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Toll-like receptor expression and activation in astroglia: differential regulation by HIV-1 Tat, gp120, and morphine

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Abstract

In this study we determine whether morphine alone or in combination with HIV-1 Tat or gp120 affects the expression of Toll-like receptors (TLRs) by astrocytes and to assess whether TLRs expressed by astrocytes function in the release of inflammatory mediators *in vitro*. TLR profiling by immunofluorescence microscopy, flow cytometry, in-cell westerns, and RT-PCR showed that subpopulations of astrocytes possessed TLR 2, TLR3, TLR4, and TLR9 antigenicity. Exposure to HIV-1 Tat, gp120, and/or morphine significantly altered the proportion of TLR-immunopositive and/or TLR expression by astroglia in a TLR-specific manner. Subsets of astroglia displayed significant increases in TLR2 with reciprocal decreases in TLR9 expression in response to Tat or gp120 ± morphine treatment. TLR9 expression was also significantly decreased by morphine alone. Exposing astrocytes to the TLR agonists LTA (TLR2), poly I:C (TLR3), LPS (TLR4) and unmethylated CpG ODN (TLR9) resulted in increased secretion of MCP-1/CCL2 and elevations in reactive oxygen species. TLR3 and TLR4 stimulation increased the secretion of TNF- α , IL-6, and RANTES/CCL5, while activation of TLR2 caused a significant increase in nitric oxide levels. The results suggest that HIV-1 proteins and/or opioid abuse disrupt the innate immune response of the central nervous system (CNS) which may lead to increased pathogenicity.

Keywords

innate immunity; opioid abuse; cytokines; free radicals; HIV-1 proteins; glia; neuroplasticity

INTRODUCTION

Alteration of the innate immune response and the corresponding release of inflammatory molecules are pivotal in the progression of many central nervous system (CNS) diseases including neuroAIDS. The inflammatory process is characterized by microglia-mediated superoxide production, nitric oxide (NO) release, and the release of pro-inflammatory cytokines and chemokines by both activated microglia and astrocytes (Akira and Kishimoto, 1992; Becher et al., 2000; Fuller et al., 2009; McGeer and McGeer, 2004). Toll like

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Receptors (TLRs), a major family of the pattern-recognition receptors (PRRs) that recognize conserved viral, bacterial, and fungal particles (Bsibsi et al., 2002; Iwasaki and Medzhitov, 2010; Medzhitov and Janeway, 1999; Takeda et al., 2003) are expressed in glia (microglia, astrocytes, and oligodendrocytes) and neurons (Bsibsi et al., 2006; Carpentier et al., 2005; Jack et al., 2005; Laflamme and Rivest, 2001; Lafon et al., 2006; McKimmie and Fazakerley, 2005; Riveccio et al., 2006). The presence of CNS pathogens activate TLRs, whose signals are amplified (Akira et al., 2003; Jack et al., 2006; Lafon et al., 2006; Zekki et al., 2002) through MyD88-dependent and/or Toll/Interleukin-1 receptor (TIR) domain-containing adapter-inducing interferon- β (TRIF)-dependent pathways (Bafica et al., 2004; Doyle et al., 2002; Yamamoto et al., 2003).

Drug abuse with injectable opioids, such as heroin, is a major cause of the spread of human immunodeficiency virus type-1 (HIV-1) (Hauser et al., 2007; Nath et al., 2002; Sheng et al., 1997). In addition to disease propagation, histopathologic evidence suggests that chronic opioid abuse intrinsically enhances the severity of CNS complications of HIV-1 by almost four-fold (Bell et al., 1998), which is noteworthy since 15–40% of non-substance abusing AIDS patients are estimated to display significant HIV encephalitis (HIVE). Morphine, acting through μ -opioid receptors (MORs) on astrocytes, can potentiate Tat-induced increases in intracellular Ca^{2+} , NF- κ B activation, and in the release of IL-6, RANTES, and MCP-1 (El-Hage et al., 2005, 2006a, 2006b, 2008a), which directly affects macrophage recruitment and activation (Takeda et al., 2003). In independent research utilizing CCR2 or RANTES (CCL5) knockout mice, we showed that deletion of either gene alone is sufficient to reduce Tat $\pm$ morphine-induced astrogliosis and microgliosis, suggesting that astroglia contribute to the intensification of HIV-1 neuropathogenesis in opioid abusers (El-Hage et al., 2008a, 2008b; Gendelman et al., 1997; Kaul et al., 2001).

Until recently, only limited studies have evaluated the role of TLR-pathogen interactions in regulating HIV-associated disease. HIV-1 may interact directly with specific TLRs (Akira et al., 2003; Bsibsi et al., 2006; Salaria et al., 2007), or indirectly by affecting the TLR response of the host to other co-infecting pathogens, while HIV-1 uses the host defense to its advantage to infect immune cells and alter the immune response (Dong and Benveniste, 2001). In addition to the highly conserved structural motifs, known as pathogen-associated molecular patterns (PAMPs) that bind TLRs, several endogenous molecules reportedly activate the innate immune system in a TLR-dependent manner (Kariko et al., 2004; Tsan and Gao, 2004). Recent studies have suggested that opioid antagonists can activate glia through direct interactions with TLR4 and that this receptor serves overlapping pain functions with opioids (Hutchinson et al., 2007, 2008), inferring an antinociceptive role for TLR4 or the overlap of opioid and TLR4 signaling pathways.

