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Toll-Like Receptor Expression in the Peripheral Nerve

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KEY WORDS

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ABSTRACT

Toll-like receptors comprise a family of evolutionary conserved pattern recognition receptors that act as a first defense line in the innate immune system. Upon stimulation with microbial ligands, they orchestrate the induction of a host defense response by activating different signaling cascades. Interestingly, they appear to detect the presence of endogenous signals of danger as well and as such, neurodegeneration is thought to trigger an immune response through ligation of TLRs. Though recent data report the expression of various TLRs in the central nervous system, TLR expression patterns in the peripheral nervous system have not been determined yet. We observed that Schwann cells express relatively high levels of TLRs, with especially TLR3 and TLR4 being prominent. Sensory and motor neurons hardly express TLRs at all. Through the use of NF-κB signaling as read-out, we could show that all TLRs are functional in Schwann cells and that bacterial lipoprotein, a ligand for TLR1/TLR2 receptors yields the strongest response. In sciatic nerve, basal levels of TLRs closely reflect the expression patterns as determined in Schwann cells. TLR3, TLR4, and TLR7 are majorly expressed, pointing to their possible role in immune surveillance. Upon axotomy, TLR1 becomes strongly induced, while most other TLR expression levels remain unaffected. Altogether, our data suggest that similar to microglia in the brain, Schwann cells might act as sentinel cells in the PNS. Furthermore, acute neurodegeneration induces a shift in TLR expression pattern, most likely illustrating specialized functions of TLRs in basal versus activated conditions of the peripheral nerve.

INTRODUCTION

The field of innate immunity changed dramatically due to the discovery of Toll like receptors (TLRs; Medzhitov, 2001). These receptors belong to a vast array of nonclonally expressed pattern recognition receptors that bind to conserved molecular structures shared by large groups of pathogens (Janssens and Beyaert, 2003). In humans, 10 different members of the TLR family coordinate the host immune response against different classes of pathogens. Recently, it has been shown that TLRs also recognize endogenous ligands, such as heat shock proteins or nuclear proteins that—when present in the extracellular milieu—are indicative of some kind of stress or "danger" (Takeda et al., 2003).

Also the peripheral nervous system (PNS) contains a defense mechanism to alert the immune system in cases of danger. In this defense response, Schwann cells play a major role. Upon neurodegeneration, they adopt a macrophage like phenotype and start secreting factors that are important in the recruitment of other inflammatory cell types (Martini et al., 2008). How Schwann cells become activated by neurodegeneration is not clear yet, but a major role for TLRs has been postulated (Boivin et al., 2007; Karanth et al., 2006; Lee et al., 2006).

While early reports on TLR expression patterns focused mainly on immune effector cells, also nonimmune cells are known to express TLRs (Muzio and Mantovani, 2001; Nishimura and Naito, 2005; Zarember and Godowski, 2002). In the nervous system, especially microglial cells appear to express a broad range of TLRs and as such are endowed with a sentinel function in the brain (Bsibsi et al., 2002; Jack et al., 2005; Olson and Miller, 2004). Astrocytes and oligodendrocytes show a more restricted profile of TLRs, with TLR3 and TLR2 being their predominant receptors (Bsibsi et al., 2002; Farina et al., 2005; Jack et al., 2005). As far as neurons are concerned, expression patterns appear to be highly dependent on cell type and developmental stage. Earlier reports suggested that neurons do not express TLRs at all, but Tang et al. (2007) demonstrated in cortical neurons that TLR2, TLR4, TLR5, and TLR9 were detectable. Most other reports focus mainly on the expression of TLR3 and TLR8 in neurons (Ma et al., 2006).

The situation in the PNS is less well studied. A few studies focus on the expression of TLR2, TLR3, and TLR4 in Schwann cells (Cheng et al., 2007; Lee et al., 2006; Oliveira et al., 2003), while in peripheral neurons up till now only expression of TLR3 and TLR4 has been reported (Li et al., 2004; Wadachi and Hargreaves, 2006). Boivin et al. (2007) recently demonstrated a major role for TLRs for recovery from axotomy, but

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despite this article a comprehensive study on TLR expression patterns in the PNS is still lacking.

We decided to address this question by looking at TLR expression patterns on individual primary cell types in the PNS, as well as by looking at tissue level, both in basal conditions and in conditions of acute neurodegeneration. Our study reveals that Schwann cells appear to function as sentinel cells in the PNS and express relatively high levels of TLRs. Furthermore, while TLR3 and TLR4 appear to play a role in immune surveillance, TLR1 might be more important in conditions of neurodegeneration, indicative of specialized functions for TLRs in the PNS.

MATERIALS AND METHODS

All animal experiments were approved by the University of Antwerp ethics committee and conducted according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA).

