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ORIGINAL PAPER

Early inflammatory profiling of schwannoma cells induced by lipopolysaccharide

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Abstract Inflammatory profiling of Schwann cells manifested as an upregulation of cytokines is present after traumatic or disease injury of the peripheral nerves. Inflammatory activation of Schwann cells via Toll-like receptors (TLRs) can be triggered by exogenous pathological molecules or endogenous ligands produced during Wallerian degeneration. We investigated the early period of inflammatory reactions by following the levels of TLR4, NFκB, IL-1β, pSTAT3, and IL-6 proteins after LPS treatment of RT4 schwannoma cells under in vitro conditions. Significantly increased levels of NFκB, IL-1β, pSTAT3, and IL-6 proteins were found 1 h after LPS action indicating their involvement in the initiation of the inflammatory reaction of schwannoma cells. This initiation was induced without increased TLR4 protein expression, but was accompanied by the appearance of TLR4 in early endosomes. The protein levels decreased within the next 6 h of treatment with a subsequent increase of NFκB, IL-1β, and pSTAT3 after 24 h of LPS treatment. In contrast, continuous decrease of IL-6 over time following LPS treatment was unexpected. Levels of soluble IL-6 protein in the culture medium also decreased with decreasing levels of LPS over 24 h.

Introduction

Wallerian degeneration (WD) induced either by traumatic injury or disease results in a rapid upregulation of inflammatory mediators like cytokines in the affected peripheral nerves. The inflammatory mediators orchestrate and facilitate communication between neurons, Schwann cells, and other cells participating in the degeneration and regeneration processes of the injured nerve (Cámara-Lemarroy et al. 2010; Rotshenker 2011; Gadient and Otten 1997). Upregulation of cytokines in Schwann cells of injured nerves reflects their inflammatory profiling triggered by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) through Toll-like receptors (TLRs) (Boivin et al. 2007; Vabulas et al. 2001). Degenerating axons produce mitochondrial and myelin sheath fragments and, together with degradation of extracellular matrix (ECM) molecules, act as potential endogenous ligands of TLRs. For example, fragments of endoneurial ECM cleaved by metalloproteinases during early stages of WD behave as ligands of Toll-like receptor 4 (TLR4) (Akira and Takeda 2004). In addition, direct inflammation induced by bacterial infection is another sort of nerve damage (Kim 2003) and the lipopolysaccharides (LPS) present in the outer membrane of Gram-negative bacteria are prototypical ligands of TLR4 (Rathinam and Fitzgerald 2013).

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The TLR4 activation signal is transduced through a myeloid differentiation primary response gene 88 (MyD88)-dependent pathway to activate the transcription factor nuclear factor-kappa B (NFκB) leading to the subsequent synthesis of cytokines such as interleukin-1 (IL-1) and interleukin-6 (IL-6) (Kawai and Akira 2007; Hao et al. 2009). Apart from having detrimental effects, the NFκB and STAT3 signaling cascades involved in the early stages of inflammation can promote cell survival (Libermann and Baltimore 1990; Kano et al. 2003; Wang et al. 2015). Moreover, STAT3 is a crucial factor for long-term survival of Schwann cells during nerve regeneration (Benito et al. 2017). The biological effects of IL-6 are mediated through IL-6 receptors belonging to the gp130 cytokine receptor family and the Janus kinase (JAK)-signal transducer and activator of transcription 3 (STAT3) signaling pathway (Bauer et al. 2007). Inflammatory profiling related to the upregulation of cytokines/chemokines reflects Schwann cell plasticity (Boerboom et al. 2017) and is associated with both promotion of the regenerative process in damaged nerve (Dubovy et al. 2013, 2014; Wong et al. 2017) and detrimental effects during neuropathic pain induction (Campana 2007; Thakur et al. 2017).

The rat RT4-D6P2T schwannoma cell line is derived from an *N*-ethyl-*N*-nitrosourea-induced rat peripheral neurotumor, RT4. This cell line is frequently used as a model for studying the molecular processes in Schwann cells. The goal of our present experiments was to study the early effects of LPS as a prototypic TLR4 ligand on inflammatory profiling of the schwannoma cells.

Materials and methods

Cell culture and treatment

Rat schwannoma cells (RT4-D6P2T), a gift of Prof. Stefano Geuna (Torino, Italy), were cultivated in Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F12) supplemented with 10% Fetal Bovine Serum (FBS), 2 mM glutamine, and antibiotics (100U/ml Penicillin and 100 μg/ml Streptomycin) (all obtained from Sigma-Aldrich) at 37 °C in 5% CO_2 atmosphere. At approximately 90% confluency, cells were seeded at a density of 1×10^4 cells per square centimeter and starved in serum-free (SF) medium overnight.

