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TLR2 Hypersensitivity of Astrocytes as Functional Consequence of Previous Inflammatory Episodes

Anja Henn, Susanne Kirner, and Marcel Leist

Precedent inflammatory episodes may drastically modify the function and reactivity of cells. We investigated whether priming of astrocytes by microglia-derived cytokines alters their subsequent reaction to pathogen-associated danger signals not recognized in the quiescent state. Resting primary murine astrocytes expressed little TLR2, and neither the TLR2/6 ligand fibroblast-stimulating lipopeptide-1 (FSL1) nor the TLR1/2 ligand Pam3CysSk4 (P3C) triggered NF-κB translocation or IL-6 release. We made use of single-cell detection of NF-κB translocation as easily detectable and sharply regulated upstream indicator of an inflammatory response or of c-Jun phosphorylation to measure restimulation events in astrocytes under varying conditions. Cells prestimulated with IL-1β, with a TLR3 ligand, with a complete cytokine mix consisting of TNF-α, IL-1β, and IFN-γ, or with media conditioned by activated microglia responded strongly to FSL1 or P3C stimulation, whereas the sensitivity of the NF-κB response to other pattern recognition receptors was unchanged. This sensitization to TLR2 ligands was associated with an initial upregulation of TLR2, displayed a “memory” window of several days, and was largely independent of the length of prestimulation. The altered signaling led to altered function, as FSL1 or P3C triggered the release of IL-6, CCL-20, and CXCL-2 in primed cells, but not in resting astrocytes. These data confirmed the hypothesis that astrocytes exposed to activated microglia acquire a different functional phenotype involving longer term TLR2 responsiveness, even after the initial stimulation by inflammatory mediators has ended. The Journal of Immunology, 2011, 186: 3237–3247.

Astrocytes are the major glial population of the brain and take predominantly metabolic roles under resting conditions (1). They constitutively express receptors for TNF-α, IL-1β, as well as IFN-γ, and can be activated by these cytokines (2–5). Microglia are the major producers of these factors in the brain (1, 6–8). These cells constitutively express most types of receptors for pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (9–11), and secrete inflammation related mediators after their activation by microbial components or by tissue damage (7, 12). Resting astrocytes express low levels of TLRs and may be considered a second line of defense, because they require activation by microglia to become responsive (10). On activation, they assume an activated phenotype, resembling that of endotoxin-stimulated macrophages (5, 10, 13), and can remain activated for a long time, for example, in stroke (14, 15), Alzheimer’s disease, and traumatic injury to the CNS (1, 16, 17).

On stimulation with proinflammatory factors, astrocytes can upregulate pattern recognition receptors (PRRs), in particular, TLR2 (9, 13, 18–20). The broad ligand spectrum of this PRR involves not only bacterial components, but endogenous ligands, such as CD14 (21), danger signals generated during tissue damage in stroke (22–24), or viruses (24–26). As TLR2 activation triggers a further release of inflammatory factors, a self-sustaining inflammatory loop may be generated in the brain and may explain glial long-term activation (10, 11, 19, 27). In brain, TLR2 upregulation has indeed been observed in several CNS disease states (6, 26, 28–30) and may be functionally involved in the damage generation (22–24, 31–35).

The signal transduction of TLR2 usually requires the formation of a complex with either TLR1 or TLR6, and possibly association with coreceptors such as CD36 (10, 31, 36). Different ligands specifically activate these receptor complexes. For instance, the model triacyl lipopeptide S-[2,3-bis(palmitoyloxy)-propyl]-N-palmitoyl-Cys-Ser-Lys (Pam3CysSk4) stimulates TLR1/2, and the Mycoplasma salivarium-derived diacyl lipopeptide S-[2,3-bis(palmitoyloxy)-propyl]-Cys-GDPKHPKSF (fibroblast-stimulating lipopeptide-1 [FSL1]) stimulates TLR2/6. The canonical signaling pathway involves the recruitment of the adaptor protein MyD88 to the receptor complex and then binding of further adaptors that trigger activation of the NF-κB and JNK signaling cascades (10, 11, 37, 38), similar to the situation observed in astrocytes activated by TNF-α or IL-1β (39). However, some TLR2 ligands may differ with respect to their capacity to trigger different signaling cascades (25, 33).

Currently, little is known about the function of upregulated TLR2 on astrocytes. The situation in animal models is complex. For instance, the cytokine response in Staphylococcus aureus-induced brain abscesses is affected by TLR2 (19, 40, 41), but the bacterial load and clearance are not (42, 43). Upregulation of TLR2 on astrocytes may also play a role under sterile inflammatory conditions, such as glaucoma (28) or stroke (24), but neither study has demonstrated the functional importance of TLR2 upregulation by inflammatory factors on astrocytes.
Research in chronic inflammation and stroke preconditioning has shown that the regulation of sequential inflammatory events is highly complex and cannot be easily deduced from knowledge on expression patterns and signaling pathways in resting cells (37, 38, 44). Frequently, a state of hyposensitization or tolerance is triggered in cells of the innate immune system, including microglia (45), on repeated stimulation (46, 47). Reasons for this may be receptor tachyphylaxis, downregulation of signaling components by other mediators (48), reprogramming (49), or upregulation of counterregulatory intracellular factors such as I-kB and A20 (37, 38, 50, 51). Mice, pretreated with a low dose of IL-1β or TNF-α, developed homologous tolerance to restimulation with the same agonist, and even cross-tolerance has been demonstrated (52, 53). Most knowledge on hyposensitization and hypersensitization was derived for homologous restimulation of TLR4, a receptor that plays no role in murine astrocytes (4, 10, 54, 55). The most relevant situation for these cells would be homologous restimulation with microglia cytokines or heterologous cross-stimulation between priming cytokines and endogenous or exogenous TLR2 ligands. Such situations arise under conditions of damage, aging, or neurodegeneration, when astrocytes are pre-exposed to TNF-α or IL-1β, and then faced with a respective microbial stimulus, virus, or components of damaged tissue (e.g., low m.w. hyaluronan [35]) that stimulates TLR2.