Cell Isolation

Primary Schwann cells were isolated from neonatal rodent sciatic nerve taken from P2-P7 pups from C57BL/6 mice or P4-P7 pups from Wistar rats (Morgan et al., 1991; Weinstein and Wu, 2001). Neonatal animals were anesthetized, decapitated, and sciatic nerves were isolated. The peri- and epineurium of the sciatic nerves was removed to reduce the amount of fibroblasts in the culture. The stripped nerves were then enzymatically and mechanically dissociated with a mixture of trypsin (2.5 mg mL^{-1}) and collagenase (3 mg mL^{-1}). Mice Schwann cells were plated on poly-L-lysin- and laminin-coated culture plates in DMEM/5% horse serum supplemented with 10 μM cytosine β-D-arabinofuranoside (Ara-C) to remove mitotic cells, mostly fibroblasts. After 72 h, medium was replaced with defined medium (Dong et al., 1997) and refreshed every two days. Rat Schwann cells were plated on Primaria tissue culture plates in DMEM/10% FBS. After 24 h, medium was replaced with DMEM/10% FBS supplemented with 10 μM Ara-C. Forty-eight hours later, the medium was replaced with DMEM/10% FBS/2 μM forskolin and 10 μg mL^{-1} pituitary extract (PEX) to stimulate Schwann cell proliferation. Purity of the Schwann cell cultures was determined using antibodies against S100B (Dako). Only cultures >95% pure were used. Motor neurons were isolated from the ventral part of spinal cord from E13 mouse embryos (Vandenberghe et al., 1998). Meninges, DRGs and the dorsal part of the spinal cord were carefully removed. The ventral part of the spinal cord was dissociated both mechanically and enzymatically. Glial cells and motor neurons were separated by centrifugation in a 6.2% Optiprep gradient. Motor neurons were plated on poly-L-ornithine- and laminin-coated plates in NB medium (Invitrogen) supplemented with 2% B27 supplement, 4 g L^{-1} glucose, 2 mM L-glutamine and 50 ng mL^{-1} NGF. Purity of motor neuron cultures was assessed by staining for neuron specific beta III tubulin (Abcam). Sensory neurons were isolated from DRGs from E13 mouse embryos (Taveggia et al., 2005). The spinal cord was dissected and DRGs were carefully removed. DRGs were enzymatically and mechanically dissociated and cells were plated on poly-L-lysin and laminin coated dishes in NB medium supplemented with 2% B27 supplement, 4 g L^{-1} glucose, 2 mM L-glutamine and 50 ng mL^{-1} NGF. During the first days of culturing a co-culture of sensory neurons, fibroblasts and a minor amount of Schwann cells was observed. To obtain pure sensory neuron cultures cycling every 2 days between the above described medium and the same medium enriched with 1% (v/v) FUDR (final 10 μM FdU and 10 μM uridine) was necessary to kill fast dividing cells. Purity of sensory neuron cultures was assessed by staining for neuron specific beta III tubulin (Abcam). Peritoneal macrophages were isolated from adult mice that were injected 4 days before euthanasia with 3% thioglycollate (Ghassabeh et al., 2006). On Day 4, mice were killed and the peritoneal cavity rinsed with PBS. Macrophages were collected and plated in RPMI/1% FCS. Erythrocytes stayed in suspension and were removed after 45 min. The next day dendritic cells in suspension were removed and remaining macrophages were kept in RPMI/10% FCS.

Induction of Acute Neurodegeneration

Axotomy experiments of the *N. ischiadicus* were conducted in 6-week-old female C57BL/6 mice according to established protocols. Briefly, mice were anesthetized with 150 mg kg^{-1} ketamine and 10 mg kg^{-1} xylazine injected peritoneally. An incision was made at the right thigh and gluteal and hamstring muscles were carefully separated to expose the sciatic nerve. The sciatic nerve was transected and the wound closed by sutures. The contralateral side was left untouched. We first evaluated the effect of sham operation and of ketamine/xylazine on the induction of several TLR and NF-κB dependent genes, but found no significant effect (data not shown).

RNA Isolation and RT-qPCR

At 4, 8, 32, or 72 h upon sciatic nerve transection, mice were killed by inhalation of isoflurane or CO_2. The transected and control *N. ischiadicus* were removed and homogenized in Trizol by the use of a Potter Elvehjem homogenisator. Small fragments were further homogenized by sonication. RNA was isolated with the Rneasy Lipid Tissue kit (Qiagen) according to the manufacturer's protocol and the quality was verified by gel electrophoresis. DNase treatment was performed with TURBO DNase (Ambion). cDNA synthesis was done with Superscript III first strand synthesis system for RT-PCR (Invitrogen). Real time qPCR (RT-qPCR) reactions were done with 10 ng cDNA in SYBR Green I mix

TABLE 1. Primer Sequence for mTLR Genes and Internal Control Genes

	For primer	REV primer
TLR1	TCAAGCATTTGGACCTCTCCT	TTGTACCCGAGAACCGCTCA
TLR2	ACAATAGAGGGAGACGCCTTT	AGTGTCTGGTAAGGATTTCCCAT
TLR3	GGGGTCCAACTGGAGAACCT	CCGGGGAGAACTCTTTAAGTGG
TLR4	AAAGTGGCCCTACCAAGTCTC	TCAGGCTGTTTGTTCCCAAATC
TLR5	GCAGGATCATGGCATGTCAAC	ATCTGGGTGAGGTTACAGCCT
TLR6	AGGAACCTTACTCATGTCCCC	TGTTGTGGGAGAGTCTCAGAA
TLR7	TCTTACCCTTACCATCAACCACA	CCCCAGTAGAACAGGTACACA
TLR8	GAAAACATGCCCCCTCAGTCA	CGTCACAAGGATAGCTTCTGGAA
TLR9	TGAAGTCTGTACCCCGTTTCT	GTGGACGAAGTCGGAGTTGT
ACT2B	GCTTCTAGGCGGACTGTTACTGA	GCCATGCCAATGTTGTCTCTTAT
B2M	ATGCACGCAGAAAGAAATAGCAA	AGCTATCTAGGATATTTCCAATTTTTGAA
HMBS	GAAACTCTGCTTCGCTGCATT	TGCCCATCTTTCATCACTGTATG
RPL13a	CCTGCTGCTCTCAAGGTTGTT	TGGTTGTCACTGCCTGGTACTT
TBP	TCTACCGTGAATCTTGGCTGTAAA	TTCTCATGATGACTGCAGCAAA

