TLR Signaling Tailors Innate Immune Responses in Human Microglia and Astrocytes[1]

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The specific signals mediating the activation of microglia and astrocytes as a prelude to, or consequence of, CNS inflammation continue to be defined. We investigated TLRs as novel receptors mediating innate immune responses in human glial cells. We find that microglia express mRNA for TLRs 1–9, whereas astrocytes express robust TLR3, low-level TLR 1, 4, 5, and 9, and rare-to-undetectable TLR 2, 6, 7, 8, and 10 mRNA (quantitative real-time PCR). We focused on TLRs 3 and 4, which can signal through both the MyD88-dependent and -independent pathways, and on the MyD88-restricted TLR2. By flow cytometry, we established that microglia strongly express cell surface TLR2; TLR3 is expressed at higher levels intracellularly. Astrocytes express both cell surface and intracellular TLR3. All three TLRs trigger microglial activation upon ligation. TLR3 signaling induces the strongest proinflammatory polarizing response, characterized by secretion of high levels of IL-12, TNF-α, IL-6, CXCL-10, and IL-10, and the expression of IFN-β. CXCL-10 and IL-10 secretion following TLR4 ligation are comparable to that of TLR3; however, other responses were lower or absent. TLR2-mediated responses are dominated by IL-6 and IL-10 secretion. Astrocytes respond to TLR3 ligation, producing IL-6, CXCL-10, and IFN-β, implicating these cells as contributors to proinflammatory responses. Initial TLR-mediated glial activation also regulates consequent TLR expression; while TLR2 and TLR3 are subject to positive feedback, TLR4 is down-regulated in microglia. Astrocytes up-regulate all three TLRs following TLR3 ligation. Our data indicate that activation of innate immune responses in the CNS is not homogeneous but rather tailored according to cell type and environmental signal. *The Journal of Immunology,* **2005, 175: 4320–4330.**

Microglia and astrocytes are endogenous cells of the CNS that are key players in the immune responses that occur within this compartment. As constituents of the innate immune system, the myeloid-derived microglia, consequent to activation, can recruit peripheral immune cells and can provide T cells with the signals necessary for differentiation and proliferation (1, 2). The neuroectodermal-derived astrocytes can also express MHC and costimulatory molecules upon activation (3, 4) and share some of the immune-related functional properties of microglia. Both cell types display activated phenotypes in the CNS inflammatory lesions that characterize the putative autoimmune disease multiple sclerosis (MS)[3] (5).

The specific signals inducing the activation states of these glial cells either as a prelude to, or consequence of, an immune response within the CNS continue to be defined. Exogenous signals with the capacity to mediate innate immune cell activation can belong to both the nonself, pathogen-associated "stranger" signals proposed by Medzhitov and Janeway (6) and to the damage-associated, altered-self "danger" signals proposed by Matzinger (7). Notably, onset and/or relapses of MS have been associated with exposure to viral infections (stranger), whereas others have proposed that the disease is consequent to primary oligodendrocyte cell death (danger) (8–11). These apparently disparate mechanisms may trigger activation and inflammation states through common means, as stranger/danger signals are increasingly recognized to ligate common receptors, namely the germline-encoded pattern-recognition receptors.

The TLR family is a major class of pattern-recognition receptor that has emerged as a central player in the initiation and tailoring of both innate and subsequent adaptive immune responses (12, 13). Ligation of distinct TLRs by different pathogen-associated molecules has the capacity to engage specific downstream intracellular signaling cascades and thus tailor the innate response to the activation stimulus. Classical TLR signaling involves the MyD88 intracellular adapter protein and results in the translocation of NF-κB to the nucleus and the production of proinflammatory cytokines and chemokines (14). A MyD88-independent pathway unique to TLR3 and TLR4 signaling can also induce the production of both the pluripotent cytokine IFN-β, and IFN-inducible protein-10 (CXCL-10), a chemokine found to selectively recruit Th1-polarized effector $CD4^+$ T cells (15). Proinflammatory Th1-polarized T cells are strongly associated with MS pathology but the mechanisms leading to the polarization of T cells infiltrating the MS brain remain unknown. Although TLR signaling has usually been reported to lead to Th1-type responses, recent findings indicate that TLR2 ligation, in particular, can polarize cytokine secretion and immune responses to the Th2-type (16).

The pattern of TLRs expressed by glial cells and the subsequent downstream signaling cascades engaged are potentially significant determinants of the inflammatory response occurring within the

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[3] Abbreviations used in this paper: MS, multiple sclerosis; GFAP, glial fibrillary acidic protein; RT, reverse transcription; qPCR, quantitative real-time PCR; C_T, cycle threshold; MFI, mean fluorescence intensity; PIC, Poly(inosinic acid):poly(cytidylic acid); PAM, palmitoyl-3-cysteine-serine-lysine-4.

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CNS compartment. The expression of TLRs varies not only between different cell types but also differs for a given cell type dependent on species, maturation, and activation state, and subsequent to autoregulation by downstream TLR signaling (17–20). Of the TLRs expressed in mice (13), Olson et al. (21) report the basal expression of all nine in mouse microglia. Murine astrocytes have also been found to express TLRs under normal conditions, albeit with lower constitutive expression levels, and with a more restricted range, lacking TLR7 and TLR8. Both cell types show functional responses to TLR ligation (19, 21). As regards human glial cells, Bsibsi et al. (22) reported variable expression of TLRs 1–8 at the mRNA level in microglia isolated from autopsy cases; astrocytes isolated from the same tissue expressed a more restricted range of TLRs. A minority of the microglia (15%) but a majority of the astrocytes expressed TLR3 and TLR4 protein. Notably, these two TLRs were shown in the same study to be upregulated within MS lesions.

