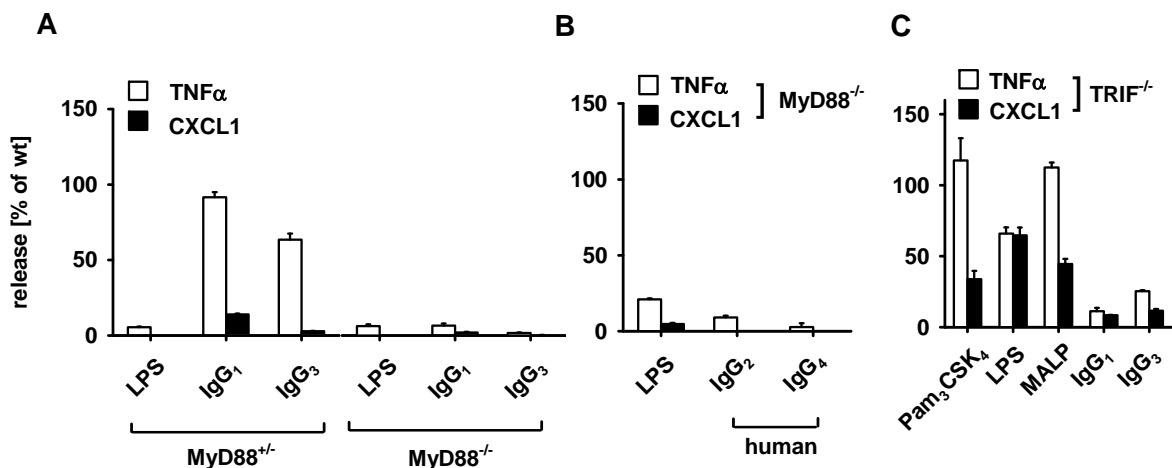
Fig. 4.18.3.: Effect of MyD88 and TRIF deficiencies on cytokine and chemokine induction in microglia. Cells were prepared from mice with **(A, B)** heterozygous (MyD88$^{+/-}$) or homozygous (MyD88$^{-/-}$) deficiency for MyD88 or **(C)** lacking TRIF (TRIF$^{-/-}$). Cells were stimulated for 18 h with **(A)** mouse IgGs (100 µg/ml, supplier A), **(B)** human IgGs (100 µg/ml, supplier E) or **(C)** Pam$_3$CSK$_4$ (10 ng/ml), LPS (1 ng/ml), MALP (10 ng/ml) or mouse IgGs (100 µg/ml, supplier A). Note that loss of MyD88 led to a complete loss of responsiveness to Pam$_3$CSK$_4$ and MALP as well (data not shown). Release of TNFα and CXCL1 was measured in the supernatant. Data are shown as percentage of the response of wildtype cells and are mean ± SEM from 6 experiments with an average of n=24 (A) or 2 experiments with an average of n=8 (B, C).

Interestingly, while LPS chemotypes, i.e. structural variants of the cell wall component as found in different gram-negative bacterial strains, were found to induce IFNβ, virtually all analyzed endogenous agonists for TLR4 (including IgGs) revealed poor or even lack of such an activity (data not shown). Compared to stimulations with microbial agonists,

DAMP-activated TLR4 signaling apparently lacks an efficient link to the production of IFNβ, although TRIF can be recruited, as just shown (see also chapters 4.5 and 4.7). IgG-triggered TLR4 consequences would thereby miss not only the (transcriptional) activities covered by IFNβ more or less directly, but also omit feedback loops falling back on their own functional spectrum. As we will see later (chapter 4.20), IFNβ can interfere with IgG-induced signaling and strongly modulate the reactive phenotype of the cells.

4.19 IgG-induced microglial responses are not caused by endotoxin contamination

Assuming TLR4-agonistic potential for endogenous molecules always bears the risk of endotoxin contaminations in the respective preparations. Endotoxin (or LPS to be more precise) could be responsible for the observed effects, or confound the TLR4 nature of the cellular response. Especially recombinant proteins produced in *E. coli* are at such a risk to be impured by LPS (Tsan and Gao, 2007, Weinstein et al., 2008b). Already traces of the highly potent PAMP are sufficient to trigger measurable reactions in microglia (e.g. LPS at 10^{-11} g/ml triggers chemokine synthesis). Studying TLR4 interactions with DAMPs we had to convincingly exclude non-protein-linked TLR4 responses. These approaches routinely included thermoinactivation and polymyxin treatment (see also chapters 4.4 and 4.7). While heat inactivation is a common method to show the protein dependence of cellular effects this procedure is not suitable for IgGs as it causes aggregation and precipitation of the protein (data not shown). Controlled thermal treatment of IgGs (increasing steps of or periods at given temperatures) could reveal a partial decline in microglial responses, yet it seemed to reciprocally also cause some activity, thus preventing an assignment to a given receptor mechanism (data not shown). Although large protein complexes or aggregates are not necessarily exerting microglia-challenging activity (chapter 4.2), the effect is even exploited for the mimicking of IC-like IgG effects (Binstadt et al., 2006). Therefore, we performed proteolytic digestions and LPS-removing affinity chromatography to prove IgG proteins as carriers of microglia-driving activity. In a series of experiments, IgGs were subjected to trypsin as well as proteinase K digestion, as shown below for representative examples of two highly active IgG preparations. Some IgG preparations turned out to be partially resistant to trypsin digestion (Fig. 4.19.1.A). They still showed intact protein bands for heavy and light chains in the SDS-PAGE, correlating with an only reduced activity on microglial cells. Interestingly, preparations being resistant to trypsin treatment turned out to be strong to very strong inducers of microglial release (Tab. 4.15.1). This

effect was independent of the IgG isotype, pointing to conformational differences among the preparations. Having still some 'residual activity' after trypsination, the contribution of entrapped endotoxin/LPS in certain IgG preparations could not be ruled out. We thus performed digestion experiments based on proteinase K.
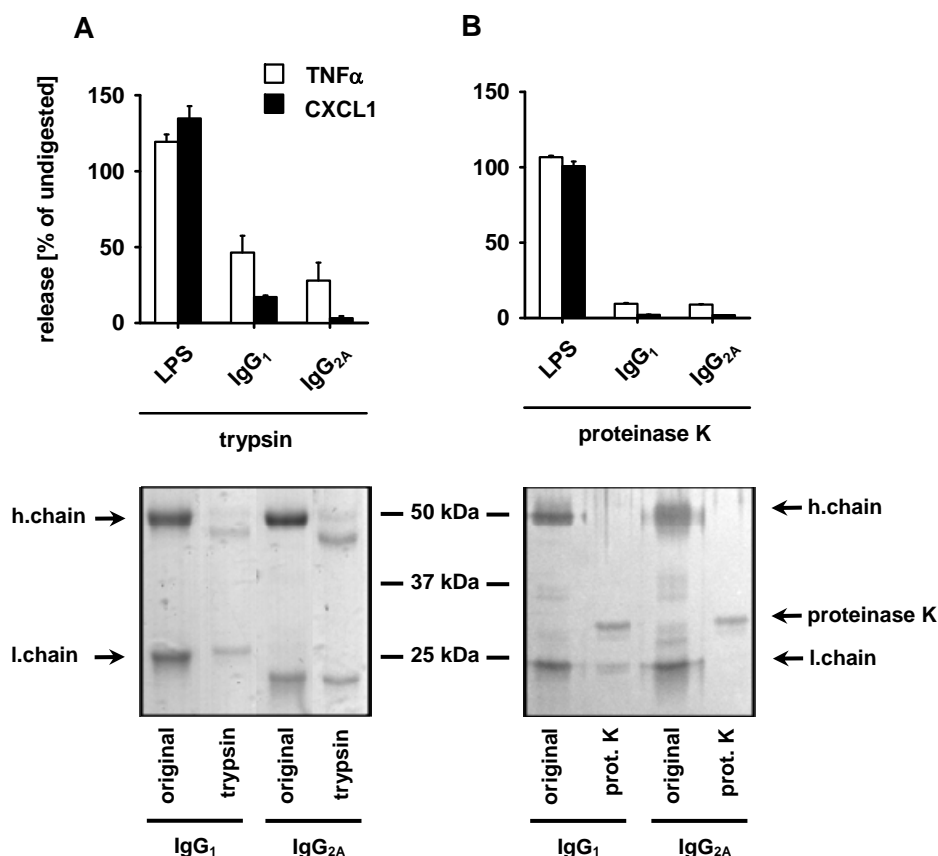
Fig. 4.19.1.: Effect of proteolysis of IgGs on the induction of cytokines and chemokines in microglia. IgGs (supplier C) and LPS (as a control) were subjected to proteolytic digestion with either immobilized trypsin **(A)** or soluble proteinase K **(B)** for 24 h at 37°C. The prote(in)ases were removed and samples were tested for induction of TNFα and CXCL1 as in Fig. 4.18.3. Data are mean ± SEM from 2 experiments with an average of n=12 (A) or n=8 (B) per group. Digested samples were also analyzed by SDS-PAGE to reveal the breakdown of the IgG heavy (h.chain) and light (l.chain) chains.

In contrast to trypsin, proteinase K treatment was more effective, showing a complete protein degradation – even for trypsin-resistant IgGs – and, consequently, a complete loss of release induction capacity (Fig. 4.19.1.B). To confirm the findings of protease digestion experiments, LPS affinity chromatography using EndoTrap™ endotoxin removal columns was performed (Fig. 4.19.2.). LPS was used at a starting concentration allowing for a final concentration on the cells within the plateau of the well-established dose-response relation for LPS-inducible release. First, this concentration would 'tolerate' unavoidable

volume variations during chromatography and thus resist some minor dilution errors on the way from the original solution to the cell assay. Second, even a small drop in microglial responses would then have required a reduction of LPS by, at least, an order of magnitude. Furthermore, an IgG_{2A} (self-made monoclonal antibody, Tab. 4.15.1., chapter 4.22) was spiked ($*IgG_{2A}$) with the same amount of LPS to check whether protein-bound LPS can also be efficiently removed by this procedure. We selected this particular IgG because it came itself with a very low release induction capacity (to allow for the observation of LPS effects).
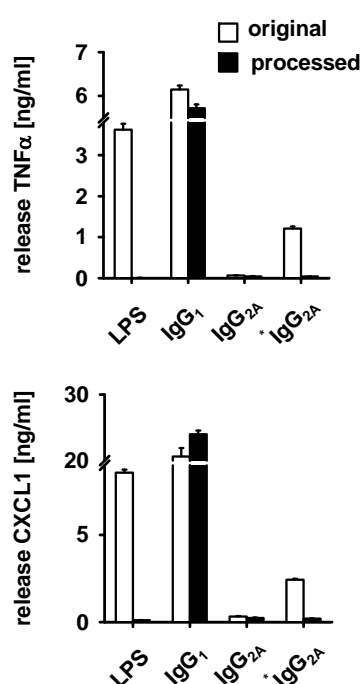
Fig. 4.19.2.: Effect of an EndoTrap^{TM} affinity-based endotoxin removal from IgG preparations on the cytokine and chemokine induction in microglia. LPS (100 ng/ml), IgG_1 (500 µg/ml, supplier C), IgG_{2A} (500 µg/ml, self-made clone) and LPS-'spiked' IgG_{2A} (500 µg/ml + 100 ng/ml, $*IgG_{2A}$) were loaded onto EndoTrap endotoxin removal columns. The eluate was collected until the column was drained. Processed and original solutions were diluted in medium to final concentrations as follows: LPS: 4 ng/ml; IgG_1: 10 µg/ml; IgG_{2A}: 20 µg/ml; $*IgG_{2A}$ 20 µg/ml/4 ng/ml and tested for release induction using stimulation for 18 h. Note that LPS was used to allow for a final concentration on the cells as being in the plateau of the dose-response curve. IgG_{2A} was used at a concentration having very low release induction capacity to analyze possible LPS-protein interactions. Data are shown for TNFα and CXCL1 are mean ± SEM with an average of n=6 per group.

LPS efficiently bound to the EndoTrap columns (Fig. 4.19.2.). It got completely removed from a pure as well as a protein-containing solution. Most importantly, the IgG_1 of very strong release induction potential (Tab. 4.15.1.) passed the LPS removal column without 'losing' microglia activation potential. Results from the LPS-spiked IgG_{2A} provided further evidence that IgGs and LPS may compete for the same receptor complex. Apparently, LPS lost activity when being paired with the IgG_{2A} of very low induction capacity (Fig. 4.19.2., compare $*IgG_{2A}$ *versus* LPS). According to the data shown for thrombin[HMWM] (see chapter 4.3), such effects can be achieved when two agonists of different induction capacity compete for the same receptor. Conceivably, IgG_{2A} was able to claim TLR4 but caused only weak signaling, acting as a partial agonist. Its presence thus lowered the TNFα and CXCL1 induction potential of LPS.

RESULTS

In summary, these results document that the monomeric IgG-triggered, apparently TLR4-dependent release activity of microglia is not a consequence of LPS contamination, but that it can be solely assigned to the protein.

4.20 IgG- and LPS-induced microglial responses are differently modulated by immunoregulatory cytokines

The data presented thus far suggest that IgGs can function as a DAMP to induce microglial responses by utilization of TLR4. A major question is how the TLR4 system would facilitate the recognition and binding to molecules having no obvious structural similarities – such as LPS and IgGs. The fact that TLR4 recruits several co-receptor and binding molecules for appropriate LPS recognition suggests that TLR4 may also associate with additional surface receptors for DAMP binding in order to provide the required binding specificity and to allow for further intracellular signaling elements to provide the molecular basis to discriminate between individual ligands. As previously shown for the involvement of the $\alpha M\beta 2$ integrin for FN binding/signaling (see chapter 4.10), a similar mechanism is likely for IgGs, although FcγRs do not seem to play an essential role. If this is true, modulating influences on the signaling – as they can be provided by cytokines – should reveal discrete consequences for the distinct agonists. Therefore, IgG$_1$ and IgG$_3$ (supplier A, Tab. 4.15.1.) and LPS were paired with certain key immune-regulatory cytokines in co-stimulation experiments. The influence on the signaling was determined by the profile of released factors (Fig. 4.20.1.). Accordingly, IFNβ would not have much influence on the induced release profile of LPS. In contrast for IgG$_1$, it suppressed chemoattractive signals for neutrophils (CXCL1, CXCL2) and enhanced production of IL-6 as well as attraction of macrophages/monocytes (CCL2), while sparing TNFα and signals for T cells (CCL3, CCL5) (Fig. 4.20.1.). The very same profile was found for IgG$_3$ and thrombin^{HMWM} and a similar modulation was obtained for a FN stimulation (see chapter 4.9). Again, the Th1 cytokine, IFNγ, could influence the microglial responses to both IgGs and LPS. Interestingly, the release pattern upon IgG$_1$ or IgG$_3$ stimulation differed dramatically under this condition. Cells showed for LPS and IgG$_3$ basically a suppression of signals for neutrophils (CXCL1, CXCL2) and to some extent also for the Th1 population of T cells (CCL3), as already previously reported for LPS (Häusler et al., 2002).
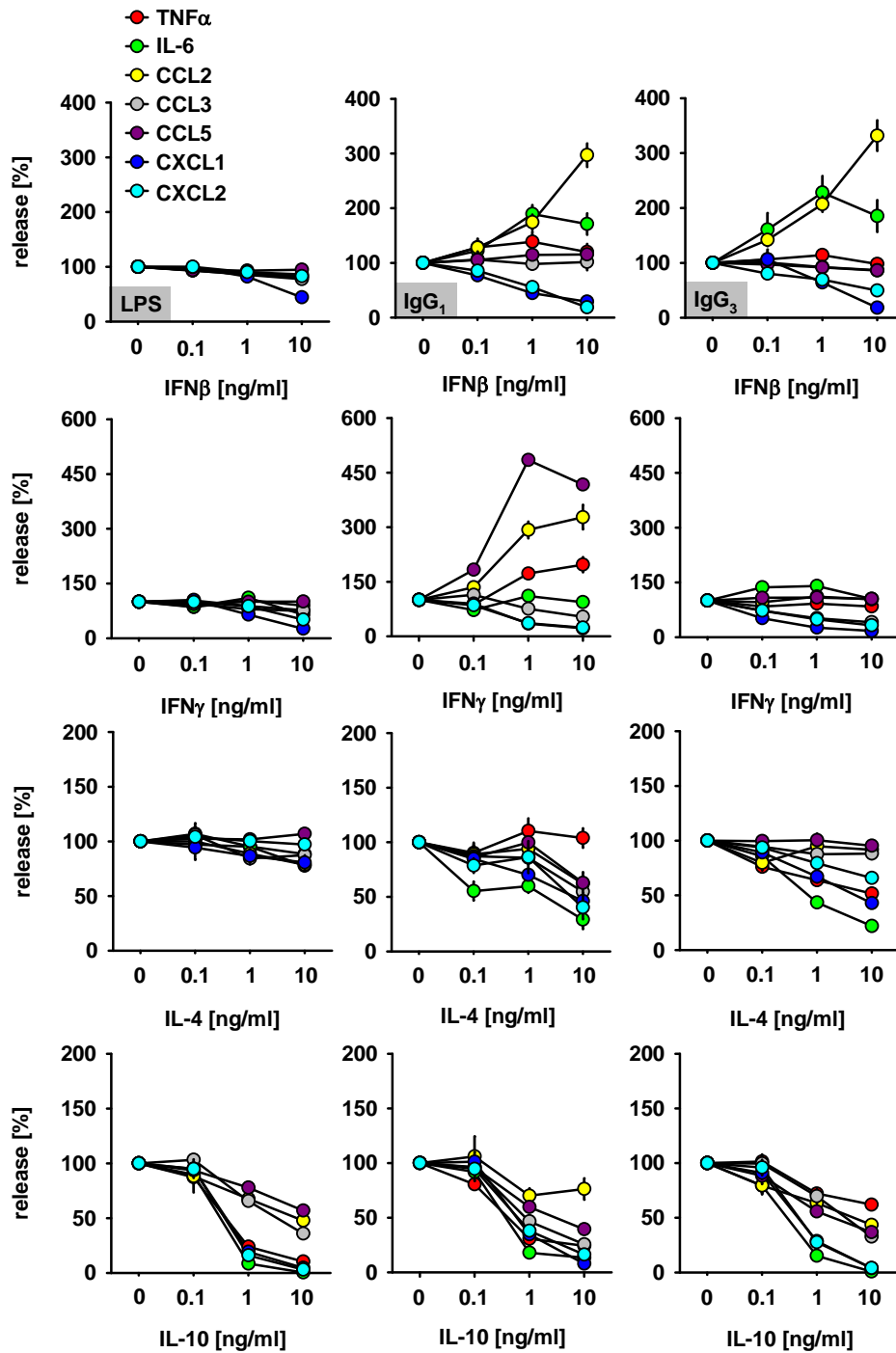
Fig. 4.20.1.: Modulation of IgG- and LPS-induced microglial release profiles by cytokines.
Cells were stimulated for 18 h with IgG$_1$, IgG$_3$ (both 100 µg/ml, supplier A) or LPS (1 ng/ml) in the presence or absence of IFNβ, IFNγ, IL-4 or IL-10. The release of certain cyto/chemokines was measured in the supernatants. Data are mean ± SEM with an average of n=12 from 3 experiments. In this study, cytokines by themselves were found to not trigger release.