and run on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). All PCR reactions were done in triplicate. Primers were designed making use of Primerbank (www.pga.mgh.harvard.edu/primerbank). Primer sequences can be found in Table 1. All primers were validated for specificity and amplification efficiency. RT-qPCR data were normalized according to the method described by (Vandesompele et al., 2002), by geometric averaging of multiple internal control genes. Processing of raw data and calculation of normalized relative quantities was done by using an improved version of the δ-delta-Ct-method (Hellemans et al., 2007).

Western Blot Analysis

Rat primary Schwann cells were seeded in 10 cm petridishes and allowed to grow to confluence. Schwann cell lysates were prepared in E1A lysis buffer (1% NP-40, 20 mM HEPES (pH 7.9), 250 mM NaCl, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 1 mM EDTA and a protease inhibitor cocktail). Protein concentration was determined by Bradford. Fifty microgram lysates were separated on NuPAGE gels, transferred to nitrocellulose membranes and analyzed by immunoblotting. Antibodies used were anti-phospho-IκB (Cell Signaling) and anti-β-actin (Sigma).

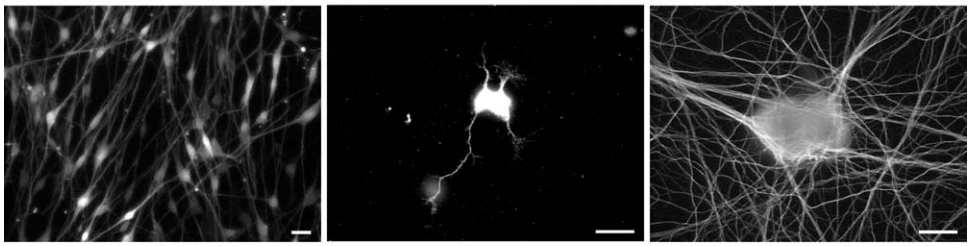
NF-κB Reporter Gene Assay

Mouse primary Schwann cells were seeded at 100,000 cells/well in a 24-well plate. Cells were transduced with lentivirus expressing pWPI-conluc, a lentiviral construct encoding an NF-κB dependent luciferase reporter gene construct (kind gift from P. Vandenabeele, University of Ghent, Belgium). Cells were cotransfected with GFP to check for transduction efficiency, which turned out to be about 100%. Three days after transduction, cells were stimulated with different TLR ligands [100 ng mL^{-1} lipopolysaccharide (LPS; Sigma)], 5 μM CpG (Invivogen), 100 ng mL^{-1} pure LPS (Invivogen), 10 μM R848 (Invivogen), 200 ng mL^{-1} Pam3Cys-SK4 (a type of bacterial lipoprotein or BLP; EMC), 10 μg mL^{-1} poly(I:C) (Amersham GE Healthcare), 1 μM poly(dT) (Alexis) or 10 ng mL^{-1} flagellin (Invivogen). The next day, lysates were prepared and luciferase activity was determined using a dual luciferase kit from Promega, according to manufacturer's instructions.

RESULTS

Since literature data on TLR expression in the PNS are scarce, we decided to isolate primary Schwann cells and primary sensory and motor neurons to study their TLR expression profile. Isolations were performed as outlined in the material and methods section and yielded very pure cultures, as revealed by immunostainings for cell-specific markers (Fig. 1A). We compared the expression levels of TLRs in the different cell types with those of primary peritoneal macrophages, cells known to express a broad range of TLRs. To this end, we evaluated the expression of nine different TLRs as well as five different housekeeping genes. We determined the most stable housekeeping gene for the four different cell types and calculated expression levels of the different TLRs relative to this one housekeeping gene [TATA-binding protein (TBP)]. As can be seen in Figs. 1B and 2, TLR expression could be detected in all three different cell types of the PNS, though at very different relative levels [as determined compared with the levels of TBP (set at 100)]. Schwann cells express the highest levels and also the broadest range of TLRs (all of them could be detected), while in motor and sensory neurons the expression levels were much lower and some TLRs were consistently absent. In independent primary cell isolations (obtained from separate mice), we noticed differences in the amount of expression of TLRs. However, the ranking of different TLRs according to their expression level was very consistent between independent experiments and is summarized in Fig. 1B. From this figure, it can be noticed that in Schwann cells especially TLR3, TLR4, and TLR7 are highly expressed. Motor neurons express predominantly TLR2, TLR3, and TLR5, while in sensory neurons especially TLR3, TLR4, and TLR5 could be found. Neither in motor neurons, nor in sensory neurons, TLR7, TLR8, or TLR9 was clearly detectable. Compared with Schwann cells or peripheral neurons, peritoneal macrophages express a much broader range of TLRs, with all TLRs except TLR3, TLR5, and TLR9 being present at relatively high level. We also compared TLR expression patterns at tissue level in spleen, sciatic nerve, and brain. As can be seen in Supporting