In this study, we examined the functional consequences of activating human microglia and astrocytes via TLR signaling. We establish that the secretion of inflammatory mediators, including those potentially impacting on the polarization of T cells, can be tailored in these CNS cells following in vitro exposure to specific TLR ligands.

Materials and Methods

Isolation and characterization of human adult microglia

Normal-appearing white matter was obtained from young adult patients undergoing partial temporal lobe resection for nontumor/noninfection-related intractable epilepsy. The protocol was approved by an institutional review board according to the guidelines of the Canadian Institutes for Health Research (CIHR). Primary microglia were isolated as previously described, based on the differential adherence of glial subtypes to tissue culture plates (23). Briefly, brain tissue was dissociated enzymatically with trypsin and DNase (both from Invitrogen Life Technologies), and mechanically by passage through a nylon mesh. A mixed glial cell suspension (oligodendrocytes and microglia) was obtained by separation on a 30% Percoll gradient (Amersham Pharmacia Biotech). The mixed cell population was cultured for two subsequent overnight periods in tissue culture flasks in MEM supplemented with 5% FCS, antibiotics, L-glutamine, and glucose (all from Invitrogen Life Technologies). Nonadherent oligodendrocytes were washed off and the remaining adherent microglia (>95% purity, as determined by CD68 immunostaining as previously described (24)) were used within 2–5 days.

Isolation and characterization of fetal microglia and astrocytes

Human CNS tissue (cerebral hemispheres) from fetuses at 17–23 wk of gestation was obtained from the Human Fetal Tissue Repository (Albert Einstein College of Medicine, Bronx, NY), following CIHR-approved guidelines. As previously described (25), astrocyte cultures were obtained by dissociation of the fetal CNS with trypsin and DNase I followed by mechanical dissociation. After washing, the cell suspension was plated at a concentration of $3–5 \times 10^6$ cells/ml on poly-L-lysine coated flasks in DMEM supplemented with 10% FCS, antibiotics, glutamine, and glucose. To obtain pure astrocytes, the mixed CNS cell culture (containing astrocytes, neurons, and microglia) was passaged upon confluency, starting at 2 wk, postisolation, using trypsin-EDTA (Invitrogen Life Technologies). Human fetal astrocytes were used between passages 2 and 4 and cultures were determined to be >90% pure, as determined by immunostaining for glial fibrillary acidic protein (GFAP; as previously described (26)). Before passaging the mixed cultures, the microglia were harvested from the supernatant. Fetal microglia were plated in flasks, and after 2 h, contaminating nonadherent cells were removed and phenotypic and functional assays were initiated within 24 h.

Isolation of peripheral cells

$CD14^+$ myeloid cells were isolated from venous blood drawn from normal consenting donors in agreement with institutional guidelines, as previously described (27). Briefly, PBMC were isolated using Ficoll-Hypaque (Amersham Pharmacia Biotech) density gradient centrifugation, and monocytes were subsequently purified using MACS (Miltenyi Biotec) $CD14^+$ beads. For functional assays, $CD14^+$ cells were plated at 1 million cells/ml in RPMI 1640 supplemented with 10% FCS, antibiotics, and glutamine.

RNA isolation, reverse transcription (RT), and quantitative real-time PCR (qPCR)

Total RNA was isolated from all cell types, including human spleen as a positive control, following lysis with TRIzol (Invitrogen Life Technologies) using the Qiagen RNeasy mini kit according to manufacturer's instructions. For microglia, Qiagen Minelute columns were used to concentrate RNA in a small volume (12 μl). All RNA samples isolated were treated with DNase (Qiagen). RT was performed on 3 μg of RNA, except for microglia where total RNA (<3 μg) was used. Briefly, cDNA was generated using random hexaprimers (Roche) with the Moloney murine leukemia virus-RT enzyme (Invitrogen Life Technologies) at 42°C. Expression levels for TLRs 1–10 for all cell types were determined by qPCR using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Primers and TaqMan FAM-labeled MGB probes (with 5′ terminal reporter dye FAM and with 3′ nonfluorescent quencher) for TLR3, TLR4, β-actin, and GAPDH designed using PRIMER express software (Applied Biosystems) and concentrations used are listed in Table I. Primers and TaqMan FAM-labeled MGB probes for TLRs 1, 2, 5, 6, 7, 8, 9, and 10 (Assays-on-Demand) and primers and VIC/TAMRA probe for 18S were

Table I. *Primers and probes for qPCR*

Gene	Forward Primer, Concentration	Reverse Primer, Concentration	Probe, Concentration
TLR3	AACCCTGGTGGTCCCATTTAT, 900 nM	AAAGCCGTTGGACTCCAAGTT, 900 nM	TCACCTCCACATCCT (FAM/MGB probe), 150 nM
TLR4	ATTCCCGGTGTGGCCATT, 900 nM	ACACCACAACAATCACCTTTCG, 900 nM	TGCCAACATCATCC (FAM/MGB probe), 150 nM
β-Actin	CGGACTATGACTTAGTTGCGTTACA, 900 nM	CATCTTGTTTTCTGCGCAAGTT, 900 nM	CCTTTCTTGACAAAACC (FAM/MGB probe), 150 nM
GAPDH	CCACATCGCTCAGACACCAT, 900 nM	TGACCAGGCGCCCAATA, 900 nM	TCAACGGATTTGGTC (FAM/MGB probe), 150 nM
18s	4319413E (Applied Biosystems), 100 nM primers, 200 nM VIC/TAMRA probe,		
TLR1	Hs00413978	Primers at 900 nM, FAM/MGB probes at 250 nM, Assays-On-Demand, Applied Biosystems	
TLR2	Hs00152932		
TLR5	Hs00152825		
TLR6	Hs00271977		
TLR7	Hs00152971		
TLR8	Hs00152972		
TLR9	Hs00370913		
TLR10	Hs00374069		
IFN-β	Hs00277188		