On the other hand, IFNγ dramatically enhanced a more general chemoattraction for T cells (CCL5) and monocytes (CCL2) as well as the production of TNFα in response to IgG$_1$.

The system would thereby even discriminate between isotypes. It supports the idea that structural features of the isotype, in addition to the idiotype (antigen recognition), could determine the tissue consequences of an (auto)antibody, for example in multiple sclerosis. TLR4 could translate the presence of an isotype as presented in the context of a cytokine milieu into a discrete reactive phenotype of microglia.

Influences of the immunosuppressive IL-10 and the Th2 'master' cytokine IL-4 were analyzed, as in chapter 4.9. Whereas IL-4 again had not much influence on the LPS-induced release it was functional on the profiles of the IgGs. Only under IL-10, microglia showed a general attenuation of the responses to all of the TLR4 agonists.

In summary, the data suggest (i) that microglia, namely its TLR4, can interpret activating and even closely related signals, in particular under the influence of modulating substances, and that (ii) further co-receptors may provide binding and signaling specificity (see also chapter 4.9). FcRs could represent potential, yet not the only candidates for such co-receptor units. In addition, these experiments further rendered LPS contamination unlikely to account for responses to IgGs as the modulating influences by cytokines revealed distinct effects.

4.21 TLR4 agonistic activity of IgGs is influenced by minor conformational changes

The propensity of IgGs to activate microglial cells via a TLR4-centered receptor/signaling mechanism seems to depend on the isotype, as indicated in Tab. 4.15.1. Yet the induction capacity still also varies among a given isotype, suggesting that its nature per se does not strictly determine the actual activity. We observed a partial trypsin resistance among the IgG preparations (Fig. 4.19.1.A, Tab. 4.15.1.). Interestingly, 'resistant' preparations turned out to be most active on microglia regarding release induction capacity, probably pointing to some impact of the tertiary and/or quartary protein structure. We addressed the questions whether the assumed TLR4 activity would correlate with subtle changes in IgG structure and whether environmental influences could be responsible for such alterations, e.g. during the purification processes. Indeed, our findings indicate that – even transient – changes in the biochemical milieu can induce long lasting changes in the protein conformation which, in turn, control the interaction with TLR4. Calibration-assisted size exclusion chromatography revealed the presence of three major protein fractions in an 'active' IgG_3 preparation (Fig. 4.21.1.A), which were all identified to contain Ig protein by SDS-PAGE (Fig. 4.21.1. B).

A

B
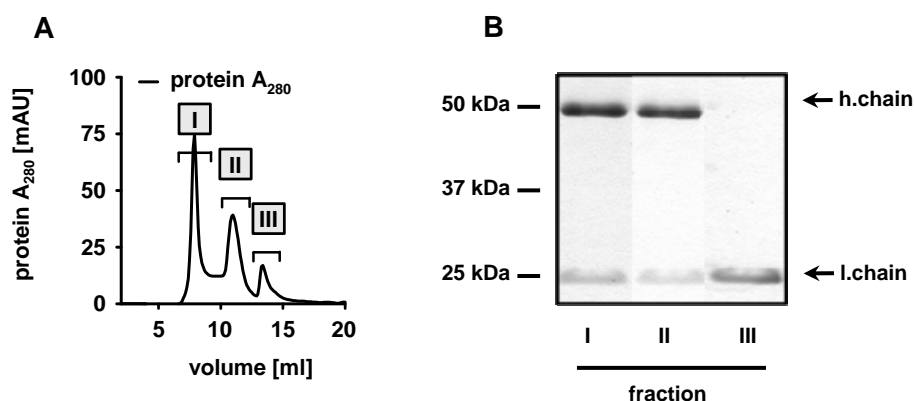
Fig. 4.21.1.: Analysis of an IgG$_3$ preparation by size exclusion chromatography. The IgG$_3$ (supplier A) was analyzed with a Superpose 12 10/300 gelfiltration column, using a constant flow of 0.5 ml/min of PBS at RT **(A)**. Absorbance was measured at 280 nm. Peaks (I, II, III) were fractionated and further analyzed by SDS-PAGE **(B)** showing heavy (h.chain) and light (l.chain) chains in fractions I and II and only light chains in fraction III.

A minor small-molecular weight peak related largely to single Ig light chains (fraction III). A second protein peak matched the expected size of 'monomeric' IgGs (fraction II). Interestingly, the major peak (fraction I) also containing IgGs eluted according to a higher molecular weight. We could rule out that this material simply represented aggregated (denatured) IgGs. Thus, we considered the fraction to contain a more defined complex of 'associated' IgGs. Most interestingly, isolated monomers (fraction II) were able to form again associates, while the associated IgGs in fraction I gave rise to monomers. As expected, the single light chain fraction was not able to build forms of a higher organization (Fig. 4.21.2.). These results indicated that associated and monomeric IgG forms were in a steady-state equilibrium. Another evidence for a relationship between protein structure and TLR4 activity was obtained from a particular lot of the very same commercial IgG$_3$ (supplier A). While this clone, used as a standard in earlier experiments, had proven for stable microglial effects, a new batch suddenly revealed a complete loss of activity. Size exclusion chromatography showed only peaks for monomeric IgGs and single light chains (Fig. 4.21.3.A). The lack of associates led us to assume that changes in the isolation and purification process had occurred. An inquiry at the manufacturer confirmed our assumption, but no details of the protocol were released. Importantly, the ability of the monoclonal IgG$_3$ to form associates and to trigger microglial responses could be recovered by a procedure involving denaturation and refolding steps.
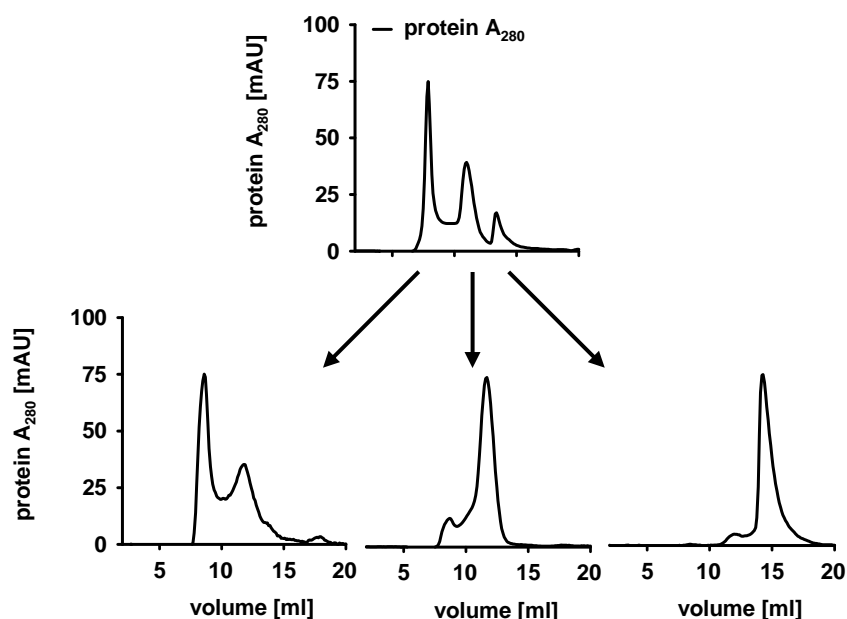
Fig. 4.21.2.: Spontaneous reformation of associates and monomers in an IgG$_3$ preparation. IgG$_3$ (supplier A) was analyzed by gelfiltration as described in Fig. 4.21.1. Proteins of the isolated peaks were immediately reanalyzed by chromatography, using the same protocol. Note that isolated associates and monomers were each able to regenerate both of their peaks. On the contrary, separated single light chain material remained as the same peak.

We tested several conditions that had been described to help missfolded proteins to successfully transform into their native conformation. A few of them (15 out of 92, data not shown) were able to restore the release-stimulating activity. Gelfiltration chromatography revealed that successfully refolded IgG$_3$ again consisted both – associates and monomers (Fig.4.21.3.B). The microglia activity of refolded IgG$_3$ was sensitive to CD14 blockade (Fig. 4.21.3.C), indicating the TLR4-dependent mechanism as described for the formerly 'functional' preparations of the clone.

These results pointed to some correlation between an associate formation and TLR4- agonistic properties. The denaturation/refolding procedure resulting in activity restoration was thereby rather mild in terms of global alterations of the protein structure, as indicated by tryptophan (Trp) fluorescence spectroscopy. The spectra of IgG$_3$ before, during and after refolding did not really differ from each other (Fig. 4.21.4.). In contrast, use of a denaturation buffer containing the reducing agent tris(2-carboxyethyl)phosphine (TCEP) resulted in a tightened fluorescence spectrum, indicating exclusion of Trp residues from the solvent and thus potential protein aggregations.
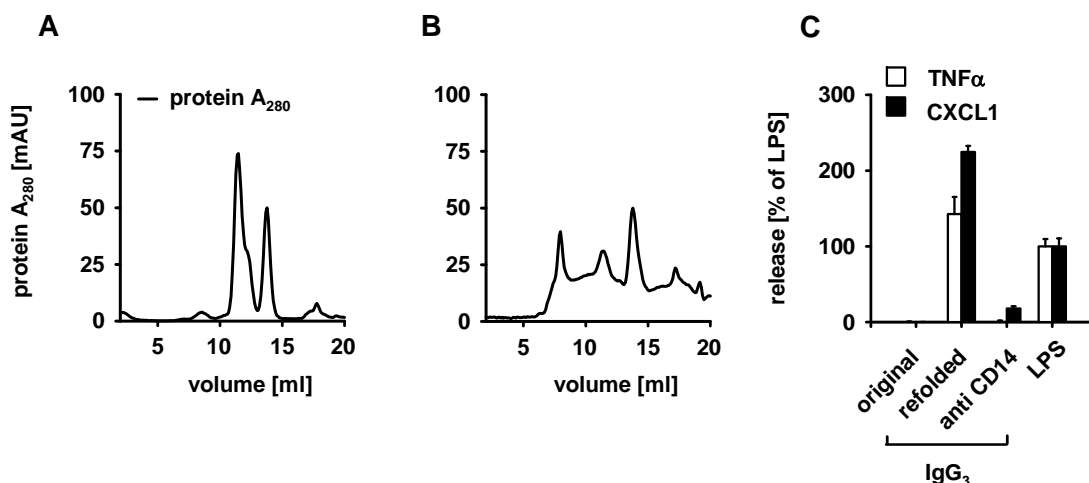
Fig. 4.21.3.: Effect of protein refolding on the release-triggering activity of 'monomeric' IgG. IgG$_3$ from a spontaneously inactive preparation (supplier A) was analyzed by gelfiltration as described in Fig. 4.21.1. **(A)** before and **(B)** after refolding. The protein was denatured (using laurylsarcosine), dialyzed, refolded by rapid dilution in refolding buffer containing L-arginine (500 mM), methyl-β-cyclodextrin (12.5 mM) in Tris/HCl buffer (50 mM, pH 8.0) and dialyzed against PBS. **(C)** Microglial cells were stimulated with IgG$_3$ (100μg/ml) of the original (inactive) preparation, IgG$_3$ (100 μg/ml) after refolding or LPS (1 ng/ml) for 18 h in the presence or absence of an anti-CD14 blocking antibody (10 μg/ml, clone 4C1). The restored cellular activity was sensitive to CD14 blockade, indicating dependence on a TLR4-centered mechanism. Data are mean ± SEM with n=4.

The concept that associate formation is important for TLR4 signaling was fueled by the observation that 'inactive' IgGs lacking associates could build up responses when being paired with 'active' associate-containing IgGs (Fig. 4.21.5.). On the other hand, pairing of two inactive' IgGs did not improve their activity on microglia. Conceivably, associates of functional preparations could serve as nucleation templates and thereby instruct non-functional IgGs to form oligomers. The principle of 'seeding' is known for other proteins, e.g. prion protein, where a fraction of missfolded protein can affect the conformation of native protein, causing the formation of fibrillary aggregates (Colby et al., 2007).
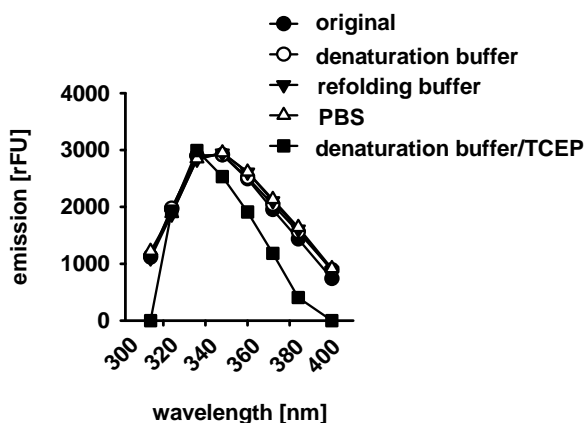
Fig. 4.21.4.: Effect of denaturation/ refolding on the tryptophan fluorescence spectrum of IgG$_3$. IgG$_3$ (supplier A) was denatured and refolded as described above. Fluorescence emission spectra were recorded using excitation at 295 nm from the unprocessed (original), denatured (denaturation buffer) and refolded (refolding buffer) IgG$_3$ and after final dialysis to PBS. Note that denaturation in a buffer containing the reducing agent TCEP resulted in a tightened fluorescence spectrum, an indication for aggregated protein.
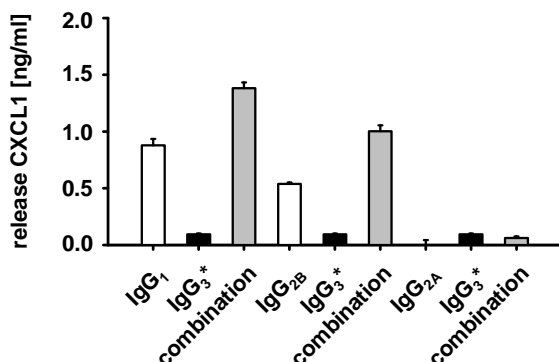
Fig. 4.21.5.: Effect of the combination of 'inactive' and 'active' IgGs. IgG_3 lacking microglial activity (IgG_3*, 100 µg/ml, supplier A) was combined with TLR4-agonistic IgG_1 and IgG_{2B} or another 'inactive' isotype, IgG_{2A} (all 100 µg/ml, supplier A) and used for microglial stimulation for 18 h. The IgG_3* could thereby add substantial effects to IgG_1 as well as IgG_{2B}, but not when being paired with the 'inactive IgG_{2A}. Cyto/chemokine release was measured in the supernatant by ELISA. CXCL1 is shown as a representative. Data are mean ± SEM with an average of n=6 per group.