LPS (Sigma-Aldrich) was diluted in water from the stock solution and stored at −80 °C till needed. Schwannoma cells were treated with LPS at 10 ng/ml concentration for 1, 6, and 24 h. Control cells were cultivated in parallel and harvested at the same time points as treated cells.

Immunostaining

Cells cultured on slides in 12-well plates were washed with phosphate-buffered saline (PBS, pH 7.2), fixed with 4% paraformaldehyde in PBS for 20 min and washed again with PBS. Cells were permeabilized with 0.1% Tween-20, washed with 0.3% bovine serum albumin (BSA) in PBS, and blocked with 3% normal goat serum in PBS for 30 min at room temperature. Then, the control and treated cells were stained simultaneously under the same conditions with a primary antibody (anti-EEA1, anti-NFκB, anti-TLR4, Santa Cruz, USA; anti-IL-1β, Life Span, USA; anti-IL-6, Invitrogen, UK; pSTAT, Abcam, UK; see also Table 1) overnight at 4 °C, washed and incubated with secondary antibodies conjugated with TRITC or FITC at room temperature for 1 h, nuclei were labeled with Hoechst 33,342, and the cells were covered with Mowiol. The cells for negative control of immunocytochemical staining were incubated with omission of the primary antibody. Cells were observed and analyzed using a Nikon Eclipse NI-E epifluorescence microscope equipped with a Nikon DS-Ri1 camera operated using NIS-Elements software (Nikon, Czech Republic).

Western blotting

Control cells and those treated with LPS at the same time periods were washed twice with ice-cold PBS and mechanically harvested from the culture dishes. The samples were centrifuged at 2000 rpm for 5 min and lysed at 4 °C in 80 mM HEPES buffer (pH 7.5) containing 8 M urea, 1 mM EDTA, 0.5% Triton X-100, and 20 mM β-mercaptoethanol. The proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad). Blots were incubated with primary antibodies (anti-β-actin, anti-STAT3, Cell Signaling, UK; anti-NFκB,

Table 1 Used antibodies and appropriate concentration

	Type/Source	Dilution	Company	Antibody registry
β-actin	mRb	1:2500	Cell Signaling, UK	AB_2223172
EEA1	mMo	1:100	Santa Cruz, USA	AB_2096823
IL-1β	pRb	1:500	Life Span, USA	AB_2650495
IL-6	pRb	1:500	Invitrogen, UK	AB_10374895
NFκB	pRb	1:500	Santa Cruz, USA	AB_650213
pSTAT3	pRb	1:500	Abcam, UK	AB_779086
STAT3	mMo	1:1000	Cell Signaling, UK	AB_331757
TLR4	pRb	1:200	Santa Cruz, USA	AB_2205016

m monoclonal, *p* polyclonal, *Rb* rabbit, *Mo* mouse

anti-TLR4, Santa Cruz, USA; anti-IL-1β, Life Span, USA; anti-IL-6, Invitrogen, UK; pSTAT, Abcam, UK; see also Table 1) at 4 °C overnight followed by a peroxidase-conjugated secondary antibody at room temperature for 1 h.

Protein bands were visualized using the ECL detection kit (Bio-Rad), imaged on a PXi chemiluminometer reader and analyzed using the GeneTools densitometry software (Syngene). All analyzed proteins were normalized to β-actin. The regulation of monitored proteins could change during the cultivation period. Therefore, results of quantitative evaluation of protein levels by WB were related to non-LPS-treated cells cultivated at the same time and expressed as the mean ratio (LPS/CTL) ± SD arising from at least three independent experiments.

Elisa

The cultivation medium was tested for soluble IL-6. Medium was collected at 1, 6, and 24 h, centrifuged at 8000 rpm for 10 min, and stored at −80 °C.

The level of IL-6 protein in the medium was assessed by ELISA using a commercial kit with a sensitivity of 0.078 pg/ml (MyBioSource, USA) in accordance with the manufacturer's instructions. Each sample was measured three times using a SUNRISE Basic microplate reader (Tecan, Austria).