from Applied Biosystems, concentrations are listed in Table I. β-actin, GAPDH, and 18S were all used as endogenous controls to account for variability in the amount of RNA transcribed and the RT reaction itself. β-actin and 18S were found to be consistent and figures show expression normalized to β-actin. qPCR cycling was performed according to the ABI PRISM 7000 Sequence Detection System default temperature settings (2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C) in a volume of 25 μl with 1× TaqMan Universal Master Mix (Applied Biosystems). For comparative purposes, estimation of the absolute mRNA levels for TLRs 1 through 10 was determined according to the δ cycle threshold (ΔC_T), where C_T is the cycle at which the detected signal is significantly above background signal, and ΔC_T is the difference between the C_T of the *TLR* gene of interest and the C_T of the endogenous control gene, *β-actin*. ΔC_T values for each TLR were categorized into different ranges of expression: high expression correlating with a small ΔC_T ($\leq$5), intermediate expression ΔC_T 5–10, low expression ΔC_T 10–15, very low expression ΔC_T $\geq$15, and $\geq$40 cycles equivalent to not detected. For relative TLR expression among individual donors, mRNA for TLRs 2, 3, and 4 was quantified by extrapolating from standard curves and normalized to endogenous controls. Standard curves were generated by making serial 10-fold dilutions using cDNA from cell types found to have high expression of the gene in question (plotting C_T vs arbitrary levels of input cDNA).

Flow cytometric analysis of TLR expression

Microglia and astrocytes were detached using trypsin-EDTA, and monocytes were isolated as described above. FcRs on myeloid cells were blocked overnight with normal mouse Igs at 3 μg/μl (DAKO Diagnostics Canada) and 10% human serum in PBS supplemented with 1% FCS and sodium azide (0.1%). The following anti-human mAbs from eBiosciences were used at 0.5 μg/μl: biotin-conjugated TLR2 (clone TL2.1), biotin-conjugated TLR4 (HTA 125), PE-conjugated TLR3 (TLR3.7). PE-conjugated streptavidin Ab (0.2 mg/ml) was used to detect biotinylated Abs. FITC-conjugated CD14 (BD Biosciences) was used to double stain for the monocyte/microglial cell surface marker, while Alexa 488-conjugated GFAP (Invitrogen Life Technologies) was used to double stain astrocytes. Isotypes matched for concentration of the primary Abs were used for all stainings. For intracellular staining, cells were fixed and permeabilized in 4% (w/v) paraformaldehyde (Sigma-Aldrich) with 0.1% (w/v) saponin (Sigma-Aldrich) in HBSS (Invitrogen Life Technologies) and then incubated with Ab in 0.1% (w/v) saponin, 1% FBS and 0.1% (w/v) NaN_3 PBS, followed by two washes and finally resuspended in 1% (v/v) FBS and 0.1% (w/v) NaN_3 PBS. Cells were acquired on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Functional assays and cytokine/chemokine ELISA

Poly(inosinic acid):poly(cytidylic acid) (polyI:C (PIC)), a synthetic dsRNA, (Amersham Pharmacia Biotech), LPS from *Escherichia coli* serotype 0127:B8 (Sigma-Aldrich), and palmitoyl-3-cysteine-serine-lysine-4 (Pam_3CSK4 (PAM); InvivoGen), synthetic lipoprotein, were used as ligands for TLR3, 4, and 2, respectively, to stimulate cells. Microglia and astrocytes were treated with the ligands for 24 h, cell supernatants were harvested, and cells were lysed using TRIzol for RNA extraction. Levels of TNFα, IL-6, IL-1β, IL-10, IL-12, and CXCL-10/IFN-inducible protein-10 were assessed using the appropriate ELISA kits (BD Biosciences) according to the manufacturer's directions. Plates were read using an EAR 400AT 96-well plate reader (SLT Labinstruments) at a 450 nm wavelength. For blocking studies, functional grade purified mouse monoclonal anti-human TLR2 (2.1), TLR3 (3.7), and TLR4 (HTA125) or the appropriate isotypes (all eBiosciences) were added to cells at 20 μg/ml (TLR4 and isotype added at 40 μg/ml in serum-free media) 30 min (room temperature) before in vitro exposure to TLR ligands.