Yet it was not clear whether formation of associates would represent a property of IgGs naturally occurring in the plasma. Therefore, we sought to detect IgGs among serum protein of higher MW. Size exclusion chromatography of mouse serum and an IgG_3 preparation (supplier A) revealed a similar peak at fraction 20 of high MW (Fig. 4.21.6.A) containing Ig, as further identified by dot blot and immunoblot analysis (Fig. 4.21.6.B). Besides the peak of monomeric Ig (fractions 41 to 45) purified IgG_3 and serum also showed an Ig protein smear over a wide range of fractions (fractions 21 to 40), indicative of molecular forms with supra-monomeric size. In serum, of course, high MW fractions containing Ig could also relate to IC, IgM or to Ig bound to other circulating proteins such as the neonatal FcR (FcRn) (Baker et al., 2009). An unequivocal exclusion of potential antigens or carriers among the high MW serum fractions can thus not be made.
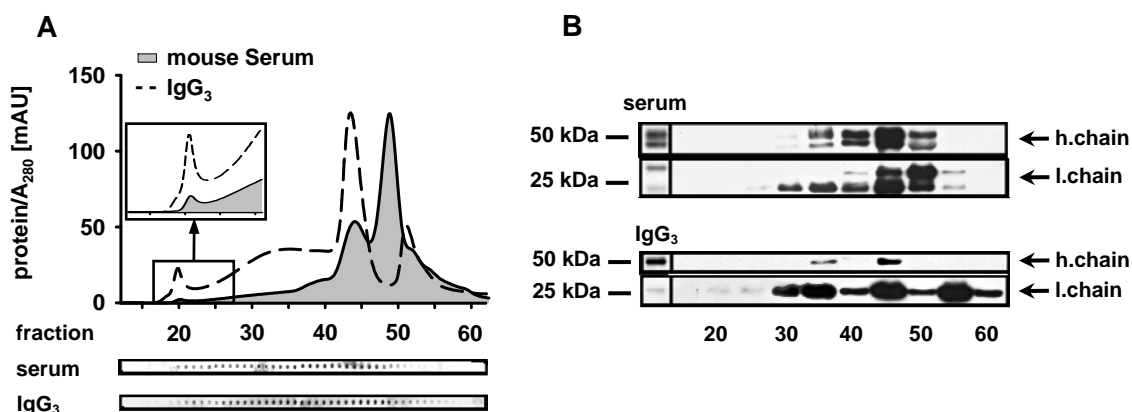
Fig. 4.21.6.: Analysis of mouse serum and IgG_3 by size exclusion chromatography. (A) IgG_3 (supplier A) and mouse serum (supplier C) were analyzed on a Superpose 6 10/300 gelfiltration column as described in Fig. 4.21.1. Fractions were tested for the presence of Ig by dot blot and immunoblot **(B)**. Detecting antibody was chosen to reveal IgG heavy chains specifically and to cover the various isotypes and light chains (κ, λ). Immunoreactive material was found in both samples at MW fractions higher than expected for monomeric IgGs.

On the other hand, purification protocols employed for the various commercial IgG preparations could also influence the propensity of forming associates. These procedures usually require separation steps that may influence the physicochemical status of the protein – intentionally only in a transient fashion. We describe further below that purification by protein G affinity chromatography, a standard method, can, indeed, have a lasting influence on the IgG protein, namely on its TLR4-agonistic activity.

4.22 Generation of monoclonal IgGs against a non-mammalian fluorescent protein

The work described thus far was based on IgGs from commercial sources. Although these preparations were devoid of detectable protein and LPS impurities and were pure as regarding the isotype, their use may still run the risk of pitfalls. Most of the IgGs analyzed in this study were distributed as isotype controls to be used in flow cytometry approaches. As the respective antigen is largely unknown, the quality control (QC) by the manufacturer does not routinely cover functional analyses of antigen binding properties, complement fixation or FcR interactions. Purification processes are rather optimized for high yields of pure IgGs, and conditions might be changed from one to the other lot without being announced. It may explain variations in the release induction potential among preparations of nominally identical isotypes or different lots of the very same clone. In the worst case, it could decide on whether an IgG has TLR4-agonistic activity at all, as described for the IgG_3 preparation with spontaneous loss of activity (see chapter 4.21). Moreover, some Ig distributed in solution include non-protein stabilizing factors, such as carbohydrates, while other preparations come as lyophilisate. Taken together, there is substantial variability in the preparations, hindering the correlation of cellular effects to isotypes or protein conformation states. Probably even more important, the treatments along the purification of IgGs which may come with a suspected impact on their structure and function could apply to the routine used by different manufacturer's. In other words, even the use of a panel of IgGs would not guarantee that they are not systematically suffering from the same production-associated artifact. The alternative of using commercial antibodies of defined antigen specificity (and QC-monitored functional performance) was prohibitedly expensive, considering the substantial amounts needed for our experiments.

To avoid uncertainties and to control all steps during a purification, monoclonal antibodies were raised against a protein with selected properties. A small monomeric

variant of the DsRed fluorescent protein (28 kDa) from the reef coral *Discosoma sp.* was chosen as the target antigen. First, it does not contain any sequence or other known structural similarity to mammalian proteins, making it also an ideal tool for *in vivo* applications based on the induction of endogenous antibodies in future studies. Anti-DsRed antibodies should thereby not interfere with CNS antigens. Second, due to its fluorescence, it can be easily identified among protein mixtures, e.g. in chromatographic analyses, and facilitates the setup of assays for antigen-antibody interactions. Third, like other fluorescent proteins, it is extremely stable against heat, pH value shifts, chemical substances and photo-bleaching. Fourth, the version chosen here would also allow to create di- or multimeric variants for analyzing the effects of monomeric IgGs *versus* IC formats both in *in vitro* as well as *in vivo*.
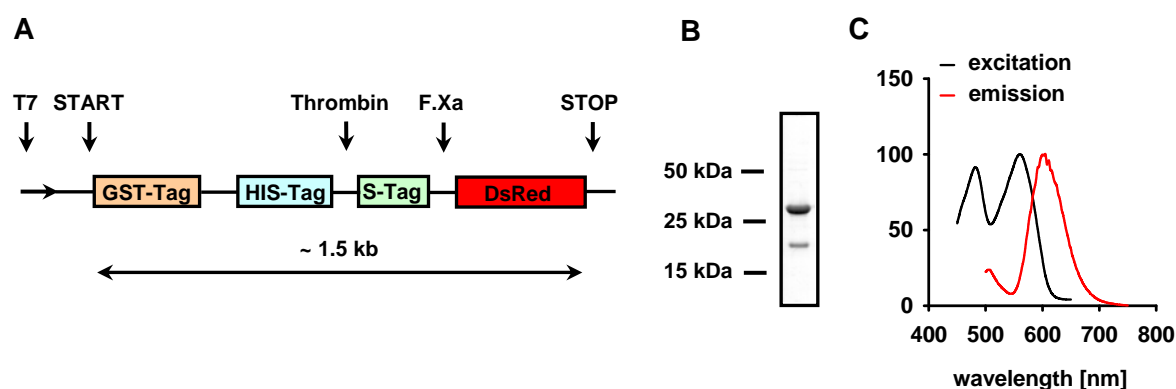
Fig. 4.22.1.: Characterization of recombinant DsRed protein for a monoclonal antibody production. The cDNA of a monomeric variant of the DsRed protein was cloned into the pET42a(+) vector resulting in a construct of about 1.5kb containing 3 N-terminal tag sequences and cleavage sites for thrombin and factor Xa (F.Xa) **(A)**. The protein was produced in *E.coli* and purified via immobilized Ni^{2+} affinity chromatography. The tag was removed by cleavage with factor Xa and a further Ni^{2+} affinity chromatography run. **(B)** The product was analyzed by SDS-PAGE, showing two protein bands at 28 kDa and 18 kDa. **(C)** DsRed absorbance and fluorescence spectra were recorded. The absorbance spectrum revealed two maxima at 482 nm and 560 nm, while emission was at maximum at 608 nm.

The DsRed protein was cloned from the original vector and produced using the pET-expression system (Fig. 4.22.1.A). The protein was produced in *E.coli* and purified via Ni^{2+}-affinity chromatography. The tag was removed by factor Xa treatment and the product was analyzed for its purity by SDS-PAGE as well as for its fluorescent properties. The purified product appeared to be highly pure and showed only two protein bands in SDS-PAGE (Fig. 4.22.1.B). The major protein band was detected at the expected size of about 28 kDa. The minor species appeared with an apparent size of 18 kDa. MALDI-TOF-MS analysis of both protein bands revealed two identical spectra (data not shown), suggesting

that the smaller protein represents a fragment of the full length version generated upon factor Xa treatment. Attempts to separate the fragment form the full length DsRed were in vain. In size exclusion chromatography, only a single peak was detected. Analysis of the absorbance spectrum revealed maxima at 482 nm and 560 nm (Fig. 4.22.1.C).
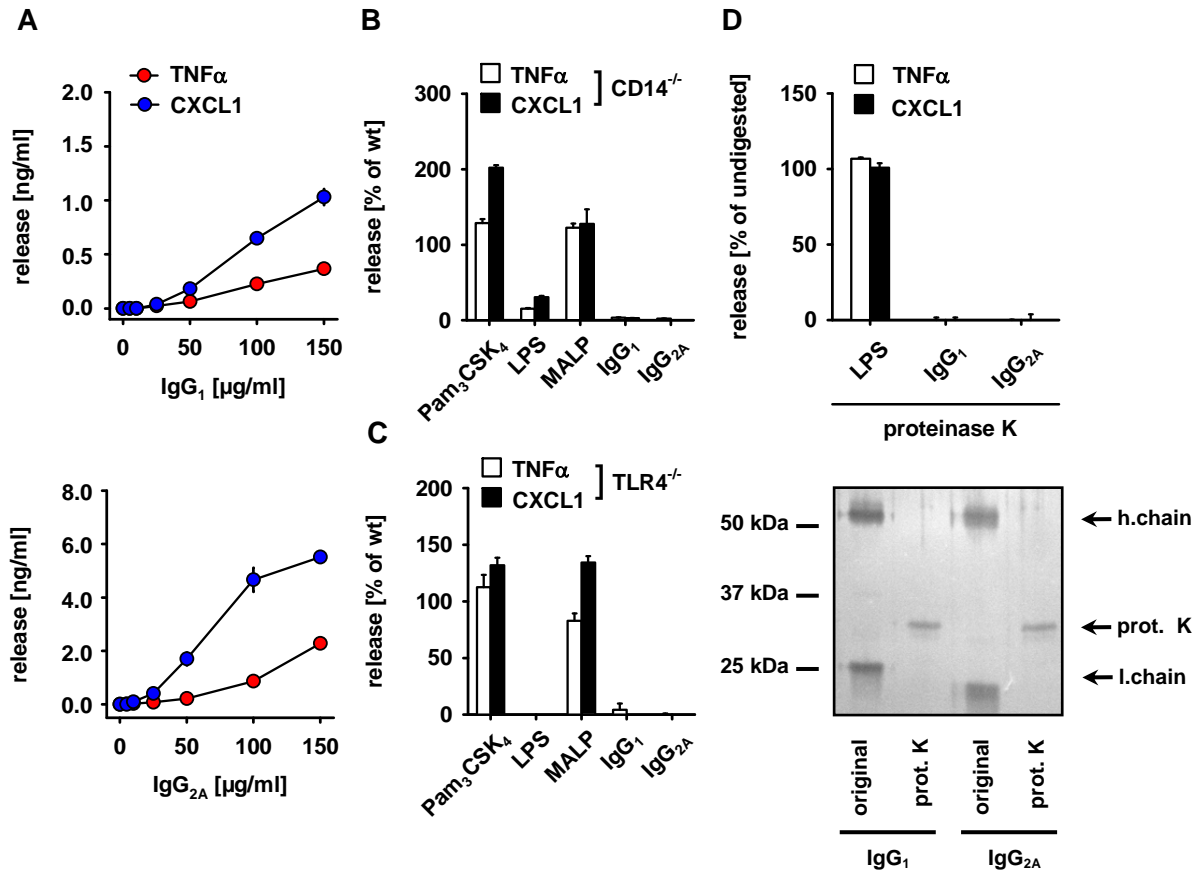
Fig. 4.22.2.: Characterization of anti-DsRed antibody clones regarding their cytokine and chemokine release induction capacity, dependence on CD14 and TLR4 as well as susceptibility to proteolysis. Microglia from **(A)** wt, **(B)** CD14-deficient (CD14$^{-/-}$) or **(C)** TLR4-deficient (TLR4$^{-/-}$) mice were incubated with agonists for TLR1/2 (10 ng/ml, Pam$_3$CSK$_4$), TLR4 (1 ng/ml, LPS) and TLR6/2 (10 ng/ml, MALP) or monoclonal anti-DsRed antibodies of the isotypes IgG$_1$ and IgG$_{2A}$ (at concentrations as indicated or 100 µg/ml) for 18h. TNFα and CXCL1 were determined in the supernatant. Data are mean ± SEM with an average of n=4 per group. **(D)** IgGs (100 µg/ml) and LPS (1 ng/ml) were subjected to proteolytic digestion with proteinase K solution for 24 h at 37°C. Samples were analyzed by SDS-PAGE. The proteinase was removed and samples were tested for induction of microglial TNFα and CXCL1. Data are mean ± SEM from 2experiments with an average of n=4 per group.

Excitation at 482 nm thereby resulted in fluorescence with maximal intensity at 608 nm. Using purified DsRed protein as antigen, monoclonal antibodies were raised (BioGenes, see chapter 3.5). Two hybridoma clones were obtained producing anti-DsRed antibodies of different isotypes, one clone for IgG$_1$ and one for IgG$_{2A}$. The IgGs were then routinely purified from hybridoma supernatants by protein G-affinity chromatography with acidic

elution at pH 2.5 and used for experiments. The observations previously made with commercial IgG preparations could be basically reproduced with these new hybridoma clones. Examples of these data illustrate the moderate capacity for release induction (Fig. 4.22.2.A), the mandatory dependence on functional CD14 and TLR4 (Fig. 4.22.2.B, C) as well as the impact of protease digestion (Fig. 4.22.2.D). Together, the findings confirmed that monomeric (antigen-free) IgGs can activate microglial cells through TLR4 and that this effect is not the consequence of endotoxin contamination.
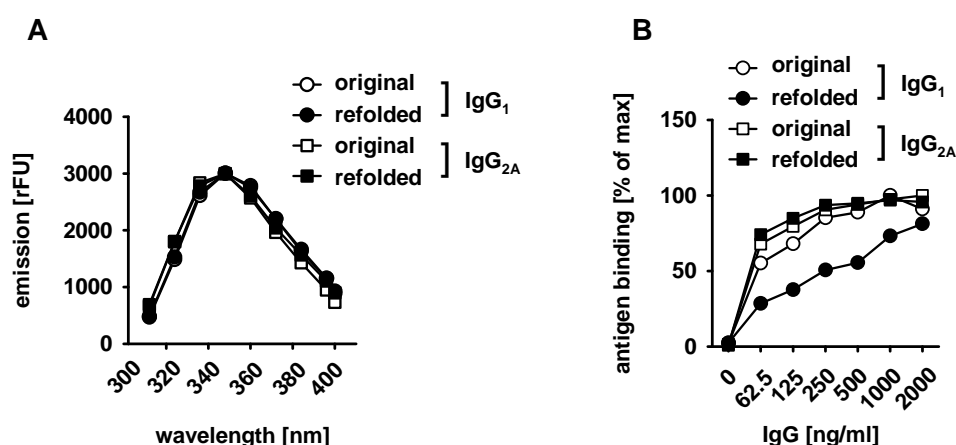
Fig. 4.22.3.: Effect of denaturation and refolding on the tryptophan fluorescence spectrum and antigen binding properties of anti-DsRed antibodies. As described in Fig. 4.21.4 IgG_1 and IgG_{2A} were subjected to denaturation and refolding. **(A)** Tryptophan fluorescence and **(B)** antigen binding properties were determined in comparison to untreated IgGs. Data are mean ± SEM with an average of n=4 per group.

One of the reasons to generate monoclonal anti-DsRed antibodies was to obtain IgGs of defined antigen specificity. The previous chapter stressed the influence of IgG protein conformation on the propensity to induce (via TLR4) microglial responses. Refolding conditions had transformed an 'inactive' into an 'active' IgG_3, along with the appearance of associates, which in turn were in a dynamic equilibrium with monomers. Yet the commercial IgGs did not allow to correlate conformational states, associate formation and TLR4 activity with the capacity to bind antigen. For most of these preparations, the antigen is not known. Therefore, the impact of refolding on the gross protein structure (indicated by the tryptophan fluorescence spectrum) and the antigen binding capacity was determined for the anti-DsRed IgG_1 and IgG_{2A} isotypes (Fig.4.22.3.). As previously shown for IgGs purchased from commercial sources, the denaturation and refolding procedure did not have an influence on the Trp fluorescence spectrum of the anti-DsRed antibodies, suggesting – if at all – only minor conformational changes. Functionally, the refolded IgGs

also maintained their ability to bind their antigen (Fig. 4.22.3.A, B). Collectively, these data show, that the release induction capacity is critically influenced by the conformational properties. Minor changes as they can be induced by mild denaturation and refolding within a suitable environment are sufficient to restore and maintain an apparently native conformation – while allowing for TLR4 signaling. The appearance of HMW IgGs in the chromatogram after refolding is, thereby, not a sign for aggregates of denatured protein – but rather an indication for native IgGs having some propensity to form labile associates (as shown above).