Collectively, the established role of TLRs in innate immunity and in the response to HIV-1, combined with reported interactions of TLRs with opioids and opioid pain pathways, prompted us to explore the possible interactive effects of opioids and HIV-1 proteins on TLR expression and evaluated the consequences of TLR activation on free radical production and cytokine release by astrocytes. The findings confirm the presence of functional TLR expression by astrocytes and further implicate astrocytes as contributing to CNS immunity and inflammation. We found that exposure to morphine, HIV-1 Tat and/or gp120 is associated with changes in TLR expression, which may partially explain how opioids mediate the response of the CNS to the effects of HIV-1 and perhaps other pathogens.

MATERIALS AND METHODS

Reagents

Morphine sulfate was obtained from the National Institute on Drug Abuse (NIDA, Drug Supply System, Bethesda, MD). Tat₁₋₇₂ recombinant protein was obtained from Phil Ray (University of Kentucky) and recombinant X4-tropic gp120 HIV_{IIIB} was obtained from Immunodiagnostics, Inc. (Woburn, MA). The following ligands were used: TLR2 ligand: lipoteichoic acid (LTA; 0.1–100 µg/ml; InvivoGen, San Diego, CA); TLR3 ligand: polyinosinic-polycytidylic acid (Poly I:C; 0.1–25 µg/ml; Sigma-Aldrich, St. Louis, MO); TLR4 ligand: lipopolysaccharide (LPS; 1–25 µg/ml; Sigma); TLR5 ligand: recombinant flagellin (1–10 µg/ml; InvivoGen); and TLR9 ligand: unmethylated CpG containing oligonucleotides (ODN; 0.5–5 µM; InvivoGen). The specific concentrations of TLR ligands used in these studies were based on previous *in vitro* studies from others (Ashkar et al., 2003, 2004; Gill et al., 2006; MacDonald et al., 2007). Antibodies directed against the TLRs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-GFAP was purchased from Chemicon (Billerica, MA). Goat anti-mouse IRDye 800CW and goat anti-rabbit IRDye 680 were purchased from LI-COR Biosciences (Lincoln, NE), and goat anti-mouse TRITC and goat anti-rabbit FITC were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The specific concentrations of the primary and secondary antibodies used in these studies were based on manufacturer recommendations.

Cell culture

Astroglial-enriched cultures were prepared using one- to four-day-old ICR mice (Charles River Laboratories, Charles River, MA) as previously described (Stiene-Martin et al., 1998). Briefly, mice were anesthetized and euthanized according to NIH and IACUC guidelines. Striata were aseptically isolated, minced in media, incubated with trypsin/DNase (37°C, 30 min), triturated through a series of decreasing bore pipettes and filtered sequentially through 135 µm and 35 µm pore nitex filters. Cells for each experiment were pooled from the striata of two to four mice. Growth medium favoring astroglial enrichment consisted of DMEM (Dulbecco's Modified Eagle's Medium, Gibco, Grand Island, NY). DMEM was supplemented with glucose (27 mM), Na₂HCO₃ (6 mM), 10% (v/v) fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS or Hyclone, Logan, UT) and penicillin/streptomycin (50 U/50 µg). Cells were grown for 10–14 days until they reached 80–90% confluency at 37°C, 5% CO₂. To obtain purified astrocytes, glial cells were incubated for 90 min in 10 mM L-leucine methyl ester (LME) (Sigma-Aldrich). After addition of LME, cultures were visually inspected to ensure maximal microglial lysis with minimal toxicity to astrocytes. Astrocytes were then washed and resuspended in glial culture medium. Purity of astrocyte cultures was determined by the percentage of GFAP-immunofluorescent cells by flow cytometry and was routinely ≥ 95%.

Experimental treatments

Recombinant Tat₁₋₇₂ was produced and purified as described previously (El-Hage et al., 2005). Briefly, the *tat* gene encoding the first 72 amino acids of HIV-1_{BRU} (obtained from Dr. Richard Gaynor, through the NIH AIDS repository) was inserted into an *Escherichia coli* Pin Point Xa-2 vector (Promega, Madison, WI). Biotinylated Tat was purified on a column of soft release avidin resin, cleaved from the fusion protein using factor Xa, eluted and desalted using a PD10 column and treated with Detoxi-Gel (Pierce, Rockford, IL). A reticulocyte amoebocyte lysate assay was used to insure the absence of trace amounts of endotoxin (Associates of Cape Cod, Inc. East Falmouth, MA). Cells were continuously exposed to untreated medium (control) or medium containing morphine sulfate (500 nM), Tat₁₋₇₂ (100 nM), or gp120 (500 pM) alone or in combination or alternatively with escalating concentrations of TLR2, 3, 4, or 9 ligands LTA (0.1, 1, 10, and 100 µg/mL), Poly

I:C (5, 10, and 25 $\mu\text{g/mL}$), LPS (1, 5, 10, and 25 $\mu\text{g/mL}$), or ODN (0.5, 1, 2.5 and 5 μM), respectively at various time points. The drug and viral protein concentrations used were based on previously published values.