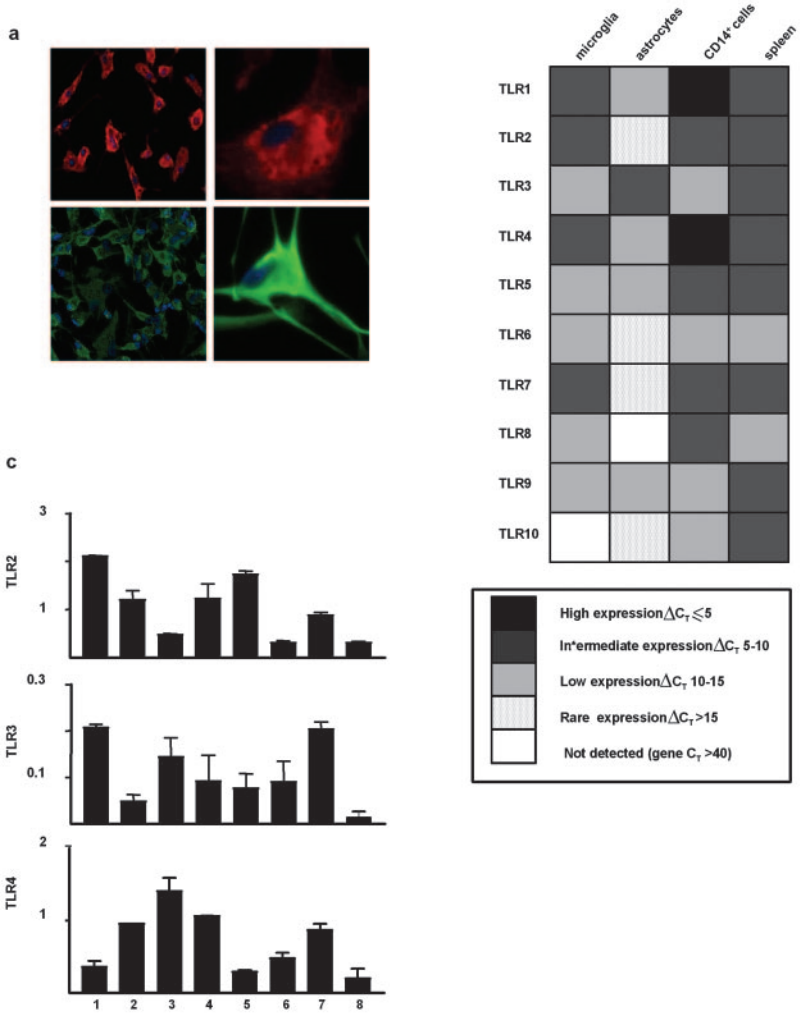

FIGURE 1. Human microglia and astrocytes express distinct profiles of TLRs. Highly purified cultures of microglia and astrocytes, as determined by CD68 (red) and GFAP (green) staining, respectively (*a*), were tested for expression of TLRs 1–10 by qPCR. Absolute mRNA levels for TLRs were estimated by determining ΔC_T (the difference between the C_T of the *TLR* gene of interest and the C_T of the endogenous control gene, β-actin). ΔC_T values for each TLR were categorized into different ranges of expression, from high (ΔC_T $\leq$5) to rare (ΔC_T $>$15) and represent the mean of multiple donors (n = 8) (*b*). Variation in the level of expression of TLRs 2, 3, and 4 among individual donors (1–8) is shown in *c*, using standard curves for qPCR mRNA quantification and normalizing to β-actin; graph indicates mean $\pm$ SEM of duplicate samples.

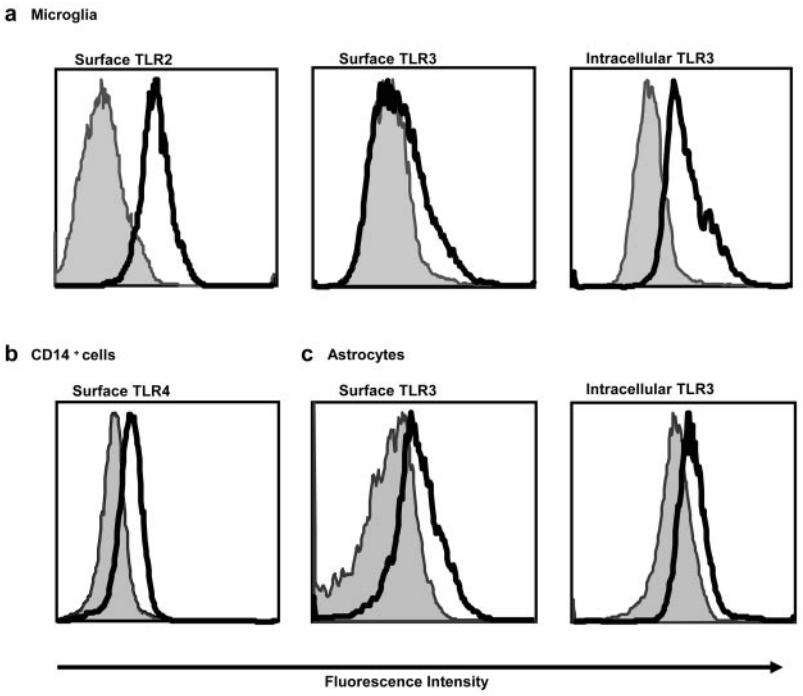

FIGURE 2. TLR protein expression by human microglia and astrocytes under basal conditions. Flow cytometry was used to detect cell surface or intracellular expression of TLRs 2, 3, and/or 4 by different cell types: human microglia (*a*), peripheral $CD14^+$ cells (*b*), and astrocytes (*c*). TLR expression by cells (gated on specific markers) is shown by the shift in fluorescence intensity of the specific Ab (thick black line) over the isotype control (filled histogram)

Statistical analyses

Data handling and analysis (Student's *t* test) were performed using Prism 3.0 (GraphPad Software).