4.23 Transient pH shifts have lasting effects on the TLR4-agonistic activity of IgGs

Antibodies from complex sources, like serum or hybridoma supernatants, can be purified by various methods. The source of the Ig, namely the amount and type of contaminating impurities, thereby determines the choice of the purification technique. As described, the handling (treatment) of IgGs could have long-lasting consequences for their structural and functional properties Ig. Therefore, anti-DsRed monoclonal antibodies were an excellent tool to study the impact of environmental parameters on associate formation, antigen recognition and TLR4-dependent activity for microglia side by side. As these antibodies were routinely produced with established hybridoma cultures in our lab, steps of the purification process could be modified and analyzed for effects on the IgG performance. Common techniques for antibody purification employ fractional precipitation, e.g. with ammonium sulfate or caprylic acid, or affinity chromatography with immobilized protein A or G. Strategies including precipitation and resolving are harsher than approaches exploiting affinity. Although rather gentle, affinity chromatography still requires elution of the bound Ig. Usually, drastic decreases of the pH value are imposed in order to break the high-affinity interaction between the Fc portion of the Ig(G) and protein A/G. It is handbook knowledge that even transient changes in the pH value can permanently influence antigen binding. The following studies on anti-DsRed IgG_1 helped to decipher the outcome of acidification on the TLR4-targeting activity.

IgG_1 from hybridoma supernatants was affinity-purified by immobilized protein G. Elution of bound IgG_1 from the column was performed with glycine buffers of varying pH value, ranging from 2.5 to 2.9. In addition, a commercial buffer system (ThermoScientific) recommended for 'gentle elution' at pH 6.6 was used. The eluate was rapidly neutralized with 1 M Tris (pH 9.5) and dialyzed against a PBS of pH 7.4. Subsequently, the anti-

RESULTS

DsRed IgG$_1$ was tested for its microglia-activating properties. Furthermore, IgG$_1$ was incubated with a 5fold molar excess of its antigen, and the mixture was analyzed by fluorescence spectroscopy-assisted size exclusion chromatography for the formation of associates and ICs.
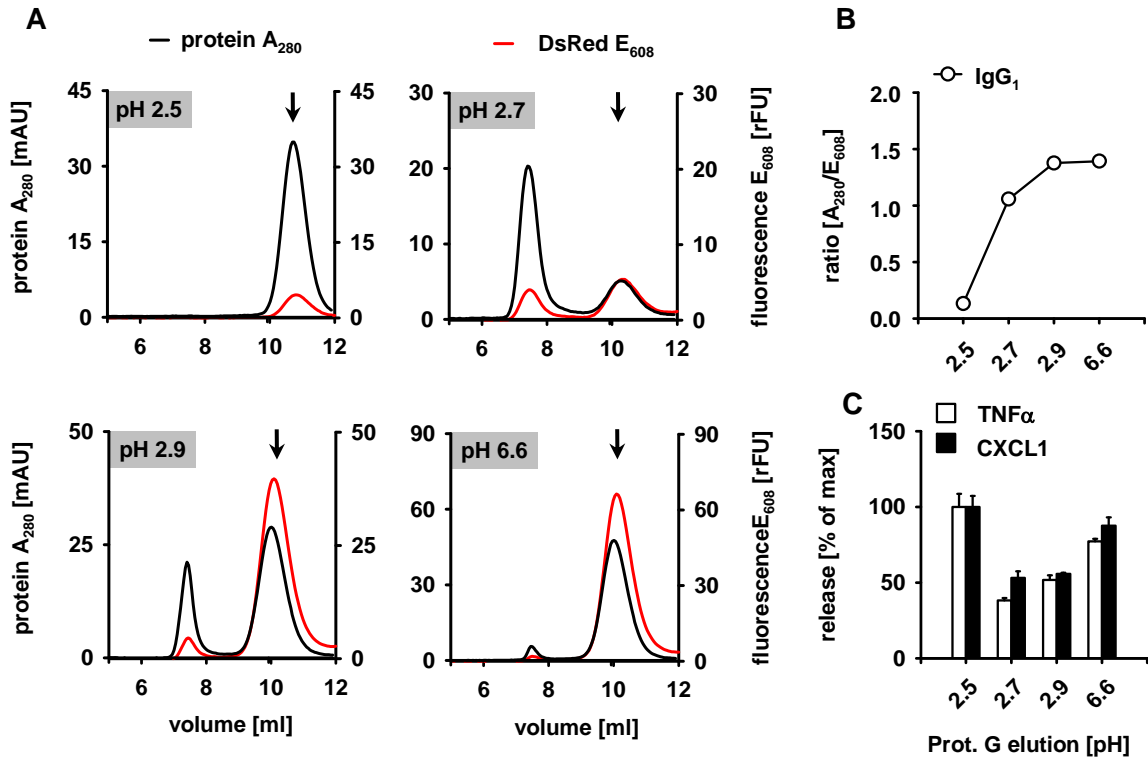
Fig. 4.23.1.: Effect of IgG$_1$ exposure to low pH values on its antigen binding, associate formation and release induction in microglia. Anti-DsRed IgG$_1$ was purified from hybridoma supernatant by protein G affinity chromatography, using buffers of different pH to elute the bound material. The pH of the eluate was immediately re-adjusted to 7.4 and the IgG was incubated with its antigen, DsRed. **(A)** Samples were analyzed by size exclusion chromatography on a Superpose 12 10/300 column. The total protein was monitored by absorbance at 280 nm and the DsRed fluorescence was recorded at 608 nm (emission maximum). Increasing amounts of higher MW forms (elution volume 7 to 8 ml) appeared upon IgG$_1$ elution from the affinity column with transient drops of the pH down to 6.6, 2.9 and 2.7. These conditions resulted in impaired binding, indicated by the fluorescence/absorbance intensities of the IgG- and DsRed-containing (IC) fractions (elution volume 9 to11 ml). (**B**) The ratio of DsRed fluorescence per total protein decreased with transient acidification to progressively lower pH values. **(C)** IgG$_1$ (150 µg/ml) from various elution conditions was tested for release induction in microglia. TNFα and CXCL1 were measure in the supernatants. Data are shown as percent of maximal induction and are given as mean ± SEM with an average of n=4 per group.

Transient reduction of the pH during the elution caused a reduction in antigen binding capacity, increasingly dramatic as the temporary acidification went from 6.6 further down to 2.5. The drop in fluorescence intensity (revealing DsRed) among the protein peak (Fig. 4.23.1.A, arrows), i.e. the decreased ratio of emission over absorbance (Fig. 4.23.1.B), indicated the loss of antigen binding capacity of IgG$_1$. At pH 2.5, antigen binding was

nearly abolished. On the other hand, IgG_1 built up more and more associates as the pH was lowered beyond the value of 6.6. The ratio of associates versus monomers reached a maximum at pH 2.7. However, no associates could be detected anymore when decreasing the pH to 2.5. In combination with the antigen binding capacity, the data show that associate formation occurs in the interphase between the native conformation with high affinity to the antigen and a 'missfolded' conformation with nearly abolished antigen binding. Interestingly, with the occurrence of associates, microglial release responses rather decreased until the pH of 2.7, but recovered again when reaching the 'missfolded' conformation at pH 2.5 (Fig. 4.23.1.C). Virtually the same results were obtained for the IgG_{2A}, yet this clone did not show associate formation at any pH value (data not shown). Together, these experiments provided crucial information about the relation between protein-structural and functional properties of IgGs. (i) The IgGs seem to strongly interact with TLR4 when presented in a still native conformation, which allows proper antigen binding. Yet also a rather missfolded format as resulting from exposure to denaturing conditions and revealing loss of antigen recognition can still drive microglial responses. (ii) Transient exposure to an acidic environment is sufficient to cause lasting changes in IgG properties. If the pH is reduced too far, the conformation can be impaired, so that antigen binding is impeded. (iii) The intermediate range of pH impact between native and impaired conformation can lead to associate formation, however, this does not necessarily correlate with the capacity to trigger microglial responses. It should be added that the interesting pH range between 7.4 and 6.6 remained obscure regarding formation of associates and TLR activity. Elution of at least minimal amounts of IgG from the affinity column was a prerequisite to study its features. Elution, however, required at least some deviation from the neutral pH. Nevertheless, these findings suggest that a purification-associated decline in pH does carry the risk of permanent alterations in IgG conformation. Moreover, a local decline in the pH level can also occur under pathological conditions to affect the functions of endogenous IgGs (as outlined in chapter 5.8).

4.24 Activation of microglial cells by IgGs depend on their carbohydrate structures

In glycoproteins, carbohydrates are either attached to the polypeptide portion via serine and threonine residues (O-glycosylation) or to the side chain of an asparagine (N-glycosylation). Not only do saccharides simply increase the size, charge or solubility of a glycoprotein. Carbohydrate moieties can influence the protein conformation and directly or

indirectly impact on its functions. Involvement in molecular recognition processes is obvious for lectin interactions. Nevertheless, via influences on conformational features, such as domain orientation, or by additional contacts, carbohydrate structures can also contribute to interactions primarily based on protein-protein recognition.
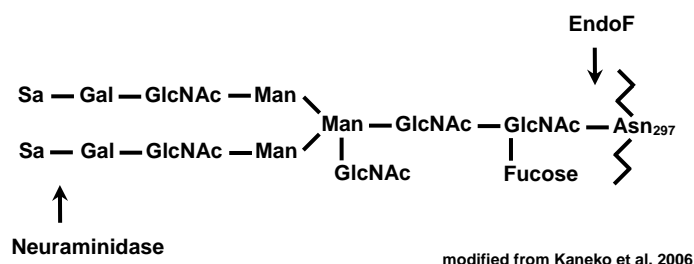
Fig. 4.24.1.: Schematic view of a fully processed IgG-G2 carbohydrate chain. IgGs contain a single N-linked complex carbohydrate moiety in each of the Cγ2 domains of the heavy chain Fc part. The carbohydrates constitute a structural family of about 32 members classified as IgG-G0, - G1 or - G2 according to the number of terminal galactose-sialic acid residues. The structure shown here is attached to Asn-297 of IgGs. Arrows indicate enzymatic cleavage sites for neuraminidase and endoglycosidase F (EndoF). Monosaccharides are abbreviated as GlcNAc (N-acetylglucosamine), Man (mannose), Gal (galactose) and Sa (sialic acid, N-acetylneuraminic acid). Scheme modified from Kaneko et al. (2006).

While Ig's of the IgG class are usually not O-glycosylated, they contain a single N-linked carbohydrate chain of the so-called complex type at Asn-297 (Arnold et al. 2007) in each of the two Cγ2 domains of the Fc part (Fig 4.24.1.). Interestingly, each Cγ2 domain can be differently glycosylated within a single molecule (Arnold et al., 2007). The bi-antennary glucan thereby belongs to a family consisting of 32 members which can be classified by the three major subsets IgG-G0, -G1 and -G2, according to the number of terminal galactose-sialic acid residues. Although intensively studied, literally nothing is known about preferential glycosylation patterns of different isotypes. X-ray crystallography and surface Plasmon resonance studies revealed that the glucan does not only stabilize the Fc part of the antibody but that it is also important for the binding of FcRs and complement (Arnold et al., 2007; Dwek et al., 1995). Moreover, it may also contribute to the affinity and specificity of antigen binding via effects on the domain orientation of the constant region (Torres and Casadevall, 2008). Furthermore, it is known that IgG variants of the IgG-G0 subtype are elevated in patients suffering from autoimmune diseases, like rheumatoid arthritis, systemic lupus erythematosus or Crohn's disease (Nimmerjahn et al., 2007). It is intriguing that agalactosylated/asialylated IgGs (IgG-G0) seem to depend on activating FcγRs, whereas IgG-G2 – containing two galactose-sialic acid residues – would rather

bind to the inhibitory FcγRIIB (Kaneko et al., 2006; Nimmerjahn et al., 2007), thus triggering anti-inflammatory responses when presented in an IC format.

Although these reports cannot explain the underlying mechanism, we raised the question whether glycosylation could affect the TLR4-dependent activity of monomeric IgGs. Indeed, enzymatic removal of either the terminal sialic acid by neuraminidase or the whole carbohydrate structure by endoglycosidase F (EndoF) abolished the microglia-activating properties of an highly active commercial IgG$_3$ (Fig. 4.24.2.A). As expected, enzymatic treatment had no effect on LPS. Surprisingly, responses to anti-DsRed antibodies – showing only a weak cyto/chemokine induction capacity (Tab. 4.15.1) – were dramatically increased upon enzymatic treatment (Fig. 4.24.2.B).
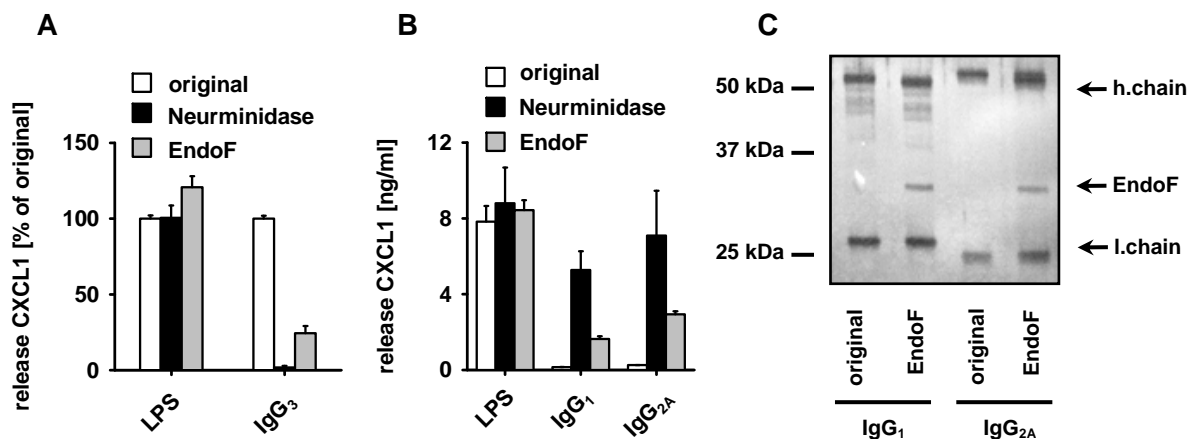
Fig. 4.24.2.: Effect of enzymatic deglycosylation on the release induction capacity of IgGs. IgG$_3$ (supplier C) IgG$_1$, IgG$_{2A}$ (anti-DsRed) or LPS were treated for 24 h with either neuraminidase to release terminal sialic acid or endoglycosidase F (EndoF) to remove the complete N-linked carbohydrate moiety. Enzymes were removed and IgGs (100 µg/ml) and LPS (1 ng/ml) were tested for the induction of cyto/chemokines using stimulation for 18 h. Data are mean ± SEM, showing CXCL1 as a representative with n=9 from 3 experiments (A) and n=3 per group (B). SDS-PAGE revealed a slight shift of the heavy chains to lower MW after EndoF treatment.

The effect of EndoF treatment was less pronounced compared to that by neuraminidase. Since EndoF removes the complete carbohydrate moiety at its link to asparagine it is more difficult for the enzyme to access the cleavage site. Therefore, most protocols use agents like SDS and/or β-mercaptoethanol in the reaction to enhance the cleavage efficacy. However, since the focus here was on the impact on TLR4 signaling, IgG proteins were not denaturized prior to enzymatic treatments. Fortunately, the digestion with EndoF removed still most of the carbohydrate moieties as heavy chains of deglycosylated IgGs appeared with reduced size in SDS-PAGE (Fig. 4.24.2.C). Sterical hindrance is less relevant for the neuraminidase, resulting in an efficient cleavage of the

terminal carbohydrate units. Upon deglycosylation as well as desialylation, the highly potent IgG_3 showed a loss of activity, whereas IgG_1 and IgG_{2A} of (very) low activity revealed a gain of function. Just the removal of the terminal sialic acid sufficed these effects. Using ICs, a gain of anti-inflammatory function upon sialylation was postulated (Kaneko et al., 2006), via signaling of the antibody-antigen complexes though specific FcγR subtypes. The present study focused on 'monomeric' IgGs and their signaling through TLR4. Together, our observations and data by others suggest that a minor carbohydrate modification may adjust the activity spectrum of antibodies – naturally occurring as a mixture of isotypes and depending on the (patho)physiological situation in monomeric or antigen-complexed form. Conceivably, the impact of sialic acid presence or absence for microglial responses to IgGs could depend on a direct contribution to TLR4 binding, similarly as considered for FcγRs. An indirect influence on receptor binding could be organized via moderate adjustments in the IgG conformation, as recently brought forward in linking Fc-structural changes to a modulation of antigen recognition (Torres and Casadevall, 2008). Alternatively, direct or indirect engagements of the sialic acid terminals could address co-receptors in a TLR4 complex that participate in the signaling. Several lectin-like receptor molecules are known to regulate cellular functions in innate as well as adaptive immune responses through sialic acid specificity and signaling (Crocker et al., 2007). The IgG/isotype-dependent opposite outcomes of carbohydrate removal on the cellular activity, however, are in favor of considering an impact on protein conformation. As shown above for the influences of pH and refolding conditions, minor changes in the IgG folding could also decide on antigen binding and TLR4-mediated cellular activity.