RT-PCR

Cells were treated with media alone or media containing morphine or HIV-1 proteins alone or in combination for 30 min, 6 h or 12 h. For semi-quantitative RT-PCR, total RNA was isolated from treated cells using GenElute™ Mammalian Total RNA kit (Sigma). cDNA was synthesized from 1 μg of total RNA using the RETROscript kit from Ambion (Austin, TX). PCR was performed using Hot Master Taq DNA Polymerase and buffers from 5-prime (Gaithersburg, MD) and primer sets for TLR2: 3'-AAG TGA AGA GTC AGG TGA TGG ATG TCG, 5'-GCA GAA TCA ATA CAA TAG AGG GAG ACG; TLR3: 3'-TCT GGA AAC GCG CAA ACC, 5'-GCC GTT GGA CTC TAA ATT CAA GAT; TLR4: 3'-CAA GTT TAG AGA ATC TGG TGG CTG TGG, 5'-TGA AAG GCT TGG TCT TGA ATG AAG TCA; TLR9: 3'-CCA CAC CAA TGC CTT TCA GAA, 5'-TGG CTT CTG ACA GCG TTG AAG. RT-PCR was performed using the PTC-200 (MJ Research). TLR mRNA was normalized to β -actin.

In-cell Westerns

Cells were grown on 24-well plates and treated with morphine or HIV proteins alone or in combination for 30 min, 6 h, or 12 h. Subsequently, media was removed and cells were washed with cold PBS, fixed in 4% paraformaldehyde, permeabilized with PBS containing 0.1% Triton-X 100, 0.1% BSA and blocked in 1.5% goat serum in PBS for 1 h. Simultaneous incubation of primary antibodies towards each TLR with anti- β -actin antibody was performed at 4°C overnight in blocking buffer. Reaction products for each TLR (visualized with IRDye 680LT; LI-COR Biosciences, Lincoln, NE) were normalized to β -actin (visualized with IRDye 800CW; LI-COR) using appropriate secondary antibodies. Fluorescence measurements were performed on the LI-COR Odyssey system.

Immunocytochemistry

Cells were grown on coverslips and treated with media alone or media with LTA, Poly I:C, LPS, recombinant flagellin, or ODN for 12 h. A prior report indicated that pre-exposure of TLR ligands could upregulate TLR antigenicity and hence their detection in astrocytes (Salaria et al., 2007). Cells were fixed in 4% paraformaldehyde, permeabilized in PBS containing 0.1% Triton-X 100 / 0.1% BSA and blocked in 1.5% goat serum in PBS for 1 h. Cells were incubated with anti-TLR and anti-GFAP antibodies overnight at 4 °C in blocking solution followed by 1 h incubation with goat anti-mouse TRITC and goat anti-rabbit FITC at room temperature. Nuclei were stained with 1 $\mu\text{g/mL}$ Hoechst 33258 in PBS for 20 min. Fluorescently labeled cells were visualized using a Zeiss AxioObserver Z.1 microscope, AxioVision software and MRm digital camera. Fluorescent images were deconvolved using AutoQuant X software (Media Cybernetics; Bethesda, MD).

Cytokine release assay

Cells were treated with media alone or media with Tat or increasing concentrations of TLR agonists for 30 min, 6 h or 12 h on 24-well plates. Conditioned medium from treated cells was collected post-treatment, after which cytokines were assayed using ELISAs for IL-6, TNF- α , RANTES and MCP-1 (R&D Systems; Minneapolis, MN) according to the manufacturer's instructions. Tetramethylbenzidine substrate (BD Pharmingen, San Diego, CA) was added for color development and microplates were read at 450 nm using a Victor 3 plate reader (PerkinElmer, Inc; Waltham, MA) immediately after terminating the reaction.

Cytokine levels were determined based on a standard curve with known amounts of cytokine.

Reactive oxygen species (ROS) assay

Intracellular ROS production was measured using dihydrofluorescein diacetate (DCFH-DA, Invitrogen) for cells treated with media alone, media with Tat, or increasing concentrations of TLR agonists. Cells grown in 48-well plates were treated with 10 μ M of cell-permeant dye for 45 min at 37°C in Hank's balanced salt solution (HBSS), followed by a wash with HBSS and exposure to Tat and TLR2, TLR3, TLR4 or TLR9 ligands for 90 min. ROS production was measured using a Victor 3 plate reader (PerkinElmer, Inc., Waltham, MA) at $\lambda_{\text{ex}} = 488$ nm and $\lambda_{\text{em}} = 525$ nm.