Limulus amebocyte lysate (LAL) assay

Aliquots of the cultivation medium collected as above were used to determine the amount of endotoxin LPS using a commercial LAL assay kit (Thermo Scientific, USA) according to manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using GraphPad Software. Our non-parametric data were tested by the Mann–Whitney U test. The level of statistical significance is shown as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Fig. 1 Immunostaining for TLR4, NFκB, and IL-1β of control RT4 cells (CTL) and cells treated with LPS for 1 h (LPS). In contrast to control cells (**a**), LPS induced an increase of TLR4 immunostaining in the endosome-like structures (**b** *arrowheads*; *inset* shows the *boxed cell* under higher magnification). LPS treatment resulted in increased NFκB and IL-1β immunofluorescence in cell nuclei (**d**, **f**) compared to control RT4 (**c**, **e**). *Scale bars* 50 μm
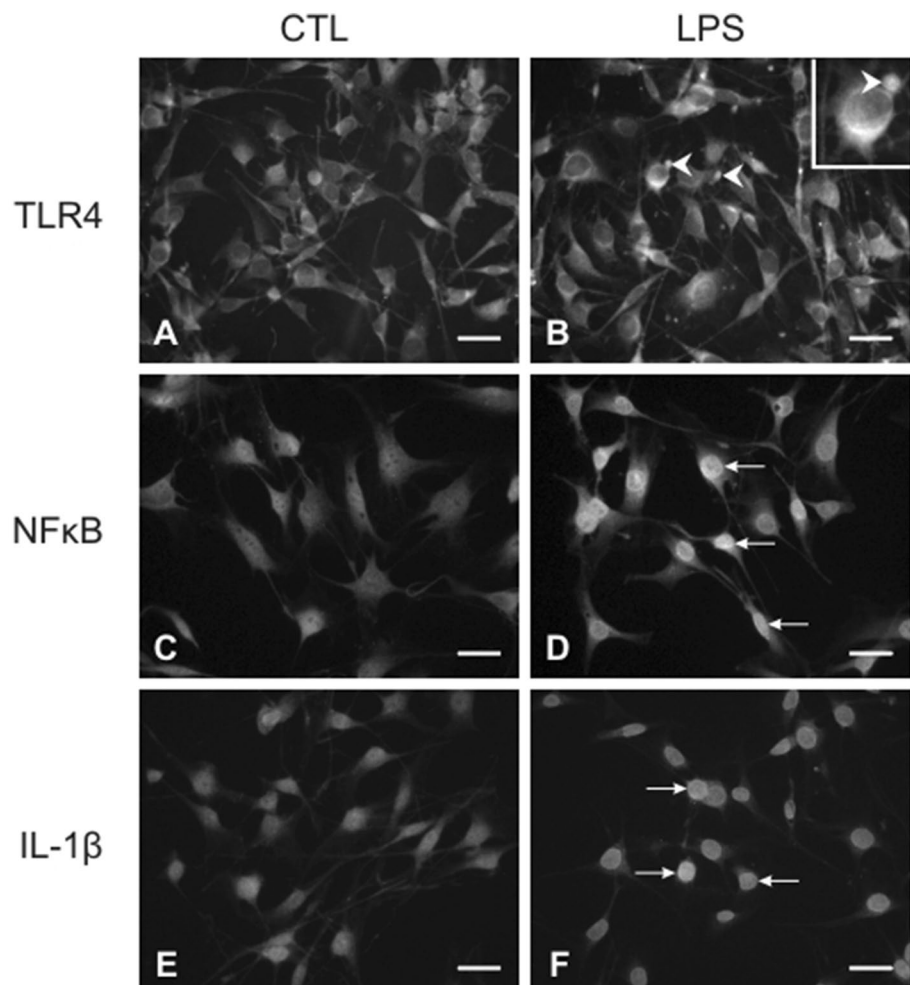
Results

TLR4 immunostaining revealed an increase in immunofluorescence intensity in RT4 cells after LPS treatment when compared with control cells. LPS-treated cells displayed TLR4 immunopositivity in their cytoplasm and mainly in large vesicular structures, but not in the plasma membrane (Fig. 1a, b). Double immunostaining demonstrated the co-localization of TLR4 and early endosome antigen 1 (EEA1) immunofluorescence indicating the presence of TLR4 protein in early endosomes of LPS-treated cells. EEA1 was not detected in control cells. In addition, TLR4 immunofluorescence was localized in small vesicular structures of the control cells (Fig. 2a–f). Western blot analysis showed no significant increase in TLR4 protein levels in RT4 cells after LPS treatment for 1 and 6 h compared to control cells. However, prolonged LPS treatment (24 h) showed a significantly increased level of TLR4 protein compared to control cells (Fig. 4a, b).

Despite mild activation of TLR4 by LPS treatment for 1 h, RT4 cells displayed increased immunostaining for NFκB and IL-1β as well as for pSTAT3 and IL-6. In comparison with control cells, increased NFκB and IL-1β immunostaining was found in the nuclei of LPS-treated cells (Fig. 1c–f). In addition, LPS treatment also induced higher intensities of IL-6 immunostaining in the perinuclear region of RT4 cells (Fig. 3a, b). Immunostaining for pSTAT3 demonstrated nuclear translocation of the activated molecules in both control and LPS-treated cells (Fig. 3c, d), but the number of pSTAT3-positive nuclei was significantly higher in treated than in control cells (Fig. 3e).

