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*J Immunol* 2011; 186:3237-3247; Prepublished online 31 January 2011;
doi: 10.4049/jimmunol.1002787
http://www.jimmunol.org/content/186/5/3237
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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 response 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 assume 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-Lys4 (Pam3CysSK4 [P3C]) 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 neuregenerative, 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. hyaluronic (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 reseeded in DMEM plus 10% FCS, containing 4.5 g/L D-glucose and 2 mM glutamine and cultured in multiwell dishes for 7–9 d before use in experiments. This protocol was shown to result in microglia or oligodendrocytes contaminations below our detection limit (<1%) by FACS, PCR, or immunostaining.

Microglia-enriched glial cultures. Primary astrocyte cultures were prepared without the Percoll gradient purification step. After 2 wk, they were briefly exposed to trypsin to remove only loosely attached cells. The detached cells were collected, washed, and reseeded for further characterization (F4/80 and CD11b staining) and for experiments (12). The cultures contained about 80% microglia.

Microglia were stimulated with 50 ng/ml LPS (from Salmonella abortus equi; BioCloth, Aidenbach, Germany). Astrocytes were stimulated with a complete cytokine mix (CCM) containing 10 ng/ml TNF-α, 10 ng/ml IL-1β, and 20 ng/ml IFN-γ (R&D Systems, Wiesbaden, Germany). For prestimulation, cells were incubated with CCM or its individual con-

stituents for 6 h, washed three times with culture medium, and further incubated for 18 h (or as indicated in the figures) in culture medium until restimulation. For restimulation, cells were incubated with CCM, the artificial triacyl lipopolysaccharide S-[2,3-bis(palmitoyloxy)-2-RS-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4-ΟΗ × 3 HCl (P3C; EMC microcollections, Tübingen, Germany) or the Mycoplasma salivarium-derived diacyl lipopolysaccharide S-[2,3-bis(palmitoyloxy)-2-RS-propyl]-(R)-Cys-GDPKHPKSIF (F5L1) (Invivogen, San Diego, CA) for 30 min (or as indicated in the figures) until measurement. Furthermore, for restimulation experiments, the following PAMPs were used: Iniquinomed-R837, polyinosinic-polycytidylic acid (poly[I:C]; Invivogen), and unmethylated CpG-containing oligonucleotide-DNA (sequence: 5′-TCCATGACGTTCCTGAGCT-3′; Microsynth, Balgach, Switzerland). IL-1 receptor antagonist (IL-1ra) and high-mobility group box 1 protein were purchased from R&D Systems (Wiesbaden, Germany); muramyl dipeptide and brefeldin A (BFA) were purchased from Sigma-Aldrich (Steinheim, Germany). The 1-β interferon (Bay11-7082) (Merck, Darmstadt, Germany) and CEP1547 (Cephalon, Westchester, PA) (39, 56, 57) were added 30 min before the stimulus and 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 (Langenselbold, 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-bound RayBio Mouse Cytokine Ab Array 3 kit (RayBiotech, Norcross, GA) as described earlier (12). Signals were visualized using a Fusion FX-7 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, Alasdair 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 percentage 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-Alexa-488 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–c-Jun Ab (clone KM-1; final dilution 1:700; Santa Cruz Biotechnology), and detected with anti-mouse IgG Alexa-488
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The threshold cycle (CT) was determined for each sample. The housekeeping gene was quantified using the Platinum SYBR Green qPCR SuperMix-UDG, as described.

The nuclear-cytoplasmic ratio of the NF-κB p65 Ag signal intensity was quantified by dividing the mean average Ag intensity in the nuclear area as 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 the experiments. All findings were reproduced at least once (usually two to three times).

The NF-κB translocation. The nuclear-cytoplasmic ratio of the NF-κB p65 Ag signal intensity was quantified by dividing the mean average Ag intensity in the nuclear area as 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 obtained automatically from the reference wells containing untreated control cells.