Nitric oxide production assay

Cells were treated with media alone, media with Tat, or increasing concentrations of TLR ligands for 24 or 48 h. At the indicated times, the supernatant was collected and the concentration of nitrite (the oxidized metabolite of NO) was assessed using the Griess reaction (Griess, 1879) (Promega, Madison) according to the manufacturer's instructions. Briefly, conditioned medium from treated astrocytes was added to 96-well plates along with known standards made with sodium nitrite solution. Equal volumes (50 μ L) of conditioned media, sulfanilamide solution and *N*-1-naphthylethylenediamine dihydrochloride (NED) solution were mixed and incubated at room temperature for 10–15 min, after which the absorbance at 540 nm was measured with a Victor 3 plate reader. The concentration of NO in samples was calculated based on the standard curve using known concentrations of nitrite.

Flow cytometry

Astrocytes were washed in PBS/0.1% BSA, and labeling was done in permeabilization buffer (PBS/0.1% BSA/0.1% Triton X-100) for detection of intracellular protein expression. TLR fluorescence associated with astrocytes was measured using a FACSCanto II (BD Biosciences) flow cytometer. TLR fluorescence intensity was assessed after gating on approximately 10,000 GFAP-positive astrocytes. Astrocyte autofluorescence was compensated by setting the phycoerythrin (PE) detector voltage to a minimum level that discriminates between autofluorescence and specific labeling in both negative and positive controls. Isotype control antibodies were used to define settings in histogram plot analyses. TLR expression is presented as a proportion of GFAP positive astrocytes.

Statistical analysis

All results are reported as mean $\pm$ SEM of at least 3 (typically 4–6) independent experiments. Data were analyzed using one-way analysis of variance (ANOVA) followed by appropriate *post hoc* tests. Resulting *p* values less than 0.05 were considered significant. Statistica Software (StatSoft; Tulsa, OK) was used for data analysis.

RESULTS

Localization of TLRs in individual GFAP-expressing astrocytes reveals phenotypically distinct subpopulations

The expression of TLRs by astrocytes has previously been described both *in vivo* and *in vitro* (Bowman et al., 2003; Hauser et al., 2007; McKimmie and Fazakerley, 2005; Park et al., 2006). However, these reports disagree greatly regarding whether a particular TLR is expressed by astrocytes, which perhaps results from differences in the brain region examined, the source of tissues, culture conditions, and/or the purity of astrocytes in different experiments. Specifically, astrocytes cultured from mouse and human brain tissues

can display multiple TLR types (i.e., TLR1, TLR2, TLR3, TLR4, TLR5 and TLR9). TLR3, which recognizes dsRNA (Bsibsi et al., 2006; Carpentier et al., 2005; Jack et al., 2005; Lafon et al., 2006; McKimmie and Fazakerley, 2005; Rivieccio et al., 2006), suggesting that astrocytes may be particularly important for anti-viral responses in the CNS. Many viral types have dsRNA genomes or produce dsRNA during their replication cycles. Representative immunofluorescence images derived from a number of experiments from cultured astrocytes showed that subsets of astrocytes can display TLR2, TLR3, TLR4, and TLR9 immunoreactivity (Figs. 1–2) with diminished levels seen for TLR5 (data not shown). When astrocytes were challenged with cognate ligands for TLR 2, TLR3, TLR4, and TLR9 for 12 h, respectively, there appeared to minor changes in TLR4 and TLR9 immunofluorescent intensity, while TLR2 and TLR3 antigenicity was slightly more pronounced than untreated controls (Fig. 1). This differed from a prior report in human astrocytes indicating that pre-exposure to its cognate ligand dramatically upregulated TLR4 antigenicity in human astrocytes (Salaria et al., 2007). The labeling pattern for TLR2 and TLR3 was punctate, although TLR2 showed a more perinuclear localization within the cytoplasm. By contrast, TLR4 and TLR9 antigenicities were more broadly distributed within the cytoplasm. In addition, antigenicity for a particular TLR was not uniformly present on individual astrocytes. Flow cytometric analysis was used to better quantify the relative level of expression of each TLR in astrocytes. As expected, our flow cytometry data (Fig. 2C–D) correlated with the immunofluorescence findings suggesting that TLR2 ($39 \pm 2\%$), TLR3 ($59 \pm 1\%$), TLR4 ($25 \pm 5\%$), and TLR9 ($21 \pm 2\%$) are heterogeneously expressed by astrocytes. Next we determined the extent to which different TLR types might be co-expressed on individual cells. Cells were double-labeled with antibodies to TLR2 and TLR9, TLR3 and TLR9 or TLR4 and TLR9, counterstained with Hoechst 33258, and analyzed (Fig. 2E). TLR9 was readily co-localized with TLR2, TLR3 or TLR4 in subsets of astrocytes (Fig. 2E). Despite the apparent overlap between TLR9 and the other TLRs assessed, not all permutations were performed and it remains uncertain whether other TLRs are co-expressed by astrocytes. All together, these results strongly suggest that phenotypically distinct subsets of astrocytes differ in the expression of one or more TLRs.