Results

TLR expression distinguishes human microglia and astrocytes

To characterize and to compare expression of TLRs 1–10 in human glial cells, we examined highly purified cultures of human microglia ($CD68^+$) and astrocytes ($GFAP^+$) under basal culture conditions (Fig. 1*a*). $CD14^+$ myeloid cells isolated from peripheral blood as well as splenic tissue were used for additional comparison. We used qPCR and categorized the range of TLR mRNA expression from high to undetected according to ΔC_T, where a small ΔC_T corresponds to high level of expression (and vice versa). Microglia obtained from multiple donors ($n = 8$) were found to basally express mRNA for TLRs 1–9, with a notable lack of TLR10 (Fig. 1*b*). Whereas TLRs 1, 2, 4, and 7 were expressed at intermediate levels, expression of TLRs 3, 5, 6, 8 and 9 was low in comparison to the housekeeper β-actin. Expression of TLRs was also found to vary considerably among individual donors, as shown in Fig. 1*c* for TLRs 2–4. Astrocytes isolated from fetal brain were found to express a more restricted range of TLR mRNA than their myeloid counterparts. Only TLR3 expression was detectable at an intermediate level though mRNA for TLRs 1, 4, 5, and 9 could be detected at low levels in basal cultures of astrocytes (Fig. 1*b*). The profile of microglial TLR expression was more akin to that found in $CD14^+$ peripheral blood cells and splenic tissue than that of astrocytes, which had a limited overlap.

We focused further investigation on TLR3 and TLR4, as they are capable of signaling through the MyD88-independent pathway, as well as on TLR2, which is restricted to the classical MyD88-dependent signal cascade. Flow cytometry was used to detect and to distinguish cell surface and intracellular TLR2, TLR3, and TLR4 protein expression. Despite relatively robust expression at the mRNA level, human adult microglia did not express detectable levels of TLR4 protein (data not shown). We were able to detect very low levels of TLR4 protein on the cell surface of peripheral $CD14^+$ cells in some donors (Fig. 2*b*). In contrast, relatively high levels of TLR2 were consistently detected at the microglial cell surface (Fig. 2*a*). TLR3 could be detected at low levels on the cell surface and was expressed more strongly intracellularly (Fig. 2*a*). Astrocytes were found to express relatively high levels of TLR3

Table II. *Expression and modulation of TLRs 2, 3, and 4 by human microglia and astrocytes*

	TLR2 Expression		TLR3 Expression			TLR4 Expression	
				Protein			
Treatment	mRNA	Protein	mRNA	Surface	Intracellular	mRNA	Protein
Microglia							
None	++	++	+	+	++	++	ND
PIC (TLR3)	++++	+++	++	++	+++	+	ND
LPS (TLR4)	++++	+++	++	++	+++	+	ND
PAM (TLR2)	+++	+++	+	+	++	+	ND
Astrocytes							
None	+/−	ND	++	+	+	+	ND
PIC (TLR3)	++	+	++++	+	++	++	ND

protein both at the cell surface and intracellularly (Fig. 2*b*), while TLR2 and TLR4 were consistently undetectable. The TLR expression pattern observed in adult microglia was also seen in cultures of their fetal counterparts (data not shown), emphasizing the distinction in TLR expression profiles between microglia and astrocytes.

The overall pattern of expression for TLRs 2, 3, and 4 by human microglia and astrocytes is summarized in Table II.

TLR signaling tailors the activation of human glial cells

To elucidate the functional significance of their TLR expression, human glial cells were treated in vitro with synthetic lipopeptide (PAM), synthetic dsRNA (PIC), and *E. coli* LPS, ligands for TLRs 2, 3, and 4, respectively. Both PIC and LPS induced microglia to secrete significant levels of TNF-α ($p < 0.005$); however, TLR3 ligation induced a more robust proinflammatory cytokine response (Fig 3, *a* and *b*). Although PAM induced very low levels of TNF-α in comparison to the two other ligands, IL-6 secretion was equally significant over control ($p < 0.05$) and comparable following ligation of either TLR2 or TLR4 (Fig 3*b*). This proinflammatory TLR response pattern was conserved in fetal microglia (data not shown) but was clearly distinct from that of monocytes; unlike microglia, $CD14^+$ cells isolated from human blood secreted comparable levels of TNF-α in response to both LPS and PAM but not in response to PIC (Fig. 3*c*). Consistent with our findings concerning TLR expression at the protein level, human astrocytes only responded to the TLR3 ligand. Whereas microglia have the capacity to secrete both cytokines in response to PIC, astrocytes responded to the TLR3 ligand with a distinct activation program, secreting high levels of IL-6 (Fig. 3*d*) but no detectable TNF-α (data not shown) compared with untreated cells. These findings indicate that the activation of human CNS cells following TLR ligation can be cell-type specific and tailored according to the activation stimulus.

As shown in Fig. 4, *a–c*, cytokine secretion by microglia in response to the ligands for TLR4 and TLR2 can be blocked using commercially available mAbs, demonstrating the specificity of LPS and PAM for their respective receptors. Although TLR3-mediated responses could not be blocked (Fig. 4*d*), this may reflect the predominantly intracellular distribution of this receptor (Fig. 2*a*).