We initiated this study to characterize the phenotypic and functional changes potentially arising under such inflammatory conditions, and asked how astrocytes differed in their response to TLR2 ligands in resting states when compared with activated, disease-relevant states.

Materials and Methods

Primary cultures

Primary cortical astrocytes. Mice (BALB/c or C57BL/6 or C57BL/6-TLR2−/− [36]; kindly provided by T. Hartung, Baltimore, MD) were bred at the animal facility of the University of Konstanz (Konstanz, Germany). All mice were housed at 22 °C and 55% relative humidity in a 12-h day/night rhythm with free access to food and water according to national regulations and EU guideline 86/609/EEC. Primary cortical astrocytes were prepared from mouse pups at 24–48 h post partum as described earlier (4, 54). In brief, brains were removed, the cortices were dissected out, and hippocampi and meninges were carefully removed before digestion with trypsin and DNase. After trituration, the cell suspension was carefully layered over a 30% Percoll solution and centrifuged at 150 × g for 10 min. The astrocyte fraction was recovered, washed, and resuspended in DMEM (high glucose), 20% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded at a density of 2 million cells (corresponding to two brains) per T75 flask, and cultured at 37 °C in a 5% CO2 atmosphere. The medium was changed after 4 d, and subsequently twice a week. After 14 d in primary culture, cells were trypsinized and incubated in DMEM for 45 min at room temperature in a T75 flask for differential adhesion of any residual microglia. Nonadherent cells (astrocytes) were resuspended in DMEM plus 10% FCS, containing 2% FCS. Anti–TNF-α antiserum, with a strong neutralizing capacity for TNF-α, remained present during the stimulation. Medium used in all experiments contained 2% FCS. Anti–TNF-α, an IgG fraction of ovine anti-murine TNF-α antiserum, with a strong neutralizing capacity for TNF-α bioactivity, was prepared and characterized as described earlier (58).

Cytokine determination by ELISA

The murine cytokine IL-6 was measured in MaxiSorp plates from Nunc (Langenfeld, Germany) using murine-specific ELISA kit Ready-SET-Go! from eBioscience (San Diego, CA) according to the manufacturer’s protocol.

Analysis of cytokine release with Ab array

Astrocytes (500,000 cells/well) of a six-well dish were cultured in DMEM plus 10% FCS for 1 wk. One day before the experiment, the FCS concentration was reduced to 2% and a total medium volume of 2 ml/well. After stimulation, the supernatant was collected and analyzed using the membrane-based RayBio Mouse Cytokine Ab Array 3 kit (RayBiotech, Norcross, GA) as described earlier (12). Signals were visualized using a Fusion Western blot imaging system (Peglab Biotechnologie GmbH, Erlangen, Germany). The pixel intensities (integrated density) of the spots were determined by ImageJ software. After background correction, the relative intensities compared with the negative control (supernatant of resting astrocytes) were calculated. Each data point presented in the results is based on three membranes, with two spots per cytokine on each membrane.

Flow cytometry

Cells were trypsinized, washed with PBS containing 2% FCS, and incubated at 4 °C for 50 min with FITC-labeled monoclonal anti-TLR2 Ab (clone 6C2; eBioscience, Alsdair Stewart, U.K.) or appropriate isotype controls in PBS containing 2% FCS. Labeled cells were washed twice and fixed in 0.5% paraformaldehyde (PFA). For each sample, a minimum of 5000 cells within the gated cell population was analyzed using a flow cytometer (Accuri Cytometers, Cambs, U.K.) and the corresponding software CFlow. The positivity of positively stained cells relative to isotype control (maximum 1% positive staining) was determined.

Immunostaining

Cells on 96-well plates were fixed with 4% PFA in PBS, pH 7.3, and blocked with 10% FCS in PBS. The primary anti-TLR2 Ab (clone 6C2), diluted in 2% FCS in PBS, was added to the cells and incubated overnight at 4 °C. Binding to the Ag was visualized after 50-min incubation with a labeled secondary anti-rat IgG-Alexa488 Ab (Invitrogen, Darmstadt, Germany) diluted in 2% FCS in PBS. Nuclei were counterstained with H-33342 (1 μg/ml) and visualized using a fluorescent microscope (Olympus IX81; Olympus, Münster, Germany).

Automated quantitative imaging

Cells were plated in 96-well plates at 15,000 cells/well and cultured in DMEM plus 10% FCS. The cells were stimulated as indicated, then fixed with 4% PFA, permeabilized with 0.1% Triton X-100, blocked with 10% FCS, immunostained with purified monoclonal mouse anti–NF-κB p65 (clone 20/NF-κB/p65; final dilution 1:300) Ab (BD Transduction Laboratories) or anti-phospho–ε-Jun Ab (clone KM-1; final dilution 1:700; Santa Cruz Biotechnology), and detected with anti-mouse IgG Alexa-488.
secondary Ab. Images were recorded with a high-resolution charge-coupled device camera on a Cellomics ArrayScan, which is based on an automated Zeiss Axiostar-100 microscope equipped with a 20× objective and an FITC/H-33342 filter set (λex = 465 nm; λem = 475 nm; λem = 535 nm). Nuclei, stained with H-33342, were imaged first (channel 1) for automated focusing and identification of valid objects. Subsequently, the stained Ags were imaged in the corresponding fields (channel 2). Based on these data sets, the nuclear translocation or intensities of NF-κB or phospho–c-Jun were quantified for each cell with the predefined algorithm “nuclear translocation,” essentially as described earlier (4, 39, 57).