In order to further elucidate the critical role of glycosylation, a carbohydrate analysis of anti-DsRed antibodies and matching isotypes of commercial sources (supplier C) was performed (in cooperation with Dr. Nicole Poulsen, GeorgiaTech, Atlanta, USA). The carbohydrates were released and hydrolyzed by incubation with trifluoroacetic acid (TFA). Monosaccharides were then analyzed by high performance anion exchange chromatography with pulsed amperometric detection. Peaks were assigned according to previously analyzed standards consisting of the monosaccharides fucose, galactose and mannose (Fig. 4.24.3.A for representative chromatograms, calibration data not shown). Absolute and relative amounts of detected sugars were then compared between the isotypes and correlated to their microglial activity (Tab. 4.24.1.).
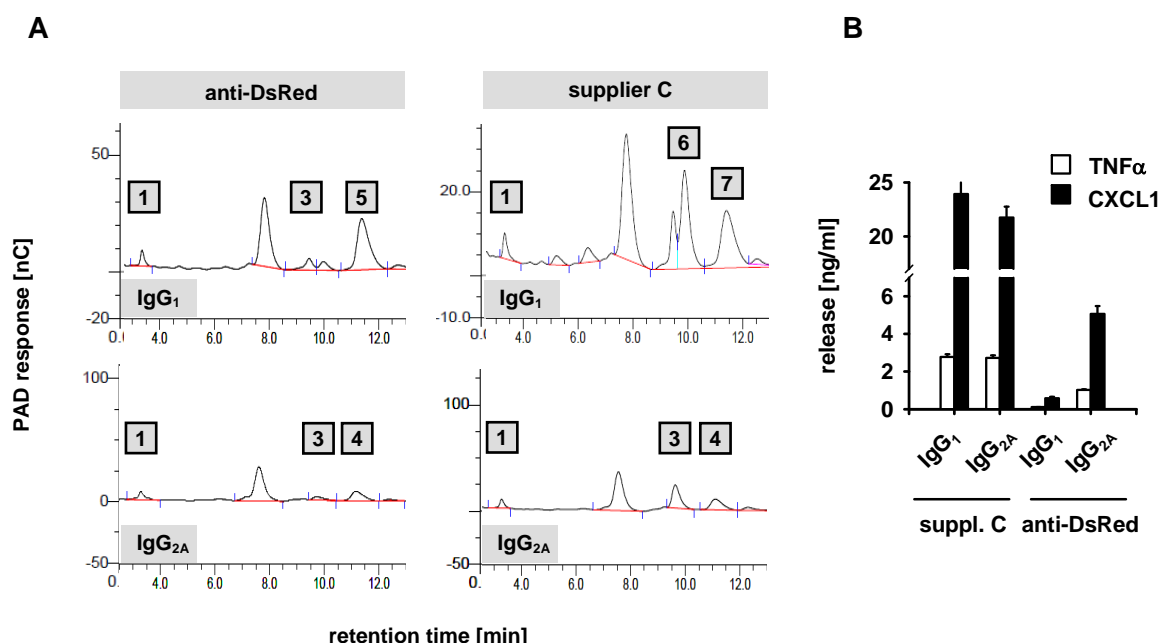
Fig. 4.24.3.: Analysis of carbohydrate moieties of anti-DsRed IgGs and isotype-matched commercial IgG preparations. IgGs (supplier C and anti-DsRed) were treated for 4 h at 100°C with TFA to release and hydrolyze the complex carbohydrate moieties. Samples were lyophilized, resolved in 15 mM NaOH and analyzed by high performance anion exchange chromatography with pulsed amperometric detection with a CarboPack PA20 column. **(A)** Separation was performed by isocratic elution with 200 mM NaOH. Monosaccharides were monitored by pulsed amperometric detection and identified by their retention times established for standards of fucose, galactose and mannose. Numbers in boxes refer to peaks assigned to monosaccharides (see also Tab. 4.24.3.). **(B)** Samples of the unhydrolyzed IgGs (100 µg/ml) were tested in parallel for induction of TNFα and CXCL1 by stimulation of microglial cells fro 18 h. Data are mean ± SEM from n=4 per group.

Importantly, the release-triggering activity of IgG seemed to correlate with the degree of sialylation. Since sialylation only occurs at the terminal galactose, the detected amount of galactose reflected the potential amount of sialic acid which can be added to the respective glycan, ranging between the extremes G0 to G2 in the individual IgG (heavy chain) molecules. Both of the commercial preparations were highly active on microglia, whereas the self-made anti-DsRed antibodies showed only moderate to week activity (Fig. 4.24.3.B). Both of the commercial IgGs showed also high amounts of galactose, whereas the anti-DsRed IgGs were significantly less galactosylated (Tab. 4.23.1, highlighted in red). Interestingly, similar amounts of fucose and mannose still suggested comparable core moiety structures by isotype. While a high sialylation potential may thus favor TLR4 activity (and removal of actual sialic acid units could thereby reduce it), such a correlation may not be strict. Neuraminidase treatment had led to a gain of function in anti-DsRed IgGs. Yet these data could not identify exact substitution sites as used in the glycans, and

there is currently information lacking as to the role of the individual sialic acids or their asymmetric introduction for the structure and function of IgGs, namely isotypes.

Tab. 4.23.1.: Analysis of carbohydrate moieties of anti-DsRed IgGs and isotype-matched commercial IgG preparations. IgGs were sorted according to their isotype and source. Peaks from the chromatogram were assigned to fucose, galactose and mannose (see Fig. 4.23.3.). Amounts of galactose (red) were taken to compare the potential of sialylation. Note that the amounts of galactose differed for the same isotype, but correlated with cellular activity. On the other hand, fucose and mannose levels were similar when comparing the same isotypes, indicative of a common glycan core moiety.

Sample	Peak	Component	Retention [min]	Area [nC*min]	Height [nC]	Amount [pmol]	rel. Amount [%]
IgG_1 supplier C	1	Fucose	3.32	1.075	5.555	52.9	6.52
	6	Galactose	9.88	8.136	21.348	329.8	**40.63**
	7	Mannose	11.40	8.265	12.397	429.1	52.85
IgG_1 anti-DsRed	1	Fucose	3.35	1.199	6.674	59.0	8.04
	3	Galactose	9.45	1.768	4.948	71.7	**9.77**
	5	Mannose	11.38	11.619	21.806	603.2	82.19
IgG_{2A} supplier C	1	Fucose	3.27	1.762	8.495	86.8	13.26
	3	Galactose	9.63	7.327	21.381	297.0	**45.39**
	4	Mannose	11.10	5.212	10.086	270.6	41.35
IgG_{2A} supplier C	1	Fucose	3.28	1.852	6.736	91.2	26.22
	3	Galactose	9.73	1.194	2.630	48.4	**13.92**
	4	Mannose	11.17	4.010	7.651	208.2	59.86

In summary, TLR4-agonistic activity of IgGs seems to depend on their conformational state that, in turn, can be sustainably influenced by subtle, even transient changes in the environment. Acidification, as part of technical procedures or as found in certain pathological processes, could switch (on) receptor preference, as shown for microglial responses. The glycoisoforms attached to the IgGs during posttranslational modification may additionally support, tolerate or deny TLR4 binding and/or signaling, especially by the sialylation degree and pattern. Conceivably, the isotype nature may rather serve as a template that defines the optional range for TLR4 function, as it does for the profile of

Fcγ R binding and complement fixation. Yet the actual TLR4 binding and signaling capacity is un/masked or tuned by physico- and biochemical conditions causing (more or less marginal) folding adjustments. TLR4 signaling may thus enable IgGs to exert, for example, innate immune functions independent of or in addition to their activities in ICs.

5. Discussion

It has been known for long that microglia respond to virtually any disturbance of the structural and functional integrity of the CNS (Kreutzberg, 1996). The role in the tissue surveillance was assumed even before the application of *in vivo* imaging techniques actually visualized microglial scanning behavior in the living brain (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009). However, the recent discoveries prove a daily activity and underline its importance for homeostatic maintenance and even nursing of synaptic connections. Importantly, these studies also reveal how fast microglia can respond to even small disruptions of the vasculature (Nimmerjahn et al., 2005). Factors usually circulating in the blood stream and denied uncontrolled entry to the CNS by an intact BBB would penetrate the parenchyma and could initiate – conceivably protective – emergency reactions of microglia. Extensive or repeated inundation by such factors may, however, contribute to secondary damage in trauma, stroke and other CNS complications compromising the vascular and BBB integrity (Gingrich and Traynelis, 2000). This work addressed several plasma proteins and their microglia-activating potential, the receptors that would convey the signaling as well as conditions that still control their efficacy.

5.1 Microglia as cellular and TLRs as molecular sensors of danger signals

Functions as a tissue macrophage have been considered since decades as relating to the 'reactive microgliosis' in most diverse neuropathologies, ranging from trauma, stroke, infection and neurodegenerative processes to autoimmune diseases (Hanisch and Kettenmann, 2007; Streit et al., 1999). Macrophages are meanwhile granted different reactive phenotypes, with the expression patterns and the functional performance rather fanning out in spectra between polarized extremes (Gordon and Taylor, 2005; Mantovani et al., 2007; Mosser and Edwards, 2008). Microglia likely reveals a similar response versatility, although the CNS provides a special environment which requires and instructs specific regulation (Hanisch and Kettenmann, 2007; Schwartz et al., 2006). In general, the innate immune system – to which macrophages like CNS microglia belong – got now recognized for more than simple assistance in the adaptive immune responses, in part due to the progress in understanding the essential tools by which infectious agents can be sensed.

DISCUSSION

PRRs are the first molecular detectors to alert the host of such a threat, and TLRs comprise one of their major families (Akira and Takeda, 2004; Creagh and O'Neill, 2006; Palm and Medzhitov, 2009). These smart molecular antennas manage to sense viruses, bacteria, fungi and protozoans by some conserved motifs of their nucleic acid, protein, carbohydrate or lipid structures. Recognition of a few conserved, functionally or vitally essential motifs, i.e. PAMPs – rather than numerous individual molecules – enables innate immune cells to cover a wide range of pathogens by a still limited set of receptors. Sharing of typical signaling pathways can then also trigger cellular and subsequently more systemic reactions that all serve the very same purpose, namely efficient host defense. Microbes will thereby often engage with more than one TLR more or less simultaneously. Bacteria are recognized by their cell wall components via surface-expressed TLRs, while their DNA activates intracellular TLRs upon phagocytosis, killing and digestion. TLRs thus cooperate among each other as well as with non-TLRs to coordinate downstream the consequences with synergistic and suppressive outcomes (Ivashkiv, 2009; Watts, 2008).

Yet it is also increasingly understood that even a given TLR can well respond with some individuality to a PAMP compound that varies by its (bio)chemical presentation (Miller et al., 2005). TLR4 responds differently to structural variants (chemotypes) of LPS, differing by acylation, phosphorylation or glycosylation (Erridge et al., 2002; Huber et al., 2006; Jiang et al., 2005). The immune system can thereby distinguish bacterial strains expressing the LPS chemotypes. Strain-specific reactions could be important to resolutely fight true pathogens while tolerating harmless germ material, as spilling from commensal flora. TLR4 properties themselves seem to differ between cellular populations, such as mast cells, DCs or macrophages. The detection of LPS in certain tissues or body compartments, such as the CNS, would have a different meaning and require adapted consequences.

TLR activation, however, does not exclusively depend on PAMPs. Danger can also arise from inside. Structures recognizing and indicating danger as well as the importance of their interaction trace back far in evolution (Matzinger, 2007). Indeed, several endogenous factors generated upon tissue injury or cell impairment can drive TLR activation (Tab. 1.4.1.). These molecules are of diverse biochemical nature. They normally serve different functions (Beg, 2002; Lotze et al., 2007; Rifkin et al., 2005). However, leaving their usual intracellular compartment (e.g., the nucleus or cytosol), losing their integration in the ECM (e.g. upon proteolysis) or being (further) modified by oxidation or aggregation – in other words, being presented in unphysiological location or format – they

gain the role of a DAMP. The TLR-induced responses to PAMPs and DAMPs overlap, because in both cases their appearance indicates a threat. DAMPs may amplify infectious responses to a PAMP (Vogl et al., 2007). They may prepare for an anti-microbial defense since wounds will unavoidably favor infection. They may, however, also organize for (innate) immune reactions in the absence of infection, by a process termed sterile inflammation (Stewart et al., 2010). TLR assemblies thereby even differ. In case of TLR4, the known homodimer mediates responses to LPS, more or less with the aid of the co-receptor CD14, whereas the recognition of endogenous material, such as oxidized low-density lipoprotein (oxLDL) and Aβ, can operate through a TLR4-TLR6 heterodimer in association with CD36 (Stewart et al., 2010).

Microglia are equipped with several TLRs (Bsibsi et al., 2002; Hanisch et al., 2008; Olson and Miller, 2004). Their implications in harmful and beneficial processes in the CNS are intensively studied. They certainly could also serve the dual responses to PAMPs and DAMPs. Among the TLRs, TLR4 claims an exceptional position because it can recruit an ensemble of accessory co-receptors, such as CD14 and LBP in the case of LPS, CD36 for responses to oxLDL and Aβ (see above), but also CD55, CXCR4, integrins or FcγR for still maldefined scenarios (Kagan and Medzhitov, 2006; Pfeiffer et al., 2001; Triantafilou and Triantafilou, 2002). Furthermore, it is the only family member using both of the TLR-associated signaling routes, i.e. MyD88- and TRIF-dependent pathways. In addition, connection to these signaling adapters is indirect since the sorting adapters MAL and TRAM are inserted, probably providing more regulatory options. Surface expression positions TLR4 to collect extracellular factors – ideal for microglial surveillance tasks. All these features and variables may allow TLR4, and microglial cells, to distinguish between agonists, to interpret their context and to translate them into tailored responses.

Blood vessels take a substantial part of the total CNS volume for the efficient supply with oxygen or nutrients and metabolites. Plasma would therefore represent an ideal nearby source of 'latent' signals for microglia. It is already assumed to contain inflammation-inducing and -regulating mediators. Besides lipids or albumin, proteases of the coagulation cascade got under investigation (Gingrich and Traynelis, 2000).

5.2 Thrombin as a potential signal for microglia

Thrombin (EC 3.4.21.5, factor IIa) is a multifunctional serine protease with distinct substrate specificity, best known for the cleavage of fibrinogen to fibrin, which finally leads

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to the formation of a fibrin clot (Davie and Fujikawa, 1975). Cleavage of the 72-kDa zymogen (prothrombin, factor II) by the factor Xa-containing prothrombinase complex delivers the active Arg-specific enzyme of 39 kDa, while several plasma and tissue inhibitors serve in its inactivation. Besides the fibrinogen conversion as a central event, thrombin tightly controls the coagulation cascade by positive and negative feedback mechanisms and is thus the most important enzyme for hemostasis (Bukys et al., 2006; Davie et al., 1991; Guillin and Bezeaud, 1987). However, thrombin's activity spectrum does not only cover cleavage of soluble peptide substrates. Thrombin controls functions of various cell types by limited proteolysis of their protease-activated receptors (PARs) (Coughlin, 2000; Ossovskaya and Bunnett, 2004).

Thrombin has been reported to act as a mitogen for lymphocytes, monocytes, platelets (Coughlin, 2000), the endothelium (Lum et al., 1994; Lum and Malik, 1996), smooth muscle and mesenchymal cells (Lum et al., 1994; O'Brien et al., 2000). Roles in the development, function, plasticity and protection of the CNS are discussed, regarding synapse remodeling, long-term potentiation or attenuation of toxic insults (Tomimatsu et al., 2002; Wang and Reiser, 2003; Zoubine et al., 1996). On the other hand, local microbleeding, BBB leakage, CNS injuries and neuropathological processes, ranging from stroke and AD to multiple sclerosis (MS) and their experimental models, associate with the complex impact of plasma proteases, with frequently thrombin being in the focus (Fujimoto et al., 2007; Gingrich and Traynelis, 2000; Han et al., 2008; Lee da et al., 2006).

The blood coagulation cascade and thrombin as its central initiator had been recognized early for a role in MS. Heparin-induced blockade of thrombin activity improved symptoms during MS relapses and EAE (Lider et al., 1989; Maschmeyer et al., 1961). In MS, AD and stroke, fibrin(ogen) could carry some neuropathogenic functions downstream of thrombin, involving also microglia, CD11b/18 and even TLR4 as cellular and molecular effectors (Adams et al., 2004; Flick et al., 2004; Smiley et al., 2001). A recent proteomic approach to the analysis of the protein compositions in defined MS lesions revealed coagulation-related proteins and concluded to the importance of thrombin (Han et al., 2008). However, the actual role of the individual components and the involved receptor mechanism(s) on CNS cells remained unclear. Microglia reveals as a source and target of various proteases in the CNS, and thrombin takes a key position in linking hemostasis to wound healing and inflammation (Möller et al., 2006; Nakanishi, 2003).