Western blotting confirmed increased levels of NFκB and IL-1β proteins in RT4 cells treated with LPS for 1 h. However, the protein levels of both NFκB and IL-1β dropped in cells treated for 6 h, while a second phase of increased levels was detected after treatment for 24 h (Fig. 4a, c, d). Similarly, Western blots also showed a significant increase in pSTAT3 and IL-6 levels in LPS-treated cells at 1 h (Fig. 4a, f, g). The levels of pSTAT3 and IL-6 proteins were the highest after 1 h treatment. This was followed by a significant drop of pSTAT3 at 6 h and a gradual reduction of IL-6 levels at 6 and 24 h (Fig. 4a, e, g). Meanwhile, the level of pSTAT3 increased again after 24 h treatment indicating a similar trend as was found for NFκB and IL-1β. In contrast to pSTAT3, protein levels of total STAT3 did not change over the period of the experiment (Fig. 4a, f). The changes in protein levels were independent of the proliferation stages of RT4 cells, because we observed no differences in proliferative cell proportions at the time points after LPS treatment (flow cytometry data not shown).
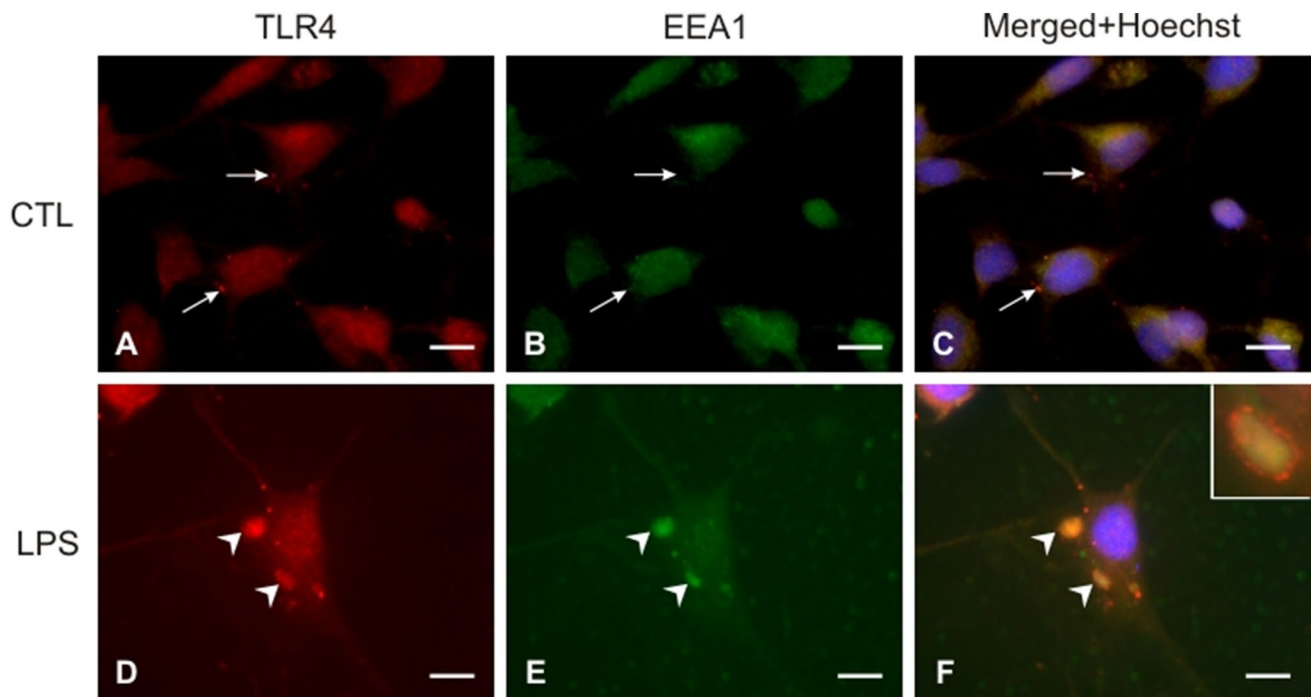

Fig. 2 The double immunofluorescence staining of control (CTL) and LPS-treated (LPS) RT4 cells for TLR4 and EEA1. Results of immunofluorescence co-localization show TLR4 in small TLR4⁺ EEA1⁻ endosomes of control cells (**a–c** *arrows*), while RT4 cells after 1 h treatment with LPS displayed a higher level of TLR4 immunofluorescence in EEA1⁺ early endosomes (**d–f** *arrowheads*; *inset* shows double immunofluorescence in enlarged early endosome). *Scale bars* 20 μm