Analysis of TLR levels in astrocytes treated with morphine and HIV-1 proteins alone or in combination

We examined Tat, gp120 and morphine's effects on innate immunity and analyzed whether particular astrocytic TLRs might respond to exposure of morphine and HIV-1 proteins. TLR expression levels were quantitatively assessed by In-cell western analysis (Fig. 3A–B). In-cell westerns are a semi-quantitative near-infrared, immunofluorescent assay (LI-COR) to assess the relative abundance of two different proteins within cells in the same culture dish. Importantly, a protein of interest is quantified by normalization to a second protein standard. Astrocytes were treated with morphine, HIV-1 Tat, or gp120 alone or in combination for 30 min, 6 h, or 12 h, TLR fluorescence was normalized to β -actin, and reported as a percent of control levels (Fig. 3A–B). These results showed that 6 h and 12 h exposure to Tat alone or in combination with morphine caused an increase in TLR2 protein levels. Although the effect of the combined treatment of Tat and morphine was less than with Tat alone, it was significantly higher than either control or morphine treatment alone. Likewise, gp120 caused an increase in TLR2 expression above levels seen in control and morphine treated groups, although only a minimal increase in TLR2 expression was detected with combined gp120 and morphine treatment. Conversely, exposure to Tat, morphine, or gp120 alone or in combination significantly suppressed the effect of TLR9 protein levels after 12 h treatment. There were no significant changes in TLR3 and TLR4 transcript levels in astrocytes exposed to Tat, gp120, morphine or a combination thereof (data not shown).

We further determined whether changes in protein levels correlated with that of mRNA. RNA was extracted from astrocytes treated with morphine and HIV-1 Tat or gp120 proteins alone or in combination for up to 12 h and TLR expression was analyzed by RT-PCR. mRNA extracted from viral protein-treated astrocytes alone or in combination with morphine showed significant increases in levels of TLR2 (Fig. 3C). Conversely, after 12 h treatment with HIV-1 proteins or morphine alone or in combination, TLR9 mRNA levels were significantly below control level (Fig. 3D). There were no changes in TLR3 or TLR4 expression in astrocytes exposed to Tat, gp120, morphine or a combination thereof (data not shown). We further explored the possibility that viral protein or opioid drug exposure might change the proportion of TLR2 or TLR9-immunopositive astrocytes using flow cytometry (Fig. 4). Approximately 10,000 events were collected for each sample and size discrimination was used as a crude method for viability determination; one representative histogram of three independent experiments is shown (Fig. 4). Flow cytometric data showed a significant increase in the proportion of TLR2 immunopositive astrocytes following exposure to Tat (42 ± 1), gp120 (46 ± 3), or combined morphine and Tat (50 ± 2) when compared to vehicle-treated controls (33 ± 2) ($p < 0.05$; one-way ANOVA; Duncan's *post hoc* test). By contrast, although the proportion of astrocytes expressing TLR9 tended to decrease following Tat (19 ± 3), gp120 (20 ± 4), morphine (16 ± 1), morphine + Tat (16 ± 1), or morphine + gp120 (20 ± 4), none of the treatments caused a significant decline compared to vehicle-treated controls (27 ± 4). The above numbers represent the average percentage of three independent studies. All together, (i) these results show that morphine $\pm$ HIV-1 proteins can influence the expression of TLR on astrocytes, and that (ii) the expression of a particular TLR by astrocytes may be plastic and modifiable by extracellular cues or environmental factors.

Induction of inflammatory cytokines and chemokines by astrocytes following TLR treatment

We next examined the functional role of TLR activation in the production of proinflammatory cytokines and chemokines by astrocytes. This included the activation of TLR2, TLR3, TLR4 and TLR9 by specific agonists and comparing the effects of the agonist to that of media alone (negative control) or media with Tat (positive control). Following 30 min, 6 h, or 12 h of ligand treatment, conditioned medium from astrocytes was collected and assayed for the cytokines; IL-6 and TNF- α , and the chemokines; RANTES and MCP-1. Similar profiles of cytokine production were seen at both 6 h (not shown) and 12 h time points (Fig. 5), although the overall cytokine accumulation within the astrocyte-conditioned medium was higher at 12 h compared to 6 h. While a range of concentrations was assessed for each TLR ligand, the response was robust for each, even at the lowest concentrations studied, suggesting there is a very low threshold for PAMP detection by astrocytes. For this reason, one representative concentration of each ligand is shown for LTA (10 μ g/mL), Poly I:C (10 μ g/mL), LPS (1.0 μ g/mL) and ODN (5.0 μ M). In comparison to vehicle-treated controls, stimulation of astrocytes with Poly I:C and LPS led to increased levels of TNF- α , IL-6, RANTES and MCP-1 (Fig. 5C–D). Treatment with LTA caused a significant increase of MCP-1 alone (Fig. 5D), while ODN only caused increases in TNF- α and MCP-1 production as compared to control treatment (Fig. 5A, D). In comparing TLR2, TLR3, TLR4, and TLR9 stimulation with Tat-mediated activation of astrocytes, TLR4 signaling was observed to induce the strongest proinflammatory response, characterized by secretion of TNF- α , IL-6, RANTES and MCP-1 (Fig. 5A–D). These results suggest that TLR agonists activate astrocytes and trigger an innate immune response that is not homogenous but rather tailored according to a particular environmental signal.