A



B

	SC	Sc.n	MN	SN
TLR1	+	++	+	+
TLR2	++	++	+++	+
TLR3	++++	++++	++++	+++
TLR4	+++++	+++++	++	+++
TLR5	++	+	++++	+++
TLR6	++	++	++	++
TLR7	+++	+++	+/-	-
TLR8	+	+	-	-
TLR9	+	+	+/-	-

Fig. 1. **A**: Immunocytochemistry pictures showing purity of primary Schwann cell, primary motor neuron, and primary sensory neuron cultures, as judged by immunolabeling with S100 for Schwann cells or β3-neuronal specific tubulin for neuronal cells. Scalebar 20 μm. **B**: Ranking of TLRs according to their relative expression levels in different PNS cell types as well as in sciatic nerve, based on three to five independent cell isolations for each cell type. − absent, +/− borderline detectable, +, ++, +++, ++++, +++++ indicate low to very strong expression. SC: Schwann cell, Sc.n.: sciatic nerve, MN: motor neuron, SN: sensory neuron.

Information Fig. 1, the expression pattern in sciatic nerve closely mimics the expression pattern in Schwann cells with TLR3, TLR4, and TLR7 being the most highly expressed receptors, but also resembles the pattern in brain where TLR3 and TLR7 are the most prominent receptors present. In spleen, just like in macrophages, a broader range of TLRs can be detected at relatively high level.

Next, we determined whether TLRs expressed on Schwann cells are also functional. To this end, we made use of two different NF-κB read-out systems, one that focuses on phosphorylation of the inhibitory protein IκB and one that measures NF-κB dependent reporter gene activation. As can be seen in Fig. 3A, from all TLR ligands tested, especially BLP, a ligand for TLR2/TLR1 heterodimers strongly triggers NF-κB activation. Other relative strong inducers (signals obtained after relative short exposure times) were LPS (nonpure mixture and as such a trigger for both TLR4 and TLR2), poly(I:C) (TLR3 ligand), and R848 (TLR7 ligand). Weaker inducers (signal obtained after more than 30 min of exposure) were pure LPS (TLR4 ligand), flagellin (TLR5 ligand), R848 + poly(D:T) (TLR8 ligand), and unmethylated bacterial DNA, CpG (TLR9 ligand). In conclusion, all TLRs expressed on Schwann cells appeared to be functional.

In a next step, primary Schwann cells were transduced with a lentiviral construct encoding a luciferase reporter gene under the control of an NF-κB dependent promotor. As can be seen in Fig. 3B, stimulation with LPS, BLP and poly(I:C) yields in relatively high transcriptional activity (>5-fold). Stimulation with pure LPS, flagellin or CpG DNA led to moderate transcriptional induction (between 2.5 and 4-fold), while R848 and R848 + poly(dT) did almost not induce NF-κB transcriptional activity. None of the used ligands resulted in a repression of basal NF-κB activity.

So far we only focused on TLR expression at basal conditions. To know whether TLR expression patterns in the peripheral nerve changed in conditions of acute neurodegeneration, we induced axotomy in the *N. ischiadicus* and isolated the sciatic nerve at several time points upon the surgical intervention. The contralateral side was left unoperated and used as a control. The *N. ischiadicus* of four mice were pooled, RNA isolated and processed for RT-qPCR. We first checked whether axotomy indeed induces an immune response, as had been reported before (Shamash et al., 2002). We checked expression levels of several inflammatory genes, such as IL-1β, COX-2, MCP-1, or CCL3. While MCP-1 and CCL3