Fig. 3 Immunofluorescence staining of RT4 cells for IL-6 as well as pSTAT3 and its quantification. Representative pictures illustrating immunofluorescence staining of control (CTL) and LPS-treated (LPS) RT4 cells for IL-6 (**a**, **b**) and pSTAT3 (**c**, **d**). Treatment with LPS increased the intensity of perinuclear immunofluorescence for IL-6 (**a**, **b** *arrows*). A few control cells contained pSTAT3 immunopositive nuclei (**c** *arrows*) in contrast to LPS-treated RT4 cells that displayed significantly higher nuclear translocation of activated STAT3 (**d** *arrows*), *scale bars* 50 μm. Positive nuclei for pSTAT3 were counted and their numbers were statistically evaluated using the Mann–Whitney *U* test (*E*, ******$p < 0.01$)
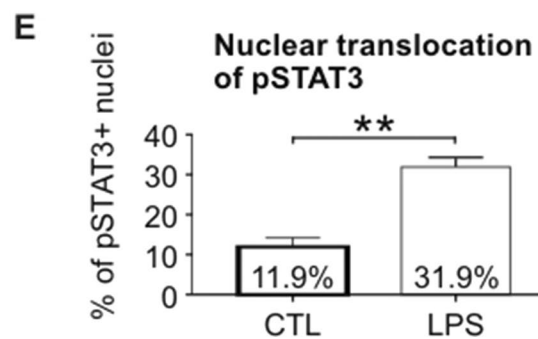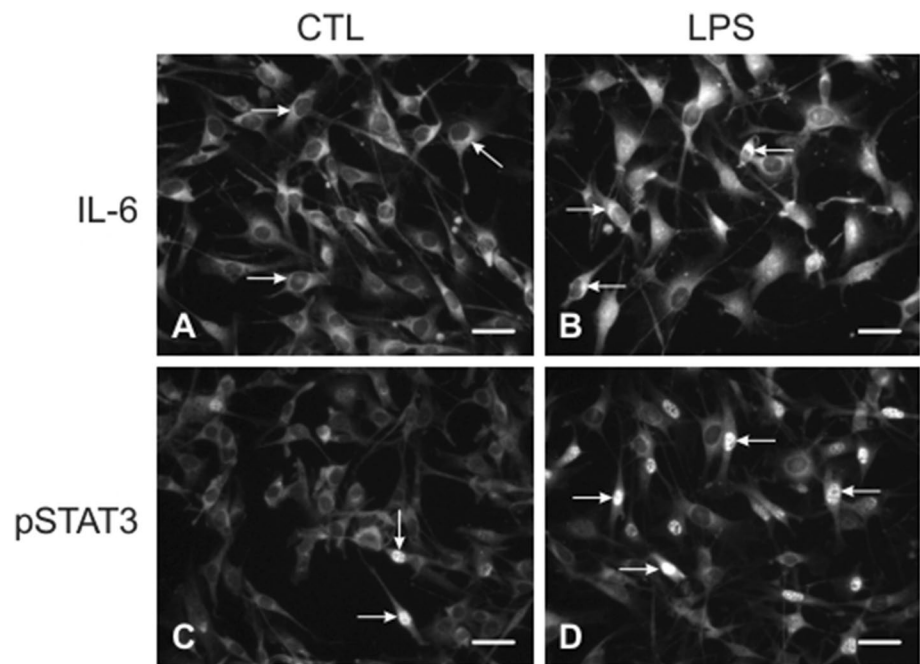
The gradual reduction of IL-6 protein levels following a robust increase after LPS treatment for 1 h was confirmed by ELISA results that showed corresponding levels of soluble IL-6 molecules in the medium. Levels of soluble IL-6 molecules also decreased after treatment for 6 and 24 h as seen in the cells. The levels of the soluble form of IL-6 molecules in medium coincided with decreased levels of LPS in the cultivation medium (Fig. 5). We tested changes of LIF levels after LPS treatment of RT4 cells because effect of IL-6 could be substituted by other members of the neuropoietic cytokine family (Kurek et al. 1996). However, no changes in LIF protein levels were found over the course of the experiment (data not shown).

Discussion

Inflammatory profiling of Schwann cells manifested as upregulation of cytokines is present after traumatic or disease injury of peripheral nerves (Ydens et al. 2013; Üçeyler et al. 2008) and can be triggered through TLRs by endogenous ligands produced during WD or by exogenous pathological molecules (Boivin et al. 2007). Proteolytic activity is elevated early after nerve injury (Hughes et al. 2002) to cleave endoneurial ECM and myelin to products that can be recognized by the TLR4 expressed by Schwann cells (Gaudet et al. 2011). LPS is a prototypical TLR4 ligand and its application induces inflammatory cytokine production by Schwann cells and accelerates myelin cleavage and phagocytosis during WD (Vallières et al. 2006). In the present experiments, we used RT4 schwannoma cells as an in vitro model to demonstrate early phases of dynamic regulation of TLR4, NFκB, IL-1β, pSTAT3, and IL-6 proteins after treatment of LPS as a prototypic ligand of TLR4.