Thrombin effects on microglia were first reported in 2000, independently by two groups, including authors from of our laboratory (Möller et al., 2000; Ryu et al., 2000).

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Microglial cells responded with proliferation as well as profound release of NO and cyto/chemokines. Thrombin elicited transient Ca^{2+} signals, with features typical for PAR activation. Thrombin preparations from various species were also found to stimulate the microglial expression of CD40 and CD95 (Weinstein et al., 2008a; Weinstein et al., 2009). The thrombin-induced damage to the CNS *in vivo* has been associated (or can be assumed to largely correlate) with an activation of microglial cells and the instruction of a neurotoxic phenotype (Fujimoto et al., 2007; Lee da et al., 2006). PARs have been the favorite (assumed) receptors, as far as signaling mechanisms were concerned. Indeed, all family members are expressed in microglia (Balcaitis et al., 2003), and Ca^{2+} signals were sensitive to hirudin blockade as well as thrombin pretreatment-mediated desensitization, typical for its protease activity (Möller et al., 2000).

However, intense research of our group demonstrated that PAR activation could not account for the cytokine and chemokine induction in microglia (Hanisch et al., 2004; Weinstein et al., 2005). Interference with (PAR) substrate binding, use of γ-thrombin (an enzymatically active, yet substrate binding-deficient form) and even active site inhibition did not affect the inflammatory response. Accordingly, the direct PAR stimulation with a panel of synthetic agonist peptides failed to trigger this effect. Still assuming the proper α-thrombin itself to promote this microglial activity, further studies then excluded an involvement of the TP508 and B-loop domains, which had been described for functions on immune cells (data not shown). In search for alternative receptors, all the constituents of CD42, also known as glycoprotein (GP) Ib/V/IX complex for the binding of von-Willebrandt factor as well as thrombin in platelets, were detected in microglia. Yet the functional interference by low MW heparin versions ruled out any contribution to the cyto/chemokine induction (not shown). Finally, this microglial activity could be identified to reside in a thrombin-associated high MW protein complex (thrombin[HMWM]) which seems to be a minor but frequent component within numerous thrombin preparations (Hanisch et al., 2004). Indeed, it occasionally comprised an almost undetectable amount of protein, while driving the inflammatory microglia response without revealing itself proteolytic activity. Use of ultrapure (GMP-approved) thrombin preparations confirmed this observation. They were able to stimulate CD40 and CD95 expression, but failed to do so for the cytokines and chemokines – and presented with the absence of thrombin[HMWM] (Hanisch et al., 2004; Weinstein et al., 2008a). Thus, some microglial effects assigned to thrombin were truly PAR-dependent, and others, namely the inflammatory part, would rather not require this mechanism. Yet the nature of the active components among the thrombin[HMWM] fraction

and their receptor(s) remained enigmatic. Their potency would, however, be impressive. Moreover, identification of an alternative carrier of activity would raise doubts as to the previous assignments of numerous cellular and tissue consequences to thrombin.

In the present study, the previous observations could be confirmed. Using size-exclusion chromatography-based approaches, we can show that, even apparently pure, thrombin preparations may contain a minor fraction of supposably aggregated HMW protein as the solely responsible non-enzymatic trigger of an inflammatory response. Thrombin[HMWM] proved its functionality on microglia of different genetic background (NMRI and C57Bl/6), inducing virtually the same profiles of comparable intensities (not shown). Moreover, thrombin[HMWM] from various species was functional on mouse microglial cells, yet differing by activity and revealing mouse thrombin[HMWM] as the most potent activator. Most importantly, we could unravel the receptor/signaling system to rely on a CD14/TLR4/MyD88/TRIF-dependent mechanism, and we identified FN as a coagulation-related protein to be a thrombin[HMWM] constituent with TLR4-agonistic capacity. Thus, thrombin-initiated downstream events could generate factors which drive indirectly inflammatory microglial responses through TLR4, while thrombin is still influencing other microglial function directly via PARs. The concept would be in agreement with the literature and could explain many findings, especially from *in vivo* work.

5.3 pFN as a coagulation factor with DAMP character for microglia

FN is a high MW glycoprotein of complex structure and diverse functions. It is expressed by numerous cell types, found in extracellular fluids and represents a major component of the ECM, where it is linked to other ECM constituents, like laminin, collagen and heparan sulphate (Astrof and Hynes, 2009; Hynes, 1986; Moretti et al., 2007; Pankov and Yamada, 2002). In humans, more than 20 isoforms are known so far, 12 variants are described in mice. The variations are a consequence of alternative splicing of a single primary transcript as well as posttranslational modifications. Processing mainly occurs at three sites, the extra domains A (EDA also known as EIII-A or EDI) and B (EDB, EIII-B or EDII) or type III homologies connecting segment (IIICS). The splicing process depends on the species, the developmental stage as well as the cell type. FN exhibits several functional domains that consist of three types of repeated regions, called FN I (12 repeats), II (2 repeats) and III (15 to 17 repeats), differently distributed within the protein and exhibiting different lengths. Type I is located at the N- as well as C-terminal parts and

contains several fibrin and collagen binding sites. Type II is found in the more N-terminal part and has basically collagen binding capability. Type III is the most abundant type and exhibits various integrin binding domains. EDA and EDB would thereby integrate between the FN III repeats.

FN occurs in two major forms. Plasma fibronectin (pFN) is the soluble form produced by hepatocytes and secreted into the blood, where it circulates in humans at 300 µg/ml of plasma (600 µg/ml in the mouse) (Moretti et al., 2007). It consists of two subunits of 220 and 240 kDa, linked near the C terminus by a pair of disulfide bonds. pFN seems to completely lack the EDA and EDB domains. While FN in general is essential for vertebrate development (its deficiency causing embryonic lethality in mice), pFN has important functions in tissue repair and coagulation processes (Astrof and Hynes, 2009; Cho and Mosher, 2006a; Cho and Mosher, 2006b). pFN stabilizes the growing thrombus though interactions with platelets as well as factor XIII and fibrin. Cellular fibronectin (cFN) is produced by fibroblasts, epithelial and other cells and forms cross-linked multimeric fibrils in the ECM. Fibrillogenesis and matrix assembly are highly regulated processes allowing for a defined ECM composition in the various tissues (Hynes, 1986; Pankov and Yamada, 2002). In contrast to pFN, cFN thus comes in various isoforms – and it contains EDA as well as EDB.

Although molecular mechanisms involved in the processing of FN are known since two decades, the resulting functional consequences remain elusive. However, studies focused on EDA-fibronectin, because it is elevated upon tissue damage as well as in wound healing and as it associates with pathologies, like rheumatoid arthritis and tumors (Okamura et al., 2001; Ridley, 2000; Rybak et al., 2007). FN interactions with a dozen or more of integrins allow for a substantial binding spectrum, $\alpha_5\beta_1$ being considered its classical partner. They mediate cell adhesion, but also link the ECM to the cytoskeleton and intracellular signaling pathways (Hynes, 1986; Hynes, 2002; Pankov and Yamada, 2002). Yet FN may also have other receptor and signaling options.

A recombinant EDA domain as produced in *E.coli* was shown to activate THP-1 monocytes/macrophages and TLR4-transfected HEK 293 cells, thus claiming a link of EDA-containing FN (fragments) to TLR4-induced inflammatory processes (Okamura et al., 2001). However, the study left several questions as to a potential contamination with bacterial LPS (Tsan and Baochong, 2007; Weinstein et al., 2008b) or to the significance of an isolated domain as dissected from a huge molecule, such as FN. Loss of effects in a TLR4$^{-/-}$ situation, as later taken to support the assumed TLR4 mechanism, would thus not

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serve as an unambiguous proof (Gondokaryono et al., 2007). Moreover, several minimal integrin and cell recognition sequences have been described as functional units, first of all the RGD tripeptide, but also LDV, REDV, IDAPS, KLDAPT and EDGIHEL for $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_9\beta_1$ (Pankov and Yamada, 2002). Yet the functional importance of a minimal site, such as RGD, depends on the flanking portions and the conformation of the parent polypeptide. The 'synergy site' PHSRN has such an influence (Livant et al., 2000a; Livant et al., 2000b). In addition, FN reveals a remarkably low number of cleavage sites for proteases, considering its size (Pankov and Yamada, 2002). Limited proteolysis, even for common trypsin-like proteases, indicates protection of the other potential cleavage sites by folding, and this suggests that functional access to FN domains is regulated. Indeed, FN fragments can unfold activities not possessed by the intact protein, or lose functions upon an excision (Barilla and Carsons, 2000; Hashimoto-Uoshima et al., 1997). Domain interactions appear rather important for FN conformation, sterical hindrance – and thus functions.

Still, later reports were based on (c)FN-EDA as a potential TLR4 agonist or did not further question its lack in pFN or ignored the risk of confounding LPS in an FN isolate. A peptide sequence within the EDA was recently identified to mediate the binding to $\alpha_9\beta_1$, suggesting that (also) other receptor interactions reside in this domain (Shinde et al., 2008). Especially the integrin binding could explain the prothrombotic activity of EDA (Chauhan et al., 2008), a potential coagulatant function of cFN upon tissue injury.

We observed microglial activity for the pFN and reported on its phagocytosis-promoting activity in synergy with TLRs, selectively sparing TLR4, an observation most relevant for bacterial meningitis (Goos et al., 2007; Ribes et al., 2010). Based on our findings here, lack of synergism can be explained by pFN and LPS sharing the use of TLR4. The significance of pFN actions on microglia (via integrins, and now also TLR4) has been emphasized for cerebral ischemia, considering extravasation of plasma proteins, especially coagulation factors, and again namely FN, as elements in the secondary injury (Milner et al., 2008). Recent findings revealed the surprisingly substantial transfer of pFN to the ECM pool of FN (Moretti et al., 2007). Depletion of the hepatocytic production of pFN not only caused delayed formation and increased instability of the thrombus, but augmented susceptibility to bacterial infection and, importantly, also ischemic and traumatic brain injury. Thus, pFN may have broad implications for tissues, including the CNS, in protective (Sakai et al., 2001; Tate et al., 2007) as well as harmful context (Han et al., 2008).

DISCUSSION

Based on FN's link to coagulation and analysis of thrombin^{HMWM} constituents, we addressed essential TLR4-agonistic DAMP features of pFN and found it capable of driving functional consequences for microglia. Mass-spectrometric analysis of thrombin^{HMWM} identified, among others, proteins of the coagulation cascade, including FN. As a likely candidate for TLR4-dependent inflammatory microglia response to thrombin^{HMWM}, it likely incorporates into a complex of coagulation factors initiated by thrombin. Most importantly, a CD14/TLR4/MyD88-dependent mechanism was shown to be responsible for the cellular effects.

Nevertheless, it is unlikely that FN solely accounts for the release-triggering activity of thrombin^{HMWM}. Thrombin preparations were routinely used at 10 U/ml (enzymatic activity units served for quantification), which corresponds to 100 µg/ml total protein, i.e. for the bovine thrombin preparation. Size exclusion chromatography, however, revealed that only about 6% of the total protein relates to thrombin^{HMWM}. Thus, estimating a size of more than 5,000 kDa, HMWM would trigger microglial responses in the picomolar range. Apparently, the amount of FN within the HMWM is even lower. FN would thus not reach threshold concentration as indicated by the dose-response curves for the purified FN. Moreover, thrombin^{HMWM} and FN exhibited differences in the sensitivity to heat exposure. While HMWM-induced responses were completely abrogated after incubation at 70°C, FN still induced release in microglia at maximum intensity. Conceivably, the complex material may dissociate at this temperature, releasing the individual constituents of thrombin^{HMWM}. However, at the level of a single molecule, factors may not trigger release by themselves or do not reach threshold concentration on the cell. Thus, assembly in a complex would enhance the DAMP action. On the other hand, microglial reactions are selective and not simply a feature of big protein complexes/aggregates. For example, cells did not respond with release to incubation with the multimeric complex KLH (4,500 – 13,000 kDa, data not shown). Nevertheless, FN might be a key element in the generation of thrombin^{HMWM}. Considering its versatile interactions with plasma/ECM molecules and its important function in the coagulation cascade by stabilizing the fibrin clot it could serve as the backbone to build up the HMWM complex during coagulation.

Our findings thus may provide a missing link to explain the observed and further suggested involvement of coagulation in diseases, like MS (Han et al., 2008; Lider et al., 1989). Besides the direct interaction of certain plasma and coagulation factors with cells of the CNS, the mechanism would involve the generation of a coagulation-associated protein complex to drive inflammatory responses in microglia. Moreover, (p)FN might be also an

important DAMP besides its involvement in coagulation. In the healthy CNS tissue, FN is exclusively found as insoluble ECM fibrils. Our data indicate that microglia would not sense immobilized FN fixed in its local environment. Effective recognition would require conformational flexibility. Besides inundation by soluble pFN – as a consequence of disrupted vasculature or breakdown of the BBB – further 'free' FN material might get generated in situations of a trauma or tumor growth and invasion, which are accompanied by ECM destruction.

5.4 Ig(G) isotypes with DAMP signal function for microglia

Ig's are a major plasma constituent and can be secreted in high amounts by plasma cells during an adaptive immune response. In autoimmune disorders, like multiple sclerosis they are considered to have a major impact in disease development and progression through the recognition of various autoantigens (Lassmann et al., 2007; McFarland and Martin, 2007; Schmidt, 1999). In multiple sclerosis, the presence of oligoclonal bands (OCB) of Ig's in the CSF of patients is one of the diagnostic hallmarks. It suggests a substantial impact of the humoral limb of the immune system in the disease. Investigations to identify relevant structures that serve as antigens for the antibody responses in MS tissue revealed several myelin proteins, lipid structures as well as non non-myelin molecules as potential autoantigenic targets (Schmidt, 1999). Conceivably, these antigens can be tagged by autoreactive Ig and trigger demyelinating attacks by activating microglia or infiltrating macrophages through the involvement of Fc- and/or complement receptors. However, only about half of the MS cases belong to the histopathological pattern that presents with Ig association and complement-mediated damage of the tissue (Lucchinetti et al., 2000). Moreover, none of the identified autoantibody targets can convincingly explain the complexity of the histopathological observations or the disease severity and course in patients. While some anti-myelin antibodies are not exclusively found in multiple sclerosis, other potential myelin targets remained obscure, because the respective screening assays – largely based on peptide sequences – did not cover folding epitops as they are expressed on natural proteins. Moreover, neither FcγRs nor the Fcγ signaling chain seemed to be critical for the development or progression of EAE – the animal model of multiple sclerosis (Breij et al., 2005; Szalai and Barnum, 2004), questioning their importance at least in the animal model (Urich et al., 2006). Because of these controversial findings we raised the hypothesis that

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additional Ig features, besides antigen complexation and FcR signaling or even complement fixation, could translate their presence into microglial activation – with either tissue damage or protection as an outcome.

The thrombin[HMWM]-associated search for the potential carrier of microglial activity with a high MW led us to postulate Ig's as a respective fraction constituent, due to their abundance in plasma and size range. In this study, we could then show that IgGs from various sources and different isotypes could trigger similar responses in microglia, although Ig's were nor found among thrombin[HMWM] protein. The activity for microglia somehow correlated with the isotype nature, suggesting a preferential interaction with the FcγR subtypes. Importantly, however, effects were observed with monomeric IgGs (i.e. commercial isotype preparations of mostly undefined antigen specificity as mainly used as negative controls in flow cytometric analyses, or with self-made monoclonal antibodies against DsRed as a defined non-mammalian protein antigen). Cellular responses were thus independent of functional antigen-antibody IC's. Even though FcγRI (CD64) can bind also monomeric IgGs, effective signaling is generally believed to require IC's. On the other hand, a recent report could demonstrate (for the first time) that monomeric IgGs can cause microglial responses and that the presence of such a – previously considered 'quiescent' – IgG pool in the CNS can exert neuroprotective activity (Hulse et al., 2008). Not only are FcγRI expressed by microglia (Kennedy et al., 1980), monomeric IgG binding may influence the recycling endocytosis (Harrison et al., 1994; Mellman et al., 1984). Yet the receptor mechanism for microglia was not further followed.

Surprisingly, we found our mouse microglia to respond to monomeric mouse (and also human) IgG isotypes by a mechanism rather independent of classical and specific FcγR interactions. It involved a CD14/TLR4/MyD88/TRIF pathway. In addition to the conformational features as they come by isotypes or as they are induced or modulated during purification routines, our data point to a critical contribution of the carbohydrate moieties. The enormous structural variety of oligosaccharides, which exceeds those of polypeptide chains by far, results from the combination of monosaccharides as building blocks (pentoses, hexoses), their variable connection in linear or branched oligomers via different carbon atoms of the ring, α- and ß-anomeric links and the optional introduction of substituent groups. The process of Ig glycosylation and the diversity of the resulting structures are not yet fully understood. However, isotype-dependent variation in the carbohydrate structure is indicated. The single sugar moieties in the constant region are now considered important for protein folding, having even unanticipated, far-reaching

consequences for the variable portions with impacts on the affinity and specificity of antigen binding (Torres and Casadevall, 2008). Affecting the conformation of the Fc part and the hinge region, where both FcR and complement interactions are organized, differences and tuning of the carbohydrate structures were shown to actually determine the functional performance of IgGs (Kaneko et al., 2006). In particular, sialic acid is assumed to play a major role as elevated plasma levels of asialylated (IgG-G0) correlate with autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus or Crohn's disease (Nimmerjahn et al., 2007). Our data indicate that manipulation of the sugar moiety dramatically influences the ability of IgGs to activate microglial cells – supposably in an isotype-dependent manner. Only recently, the impact of the IgG glycosylation in a variety of autoimmune models has been addressed in more detail. Albert et al. (2008) could show that specific removal of IgG-associated carbohydrate moieties could interfere with antibody-mediated inflammatory processes. Most importantly, these effects seemed to be specific for certain isotypes, supporting our findings.