NF-κB translocation. The nuclear-cyttoplasmic ratio of the NF-κB p65 Ag signal intensity was quantified by dividing the mean average Ag intensity in the nuclear area defined as “circ” by the mean average Ag intensity of a “ring” around this area, which covered a cytoplasmic region. The distance of the circ to the nuclear outline was 5 pixels (3.3 μm). The ring had a width of 3 pixels (1.9 μm) and a distance of 5 pixels (3.3 μm) from the nuclear outline. For determination of reference values, resting cells in three reference wells were imaged. In each reference well, the cells of three fields were analyzed. “Activated astrocytes” were defined as cells whose circ/ring ratio was at least one SD above the average circ/ring ratio observed in each individual cell, and frequently is a good indicator of downstream cellular responses. NF-κB p65 was easily stained in astrocytes, and visual observation suggested that in quiescent astrocytes, most NF-κB is found in the cytosol. Stimulation with a CCM (4), consisting of TNF-α, IL-1β, and IFN-γ, resulted in translocation of the transcription factor into the nucleus and into the perinuclear area (Fig. 1A).

The NF-κB translocation state of astrocytes stimulated with CCM was monitored over 24 h, using an unbiased imaging and quantification procedure established on an automated microscope (Fig. 1A). Already after 30 min, a stable plateau of activation involving 60–90% of the cells was observed. The number of activated cells decreased very slowly, and was still significantly increased (40–50% of the cells) after 24 h. Although this long activation time is useful for measurements of receptor activation, it is unsuitable for restimulation experiments, as the signal did not return to baseline (Fig. 1B). Introduction of a washing step after 6 h, to remove remaining cytokines, caused NF-κB to return to the cytosol within 60 min after the wash and to remain at baseline. Under this condition, it seemed feasible to measure the response to a second stimulatory event, for example, after 24 h.

Initial experiments involved both stimulation and restimulation with CCM 24 h apart, with a washing step in between. Staining of the cells after 24 h (before the second exposure to CCM) showed that their NF-κB was cytosolic (Fig. 1C). The extent of nuclear translocation was similar after the first and second stimulation (24 h, CCM), provided that a washing step was performed 6 h after the first stimulus (Fig. 1D). Thus, we established a system allowing a quantitative and time-resolved measurement of restimulation effects in astrocytes.

*Specifically altered TLR2 signaling and expression in astrocytes prestimulated with CCM*

The major question of this study was whether astrocytes behave differently to danger or damage signals after exposure to a proinflammatory environment or certain cytokines. Therefore, we tested whether astrocytes, preconditioned with CCM, showed an altered response to ligands of PRRs. The cells were prestimulated with CCM, washed, and restimulated 18 h later with different inflammmogens. The TLR2/6 ligand FSL1 and TLR1/2 ligand P3C triggered NF-κB translocation under these conditions, whereas no significant NF-κB translocation was detectable when LPS (TLR4 ligand), poly(I/C) (TLR3 ligand), imiquimod (TLR7 ligand), unmethylated CpG-containing oligonucleotide (TLR9 ligand), muramyl dipeptide (nucleotide-binding oligomerization domain containing 2 ligand), or high-mobility group box 1 protein (TLR4 and receptor for advanced glycation end product ligand) were used (Table I).

The increased sensitivity of preactivated primary astrocytes to the TLR2 ligands was characterized in more detail. High concentrations of FSL1 (Fig. 2A) or P3C (not shown) triggered only a minor response in resting astrocytes, but this was greatly increased and shifted to lower concentrations after prestimulation of

**Results**

**NF-κB as sharp indicator of repeated homologous restimulation of astrocytes with proinflammatory cytokines**

To explore the response of murine astrocytes on restimulation (59), we chose translocation of the transcription factor NF-κB from the cytoplasm to the nucleus as our primary readout. This end point was chosen because it is coupled to several different inflammation-relevant receptors. Moreover, the signal is well correlated with receptor activation in most physiological situations, inactivates quickly on termination of the stimulation, can be observed in each individual cell, and frequently is a good indicator of downstream cellular responses. NF-κB p65 was easily stained in astrocytes, and visual observation suggested that in quiescent astrocytes, most NF-κB is found in the cytosol. Stimulation with a CCM (4), consisting of TNF-α, IL-1β, and IFN-γ, resulted in translocation of the transcription factor into the nucleus and into the perinuclear area (Fig. 1A).
CCM. All data are means of stimulations of naive cells (none) or cells prestimulated 24 h earlier with 0.001.

Sequential stimulation of astrocytes after 0 min and 24 h with washing 6 h after the first stimulation with CCM. Representative images show NF-κB translocation, average pixel intensities in these areas were used to calculate the circ/ring ratio of each valid cell. Cells were defined as activated (NF-κB translocation) when their circ/ring ratio exceeded a threshold value identified from reference resting cells. A. Astrocytes were stimulated and either left undisturbed together with the stimulus or washed after 6 h and left in cytokine-free medium. B. Nuclear outline was identified (marked in blue). Then a nuclear subarea (circ) was defined. The cellular outline was defined based on NF-κB background staining. The nuclear coordinates were used to define a cytoplasmic area around each nucleus (ring). For quantification of the NF-κB translocation, average pixel intensities in these areas were used to calculate the circ/ring ratio of each valid cell. Cells were defined as activated (NF-κB translocation) when their circ/ring ratio exceeded a threshold value identified from reference resting cells. B. Astrocytes were stimulated and either left undisturbed together with the stimulus or washed after 6 h and left in cytokine-free medium. C. Nuclear DNA NF-κB Quantification