Intracellular detection of TLR4 and its activation by LPS

Under normal conditions, TLR4 is localized to the endoplasmic reticulum and the Golgi region, the plasma membrane,

Fig. 4 a Representative Western blots of control (−) RT4 cell lysates and lysates of cells after LPS treatment (+) for 1, 6 and 24 h. Quantitative changes of individual proteins expressed as a density ratio of LPS-treated to control cells (LPS/CTL) are shown in **b–g**. Statistical significance is indicated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$
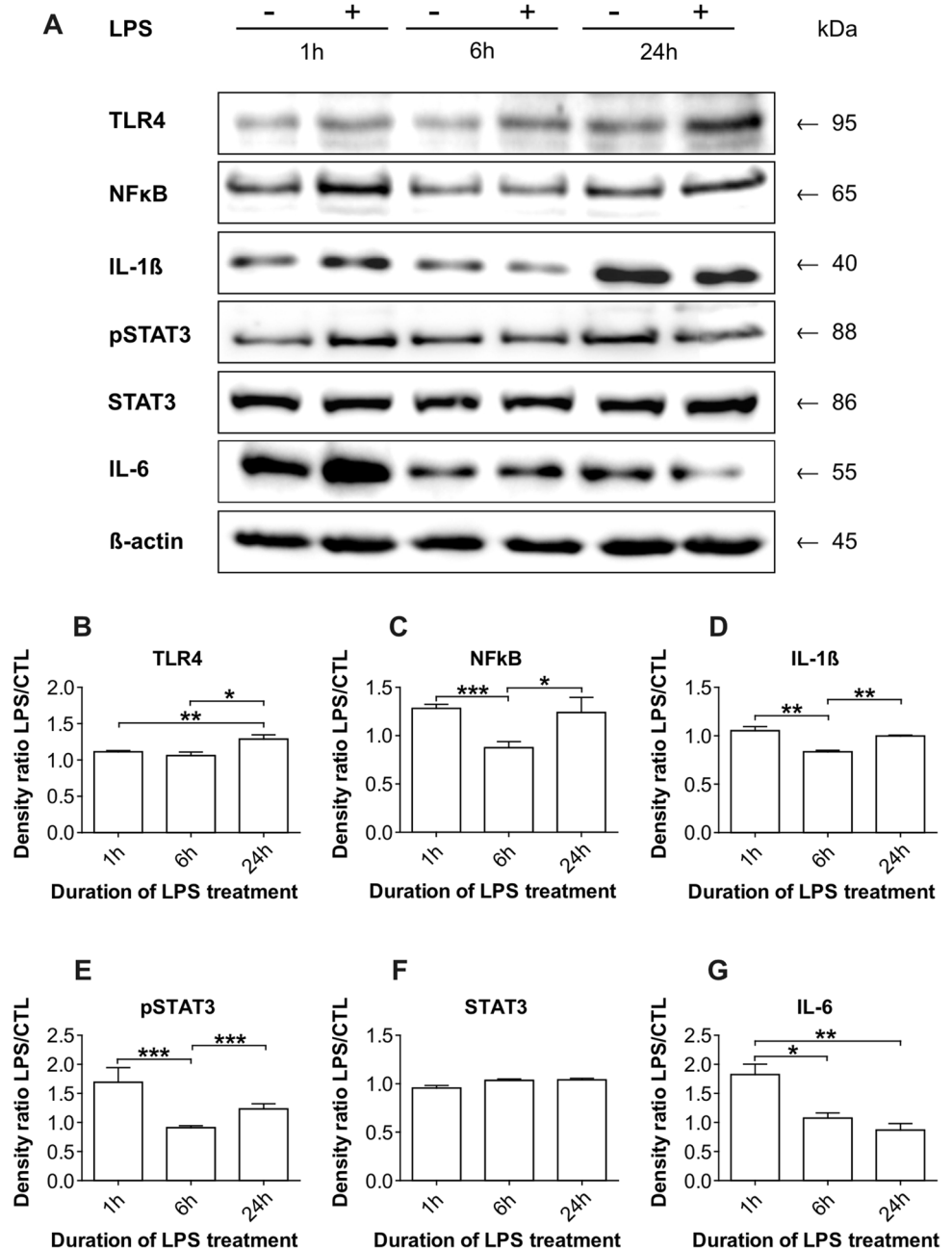
and sporadically in endosomes. If the cells are activated by appropriate stimuli, TLR4 creates a receptor complex localized in endosome structures (Husebye et al. 2006; Chow et al. 1999). Results from HEK296 cells and monocytes demonstrated the co-localization of LPS and TLR4 in early endosomes 15 min after LPS treatment suggesting rapid and active endocytosis. In the late phase of LPS treatment, the TLR4–LPS complex was accumulated close to the perinuclear area and was followed by constitutive ubiquitination of TLR4 3 h after LPS treatment (Husebye et al. 2006).