Nitrite accumulation in astrocytes is largely unaffected by TLR agonists

NO release is involved in many biological functions such as normal and aberrant signaling (Lander et al., 1995). TLR ligands can induce NO production as part of an antiviral response in infected cells (McKimmie and Fazakerley, 2005). Therefore, we evaluated the consequences of TLR-induced activation by examining nitrite formation in astrocytes (Fig. 6). NO production was measured by the Griess assay, which is based on the accumulation of nitrite as the end product of NO metabolism. We previously found that treatment with gp120 or morphine alone or in combination had no significant effect on NO production and therefore their actions were not pursued further in the present study. After 24 h of exposure, Tat or LTA treatment caused significant accumulation of nitrite in astrocyte cultures, while cultures treatment with other TLR ligands had undetectable amounts of nitrite after 24 h of treatment. In astrocyte cultures, nitrite production was only seen following exposure to 1.0 µg/ml LTA (Fig. 6A). Thus, the results show that the TLR2 alone appears to be coupled to NO production.

TLR agonists induce ROS formation in astrocytes

ROS can control the production of the innate proinflammatory immune response through the activation of redox-sensitive signal transduction pathways such as MAPK, AP-1 and through TLR4 via their effects on NF-κB activation (Matsuzawa et al., 2005; Rao, 2001; Ryan et al., 2004; Suzuki et al., 1997). Since the TLR ligands were able to induce secretion of cytokines and chemokines in astrocytes, we evaluated the consequences of TLR agonist-induced activation of astrocytes by intracellular ROS formation. Furthermore, increases in ROS are reported to be downstream of TLR activation (Matsuzawa et al., 2005; Mustafa et al., 2009; Ryan et al., 2004). We previously found that treatment with gp120 and morphine alone or in combination had no significant effect on ROS production in astrocytes, and their actions were not examined in the present study. Compared to vehicle-treated control cells, exposure to Tat and as little as 1 µg/mL of LTA, 5 µg/mL of LPS or poly (I:C), or 1.0 µM ODN significantly increased the level of oxyradicals in astrocytes (Fig. 7).

DISCUSSION

The present findings show (i) that individual TLRs are not uniformly expressed in astrocytes and (ii) that the expression of a particular TLR may change dynamically with exposure to pathogenic insults or drug abuse. In addition, although TLR expression levels are reportedly low in astrocytes and some controversy whether astrocytes express TLRs at all (Bowman et al., 2003; Carpentier et al., 2007, 2008; El-Hage et al., 2006a; Farina et al., 2005, 2007; Hauser et al., 2007; McKimmie and Fazakerley, 2005; Riveccio et al., 2006), our results suggest that TLR expression by astrocytes can be highly dynamic and modifiable by extracellular cues. Moreover, the ability of cognate PAMP ligands to induce ROS and cytokine production indicates the TLR receptors on astrocytes are functional. Our findings corroborate recent data indicating that TLR ligands (i.e., LTA, Poly I:C and LPS), can upregulate cytokine and chemokine expression in cultured murine and rat astrocytes (Akira et al., 2000; Borysiewicz et al., 2009; De Miranda et al., 2009). Moreover, the sheer number of astrocytes in the brain and the fact that subsets of astrocytes appear to express functional TLRs, dramatically underscores the potential importance of astroglia in CNS immunity. Clearly, additional studies are needed to determine the extent to which these *in vitro* findings mimic patterns present *in vivo* and to ascertain the exact role of astroglial TLRs in neuroimmune function. However, if disrupted TLR signaling can indeed be established as a potent driving force for opioid drug-HIVE comorbid disease progression, then understanding the origins of aberrant TLR signaling may be of therapeutic benefit.

TLR activation can affect HIV-1 replication *in vitro* (Equils et al., 2001, 2003; Laflamme and Rivest, 2001; Liu et al., 2006; Scheller et al., 2004; Schlaepfer et al., 2004, 2006; Sundstrom et al., 2004), although the underlying mechanisms are not completely understood. Therefore, defects in normal TLR activation might be expected to compromise antimicrobial defenses and thereby modify disease progression. In the present study, we found enhanced TLR2 mRNA and protein levels in astrocytes treated with HIV-1 Tat, alone or in combination with morphine. Alternatively, gp120 by itself increased TLR2 protein levels at 6 h and 12 h; however in this instance, concurrent morphine exposure negated the gp120-elicited increases in TLR2. The recent demonstration that NF- κ B and TNF- α can regulate TLR2 expression in astrocytes through autocrine/paracrine feedback may provide an explanation for the increased expression of TLR2 (Phulwani et al., 2008), since both Tat and gp120 can elevate NF- κ B-dependent cytokine expression (El-Hage et al., 2008b). TLR2 upregulation may contribute to the pathophysiology of transient cerebral ischemia (Ziegler et al., 2007). Outside the CNS, Heggelund et al. (2004a), showed increased surface expression of TLR2 on monocytes of HIV-infected patients and enhanced TNF- α release in response to TLR2 ligation, while others have shown levels of soluble TLR2 disrupted in HIV-1 infection (Heggelund et al., 2004b). Lastly, our findings differed somewhat from another study by Salaria et al. (2007), who found that gp120 increased TLR4 expression by human astrocytes. The reasons for the discrepancy are uncertain but may result from (i) species differences, (ii) astroglial heterogeneity among different brain regions (Fitting et al., 2010), or (iii) inherent differences in the effects of X4, R5, or dual-tropic gp120 variants. X4 and R5-tropic variants are reported to possess unique neurotoxic profiles (Kaul et al., 2007; Bachis et al., 2010; Medders et al., 2010), and each tropic variant differentially affects TLR function. Based on the present findings X4-tropic gp120_{III}B, it is uncertain whether R5 or dual tropic gp120 variants might yield different results.