**FIGURE 1.** Quantification of single and repeated NF-κB translocation in individual astrocytes. Astrocytes were stimulated with CCM, immunostained for NF-κB (p65), and counterstained for DNA (H-33342). Fluorescent images were captured and then analyzed by an unbiased algorithm. A. First the nuclear outline was identified (marked in blue). Then a nuclear subarea (circ) was defined. The cellular outline was defined based on NF-κB background staining. The nuclear coordinates were used to define a cytoplasmic area around each nucleus (ring). For quantification of the NF-κB translocation, average pixel intensities in these areas were used to calculate the circ/ring ratio of each valid cell. Cells were defined as activated (NF-κB translocation) when their circ/ring ratio exceeded a threshold value identified from reference resting cells. B. Astrocytes were stimulated and either left undisturbed together with the stimulus or washed after 6 h and left in cytokine-free medium. NF-κB translocation was determined by automatic imaging as in A at the time points indicated. C. Sequential stimulation of astrocytes after 0 min and 24 h with washing 6 h after the first stimulation with CCM. Representative images show NF-κB at key time points. D. NF-κB translocation was quantified 30 min after stimulations of naive cells (none) or cells prestimulated 24 h earlier with CCM. All data are means ± SEM of triplicate determinations. ***p ≤ 0.001.

the cells with CCM. The percentage of cells activated by re-stimulation with a TLR2 ligand was nearly as high as that activated by CCM re-stimulation. Knockout of TLR2 completely blunted the response to FSL1 (Fig. 2B) or P3C (not shown), but had no effect on the response to CCM. Thus, a role for TLR2 in the “hypersensitivity” response to P3C and FSL1 was corroborated. In line with this, we observed a strong induction of TLR2 expression above background staining on the cell surface on stimulation with CCM for 24 h (Fig. 2C, 2D). Notably, augmented expression of TLR2 was also observed when cells were exposed to CCM for only 6 h, washed, and then left unstimulated for a further 18 h. This increased TLR2 expression decreased over time (57% positive cells after 24 h, 20% after 48 h, 10% after 72 h), and was only slightly elevated (4%) above the controls (0–2%) 96 h after the priming stimulus (Fig. 2E). Increased TLR2 expression after CCM stimulation was also observed on mRNA level in this (~200-fold after 4 h; 80-fold after 24 h) and other (13) studies, and we now have demonstrated its functional role in cells.

**Heterologous sensitization to TLR2 stimulation by CCM under continuous inflammatory conditions**

In the next steps, we examined how robust the “TLR2 hypersensitization” in astrocytes was under changing experimental conditions. First, the system dynamics of heterologous or homologous restimulation were compared. We tested a potential role of TLR2 stimulation under continued inflammatory conditions, that is, in the absence of a washing step separating the first and second type of stimulus. Using P3C (Fig. 3A) or FSL1 (not shown), respectively, we observed that astrocytes were also fully stimulated under these conditions, even though the signal level of already activated cells was still higher than the initial baseline because of the continued presence of the priming stimulus (CCM). This type of response differed completely from that observed for CCM restimulation, when astrocytes behaved refractorily on restimulation. This suggests that the NF-κB system, under general inflammatory conditions, remains responsive for a second, heterologous stimulus, even when it is partially activated already (Fig. 3A). To further explore the robustness of heterologous TLR2 sensitization, we chose three approaches in which cells were rechallenged as late as 72 h after initiation of the pretreatment. First, when the delay period after the washing step was prolonged to 66 h, sensitization to FSL1 was still observed (Fig. 3B). Second, CCM was left on astrocytes for 54 h before washing and delayed restimulation. Third, astrocytes were challenged three times with CCM and intermittent washing steps within 72 h, and then rechallenged with FSL1. In the latter two cases, a maximal response was observed, comparable in strength with the standard conditions with FSL1 (24 h) or the stimulation of untreated cells with CCM (Fig. 3B). Thus, hypersensitivity of TLR2 is a very prolonged feature and differs strongly from tolerization, which is observed in macrophages (49).

We also examined whether cross-sensitization between TLRs occurs. Although TLR2 was the astrocyte receptor most strongly upregulated by inflammatory stimuli, TLR3 showed the strongest basal expression of all examined receptors (Fig. 3C). Stimulation of astrocytes with the TLR3 ligand poly(I:C) led to a >100-fold upregulation of mRNAs for Ifn-β and Ccl2, and an 52 ± 6-fold increase of Tlr-2 (not shown). The functional consequences of this upregulation were tested by restimulation of poly(I:C) pretreated astrocytes with a TLR2 ligand. We found a strong upregulation of the resultant NF-κB response. Thus, cross-sensitization between TLR3 and TLR2 behaved similarly as that of CCM and TLR2 (Fig. 3D).

**Pharmacological modulation of TLR2 sensitization**

To test whether the altered reactivity (TLR2 hypersensitization) involved also signaling cascades different from the NF-κB system,
we examined the phosphorylation of c-Jun in the nucleus as another downstream signal linked to TLR2 and many inflammatory receptors. According to this readout, prestimulation with CCM resulted in increased reactivity to FSL1 compared with non-stimulated cells (Fig. 4A). We used pharmacological inhibitors to verify that we, indeed, measured two different and independent responses to TLR2 stimulation. The mixed lineage kinase inhibitor CEP1347 (39, 57) blocked the c-Jun response without affecting responses to TLR2 stimulation. The prevention of TLR2 surface expression by BFA, as measured by specific intervention during the induction phase, we used pharmacological inhibitors to verify that we, indeed, measured two different and independent responses to TLR2 stimulation. The mixed lineage kinase inhibitor CEP1347 (39, 57) blocked the c-Jun response without affecting NF-κB translocation (Fig. 4B, 4C). Conversely, the NF-κB translocation was inhibited by the IκB kinase inhibitor Bay 11-7082 (Fig. 4D) or the proteasome inhibitor MG132 (not shown), but completely abolished its translocation after restimulation with FSL1 (not shown). Thus, it appears that enhanced TLR2 expression by inflammatory preconditioning translates to functional signaling via several independent downstream signal transduction pathways on stimulation with TLR2 ligands.