MyD88-independent signaling in microglia and astrocytes

We next sought to determine whether signaling through the MyD88-independent pathway contributes to this divergence in human glial activation programs following exposure to different TLR ligands. Both IFN-β and CXCL-10 are produced downstream of the MyD88-independent TLR signal transduction pathway, as shown in peripheral cells following the ligation of TLR3 and TLR4 (12, 13). IFN-β was assayed by qPCR due to the inherent difficulty in testing for IFN-β protein. Human adult microglia expressed IFN-β mRNA and secreted significant levels of CXCL-10 ($p < 0.0004$) following ligation of both TLR3 and TLR4 compared with baseline (Fig. 5, *a* and *b*). Induction of IFN-β mRNA was clearly stronger following ligation of TLR3 than TLR4 (Fig. 5*b*), whereas comparable levels of CXCL-10 are produced downstream of both receptors (Fig. 5*a*). Microglia treated with the TLR2 ligand lack both responses, but produce proinflammatory cytokines as shown previously (Fig. 3), consistent with the restriction of TLR2 signal transduction to the MyD88 intracellular adaptor. As shown in Fig. 5, *c* and *d*, ligation of TLR3 on astrocytes induced dose-dependent IFN-β mRNA expression as well as the secretion of high levels of CXCL-10. Thus, astrocytes parallel microglia in their capacity to signal through the MyD88-independent pathway downstream of TLR3. In contrast, human astrocytes were refractory to the ligands for TLR2 and TLR4.

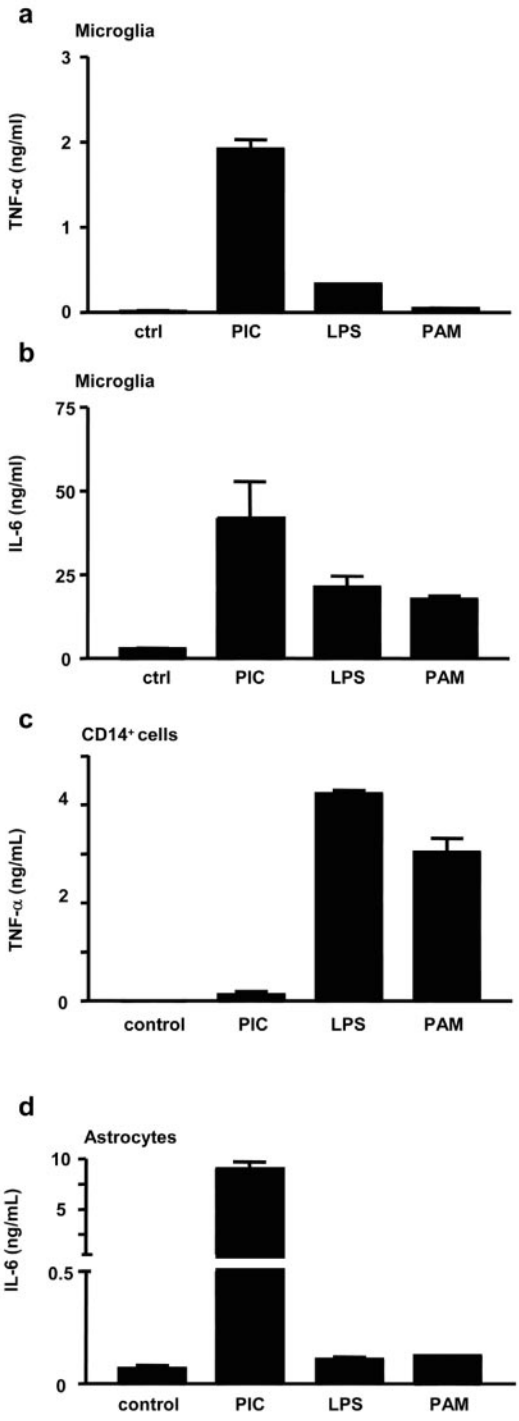

FIGURE 3. Ligation of TLRs induces divergent proinflammatory cytokine secretion by human microglia and astrocytes. Cell cultures were treated with PIC (50 μg/ml (microglia), 10 μg/ml (astrocytes)), LPS (100 ng/ml), or PAM (100 ng/ml), ligands for TLRs 3, 4, and 2, respectively, for 24 h and cell supernatants were tested by ELISA for the cytokines TNF-α (*a* and *c*) and IL-6 (*b* and *d*). Microglia (*a* and *b*), $CD14^+$ cells (*c*), and astrocytes (*d*) have distinct cytokine responses to the TLR ligands; graph shows mean ± SEM of duplicate samples from a representative of three independent experiments.

Secretion of Th1/Th2-polarizing cytokines following TLR-mediated activation

Having demonstrated that microglia respond with distinct activation patterns following ligation of TLRs 2, 3, and 4, we next