C-Jun phosphorylation. The signal intensity of fluorescently labeled phospho–c-Jun Ag in the nucleus was quantified by determining the mean average Ag intensity of the nucleus. The signal intensity of the cytoplasmic area (ring) was not determined because it was frequently close to background. Quantifications were performed in at least 200 cells per field.

BV-2 conditioned medium
Conditioned medium (CM) from the murine microglia cell line BV-2 has been characterized earlier (54). Cells were seeded in a T175 flask and incubated overnight in DMEM and 2% FCS. Cells were treated with 50 ng/ml LPS in 10 ml DMEM plus 2% FCS for 4 h. After incubation, the supernatant (i.e., CM) was filtered with a 0.22-μm sterile filter to remove cells and cell debris. For LPS-control medium, BV-2 were incubated for 4 h with medium only. LPS (50 ng/ml) was then added to the medium after removal and filtering. Aliquots were frozen in liquid nitrogen and stored at −80°C.

RNA extraction, RT-PCR, and quantitative PCR
Total RNA was extracted with TRIzol from Invitrogen, and 1 μg total RNA was reverse transcribed with Superscript II Reverse Transcriptase (Invitrogen) using random hexamers and Oligo-dT-Primer in a 20-μl reaction according to the manufacturer’s protocol. PCR amplification of the cDNA was quantified using the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). With the iCytoMyq qPCR detection system (BioRad Laboratories, Hercules, CA) and corresponding data analysis software, the threshold cycle (Ct) was determined for each sample. The housekeeping gene was control, gapdh. The relative cDNA levels were compared among different groups using the δCt method. Absolute levels were calculated by using as Ct for calculations (57). Primers used were gapdh sense (NM_008084): 5'-TGC ACC ACC AAC TCA G-3', anti-sense: 5'-GGA TGG ACC GGT CAT GCT C-3'; filha sense (NM_013693): 5'-CTA TGG ACC AGA CCC TCA CAC TCA-3', anti-sense: 5'-CAT TCC AGC TGT GCC TTC-3'; c-Jun sense (NM_031252): 5'-CAT GCA CCC GGA CAT ATG-3', anti-sense: 5'-CAG ACC TTC GCG GGC CCT-3'.

Statistics
NF-κB translocation data and phospho–c-Jun data are based on at least 200 valid cells per well and are indicated as the means ± SEM of at least three wells. Flow-cytometry analysis was done in duplicates. Cytokine arrays were performed in triplicates using material from independent biological experiments. All findings were reproduced at least once (usually two to four times) in an experiment using an independent cell preparation, and representative data sets are shown. Statistical analysis was performed with GraphPad Prism software (version 4.03), using t test or one-way ANOVA with Bonferroni’s post hoc test, as appropriate.

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).

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 pretimulation of
the cells with CCM. The percentage of cells activated by restimulation with a TLR2 ligand was nearly as high as that activated by CCM restimulation. 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 *Iifn-β* and *Ccl2*, and an 52 ± 6-fold increase of *Ifr-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 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), whereas these two inhibitors did not reduce the c-Jun response (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).
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.”

![FIGURE 4. Independent sensitization of two signaling pathways.](image)

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 co-incubated 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.](image)

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.

**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
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.

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.
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 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 naïve astrocytes apparently devoid of receptor expression. This translocation 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 duplicate determinations on each array. *p ≤ 0.05; **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 (TYKi/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 all respective readouts, 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.

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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, 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 absciss formation. A continued immune surveillance would be ensured by the high turnover of microglia, and invasion of peripheral bone marrow-derived cells (6). 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 clearly differed from other activation responses in astrocytes, with respect to stimulation (5). It is remarkable that TLR2 hypersensitization

References

Acknowledgments
We are indebted to many colleagues for valuable contributions and insightful discussions during the course of this work. B. Schimmelpennig provided excellent technical assistance.

Disclosures
The authors declare no conflict of interest.