To examine whether TLR2 hypersensitization was also modifiable by specific intervention during the induction phase, we used several inhibitors of cell biological processes. BFA, an inhibitor of intracellular protein trafficking, was nontoxic under the test conditions and showed a clear distinction between its effect on the priming with CCM and the secondary challenge of astrocytes with FSL1. BFA did not inhibit NF-κB translocation triggered by CCM (data not shown), but completely abolished its translocation after restimulation with FSL1 (Fig. 4E). This effect correlated well with the prevention of TLR2 surface expression by BFA, as measured by FACs and immunocytochemistry (not shown). We conclude that specific processes, such as the translocation of proteins from the Golgi apparatus, are required for the sensitization to TLR2 ligands.

**Triggering of hypersensitized astrocytic TLR2 signaling by microglia**

As CCM is only an artificial experimental tool, we investigated whether microglia, or their secreted products, would be able to sensitize astrocytic TLR2 responses similar to CCM. CM was produced by stimulation of murine BV-2 microglia cells with LPS and harvesting of their culture supernatants. Transfer of CM to astrocytes produced a specific inflammatory response not observed with supernatants from resting microglia (54). The upregulation of most mRNAs coding for inflammation-related cytokines by CM was in a similar range as that triggered by CCM (Fig. 5A). For some genes (e.g., IFN-β and nos-2), significant upregulation by CM was observed, but CCM had a 20- to 30-fold stronger effect (not shown). The upregulation of TLR2 was of particular interest. CM triggered both a strong upregulation of the mRNA (Fig. 5A) and of the cell surface expression of the cognate receptor (Fig. 5B). Having established this bioactivity of CM, we tested its ability to sensitize astrocytes for TLR2 ligands. A direct comparison of CM and CCM according to our standard protocol indicated that CM-pretreated cells became similarly responsive to FSL1 (Fig. 5C) and P3C (not shown) as CCM-treated astrocytes. As an independent approach to elucidate a potential role of microglia in the TLR2 sensitization, we set up direct cocultures of primary astrocytes and microglia. For the selective activation of microglia in such a coculture (4), we used LPS. At 24 h after this initial trigger, the reactivity of astrocytes to FSL1 was tested. NF-κB translocation in cocultures was selectively measured in the astrocytes, as the cells were easily discriminated by their different nuclear size. Pure astrocytes were not affected by LPS alone, and microglia, without LPS stimulation, were not able to sensitize astrocytes. Hypersensitization of astrocytes to FSL1 occurred only by coculture with microglia and additional LPS stimulation (Fig. 5D). Similar data were obtained in cultures in which the cell populations were separated in transwells (not shown). These results suggest that TLR2 sensitization of astrocytes occurs in brain via their interaction with activated microglia.

**TLR2 sensitization by IL-1β**

To define some of the functional mediators in CM responsible for activation of astrocytes, we used IL-6 release as an easily quantifiable end point. CM was preincubated for 1 h with a neutralizing anti-TNF-α Ab, or an IL-1ra, or both, before it was used to stimulate astrocytes. We found that IL-6 release of astrocytes was significantly reduced by either anti-TNF-α Ab or IL-1ra, respectively, and neutralization of both TNF-α and IL-1 prevented IL-6 release completely (Fig. 6A). As follow-up of these experiments, we directly investigated the role of these cytokines for TLR2-ligand hypersensitization. Astrocytes were prestimulated with TNF-α (10 ng/ml), IL-1β (10 ng/ml), IFN-γ (20 ng/ml), or mixtures thereof, before restimulation with FSL1. Stimulation with TNF-α plus IL-1β, and even with the individual cytokines, was sufficient to sensitize astrocytes to 10 or 100 ng/ml FSL-1 (Fig. 6B). Prestimulation with IFN-γ alone had no effect. As TNF-α has been ascribed a dominant and essential role for TLR2 up-regulation on astrocytes (27) exposed to S. aureus, we investigated here whether the apparently independent sensitization by IL-1β may be caused by the triggering of autocrine TNF-α release. Anti-TNF-α Ab was used to neutralize any such effect during the IL-1β stimulation. The Ab was used at a concentration known to neutralize TNF-α concentrations of up to 50 ng/ml and to block all TNF-α responses in vivo (60, 61). The direct comparison of IL-1β prestimulation effects in the absence or presence of anti–TNF-α indicate that IL-1β alone is sufficient to fully sensitize resting astrocytes (Fig. 6C). To examine whether these functional data correlated with TLR2 expression on the cell surface, astrocytes...
were stimulated for 6 h with IL-1β in the presence of anti–TNF-α before they were washed, left for a further 18 h in the presence of anti–TNF-α, and then stained for TLR2. A significant upregulation was observed under this condition, which further supports the notion that IL-1β alone is sufficient to sensitize astrocytes to TLR2 ligands (Fig. 6D).

**FIGURE 2.** Sensitization of astrocytes to the TLR2 ligand FSL1. Astrocytes were prestimulated with CCM or not prestimulated, washed after 6 h, and restimulated or analyzed after a total time of 24 h. A, NF-κB translocation was quantified 30 min after restimulation with FSL1. B, Wild-type (wt) or TLR2−/− astrocytes, respectively, were prestimulated with CCM, washed and restimulated with 100 ng/ml FSL1, medium alone (control), or CCM. All data are means ± SEM of triplicate determinations. ***p ≤ 0.001. C, wt astrocytes and TLR2−/− astrocytes were stimulated for 24 h with or without CCM, fixed, and stained for TLR2 (green). Nuclear DNA was stained with H-33342 (red). The TLR2 expression on the cell surface was determined by immunofluorescence microscopy. D, wt or TLR−/− astrocytes, respectively, were stimulated without any washing steps with CCM or left untreated (−). TLR2 surface expression of stimulated or nontreated cells, respectively, relative to unstained cells (secondary Ab control) was determined by flow cytometry. The graph is representative for two experiments (data are means ± SD). E, wt astrocytes were stimulated with CCM and washed with medium after 6 h. TLR2 surface expression was determined by flow cytometry after a total time of 24 or 98 h, respectively. The graph is representative for two (98 h) to five (24 h) experiments. n.s., not significant.