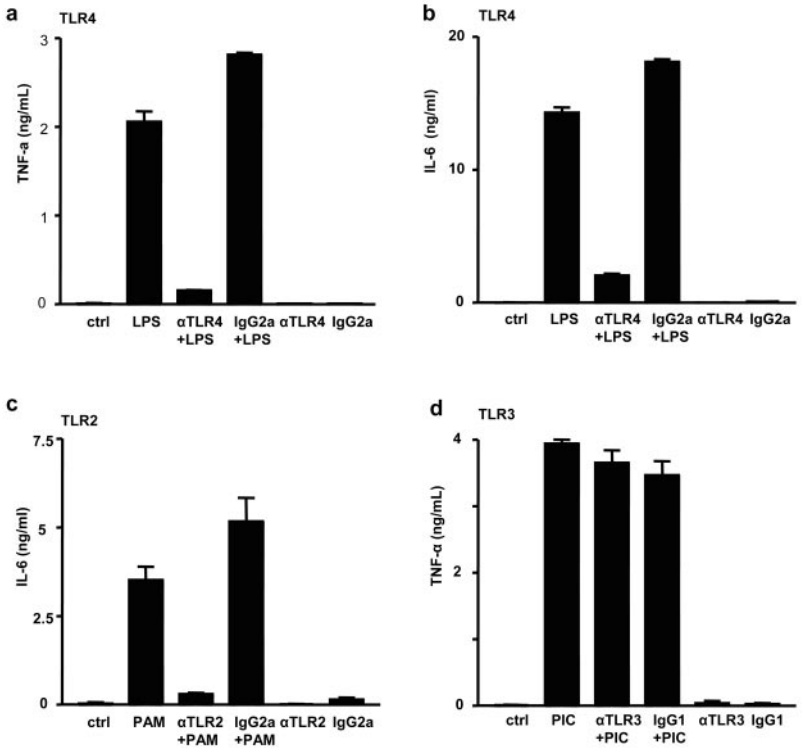

FIGURE 4. Specific Abs can block TLR2- and TLR4-, but not TLR3-, mediated proinflammatory responses by human microglia. Cells were preincubated with 20 μg/ml Ab or isotype (40 μg/ml for TLR4 and isotype), followed by stimulation for 24 h with ligands for TLR4 (*a* and *b*), TLR2 (*c*), and TLR3 (*d*). For TLR4, blocking was performed in serum-free media. TNF-α (*a* and *d*) and IL-6 (*b* and *c*) ELISA graphs show mean $\pm$ SEM results for duplicates from a representative of three independent experiments.

investigated their capacity to secrete Th1- vs Th2-polarizing cytokines (IL-12 and IL-10, respectively) in response to TLR ligation. We found that the secretion of IL-12 by human microglia was highly selective for the TLR signal. Neither TLR4 nor TLR2 ligation induced any significant IL-12 response, while TLR3 ligation resulted in the secretion of high, though variable, levels of the cytokine (Fig. 6*a*). In contrast, microglia were found to secrete IL-10 following ligation of all three TLRs (Fig. 6*b*). Astrocytes did not secrete detectable levels of either IL-12 or IL-10 following ligation of TLR 2, 3, or 4 (data not shown). Thus, distinct TLR ligands have a graded polarizing effect on human microglial responses, with PIC leading to the strongest Th1-inducing cytokine profile of the three ligands tested.

The functional tailoring of cytokine and chemokine responses by human microglia and astrocytes following TLR ligation is summarized in Table III.

Functional regulation of receptor expression following TLR ligation

TLR expression in peripheral immune cells has been shown to be regulated following exposure to maturation signals and to specific TLR ligands and varies from species to species (17–20). We examined whether human glial cells could also regulate the expression of individual TLRs consequent to in vitro stimulation with the ligands for TLRs 2, 3, and 4. We found that the ligation of all three

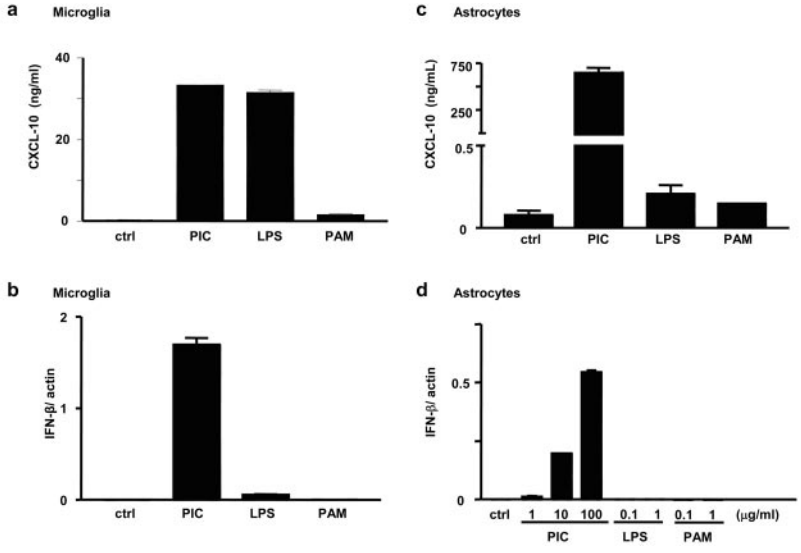

FIGURE 5. TLR ligation induces MyD88-independent signaling in human glial cells. The secretion of CXCL-10 and the mRNA expression of IFN-β relative to the housekeeper β-actin, as determined by qPCR, were used as read-outs for the MyD88-independent signal transduction pathway. Cultures of microglia (*a* and *b*) or astrocytes (*c* and *d*) were treated with TLR ligands for 24 h. Cell supernatants were analyzed for CXCL-10 by ELISA and cDNA was extracted from the cells for qPCR. Graph shows mean $\pm$ SEM of duplicate samples from a representative of three (*a*–*c*) or two (*d*) independent experiments.