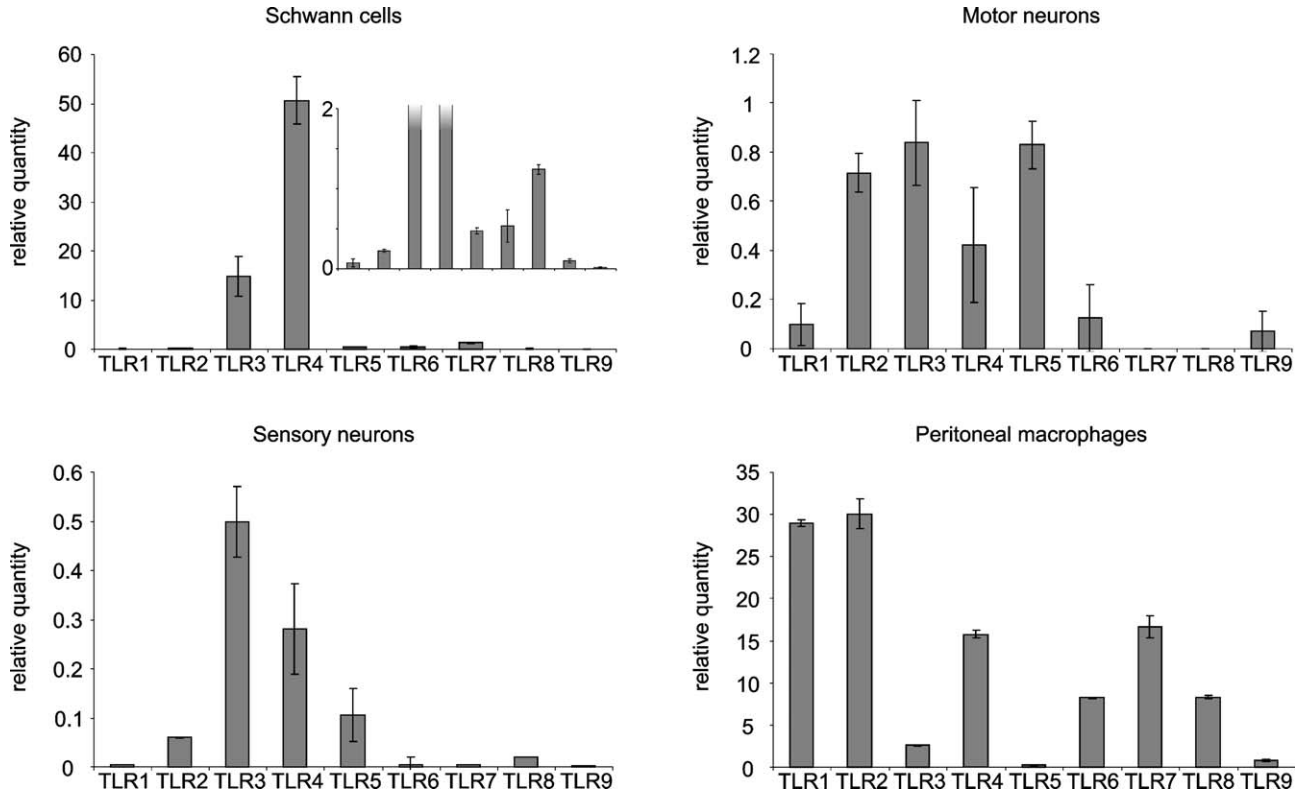


Fig. 2. TLR expression in primary Schwann cells, motor neurons, sensory neurons, and peritoneal macrophages, as determined by RT-qPCR. TLRs are expressed relative to TBP levels, the most stable housekeeping gene out of five different housekeeping genes measured in the different cell types. Note the different *Y*-axis scales used. For Schwann cells, an additional inset was added with adapted scalebar set to 2, showing that each TLR is expressed. A representative experiment is shown out of three to five independent experiments based on different cell isolations for each cell type. All measurements are done in triplicate.

reached peak amounts 32-h postaxotomy, IL-1β and COX-2 were early response genes and reached their plateau levels at 8-h postsurgery (Supp. Info. Fig. 2). Next, we determined expression levels of TLRs. Confirming earlier results shown in Fig. 1B; TLR3, TLR4, and TLR7 appear to be the most prominent receptors in the peripheral nerve in basal conditions (Fig. 4B). Upon neurodegeneration, TLR1 becomes strongly induced, while TLR9, TLR2, and TLR6 appear to be more weakly upregulated (Fig. 4A). This results in a shift in TLR expression profile with TLR1 becoming the most prominent receptor in conditions of neurodegeneration (Fig. 4B).

In conclusion, our data suggest specialized functions of TLRs in basal versus activated conditions of the peripheral nerve. TLR3, TLR4, and TLR7 are highly expressed in basal conditions and might play a role in immune surveillance. TLR1 becomes strongly induced upon neurodegeneration, suggesting a possible involvement for this receptor in conditions of neuronal stress.

DISCUSSION

Since the discovery of mammalian TLRs, more than 10 years ago, many research groups showed their expression on a variety of immune and nonimmune cell types and tissues. In the CNS, expression of TLRs was first demonstrated in the early 2000s (Laflamme and Rivest, 2001; Laflamme et al., 2001; Lehnardt et al., 2002). Since then, the number of studies describing the role of TLRs in both infectious and noninfectious conditions inside the brain is growing steadily. In striking contrast, the situation in the PNS remains less clear and despite their established role in immunity, TLR expression patterns in the PNS have never been determined. Earlier studies showed expression of TLR2, TLR3, and TLR4 in Schwann cells, while in peripheral neurons only expression of TLR3 in DRGs and TLR4 in DRGs and in trigeminal nocireceptors has been shown (Cameron et al., 2007; Li et al., 2004; Wadachi and Hargreaves, 2006). Therefore, we decided to look both in primary Schwann cells as well as in motor and sensory neurons whether they express TLRs and if the expression pattern changes upon neurodegeneration.

Just like microglia in the CNS, Schwann cells adopt a macrophage-like phenotype upon injury of the axon (Reichert et al., 1994). They dedifferentiate and return to a proliferating state, start secreting chemokines, cytokines and neurotrophic growth factors, and aid in the phagocytosis of myelin débris (Martini et al., 2008). Therefore, it might be of no surprise that they express the highest level of TLRs. All TLRs tested could be detected at mRNA level and also appeared to be