**FIGURE 3.** TLR2 sensitization of astrocytes under continuous inflammatory conditions. A, Cells were prestimulated with medium (−) or CCM (+). They were washed or not, as indicated, 6 h after the start of the incubation. After a total of 24 h, cells were restimulated with 100 ng/ml P3C or CCM, respectively. NF-κB translocation was measured 30 min after restimulation. B, Four preincubation schemes of astrocytes (a–d) were tested. They were (a) left untreated for 72 h, (b) stimulated with CCM (hatched boxes) for 6 h, (c) 54 h, or (d) 3 × 6 h as indicated in the scheme. After CCM exposure, they were always washed and left in fresh medium (black lines). At the end of the preincubation, the cells were stimulated with 100 ng/ml FSL1 (black bars) or CCM (hatched bar). NF-κB translocation was quantified 30 min later. C, Astrocytes were stimulated with CCM for 16 h. The mRNA from stimulated and control astrocytes was extracted and analyzed by quantitative PCR. The basic expression of Tlr2 is indicated relative to Gapdh levels (white bars, left axis). The upregulation by CCM is indicated relative to unstimulated controls (black bars, right axis). D, Astrocytes were stimulated with 10 μg/ml poly(I:C), washed with medium after 6 h, and restimulated with FSL1 18 h later. NF-κB translocation was quantified 30 min thereafter. All data are means ± SEM of triplicate determinations. ***p ≤ 0.001. n.s., not significant.
Functional consequences of TLR2 hypersensitization in inflammatory astrocytes

The signaling-based, single-cell experimental system allowed us to examine for the first time the consequences and mechanisms of sequential inflammatory stimulation in glial cells. However, because of the apical signal transduction end points chosen here, it is not clear whether the differences we observed (NF-κB translocation or c-Jun phosphorylation) have further functional consequences. To address this, we investigated whether TLR2 hypersensitization of astrocytes alters their production of inflammatory mediators. The pattern of protein release of astrocytes, prestimulated with CCM and restimulated with FSL1 for 8 h, was characterized with a "cytokine antibody array." The supernatant of

FIGURE 4. Independent sensitization of two signaling pathways. A, Astrocytes were prestimulated with CCM, washed, and restimulated with 10 ng/ml FSL1 after 24 h. After the times indicated, the cells were fixed, immunostained for phospho-c-Jun and imaged. The mean pixel intensity in the nuclear region was determined by quantitative imaging. *p ≤ 0.05 versus 0 min time point. B, Astrocytes were prestimulated with CCM, washed, and restimulated with FSL1 in the presence (dashed) or absence (solid line) of CEP 1347 (500 nM). The cells were fixed 1 h later, immunostained for phospho-c-Jun, and the mean nuclear pixel intensity was quantified. C, Astrocytes were treated as in B. NF-κB translocation was quantified 30 min after restimulation with FSL1. D, Astrocytes were prestimulated with CCM, washed, and restimulated with FSL1 in the presence (dashed) or absence (solid line) of Bay11-7082 (5 μM). NF-κB translocation was quantified 30 min later. E, Prestimulated astrocytes were coincubated with BFA (10 μg/ml) or solvent control until restimulation. NF-κB translocation was determined by automated imaging 30 min after restimulation with FSL1. All data are means ± SEM from triplicate determinations.

FIGURE 5. TLR2 sensitization of astrocytes by microglia. A, Astrocytes were stimulated for 4 h with CCM or CM from BV-2 cells (stimulated for 4 h with LPS). Then the mRNA was extracted from the astrocytes and analyzed by quantitative PCR. B, Astrocytes were stimulated for 24 h with CM. TLR2 expression of stimulated or nontreated cells relative to unstained cells (isotype control) was determined by flow cytometry. C, Astrocytes were prestimulated for 6 h with CM or CCM, respectively, washed, and restimulated with FSL1 18 h later. NF-κB translocation was determined 30 min after restimulation. D, Astrocytes were cultured alone (white bars) or cocultured with primary microglia (black bars). For microglia activation, LPS (50 ng/ml) was added for 6 h, as indicated. Cells were washed after 6 h and further incubated for 18 h, followed by restimulation with 30 ng/ml FSL1. NF-κB translocation was determined after 30 min only in astrocytes. Data are means ± SEM from triplicate determinations. ***p ≤ 0.001. n.s., not significant.

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resting nontreated astrocytes was used as baseline for normalization of the relative increase in secretion. Across three separate experiments, we consistently observed the same three types of inflammatory response: the release of the first group of cytokines/chemokines, comprising IL-6, CCL-20 (Mip-3a), and CXCL-2 (Mip-2), was highly upregulated in supernatants from astrocytes prestimulated with CCM and challenged with FSL1. Stimulation with FSL1 only, or the CCM treatment alone, had only small effects. This response mirrored the NF-κB signaling data of this study and showed that TLR2 sensitization has downstream functional consequences (Fig. 7A). The second type of response was seen for CXCL1 and CCL2 (MCP-1). These factors were strongly secreted on TLR2 stimulation, irrespective of whether the cells had been presensitized (Fig. 7B). Secretion of a third group of proteins, comprising CCL5 (RANTES) and soluble CD106 (VCAM-1), was not triggered by FSL1 alone. The stimulation with CCM provoked a prolonged maximal response that was still ongoing when cells were restimulated, so that sensitizing effects were not measurable (Fig. 7B). To examine the consequences of TLR2 sensitization also on transcriptional level, we measured the mRNA for three inflammation-related enzymes. To avoid interference from the first stimulus, we performed restimulation after 3 d. The levels of Nos-2, Cox-2, and Tyki were strongly upregulated in cells prestimulated with CCM and then challenged with FSL1, compared with cells that had not been prestimulated (Fig. 7C).