In agreement with these findings, our results confirmed the localization of TLR4 in enlarged early endosomes of RT4 schwannoma cells after LPS treatment. In addition,

the lower levels of TLR4 protein in RT4 cells after LPS treatment for 1 and 6 h also suggested swift ubiquitination of this receptor complex that was not recognized by used TLR4 antibody. The significant increase in TLR4 protein was found in RT4 cells after treatment for 24 h, and coupled with a decrease in LPS level in the medium suggested that LPS was probably degraded.

TLR4 activation and signaling for cytokine synthesis

Activation of TLR4 by the cognate ligand triggers downstream signaling cascades leading to the activation of the nuclear transcription factor NFκB, which controls the
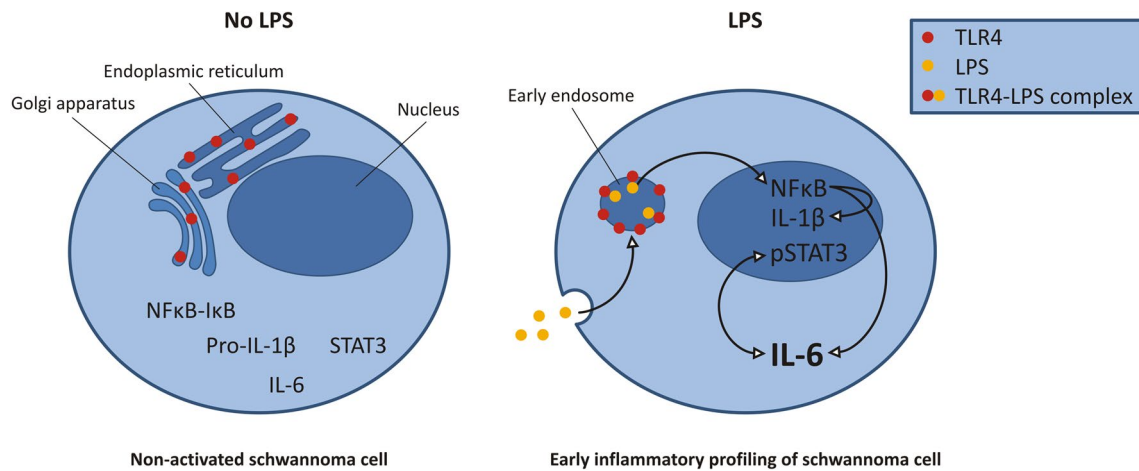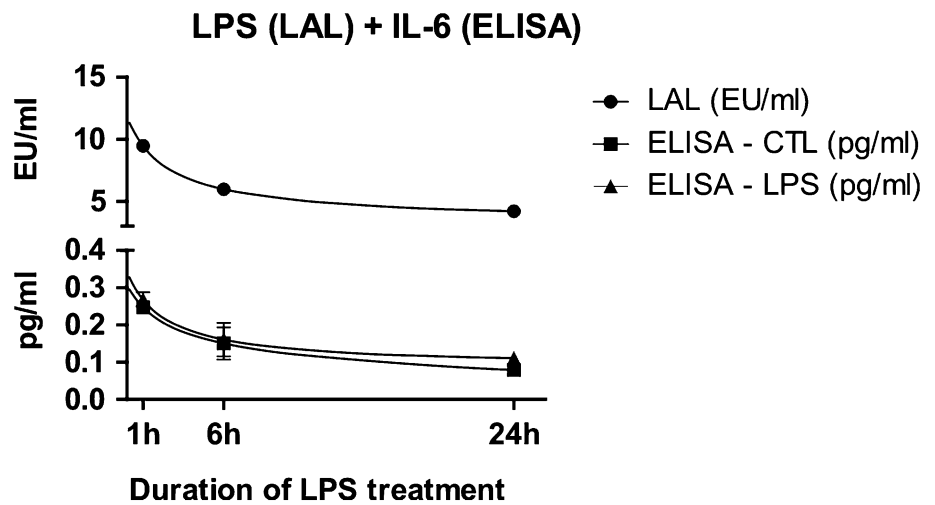
Fig. 6　Schematic drawing illustrating a possible process of very early (1 h) TLR4 activation and subsequent inflammatory profiling of schwannoma cells based on our results. Non-activated schwannoma cells contain TLR4 mainly in the endoplasmic reticulum and Golgi apparatus. Inactive NFκB-IκB, pro-IL-1β, and STAT3 as well as a low level of IL-6 are in the cytoplasm. During early periods of LPS treatment, LPS molecules are internalized and bind with TLR4 in early endosomes to increase production of IL-1β and IL-6 via activated NFκB. Increased level of IL-6 induces phosphorylation of STAT3 (pSTAT3) that contributes to swift increase of IL-6 via amplification loop

synthesis of pro-inflammatory cytokines (Kawai and Akira 2007).