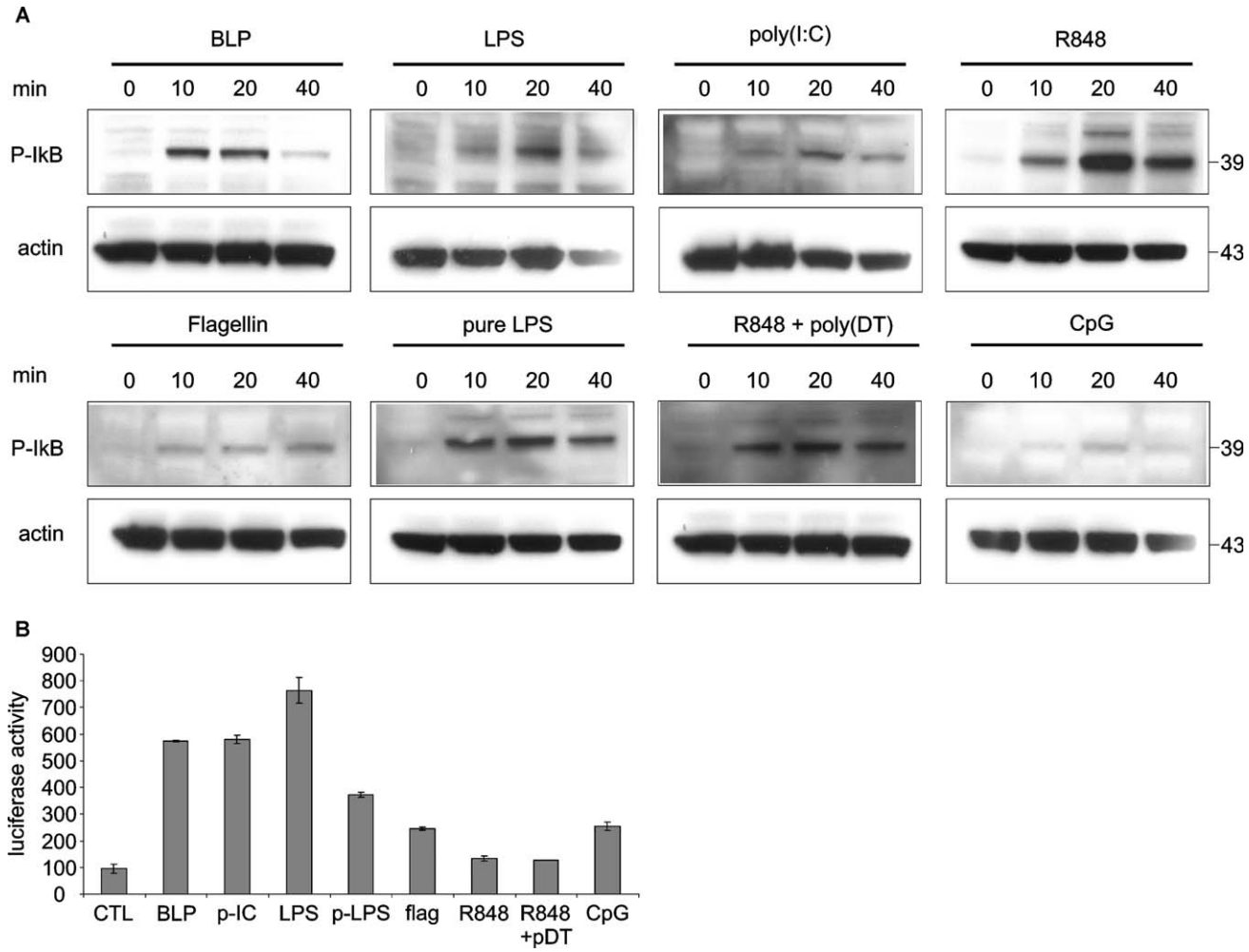


Fig. 3. TLRs are functional in primary Schwann cells. **A**: Western blot analysis showing activation of P-IκB by stimulation of Schwann cells with different TLR ligands for different time periods. Upper panel: signals obtained after relative short exposure times (±10 min). Lower panel: signals obtained after relative longer exposure times (>30 min). MW values expressed in kDa are shown at right side of blots. **B**: NF-κB reporter gene assay in primary Schwann cells stably transduced with an NF-κB dependent reporter plasmid, showing transcriptional activation of NF-κB upon stimulation with different TLR ligands. LPS (TLR2 and TLR4 ligand), CpG (TLR9 ligand), pure LPS (TLR4 ligand), R848 (TLR7 ligand), BLP (= Pam3Cys-SK4) (TLR2/1 ligand), poly(I:C) (TLR3 ligand), R848+poly(dT) (TLR8 ligand) and flagellin (TLR5 ligand). Representative experiment out of two independent experiments shown. All measurements are done in triplicate.

functional, which confirms their expression at protein level. In contrast, the TLR expression levels detected on both motor and sensory neurons were much lower than those on Schwann cells, and even at mRNA level, we could not detect expression of all TLRs. TLR expression patterns detected on primary Schwann cells were very similar to those on sciatic nerve tissue, again pointing to a major contribution of Schwann cells in TLR expression in the PNS. This is consistent with the broad expression pattern of TLRs detected in microglia in the brain (Bsibsi et al., 2002) and suggests that Schwann cells might have a similar sentinel function in the PNS, which could be needed for immune surveillance. However, while other sentinel cells like peritoneal macrophages express several receptors at relative high levels, Schwann cells express only a very restricted set of TLRs at high level. This could be well related to the fact that they are exposed to a more limited range of pathogen classes than macrophages, due to the presence of the blood nerve barrier. In this respect, it is interesting to note that TLR3 and TLR7, the two receptors that control the host response against viruses, appear to be strongly expressed in sciatic nerve at basal level. Since axons are known to be vulnerable to hijacking by viruses (Antinone and Smith, 2010; Chen et al., 2007; Wang et al., 2009), this might be physiologically relevant.