To confirm the functional sensitization of astrocytes by an independent approach, we chose to measure the well-quantifiable IL-6 production of astrocytes after prestimulation with CCM and restimulation with the TLR2 ligand P3C. The standard incubation scheme of the signaling studies was used, and IL-6 release was determined by ELISA at several time points after the restimulation. Preactivated astrocytes exposed to P3C secreted high amounts of IL-6 into the medium, whereas CCM or P3C alone had only minor effects (Fig. 7D). This experiment was repeated for the 8-h time point also with FSL1 (not shown), and the results were essentially similar to those shown for P3C. Taken together, these data suggest that the TLR2 hypersensitizing effects of cytokines on astrocytes, characterized in detail for NF-κB translocation, correlate with functional changes such as the augmentation of IL-6 release.

Discussion

All cells react to their environment with plastic changes that eventually alter their phenotype and function. Although astrocytes constitute the major cell population in the brain capable of a strong inflammatory response, surprisingly little is known about their response to stimuli after previous exposure to cytokines derived from microglia. We found in this study that sensitization to TLR2 ligands represents a specific, sensitive, and robust functional “memory” of previous exposure to microglia-derived TNF-α or IL-1β, which lasts for several days. Molecularly and mechanistically, this was reflected by upregulated TLR2, which, when activated by adequate ligands, augmented NF-κB translocation, increased c-Jun phosphorylation, and enhanced secretion of cytokines and chemokines. In contrast with the more common studies on the direct downstream effects of inflammatory activation, we had to overcome technical issues arising from the overlap of first and second signal to obtain unambiguous data from repeated stimulation. For instance, using transcriptional regulation as end point turned out to be difficult, as the activated cytokine responses in astrocytes take longer than 24 h to return to baseline (13) (not shown). This is also true for the secretion of some factors, such as RANTES and VCAM, which remained fully active.

**FIGURE 6.** The role of IL-1β in TLR2 sensitization. A, CM was preincubated for 1 h with anti–TNF-α Ab in the additional presence (white bars) or absence (black bars) of IL-1ra (10 μg/ml). Astrocytes were then stimulated for 8 h with these mixtures before IL-6 release was determined by ELISA in the supernatant. B, Astrocytes were prestimulated for 6 h with medium (−) with CCM or with combinations of its components. The cells were washed and restimulated 18 h later with FSL1. The activation of the cells was determined by measurement of NF-κB translocation after 30 min. C, Astrocytes were pretreated with CCM, IL-1β, or IL-1β together with neutralizing anti–TNF-α Ab, respectively, and washed after 6 h. Anti–TNF-α Abs were readded to the medium after wash. Cells were restimulated with FSL1 after a total time of 24 h. The NF-κB translocation was determined 30 min later. Data are means ± SEM of triplicate determinations. D, Astrocytes were treated with CCM or 10 ng/ml IL-1β plus anti–TNF-α Abs, respectively, and washed after 6 h. TLR2 expression was determined by flow cytometry after a total incubation time of 24 h. The graph is representative of two to five experiments. n.s., not significant.
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for more than a day (Fig. 7). The setup of the NF-κB translocation assay on a single-cell level allowed a high time resolution, distinction between responses of different cell types in cocultures, and a high throughput, also under conditions of limited supply of relatively pure primary astrocytes. After optimization of the system with help of the NF-κB readout, we also evaluated functional implications, for example, IL-6 secretion and the release of CCL-20 and CXCL-2. To our knowledge, this is the first study in brain cells that addresses the altered signaling and functional response to stimulation of a TLR after pre-exposure to inflammatory cytokines.

Chronic neurodegenerative diseases are usually accompanied by long-lasting neuroinflammation, and even short insults such as stroke or trauma trigger an inflammatory reaction that may last up to several months (15, 16, 29). Under such conditions, glial cells alter their phenotype, and some of these changes may determine whether the inflammatory response is terminated or continues. As feedforward cycles between microglia and astrocytes may lead to neuronal death (62), understanding of the functional implications of the altered phenotype is of utmost importance to understand disease pathology and pharmacological intervention possibilities. For instance, it has been demonstrated that the signaling of CD95 or of adenosine receptors is altered on preactivated astrocytes, in a way that would provide mechanisms to limit an inflammatory response (4, 59). Our new findings of TLR2 sensitization provide a potential explanation why inflammatory events may continue and even become enhanced. Interestingly, sensitization to other TLR ligands was not observed, and the response to CCM was desensitized in continuously exposed cells. Some TLR2 specificity is suggested by the observation that it was the most strongly up-regulated receptor. This suggests that it may take an endogenous role (29) apart from sensing of PAMPs. For instance, involvement of TLR2 surface expression by BFA completely abolished astrocyte translocation. Currently, the sensitizers IL-1β, CM, and CCM all upregulated TLR2 transcription and surface expression. Moreover, prevention of TLR2 surface expression by BFA completely abolished astrocyte sensitization. However, some discordance between FACS data and sensitization data were also observed. For instance, high concentrations of TLR2 ligands triggered some NF-κB response also in naive astrocytes apparently devoid of receptor expression. This desensitization data were also observed. For instance, high concentrations of TLR2 ligands triggered some NF-κB response also in naive astrocytes apparently devoid of receptor expression. One of the key findings triggering this study was the strong transcriptional regulation of TLR2 by CCM and other stimuli (13, 24). However, a functional role of this needed to be shown, as also factors repressing TLR signaling, such as IκB and SOCS1-3, were upregulated (13) and receptor mRNA expression is a poor predictor for activity. For instance, in vivo TLR2 can be strongly upregulated on microglia but not contribute to the functional response (65). In the current study, we see some correlations of receptor upregulation with the sensitization response. For instance, the sensitizers IL-1β, CM, and CCM all upregulated TLR2 transcription and surface expression. Moreover, prevention of TLR2 surface expression by BFA completely abolished astrocyte sensitization. However, some discordance between FACS data and sensitization data were also observed. For instance, high concentrations of TLR2 ligands triggered some NF-κB response also in naive astrocytes apparently devoid of receptor expression. This sensitization was paralleled by a functional response for certain readouts (e.g., CXCL-1 induction). When cells were followed for 6 d after priming by CCM, FACS analysis indicated a return of surface TLR2 levels close to controls, whereas the NF-κB response was still significantly enhanced after restimulation with FSL1 (data not shown, n = 2 experiments with all respective controls). Finally, IL-1β triggered TLR2 surface expression only in a subpopulation of cells, whereas nearly all individual astrocytes responded with enhanced NF-κB translocation. Currently, we can only speculate about an explanation for these apparent discrepancies. Most likely, a subpopulation of functional TLR2 receptor complexes is accessible to small ligands but masked for immunostaining. In addition, TLR2 receptor complexes may take on different functionality depending on the cellular context.