TLR4-mediated activity of IgG isotypes could arise from technical processes of harvest and purification from hybridoma supernatants or ascites. These steps commonly involve affinity chromatography on immobilized protein G/A and elution at preferentially low pH. Our data suggest that even a transient exposure to low pH can affect the IgG properties, importantly with lasting consequences. A transient shift to low pH values not only affected the antigen binding properties of the IgGs, but led to the formation of labile associates. Although associate formation turned out not to be a strict requirement for microglial activity, it indicated conformational alterations which determined the microglial activity. We could demonstrate that a rather missfolded IgG$_3$, revealing loss of antigen binding properties as resulting from the exposure to denaturing pH conditions, can drive microglial responses. Yet, IgGs can activate microglial cells also when being presented in a native conformation, which allows proper antigen binding. However, the peri-neutral pH range by which IgG molecules would start gaining such a function remains elusive. Elution of IgGs from an affinity column required at least some deviation from neutral pH. We need to adapt isolation protocols for our home-made anti-DsRed antibodies to determine IgG changes in the close-to-neutral pH range, yet avoiding a manipulation by any other environmental parameter.

Generated during the process of IgG production, TLR4 activity would potentially not only interfere with laboratory applications *in vitro* and *in vivo*. It could prove significant for the growing market of antibodies which are generated for clinical use. The *in vitro*

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design and manipulation of the Fc portion might come with unpredictable side effects, including undesired receptor activations, such as for the TLR4. Especially the industrial need for maximal yield favors isolations which would bear the risk of inducing unnoticed 'side activities'. Recent reports demonstrate that the constant region of an antibody does not only have an influence on the effector functions, but on binding features as well (Torres and Casadevall, 2008). We see, in addition to a technical 'artifact', the pH-controlled receptor switch – or gain of a TLR-agonistic property – also as a likely natural process. Conceivably, an IgG protein could be subject to such subtle conformational rearrangements when entering a similar low pH environment in inflamed tissues or at sites of bacterial infection (Lardner, 2001).

Metabolic disturbance or substances released from immune cells and germs condition a local drop in pH, reaching ranges well below 6, which we found efficient for instructing structural changes in the IgG molecule. One may speculate that such a mechanism may enable Ig's to drive innate immune responses, especially in early phases of host responses to infection – when the humoral immunity is not yet fully blown by production of high-affinity antibodies. A TLR recruitment could add to the options of microbial opsonization, as already based on the PAMP-PRR recognition, complement and antibody-FcR binding. Thus, the pH sensitivity of IgGs as it reveals under laboratory conditions of purification may have a true *in vivo* correlate.

Our data indicate, that microglial cells can readily respond to certain plasma-derived proteins, which are either connected to coagulation or have an important function in adaptive immunity. Importantly, observations as derived from primary cell cultures could be confirmed in the more complex situation of an organotypic slice culture based on mouse hippocampus (OHSC). In collaboration with Dr. Oliver Kann (Charité, Berlin) we could demonstrate that incubation of OHSCs with thrombin[HMWM] (data not shown) or monomeric IgGs induce cyto- and chemokine responses similar to that obtained with primary microglial cultures. Fluorescence-conjugated proteins revealed single cells exhibiting microglial morphology to be the target and response element in the tissue. Moreover, these experiments indicate that larger molecules, e.g. IgGs or even the huge HMWM complex, readily inundate the tissue to induce cellular responses. Thus, the blood compartment represents an ubiquitous and important source of potential DAMP signals for an activation of microglia upon vascular impairment (Hanisch and Kettenmann, 2007).

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5.5 Thrombin^{HMWM}, FN and IgGs as agonists for a CD14/TLR4 receptor complex

Plasma-derived thrombin^{HMWM}, pFN and IgGs triggered a release of proinflammatory cyto- and chemokines in microglia. Protein preparations of different species showed such functionality for microglia of different mouse strain background (NMRI and C57Bl/6), indicating conserved ligand recognition and subsequent receptor signaling. TLR4 as a PRR revealed to be the central element for sensing these factors as DAMP signals, requiring, however, also the assistance of CD14.

Microglia physically (TLR4$^{-/-}$) or functionally (C3H/HeJ) lacking a TLR4 failed to mount a response. Using specific antibodies against CD14, the co-receptor for TLR4 in responses to LPS, or cells from CD14$^{-/-}$ animals, responses to thrombin^{HMWM}, pFN and IgGs were drastically impaired. Similar observations were obtained by using a TLR4/MD2-blocking antibody. Referring to some IgG isotypes as triggering release by themselves it is important to emphasize that all blocking antibodies were used at 'subthreshold' concentrations and, furthermore, were proven to cause no response on their own. Nevertheless, we occasionally found commercial antibodies employed in other work to induce microglia cyto/chemokines – at concentrations of 10 μg/ml.

The demonstration of a DAMP activity with a TLR-based mechanism is always under the suspicion that contaminating microbial agonists cause the cellular or systemic effects (Gao and Tsan, 2003; Tsan and Baochong, 2007). LPS impurities are frequent and can be substantial, even occurring in preparations that claim a 'low endotoxin' level (Weinstein et al., 2008b). Therefore, we demonstrated the LPS-independence for the observed microglia activities for all three candidates, thrombin^{HMWM}, FN and IgGs, by several approaches. They included protocols of heat inactivation, combining profiles of varying treatment periods and temperatures. We thereby ruled out that a reported phenomenon of LPS heat sensitivity could delude conclusions as to a protein nature of the activity carrier (Gao et al., 2006). Since the thermoinactivation regimes are not entirely suitable for all proteins (e.g. heat treatment of IgGs caused aggregation and precipitation with uncontrollable effects on the cells), we also performed experiments with polymyxin treatment, protease digestion or affinity-based endotoxin removal to convincingly exclude LPS effects in all three DAMP cases. Moreover, thrombin^{HMWM} and – analyzed in more detail – FN could be shown to compete and even displace LPS from the receptor to induce their own signaling.

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When pairing LPS (at saturating concentration) with FN for a combined stimulation of microglia, we detected a characteristic change in the resulting release pattern. While chemoattractive signals for neutrophils (CXCL1) were dramatically increased (compared to amounts as triggered by LPS alone), signals for T cells and monocytes/macrophages (CCL2, CCL3, CCL5) or TNFα remained unchanged. On the other hand, the inflammatory cytokines IL-12 or IL-6 were reduced. Importantly, at a maximal concentration of added FN (200 µg/ml), the resulting pattern perfectly correlated with the profile obtained with FN at this concentration alone, indicating that FN had functionally displaced LPS from TLR4. At lower concentration of FN, however, the release pattern displayed characteristics of a mixed signaling, with contributions of both agonists. Such a competition likely applies to thrombin[HMWM] and the IgGs as well, since their combination with LPS similarly affected the resulting release response. For instance, pairing LPS with bovine thrombin[HMWM] – having a lower activity on microglia compared to LPS – apparently led to a down-regulation of TNFα and CXCL1 release. In contrast, pairing of LPS with the mouse thrombin[HMWM] – which proved more potent than LPS – resulted in an enhanced release. Mouse thrombin[HMWM] could also 'down-regulate' the joint response as long as it was applied at concentrations being not yet agonistic themselves. However, they were sufficient to already claim TLR4 from LPS. Combining the mouse thrombin[HMWM] exactly at a concentration of equivalent effect to LPS, their combination was equally effective as each agonist alone. Only exceeding the mouse thrombin[HMWM] above this threshold, it turned out to augment the responses. In other words, thrombin[HMWM] can bind TLR4 efficiently at concentrations which may not yet – or barely – trigger significant microglial reactions, thus reducing for LPS the access to its receptor, a behavior best described as partial agonism. This notion is nicely supported by the creation of a stable antagonist version of thrombin[HMWM]. The bovine thrombin protein revealed complete loss of release-inducing capacity upon defined exposure to 70°C. In a LPS combination, however, this inactivated material exhibited even enhanced release attenuation. Of course, it could not contribute any agonistic function, while maintaing sufficient affinity for TLR4 to exclusively act as an antagonist. Receptor binding still required some native conformation of the responsible domain, since the exposure to higher temperatures finally abrogated the 'inhibitory' effect completely.

To even show a ligand competition directly, we confirmed a fluorescent LPS derivative to be functional for release induction and also determined its dependence on CD14 (not shown). However, microscopic attempts to reveal its surface binding gave

unsatisfying results, due to a low signal intensity. We performed immunostaining in combination with confocal microscopy, FACS and self-made ELISA to find TLR4 in sparse expression only (not shown). Additional experiments (based on staining or manipulation of 'raft'-like structures) suggested a clustered distribution in membrane-domains, but all together at very low expression level. These oberservations rendered also techniques, such as fluorescence resonance energy transfer (FRET), less likely to deliver a convincing prove, as long as focusing on microglia. However, a switch to model systems with overexpression of TLR4 was not favored as it would come with an undefined set of coreceptors and intracellular signaling organization, which both revealed to be essential in controlling the specific DAMP activities. Still the present data clearly demonstrate that thrombin^{HMWM}, pFN or IgGs can recruit CD14/TLR4 to induce an inflammatory response in microglia.

For thrombin^{HMWM}, the activity can be partially explained by 'entrapped' pFN. For pFN, further research will be required to reveal the functional domains within the individual DAMP ligands and the receptor. The use of recombinant, isolated single domains, as discussed above for FN-EDA, may not adequately reflect the complex interaction of a large protein (not stressing again the risk of contamination). We used full-length, naturally derived pFN, characterized by FPLC, SDS-PAGE for purity and proper constitution. pFN is assumed to be devoid of EDA, in contrast to cFN. We performed immunoblot and mass spectrometric analyses to exclude the EDA as the carrier of cellular activity. We could not identify the domain in our FN preparations, suggesting that TLR4 effects of full-length pFN do not necessarily depend on EDA, but on other individual or combinations of domains. CD14/TLR4 may also recruit accessory partners for a TLR4-centered receptor complex. In the case of FN, members of the integrin family would be prominent candidates. For IgGs, interaction with their professional FcγR could also play a role. A concept of TLR4 as a sophisticated sensor for the recognition of distinct agonist structures could, in fact, include optional engagement with surface molecules to adapt binding and signaling.

5.6 TLR4 – decision maker in microglial responses to PAMPs and DAMPs

Recognition of LPS revealed not to be simple at all. For functional interaction, TLR4 requires association with MD-2 to form homodimers. Furthermore, activation of TLR4 is preceded by the transfer of LPS to membrane-bound or soluble CD14 by LBP. Yet, LPS is not a single molecule, but exists as a range of structural variants, i.e. chemotypes. LPS

comprises the lipid A moiety, a core oligosaccharide, and the O-polysaccharide (O-antigen). Depending on its complexity, different rough (R)-, semi rough (SR)- and smooth (S)-forms of LPS can be distinguished (Jiang et al., 2005). The chemotypes vary by the bacterial strains (which thereby reveal a rough or smooth appearance of their colonies). Based on these structural differences, the various LPS forms are assumed to differ by the kinetics of blood clearance, cellular uptake and activation of granulocytes, mast cells, macrophages and complement (Huber et al., 2006). Moreover, chemotypes seem to differ by CD14 dependence. In addition, LPS may induce the formation of heterologous, supramolecular receptor clusters which vary in composition depending on the cell type and, most importantly, the ligand (Triantafilou and Triantafilou, 2002). Such versatile association with other surface molecules/receptors may not only explain how the TLR4 system can even distinguish between different LPS forms. It may also be a requirement to recognize and interpret the diversity of assumed endogenous ligands. Such a TLR4-centered receptor complex would further expand and diversify the intracellular signaling options.

Following this idea, the release activity of microglia in response to certain TRL4 ligands should be differently influenced when paired with a second signaling factor. A modulatory influence could be organized by diverse cytokines emitted from other cells of the CNS and/or the immune system. We focused on the Th1-related IFNγ as well as the Th2-associated and immunosuppressive cytokines, IL-4 and IL-10. IFNβ was also included as a regulator of macrophage functions and because we found it to be induced by LPS, but not by our DAMPs. It is also used as a therapeutic agent in the treatment of multiple sclerosis. The microglial responses, especially to the endogenous ligands, can be dramatically influenced by these cytokines, while LPS-mediated reactions were not or only partially affected. Influences came as a shift in the release pattern by promoting or suppressing the production of individual factors. Importantly, under the influence of these cytokines, the system can not only distinguish between the microbial ligand, i.e. LPS, and the endogenous DAMPs, but also between even closely related DAMP signals, i.e. IgG$_1$ and IgG$_3$. The different influences of these key cytokines on the release patterns of microglia as triggered by DAMPs or LPS can thus be taken as a hint for an individual organization of their TLR4-centered signaling. If all agonists would trigger an absolutely identical TLR4 signaling, modulating factors (like the IFNγ or IL-4) should also cause indistinguishable influences. In contrast, if a given modulator affects responses to the various TLR4 agonists differently, their primary signaling should have been distinct to start

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with. Our data suggest that TLR4 could dissociate its responses to individual agonists even more when an enriched signaling context is in place.

Furthermore, we could demonstrate that FN-mediated responses involve integrin (CD11b) assistance, while IgGs may recruit FcγRs. Interestingly, when the interaction of CD11b with FN was disturbed either by blockade with an antibody or citrullination of FN microglial responses to FN, but not to LPS, were enhanced. These observations fit well to a study from Pfeiffer et al. who suggested a pre-formed cluster of CD14, CD55, the FcγRs CD32 and CD64 and CD47 (Pfeiffer et al., 2001). Stimulation with LPS induced co-clustering with TLR4, FcγRIIIa (CD16a) and CD81, while CD47 was dissociated. In contrast, the composition of the cluster induced by ceramide was found to differ by the absence of CD16a, TLR4 and CD81, but the presence of CD47. The clustering would occur in membrane microdomains, i.e. lipid rafts, and bring these co-receptors into proximity to CD14. This model implies a central role for CD14 as it collects the various signals and initiates ligand-specific formation of receptor clusters within lipid rafts. Own studies on the participation of lipid rafts revealed that depletion of cholesterol, meaning destruction of the lipid raft architecture, had similar effects on FN-induced release as the CD11b blockade with an antibody or citrullination of FN. However, also LPS-induced responses were enhanced by cholesterol depletion, while responses to thrombin^{HMWM} and TLR1/2 or TLR6/2 ligands remained unaffected (data not shown). Importantly, the involvement of β_2 integrin as a building block of $\alpha_M\beta_2$ (CD11b/18, CR3) was previously shown to enhance LPS responses (Kagan and Medzhitov, 2006). Our data on microglial CD11b (α_M), in contrast, revealed an opposite outcome for pFN, no consequence for LPS (as well as thrombin^{HMWM}) – and addressing β_2 directly, we did not obtain any effect. The sharp differences may well relate to the recently described dissimilar functional properties of CR3 in macrophages and microglia (Hadas et al., 2010) and underline its distinct role by cell type and agonist. Finally, we also tested the role of CXCR4, the receptor of stroma-derived factor (SDF, CXCL12) with a reported link to TLR4. A blocking antibody did, however, not interfere with FN, neither had SDF application an effect.

Taken together, our data provide further evidence that CD14/TLR4 could act as a receptor module which is shared by various exogenous (LPS) and endogenous factors. However, the agonists maintain individuality of their inducible consequences through recruitment and activation of private co-receptors. CD14/TLR4 could rather function as a 'centered' element in variable receptor complexes, sharing recognition of danger-spelling

molecules. Still, the variability of TLR4 insertion can go as far as exchanging CD14 for CD36 in a TLR4/6-heterodimeric binding of amyloid-β and LDL (Stewart et al., 2010).

5.7 TLR4 signaling to PAMPs and DAMPs

TLR4 signaling is considered to occur through the signaling adaptor proteins MyD88 and TRIF. MyD88 needs assistance of the sorting adapter MAL for an appropriate receptor function (Akira and Takeda, 2004). It leads to the early phase of NFκB activation, certain MAPK pathways involving JNKs, p38^{MAPK} and p42/44^{MAPK} and results in the induction of multiple proinflammatory cytokines and chemokines. The pathway via TRIF requires TRAM as the sorting adaptor. It leads primarily to the expression of IFNβ and subsequently to the induction of interferon-regulated genes and accounts for the late phase activation of NFκB and MAPKs (Akira and Takeda, 2004; Yamamoto et al., 2003). Such a sequential signaling could be explained in part by MyD88 activation through TLR4 at the plasma membrane, followed by activation of the intracellular TRIF upon receptor internalization, providing access to distinct pools of signaling elements (Kagan et al., 2008). Moreover, the signaling of different LPS chemotypes apparently differs in the dependence and use of MyD88 and TRIF. In agreement, our research on microglia also indicates a time- as well as pathway- selective dependence of certain cyto- and chemokine inductions upon various TLR4 stimulations (data not shown).