Whether or not stimulation with TLR ligands induces NF-κB dependent signaling cascades in primary Schwann cells remains controversial. In a study published in 2007, nuclear translocation of NF-κB in Schwann cells could not be detected upon stimulation with LPS, peptidoglycan (PGN) or *E. coli* (Vincent et al., 2007). In contrast, Lee et al. (2007) demonstrated that stimulation of Schwann cells with dsRNA does induce NF-κB activation (as detected through degradation of IκB), while Oliveira et al.

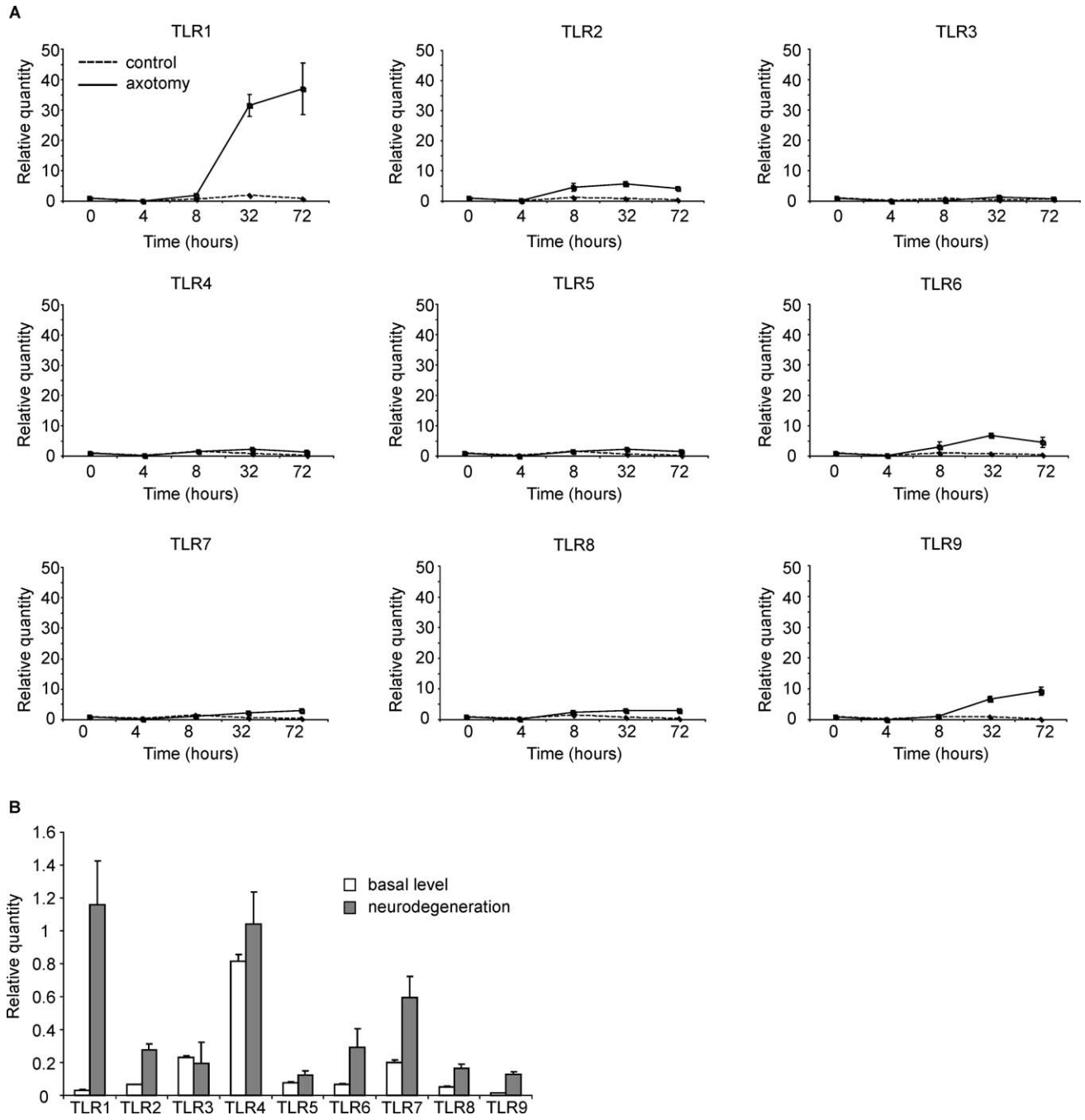


Fig. 4. Induction of TLR expression by acute neurodegeneration in the sciatic nerve. **A**: RT-qPCR analysis showing induction of TLR mRNA expression at different time points upon injury of the sciatic nerve. mRNA expression levels are expressed relative to the basal condition (= 0-h time point). A representative experiment out of three independent experiments is shown. All measurements are done in triplicate. **B**: Histogram summarizing the expression levels of the different TLRs in basal conditions (0 h untreated) versus conditions of neurodegeneration (72 h, treated). mRNA expression levels are expressed as relative values using the ΔΔCt method.