FIGURE 7. Functional consequences of TLR2 sensitization in astrocytes. Astrocytes were prestimulated with CCM for 6 h, washed, and restimulated after 24 h with TLR2 ligand for 8 h. The corresponding controls were stimulated with medium only (solvent). A/B. The supernatants were analyzed with Ab arrays for different cytokines. The relative cytokine expression compared with supernatants of nontreated samples (negative control) is displayed. Examples for a hypersensitized response are shown under A. Examples for cytokines with different behavior are shown under B. Data are means ± SEM from three experiments, with ***p ≤ 0.001. C. Astrocytes were prestimulated with CCM for 6 h, washed, and restimulated 72 h later with 10 ng/ml FSL1 for 4 h. The mRNA was extracted and analyzed for inducible NO synthase (iNOS/Nos-2), inducible cyclooxygenase (Cox-2/PtgS2), and LPS-inducible thymidylate kinase EC 2.7.4.14 (TYK2/Cmpk2) by quantitative PCR. The relative expression was evaluated for inducible NO synthase (iNOS/Nos-2), inducible cyclooxygenase (Cox-2/PtgS2), and LPS-inducible thymidylate kinase EC 2.7.4.14 (TYK2/Cmpk2) by quantitative PCR. The relative expression compared with nontreated samples is displayed. D. IL-6 was measured by ELISA in supernatants of astrocytes at different times after restimulation with 10 ng/ml P3C. Data are means ± SEM from three experiments, with ***p ≤ 0.001.
Receptors that do not signal are commonly observed in many situations. However, it is known from apoptosis research that normally ineffective receptor stimulation can be sufficient to trigger a maximal response when the transduction machinery is sensitized (58, 60, 66, 67).

We found in this study that both TNF-α and IL-1β can sensitize astrocytes, whereas IFN-γ plays no role. In contrast, IFN-γ acts strongly synergistically with TNF-α and IL-1β, and IL-1β alone shows only minor effects in the induction of IL-6 or the sensitization to CD95L (4). Thus, IL-1β takes a special role in TLR2 hypersensitization in contrast with other astrocyte activation reactions. One concern on that observation may be the triggering of autocrine TNF-α by IL-1β, as suggested for TLR2 upregulation by S. aureus in later passage astrocytes (27). However, we found that IL-1β still sensitized astrocytes and upregulated TLR2 when TNF-α was blocked by neutralizing Abs. This is in agreement with several studies claiming a dominant role of IL-1β in astrocyte stimulation (5). It is remarkable that TLR2 hypersensitization clearly differs from other activation responses in astrocytes, with respect to the optimal combination of cytokines, and that maximal TLR2 sensitization can occur under conditions where inducible NO synthesis is not induced at all and IL-6 triggered to only a very small degree (4).

Sensitization to immune mediators is more rarely observed than the opposite response, that is, tolerance. In cells of the macrophage lineage, prestimulation or a tumor environment can trigger reprogramming to a rather anti-inflammatory M2 state. This has also been observed for microglia (46, 47, 49) and would make sense to avoid chronic inflammation, tissue damage, or abscess formation. A continued immune surveillance would be ensured by the high turnover of microglia, and invasion of peripheral bone marrow-derived cells (5). Astrocytes, presensitized to be activated by damage-associated molecular patterns via TLR2 (24), would contribute to the attraction and accumulation of microglia/macrophages via their particularly high capacity to secrete chemokines (13). Although tissue astrocytes are difficult to phenotype because of their extreme heterogeneity and the absence of a good marker for all subtypes (68), TLR2 upregulation in disease situations has been described (24, 26). More functional studies on the in vivo role will be required.

Up to now, the many direct consequences of a primary stimulation of astrocytes have been characterized in great detail (5, 27, 69). The next logical step toward an understanding of chronic neuroinflammation and the multiple activation states cells can take is the study of the reactions of astrocytes in different preactivation states. Large phenotypic alterations are suggested by the few existing studies showing an increased resistance to oxidative stress, altered CD95 signaling, or an altered inflammatory modulation by adenosine (4, 59, 69). However, much more work will be required to examine whether prestimulated astrocytes show different pharmacological properties or whether their response to endogenous damage signals is altered. Different behavior in disease models and human brain will need investigation, and the elucidation of the molecular correlates of altered responses will require robust models, such as the one developed for this study.

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Disclosures
The authors declare no conflict of interest.