Unlike TLR2, TLR9 transcript levels decreased in astrocytes treated with Tat, gp120, and/or morphine. Likewise, both in-cell western and cytometric analyses showed corresponding declines in TLR9 proteins at 12 h after treatment. Notably, the proportion of astrocytes expressing TLR9 appeared to be less compared to TLR2 and TLR3 (Fig. 1B). The dynamic nature of TLR9 expression may partially explain why studies of TLR9 expression in astroglia have been controversial (Bowman et al., 2003). TLR9 participates in a coordinated response with TLR3 and TLR7/8 to suppress viral infections in the CNS (Mishra et al., 2006; Suh et al., 2009). Other investigators report aberrant TLR9 signaling in HIV-infected innate immune cells (Martinelli et al., 2007; Martinson et al., 2007), while polymorphisms in TLR9 affect HIV-1 progression clinically (Bochud et al., 2007). Then again, increased TLR3 expression was found in the lymph nodes of simian immunodeficiency virus (SIV)-infected macaques (Sanghavi and Reinhart, 2005), and Tat exposure was found to reduce TLR4 expression in cholangiocytes (O'Hara et al., 2009), suggesting that the virus or specific viral proteins can intrinsically affect TLR function—as seen in the present study. Alternatively, morphine can enhance LPS-mediated increases in vascular permeability suggesting additional modes of interaction with TLR signaling pathways (Liu et al., 2004). Collectively, HIV-1 infection may disrupt the innate immune response by systematically altering the function of a subset of TLRs, which appear to include an increase in deleterious TLR2 signaling coordinated with a loss in beneficial antiviral functions through reductions in TLR9 expression.

Even though morphine's effects were more subtle than either viral protein, this should not discount morphine's interactions with the TLR system. In the current studies, morphine alone caused a significant reduction in TLR9, while morphine attenuated gp120-induced increases in TLR2 transcript and protein levels. In combination, morphine and gp120 caused marked reductions in TLR9 protein levels at 6 h that were not seen with either substance alone. Thus, the immunosuppressive effect of morphine appears may be in part linked to

alterations in TLR signaling. Wang et al. (2008) showed that morphine, in the presence of *S. pneumoniae* infection, inhibits NF- κ B gene transcription through actions on TLR9 signaling in alveolar macrophages. Similarly, in human peripheral blood mononuclear cells (Bonnet et al. 2008), MOR activation mediates morphine-induced TNF- α and IL-6 inhibition in TLR2-stimulated monocytes and has been shown to suppress cytokine production by the TLR2 agonist, peptidoglycan (PGN). TLR2 mediates morphine-induced apoptosis in HEK293 cells in a MyD88-dependent manner (Li et al., 2009). Thus, morphine may potentially act at several direct and indirect levels to modify TLR expression astrocytes and to modify the host immune response.

Induction of neuropathic pain by opioid agonists was reversed using opioid receptor inhibitors, naloxone and naltrexone, through blockade of TLR4 activation in glia (Hutchinson et al., 2007; Watkins et al., 2009), suggesting TLR4 is an important mediator of functions outside of strict host-pathogen recognition and defense. Certainly, the upregulation of proinflammatory target genes encoding cytokines, chemokines and the production of ROS, as seen with activation of TLR 2, TLR3, TLR4, and TLR9 in the present study, are essential for pathogen elimination; however, these molecules can also mediate neuronal cell death (Brosnan et al., 1994; Chao et al., 1992). We hypothesize that the production of ROS, resulting from the activation of TLRs, may affect nociception in vivo as well. Alternatively, stimulation of astrocytes with TLR ligands can trigger the release of cytokines and downregulation of the glutamate transporter gene, excitatory amino acid transporter-1 (EAAT1 or GLAST), thereby limiting the ability of astrocytes to uptake excess glutamate (Bal-Price and Brown, 2001; Scumpia et al., 2005). The inability to sequester excess glutamate may enhance host defenses, but alternatively may contribute to excitotoxic insults in the CNS. Unfortunately, a trade-off for a heightened inflammatory response can be increased excitotoxicity and neuronal injury. Therefore, TLR stimulation on astrocytes has the ability to not only affect innate immunity, but astrocyte performance in general, which could have significant consequences for brain function.

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Figure 1.

Cellular localization of TLR immunoreactivity in astrocytes in the presence and absence of cognate agonists for TLR2 (LTA), TLR3 (Poly(I:C)), TLR4 (LPS), and TLR9 (ODN). TLR antigenicity was co-localized with GFAP immunofluorescence in astrocytes. TLR2, TLR3, TLR4, or TLR9 was co-localized in subsets of GFAP-positive astrocytes. Although TLR activation is reported to reveal some TLR types, we found only modest qualitative differences in the pattern and intensity of TLR immunofluorescence at 12 h post-treatment. Arrows indicate TLR immunoreactivity.