This study reveals that all analyzed DAMP molecules, i.e. thrombin^{HMWM}, pFN and IgGs, signal through the classical CD14/TLR4-assigned intracellular elements. Their signaling would strictly depend on the MyD88 pathway, as shown by the knockout experiments. These factors triggered the phosphorylation of NFκB and p38^{MAPK}, as shown for FN as a representative example. Nevertheless, the agonists differed by the strength of activation as well as the kinetics. Interestingly, thrombin^{HMWM} as well as IgGs, but not pFN, may also involve TRIF signaling, as indicated by the behavior of the TRIF$^{-/-}$ microglia. This is not surprising in light of growing evidence for intimate crosstalk between MyD88 and TRIF. However, all DAMP molecules failed to induce detectable amounts of IFNβ, while LPS led to such a response. TRIF may have more contributions than governing IFNβ. However, selective IFNβ induction by LPS could represent a mechanism by which the PAMP can modulate DAMP responses, while not being much influenced itself. It will be a task of follow-up studies to dissect the individual involvement of MyD88- and TRIF and its controlled intracellular elements in the process of converting a specific agonistic signal

into selective and appropriate cellular consequences. Work in this direction can already determine contributions of Bruton's tyrosine kinase, a protein tyrosine kinase with a particular association to B cell receptors as well as certain TLRs.

5.8 Environmental factors controlling the character of DAMPs

Many DAMPs are released upon tissue injury and cellular death. They are cytosolic or nuclear proteins exhibiting a defined function within a special local environment. The cytosolic milieu is a highly reducing environment due to the presence of several reducing enzymes that regulate the thiol redox status of proteins. In contrast, the extracellular space is highly oxidizing, because of the absence of reducing enzymes and the presence of oxidizing agents, like oxygen or transiently produced mediators, like NO (Lotze et al., 2007). Conceivably, released cytosolic proteins change their redox status, which alters their conformation and finally leads to their functional denaturation. The disordered redox environment might promote the DAMP nature. On the other hand, necrotic cell death leads to the release of various enzymes that are usually contained within the cell. These enzymes may maintain their functional properties, get even activated and promote the modification of extracellular proteins. Prominent examples are members of the PADs, which catalyze the posttranslational modification of arginine residues into citrulline. Such a conversion is accompanied by dramatic changes in charge, as the resulting citrulline lacks the basic guanidine group of arginine. At the protein level, it affects the net charge distribution and interferes with ionic as well as hydrogen bonds. Thus, citrullination can induce conformational changes. PADs are widely distributed in mammalian tissues and are expressed in various cells types (György et al., 2006). The catalytic function of the enzyme strictly depends on Ca^{2+}. Under physiological conditions, the intracellular Ca^{2+} concentration is not sufficient to activate PADs (Takahara et al., 1989). It suggests that citrullination occurs when the intracellular Ca^{2+} is significantly increased or during necrosis, when the enzyme is exposed to the extracellular Ca^{2+} levels which are much higher. Indeed, citrullination is a process that often occurs in the context of tissue injury and, importantly, it is associated with certain diseases, like rheumatoid arthritis, MS and AD (György et al., 2006). It is suggested, that deiminated proteins are highly immunogenic and induce strong B cell responses since antibodies against citrullinated proteins are a hallmark in arthritis. Citrullination may also induce or promote a DAMP character of extracellular proteins due to the severe changes in the protein conformation that can

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affect protein function and protein-protein interactions. We discussed the possibility that citrullination interferes with the integrin binding of pFN. Deimination of pFN had the same consequence of release enhancement as a direct integrin blockade with an antibody. Upon a (even minor) blood vessel leakage, citrullination could thus boost the DAMP activity of FN. It could lift the calming influence of integrins on FN-TLR4 signaling.

Modifications of protein conformation may also arise from a pH shift at sites of inflammation, infection and cell death. Under physiological conditions, the pH inside a cell is 6.8 to 7.0, around the neutral point of water at 37°C (Lardner, 2001; Mahnensmith and Aronson, 1985). However, the intracellular pH varies by the compartment, being 6.4 in the Golgi apparatus and about 5.0 in lysosomes. Enhanced proliferation, RNA and protein syntheses, however, associate with an intracellular alkalinization due to increased sodium-hydrogen exchanger activity (Mahnensmith and Aronson, 1985). For instance, lymphocytes stimulated with LPS exhibit abrupt elevations of the intracellular pH along with a burst of RNA and protein synthesis, followed by a second rise correlating with the rate of RNA synthesis (Gerson and Kiefer, 1982). Usually, the extracellular milieu is with a pH of 7.4 slightly basic. However, enhanced metabolic activity with the production of metabolites, like CO_2 or lactate (i.e. under anaerobic conditions), or presence of short chain fatty acids from bacteria can acidify the cellular environment. Necrosis and inflammation can even lead to a local acidification with pH values below 6.0 (Lardner, 2001). Because of the logarithmic character of the pH value, a reduction from 7.4 down 6.0 stands for a more than 20fold increase in the H^+ concentration.

We discussed pH influences on IgGs and their postulated TLR4 activity. The experiments illustrated a relationship between transient pH shifts, protein structure and functional properties of IgGs. Transient IgG exposure to a low pH had dramatic and long-lasting consequences not only for the antigen binding, but also induced conformational changes which could be accompanied by associate formation. An acidification in the inflamed tissue, of course, would not reach pH values of 3.0 or less. However, our data provide evidence that even a small change by 0.2 units can be sufficient to affect IgG properties. Yet, the interesting pH range between 7.4 (healthy tissue) and 6.6 (inflamed tissue) remained obscure as regarding associate formation and TLR4 activity (because of technical limits in affinity chromatography elution). Nevertheless, exposure to a low pH environment as a mechanism for Ig(G)s to gain TLR4 activity is entirely conceivable. Ig molecules reaching an inflamed tissue may thereby acquire the option to interact with TLRs on effector cells, in addition to the repertoire of FcRs or complement interactions.

DISCUSSION

5.9 Microglial reactive phenotypes as controlled by DAMPs

The previous chapters discussed how microglial cells would react to the appearance of certain DAMP molecules – as derived from the blood – via a TLR4-centered mechanism. The induced cytokine and chemokine profiles point to an inflammatory phenotype, similarly as obtained by stimulation with the microbial ligand LPS. Yet, there is evidence that activation of microglia can drive distinct functional behavior as it is already established for situation-adapted responses of macrophages (Gordon and Taylor, 2005; Mantovani et al., 2004). Considering the complex TLR4 system, by its ability to recruit additional co-receptors and its link to diverse intracellular signaling options, a TLR4 engagement by DAMPs may not necessarily lead to a totally defense-oriented phenotype, as it is instructed by LPS. Indeed, a comparison of the gene induction profile of FN- and LPS-stimulated microglia revealed similarities but, most importantly, also significant differences. FN itself associates with M2 macrophages (Gratchev et al., 2001; Martinez et al., 2006) and revealed to be most differently regulated by FN versus LPS. Factor XIII, as the enzyme linking FN to fibrin and as a 'marker' of alternative macrophage activation (Torocsik et al., 2005), was also among the most differently regulated genes in our array comparison of FN and LPS. Thus the DAMP FN may organize for a more repair-oriented and pro-coagulant profile in microglia, which is opposed by the PAMP LPS, while both include proinflammatory cytokines in each of their portfolios. This suggestion is even more underlined by recent findings reporting a neuroprotective function of pFN following traumatic brain injury and ischemia (Sakai et al., 2001; Tate et al., 2007). Moreover, the microglial responses to DAMP molecules, like FN, were easily modulated and shifted depending on the cytokine milieu, while LPS-induced responses revealed to be more stable. LPS inducing IFNβ could modify responses to DAMPs, while sparing its own profile of induced cyto/chemokines. Our DAMPs apparently cannot trigger much IFNβ, whereas they can the displace LPS from its receptor. Such a regulation of macrophage/microglia phenotypes by DAMPs, namely by FN, could have major implications in wound healing, diabetes, infection, tumor growth as well as invasion or multiple sclerosis (Bouma et al., 2004; Gaggioli et al., 2007; Han et al., 2008; Mantovani et al., 2008; Ribes et al., 2010; Schäfer and Werner, 2008).

Translated into an *in vivo* situation, microglial activation through TLR4 in infectious as well as non-infectious (sterile) inflammation scenarios could vary in outcomes by the individual agonist and the specific environment, allowing for a tight control of appropriate

responses. The reactive phenotypes may bias for defense or repair, but missing the situational context could be disastrous in either way. The excessive triggering of fighting forces in non-infectious challenges could result in otherwise avoidable tissue damage. On the other hand, repair programs may fail in preventing primary tumor growth or metastasis, and could even promote their tissue infiltration and invasion. FN could well integrate in these regulations, but is certainly not the only plasma- or ECM-derived candidate (Kim et al., 2009; Midwood et al., 2009).

DAMPs and PAMPs may not only play roles as opponents. Evidence arose from observations that a combination of two, by themselves inflammatory, signals can reverse the phenotype of macrophages. For instance, incubation with IC can instruct enhanced phagocytic performance and induce production of inflammatory cyto- and chemokines by FcR ligation. Surprisingly, combination with LPS reverses this phenotype in macrophages, turning them into an alternative-like 'type II' or M2b behavior with a IL-10^{high}/IL-12^{low} signature (Anderson et al., 2002). The dampening of FcR-induced inflammatory activity was also observed with other proinflammatory stimuli (Gerber and Mosser, 2001).

In autoimmune diseases, like multiple sclerosis, autoantibodies are thought to be harmful and to promote demyelination and neuronal damage due to their reactivity to myelin and other CNS molecules. However, there is evidence that Ig can contribute to remyelination and CNS repair, even if they are reactive to CNS antigens (Warrington et al., 2001). Moreover, intravenous immunoglobulin (IVIG), a mixture of monomeric, mostly IgG (isotypes) from thousands of donors, have proven beneficial effects in autoimmune and inflammatory diseases (Arnson et al., 2009). The mechanism(s) of action is not yet really understood, but is assumed to involve the contribution of the inhibitory FcγRIIb (Ballow, 1997). As an alternative and additional mechanistic option, IgG-triggered tissue consequences could depend also on non-FcR receptors, such as TLR4. We could show that IVIGs consistently modulated IgG-driven microglial reactions (data not shown). Yet, the precise conditions to generate endogenously TLR4-active IgGs and their resulting consequences for the tissue remain enigmatic. However, our data regarding the influence of endogenously produced IgGs against a non-CNS-related antigen or of applied exogenous isotypes indicated some beneficial effect of the IgGs on the disease outcomes in EAE (data not shown). On the other hand, the phenomenon of IgG-TLR4 interaction may contribute to the worsening of multiple sclerosis courses upon infection. The assumed 'activation of the immune system' may thus also come with Ig-driven consequences via innate immune effector cells, such as microglia.

6. SUMMARY AND CONCLUSIONS

Microglial cells represent the resident tissue macrophages of the CNS. Constantly monitoring their environment they can rapidly respond to signs of a disturbed homeostasis with transformation to alerted, activated and finally reactive states. Depending on the activating signal and its context, microglia could commit to diverse reactive phenotypes, characterized by distinct gene induction patterns and tailored functional properties – a principle that has been currently established for several macrophage populations throughout the body. The activation process can be triggered by factors which indicate an infectious threat, i.e. by exogenous signals relating to microbial structures, formally known as PAMPs. On the other hand, microglia can also sense endogenous signs of cellular impairment and tissue injury, thereby serving as DAMPs. Such signals may derive from molecules which are released from damaged cells, the disrupted ECM or from the blood compartment. Usually, most circulating proteins are denied to enter the CNS parenchyma by the BBB. Upon BBB impairment or vascular injury, however, even larger proteins gain access to CNS cells, including microglia. It is well documented that microglia can rapidly respond to blood vessel lesions and that inundating blood content has multiple consequences for neurons and glial cells. While microglial functions may in general aim at the protection of the structural and functional integrity of the CNS, excessive acute or chronic activation bears the risk of substantial damage of the vulnerable tissue. However, little is still known about the nature of plasma factors with microglia-activating and – modulating potential, the induced cellular responses as well as their receptor and signaling mechanism.

Thrombin, the central blood coagulation factor, exerts also a multitude of cellular effects through limited proteolysis of PARs. This mechanism has also been considered for the induction of proinflammatory mediators in microglia. Challenging this notion, we found that thrombin proper does not directly trigger the proinflammatory responses. By FPLC-based analysis, we identified a minor fraction of thrombin-associated high molecular weight protein (thrombin[HMWM]), containing thrombin and additional plasma/coagulation factors among a presumably complex protein aggregate, as the sole and extremely potent carrier of this activity. The thrombin[HMWM]-mediated induction of cytokines and chemokines in microglial cells of different strains involves a CD14/TLR4/MyD88/TRIF receptor and adapter protein complex. This has been derived from studies on cells from the respective knockout and corresponding wild type models in combination with blocking antibodies,

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TLR agonists and thrombin preparations from different species. Biochemical fractionation and MS analyses revealed FN as a constituent and potential carrier of activity among the thrombin^{HMWM}. Furthermore, the complex contains several additional coagulation factors which are known to be generated or recruited upon thrombin-catalyzed activation of the coagulation cascade, suggesting also an *in vivo* relevance of the observed mechanism. Importantly, FN of various species induced a very similar response profile in microglia and required typical TLR receptor elements as described for the thrombin^{HMWM}. FN could even functionally compete and displace LPS from the TLR4. Yet both agonists also presented with some distinct cellular response features, suggesting distinct intracellular signaling and the potential contribution of additional co-receptors. Indeed, the $\alpha M\beta 2$ integrin revealed to have influences on TLR4 effects. Since FN is not only a plasma factor, but also a constituent of the ECM, the question was addressed why the soluble protein would activate microglia and – most likely – the ECM-bound format not. Flexibility of the soluble FN could be a key feature as the immobilized form had no activity. Finally, a gene array-based study supported the findings from analyses of individual microglial responses to LPS or FN and revealed an partially opposite orientation of the reactive phenotype, i.e. defense versus repair.

Along with these studies, we found that IgGs of certain isotypes could apparently stimulate microglia without being presented as an immune complex. A screening of a larger number of IgG preparations – from commercial sources or self-made/purified monoclonals from hybridoma cultures – thereby revealed an isotype-dependent pattern. Even though mouse and human IgGs differ by isotype classification, FcγR preference and the functional consequences of the FcγR subtypes, selected human IgG isotypes were found to also stimulate the cells. Similar to the observations on thrombin^{HMWM} and FN, TLR4 and its associated receptor/signaling partners were essential for microglial responses. Contributions of FcγR (receptors and signaling chain) may further enrich binding and signaling options but were not mandatory. IgGs would (in an isotype-dependent manner) gain the ability to activate TLR signaling when being modified in their conformational status and association state as identified and analyzed by FPLC procedures and as further characterized by various biochemical techniques. Our data suggest that even a transient exposure to low pH, as used during purification procedures or as found in inflamed tissues, can have lasting effects on IgG properties, including antigen binding and TLR4 interaction. Furthermore, the carbohydrate moieties of the Fc portion seemed to have a significant contribution. Conceivably, production-associated

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alterations and a resulting TLR4 interaction could affect IgG applications in research and diagnostics, hiding as part of the 'non-specific' binding or underlie the 'noise' in functional studies (as unexplainable effects of control treatments). Introduced by production, TLR4 activity of IgGs would potentially not only interfere with laboratory applications *in vitro* and *in vivo*. It could also have a significant impact for antibodies which are generated for clinical use. Engineering of therapeutic antibodies with manipulations in Fc portions may have unpredictable side effects, including undesired receptor activation. However, the conditions which generate endogenously TLR4-active IgGs and their resulting consequences for the tissue remain enigmatic. Yet, IgGs reaching lesions with low pH may acquire the option to interact with TLRs on effector cells, in addition to the repertoire of FcγRs and complement activation. Because the observed TLR4 function does not require presence of the antigen it could be available also for antibodies not presented as ICs. Thus, TLR4 interactions could conceivably contribute to pathogen opsonization. On the other hand, a TLR4 engagement in chronic diseases, such as multiple sclerosis, may influence the disease progression.

TLRs are essential for an initiation and regulation of appropriate innate and adoptive immune functions in response to pathogenic challenges. In the CNS, TLR activation can lead to excessive inflammatory responses or contribute to neurodegenerative processes. However, there is also growing evidence that immune activities and inflammation are important for neuroprotection and tissue repair – and probably beyond. TLR signaling is even suggested for roles in adult neurogenesis (Hanisch et al., 2008). This study focused on microglia and TLR4 as central response elements to sense and interpret endogenously derived signals. We could show that microglia can readily respond to such signals with distinct functional consequences, proving the versatility of the TLR4 receptor system for 'understanding' signs of danger.

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