(2003) reported that TLR2 on Schwann cells is functional but would lead to apoptosis rather than cytokine activation. Finally, Cheng et al. (2007) showed that stimulation of TLR4 on Schwann cells with LPS leads to an activation of MAPK signaling cascades. Quite strikingly, in our hands, all TLRs tested appeared to be functionally active in Schwann cells, although different TLR ligands showed different potency. Consistently, the strongest response was obtained with BLP, a TLR1/TLR2 ligand, though TLR1 and TLR2 are only expressed at very modest levels in Schwann cells. TLR3, TLR4, and TLR7 stimulation also yielded robust NF-κB responses, while ligands triggering TLR5, TLR8, or TLR9 were much weaker, reflecting the differences in expression levels we observed. Since TLR2 stimulation with *Mycobacterium leprae* on Schwann cells has been reported to induce apoptosis and

since NF-κB can have proapoptotic activities when it is acting as a transcriptional repressor (Campbell et al., 2004), we tested whether or not stimulation of Schwann cells with TLR ligands leads to NF-κB dependent gene expression. Corroborating our expression data, ligands for TLR2, TLR3, and TLR4 appeared to be the strongest ones, while ligands for TLR7 and TLR8 hardly induced any further gene expression above background. What was clear, however, is that we did not observe TLR-dependent NF-κB transcriptional repressing activity. Summarizing these data, it appears that all TLRs expressed on Schwann cells are functional and that the potency of their response more or less reflects their expression levels. One important exception is TLR1/TLR2, which despite its low abundance, yields in very strong NF-κB responses upon stimulation.

Recently, TLRs have been shown to recognize both exogenous as well as endogenous stresses, such as infections, injury, the presence of dying cells or degradation of extracellular matrix. Also in conditions of neurodegeneration, they are prime suspects to detect neurodegeneration and trigger an immune response although, until now, no endogenous ligands released by dying neurons or Schwann cells have been identified yet. In many neurodegenerative diseases, TLR expression is strikingly induced (Babcock et al., 2006; Bsibsi et al., 2002; Jackson et al., 2006; Mishra et al., 2006) and TLR knockout animals often show abrogated responses (Chen et al., 2006; Prinz et al., 2006; Tahara et al., 2006), implying a role for TLRs in neurodegenerative disorders. Also in conditions of injury, TLR expression levels appear to be induced in the CNS. In models of spinal cord injury, Kigerl et al. detected upregulation of TLR1, TLR2, TLR4, and TLR5 (Kigerl et al., 2007), while in response to stereotactic transection of axons in the enthorhinal cortex, TLR1 and TLR2 were induced (Babcock et al., 2006). In the peripheral nerve, the situation remains less well studied. Boivin et al. (2007) recently showed that TLR2 and TLR4 have protective effects in conditions of acute neurodegeneration, but expression of TLRs in these conditions has not been evaluated.

We found that acute neurodegeneration in the sciatic nerve leads to strong upregulation of TLR1, while TLR2, TLR6, and TLR9 expression levels appeared to be modestly induced. Interestingly, expression levels for TLR3, TLR4, and TLR7, which are already highly expressed in basal conditions, were not affected by neurodegeneration. These data therefore suggest that TLR1 might have a particular function in conditions of neurodegenerative stress, which correlates well with the fact that TLR1 appears to be the most sensitive receptor present on Schwann cells. However, since circulating monocytes/macrophages are known to be recruited to the sciatic nerve in conditions of nerve lesions (Martini et al., 2008), it is unclear at the moment what the contribution of macrophages is to the TLR levels we detect. In several independent axotomy experiments, we noted that TLR1 levels reach their peak between 32 and 72 h upon axotomy, which is well before the peak of macrophage infiltration (start infiltration: 1–3 days postinjury, peak levels: 14 days postinjury), (Martini et al., 2008). Still, further experiments are needed to establish the contribution of macrophages. The induction of TLR1 in conditions of acute neurodegeneration is in line with earlier observations of Boivin et al. (2007), who showed defective responses upon axotomy of the sciatic nerve in mice lacking TLR2, the obligate heterodimer for TLR1 (Takeuchi et al., 2002). However, it was clear from their paper that TLR2 is not solely responsible for the observed defects. Studies on TLR1 knockout mice have not been published yet, but are needed to confirm a role for TLR1 in acute neurodegeneration.

How TLRs detect neurodegeneration still remains unanswered, but it has been shown before that immune responses generated upon acute neurodegeneration are very fast responses, with inflammatory cytokine levels starting to accumulate as early as 1.5 h upon the time of injury (Boivin et al., 2007). In this respect, it is interesting to note that TLR1 recognizes lipoproteins (Takeuchi et al., 2002). It seems plausible that Schwann cells detect changes in lipid composition of the axonal membrane rather than secreted proteins as signals for neurodegeneration and we are currently following up this hypothesis.

In conclusion, the present study shows that Schwann cells appear to function as sentinel cells in the peripheral nerve, although, compared with peritoneal macrophages, they express a much more restricted set of TLRs at high level. Furthermore, TLRs might show functional specifications in the PNS, with TLR3 and TLR4 being highly expressed in basal conditions, pointing to their possible role in immune surveillance, while TLR1 might be important in conditions of neurodegeneration. Since TLRs have been shown to confer neuroprotective effects in conditions of acute neurodegeneration, these results are interesting for the development of novel therapies and indicate that TLR1 might be an important target.

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