Figure 2.

Flow cytometric analysis of TLR immunofluorescence in astrocyte-enriched cultures. A) A plot of forward scatter (FSC-A) vs. GFAP (A). B) A histogram showing the relative differences in TLR2, TLR3, TLR4, and TLR9 fluorescence intensity associated with astrocytes (B); isotype controls (dashed line), TLR2 (red line), TLR3 (blue line), TLR4 (green line), TLR9 (purple line) and GFAP (black line). C–D) Data are plotted to show the intensity of the phycoerythrin (PE) fluorescent signal in isotype-control and GFAP to indicate negative and positive staining, respectively (PE was used for TLRs 2–4). E) TLR9 could be co-localized with TLR2, TLR3, or TLR4; the extent to which other TLRs are co-expressed in the same cell was not explored. Cell nuclei were counterstained with Hoechst 33258. Approximately 10,000 events were analyzed per treatment condition in each experiment and size discrimination was used as a crude method for viability determination. Representative histograms of three independent experiments are shown; values indicate the mean percentages of GFAP-immunopositive astrocytes $\pm$ SEM in which fluorescein isothiocyanate (FITC) labeled TLRs could be co-detected.

Figure 3.

Effects of morphine, HIV-1 Tat, and gp120 on the expression of TLR2 and TLR9 protein and mRNA in astrocyte cultures. A–B) Astrocyte-enriched cultures were treated with morphine, Tat, and gp120 alone or in combination for 30 min, 6 h and 12 h. Cells were fixed, permeabilized and labeled with antibodies to TLR2, TLR3, TLR4 or TLR9 (IRDye 680LT), and normalized against β -actin (IRDye 800CW), and quantitated by In-cell western analysis (LI-COR) (A–B). C–D) After 12 h treatment with morphine, Tat, and/or gp120, total RNA was extracted, RT-PCR followed by gel electrophoresis was performed, TLR mRNA levels were quantitated densitometrically, and normalized to β -actin. A–D) Collectively, TLR3 or TLR4 expression was unaffected by exposure to Tat, gp120, morphine or combinations thereof (data not shown), while TLR2 (A) and TLR9 (B) protein or transcript levels (C–D), respectively, showed reciprocal increases and decreases in response to most treatments (* $p < 0.05$ vs. controls); protein and mRNA determinations are the mean $\pm$ SEM of 4 independent experiments (* $p < 0.05$ vs. controls).

Figure 4.

Effects of morphine, HIV-1 Tat and/or gp120 on the proportion of TLR2 and TLR9 immunofluorescent astrocytes at 12 h following continuous exposure. Dot plots show that exposure to Tat (42 ± 1), gp120 (46 ± 3), or combined morphine and Tat (50 ± 2) increased the proportion of TLR2-immunopositive astrocytes compared to vehicle-treated controls (33 ± 2) ($p < 0.05$; one-way ANOVA; Duncan's *post hoc* test), while the proportion of TLR9-immunofluorescent astroglia was unaffected by these treatments at 12 h. Approximately 10,000 events were analyzed per treatment condition in each experiment. Representative dot plots are shown (x-axis, PE; y-axis, FITC) on the x-axis; values are the mean percentage of TLR2 (red) or TLR9 (green) positive astrocytes $\pm$ SEM from 3 independent experiments.

Figure 5.

TLR agonists induce cytokine/chemokine secretion by astrocytes at 12 h following exposure. A–D) HIV-1 Tat (positive control) and TLR2 (LTA), TLR3 (Poly(I:C)), TLR4 (LPS), and TLR9 (ODN) agonists differentially increased the release of TNF- α (A), IL-6 (B), RANTES (C), and/or MCP-1 (D) by astrocytes as determined by ELISA. Values represent the mean $\pm$ SEM of 4 independent experiments (* $p < 0.05$ vs. control).

Figure 6.

Effects of TLR agonists on NO production by astrocytes at 24 h following exposure. A–D) With the exception of the lowest concentration of the TLR2 agonist LTA (A), TLR3 (Poly(I:C)) (B), TLR4 (LPS) (C), and TLR9 (ODN) (D) agonist exposure had no effect on the accumulation and/or release of nitrites by astrocytes. Nitrite levels were assessed using the Griess reaction; HIV-1 Tat was included as a positive control; values represent the mean $\pm$ SEM of 4 independent experiments (* $p < 0.05$ vs. control).

Figure 7.

TLR agonists stimulate ROS formation in astrocytes. A–D) Intracellular ROS production was measured by treating cells with DCF prior to treatment with media alone (control), HIV-1 Tat (positive control), or increasing concentrations of TLR2 (LTA) (A), TLR3 (Poly(I:C)) (B), TLR4 (LPS) (C), or TLR9 (ODN) (D) agonists, and measuring DCF fluorescence after 90 min. treatment. Values represent the mean $\pm$ SEM of 4 independent experiments (* p < 0.05 vs. control).