Despite a low level of TLR4, LPS treatment for 1 h induced a higher level of NFκB, IL-1β, as well as pSTAT3 and IL-6 proteins in RT4 schwannoma cells compared to control cells. These results suggest that the low pre-existing level of TLR4 is sufficient to trigger signaling cascades leading to increased synthesis of cytokine IL1β and IL-6. TLR4 expression after LPS treatment of primary Schwann cell culture is also followed by cytokine production (Hao et al. 2009).

A growing body of evidence implicates IL-6 as a key component in the injury response of the nervous system. Moreover, IL-6 is also the one of the best-characterized pro-tumorigenic cytokines (Taniguchi and Karin 2014). IL-6 and other members of the IL-6 family activate the JAK/STAT3 pathway through engagement of its IL-6R and the common signaling receptor subunit gp130 (Garbers et al. 2012). STAT3 is activated in reactive astrogliosis (Sriram et al. 2004) and in reactive Schwann cells distal to nerve injury (Ben-Yaakov et al. 2012). The increase in IL-6 via pSTAT3 may also be related to GFAP induction as was demonstrated in Schwann cells during early WD of injured peripheral nerve (Lee et al. 2009a). In our in vitro experiments, LPS treatment of RT4 cells for 1 h increased levels of IL-6 and activated STAT3 that was translocated in the nuclei. IL-6 production was highly correlated with the activation of STAT3 suggesting that IL-6 promoted its own

production through pSTAT3. This amplification loop was identified in B cells, as B-cell lymphoma with high STAT3 expression exhibited elevated production of IL-6 (Lam et al. 2008). The amplification loop was probably limited only to within 1 h of LPS treatment in RT4 schwannoma cells.

Six hours of LPS treatment did not induce any increase of studied proteins. However, protein levels were again increased after LPS treatment for 24 h excluding IL-6 continuing with decrease till 24 h. This dynamic of the pSTAT3 level coincided with the levels of IL-1β and NFκB over the period of our experiment, but did not correspond with the continuous decrease of IL-6 protein till 24 h. Nevertheless, the decreasing level of IL-6 correlated with LPS levels in the medium over the cultivation time indicating that IL-6 mediates the acute response to LPS stimuli. It is known that the action of IL-6 in late periods could be substituted by other members of the neuropoietic cytokine family e.g., LIF (Kurek et al. 1996). However, our results revealed no changes of LIF levels after LPS treatment of RT4 cells indicating involvement of other members of neuropoietic family (Taga 1996) or neurotrophins (Pellegrino and Habecker 2013) in STAT3 activation during the early reaction of schwannoma cells to LPS treatment.

Both NF-κB and STAT3 are rapidly activated in response to various stimuli including cytokines and cell stresses (Battle and Frank 2002), although they are regulated by different activating and signaling mechanisms (Grivennikov and Karin 2010). We observed very similar patterns of pSTAT3 and NFκB protein levels in RT4 schwannoma cells after LPS treatment for 1, 6, and 24 h. The simultaneous activation of STAT3 and NFκB induced similar genes and was detected in tumor cells (Grivennikov and Karin 2010). NFκB can directly regulate IL-6 level via MyD88 in hepatocellular carcinoma (Naugler et al. 2007), while STAT3 can prolong nuclear retention of NFκB in prostate cancer cells (Lee et al. 2009b).

In conclusion, RT4 schwannoma cells treated with LPS for 1 h showed a significant elevation of IL-6, pSTAT3, IL1β, and NFκB protein levels, but this was not accompanied by any significant increase of TLR4 regulation. In addition, detection of TLR4 in early endosomes of RT4 schwannoma cells after LPS treatment in the early time points indicates intracellular TLR4 traffic in peripheral glial cells. The results of our in vitro experiments demonstrated a dynamic early inflammatory profiling of Schwann cells that can also occur in vivo via activation of TLR4 by endogenous ligands during WD or exogenous ligands after inflammatory nerve injury. A possible mechanism of early inflammatory profiling of Schwann/schwannoma cells after LPS treatment is summarized in Fig. 6.

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