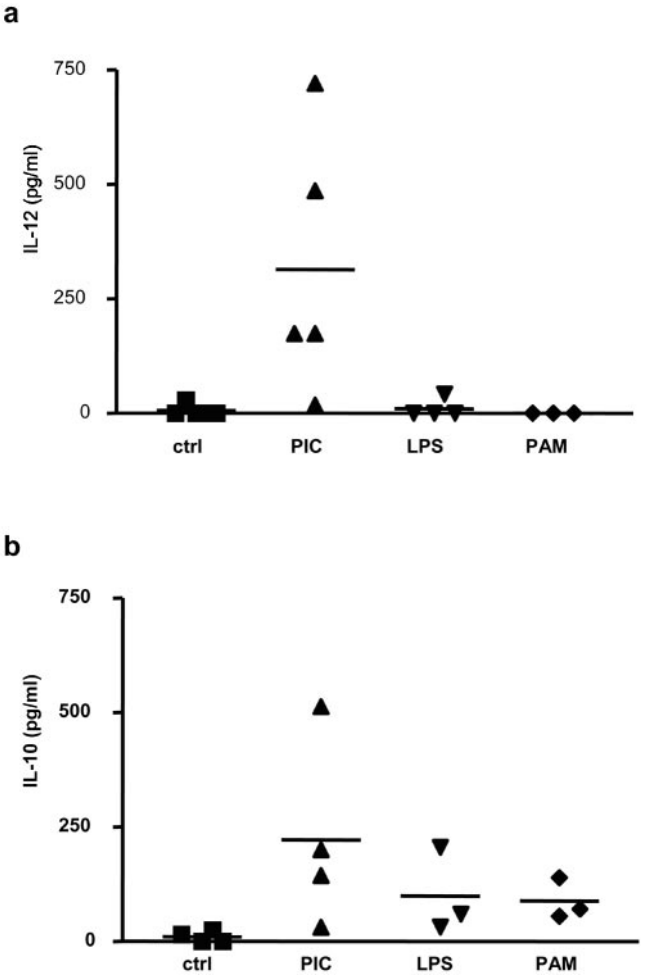

FIGURE 6. Microglial secretion of IL-12 and IL-10 is polarized by TLR ligands. Cultures of human microglia were treated with TLR ligands (concentrations as in Fig. 3) for 24 h and cell supernatants were tested for IL-12 (*a*) and IL-10 (*b*) by ELISA. Graph shows results of individual independent experiments, indicating mean of each treatment, with $n = 5$ (ctrl, PIC), $n = 4$ (ctrl, LPS, PAM) for IL-12, and $n = 4$ (ctrl, PIC), $n = 3$ (LPS, PAM) for IL-10.

TLRs, particularly PIC, resulted in the up-regulation of TLR2 expression ($p = 0.0025$) compared with baseline by human microglia at 24 h, as shown by qPCR (Fig. 7*a*), indicating a general activation-induced positive feedback loop. TLR3 expression was also governed by a positive feedback loop, but significant up-regulation was restricted to signaling through TLR3 and TLR4, as TLR2 ligation had no effect compared with baseline (Fig. 7*b*). In contrast to both TLR2 and TLR3, TLR4 expression was found to be strongly down-regulated following stimulation with ligands to all three TLRs (Fig. 7*c*), especially by PIC and LPS ($p < 0.05$ compared with baseline) indicating a negative feedback loop governing the expression of this receptor in human microglia.

We conducted flow cytometric analysis to examine whether this regulation of individual TLRs at the mRNA level correlates with ligand-induced regulation of TLR protein in human microglia. As shown in Fig. 8, our findings at the protein level are in agreement with regulation at the mRNA level. Expression of TLR2 at the microglial cell surface increases relative to control following exposure to all three TLR ligands, namely PIC, LPS, and PAM (Fig. 8*a*). TLR3, in correlation with our qPCR data, was found to be up-regulated, both intracellularly (Fig. 8*b*) and at the cell surface (Fig. 8*c*), in response to both PIC and LPS, indicative of a positive feedback loop. Due to the fact that microglial TLR4 expression levels are below detection threshold, we used a functional assay to determine whether the down-regulation we observed at the mRNA level coincided with a decrease in LPS responsiveness. As shown in Fig. 8, *d* and *e*, for TNF-α and IL-6, respectively, pretreatment of microglia with LPS results in a functional abrogation of TLR4 signaling in response to a secondary exposure to the ligand.

We sought to determine whether astrocytes were subject to similar regulation by TLR signaling. Following exposure to the ligand for TLR3, qPCR was performed on astrocytes to determine whether the expression of mRNA for TLRs 1–10 was modulated. As shown in Fig. 9*a*, treatment of astrocytes with PIC leads to the up-regulation of TLRs 1–5, as well as TLR9. Notably, the increase in TLR3 expression relative to untreated cells was the greatest downstream effect of PIC exposure, indicative of a strong positive autoregulation in astrocytes. We correlated this TLR3-mediated regulation of TLR expression with protein levels using flow cytometric analysis. As shown in Fig. 9*b*, we found that exposure to PIC increased intracellular TLR3 expression relative to untreated astrocytes, while surface expression was essentially unchanged. Notably, we were also able to detect TLR2 expression (mean fluorescence intensity (MFI) increase to above isotype) on astrocytes exposed to PIC, in contrast to untreated cells.

Discussion

In this study, we demonstrate the role that TLRs play in triggering human microglia and astrocytes to mount functional responses that will impact neuroinflammation. Our results point to a distinction in TLR expression between these two glial cell types and illustrate their divergent functional responses to TLR ligands. We have established that immune responses triggered by human CNS cells can be tailored to the environmental signal, i.e., the TLR ligand. In particular, our findings indicate that microglial activation can be

Table III. *Cytokine/chemokine responses by human microglia and astrocytes are tailored to the TLR ligand*

	MyD88-Dependent Responses		MyD88-Independent Responses		Th1/Th2 Polarizing Responses	
Ligand	TNF-α	IL-6	CXCL-10	IFN-β mRNA	IL-12	IL-10
Microglia						
None						
PIC (TLR3)	+++	+++	+++	+++	+++	+++
LPS (TLR4)	++	++	+++	+	+/−	++
PAM (TLR2)	+/−	++	+/−	−		++
Astrocytes						
None						
PIC (TLR3)		++++	++++	+++		
LPS (TLR4)			+/−			
PAM (TLR2)			+/−			

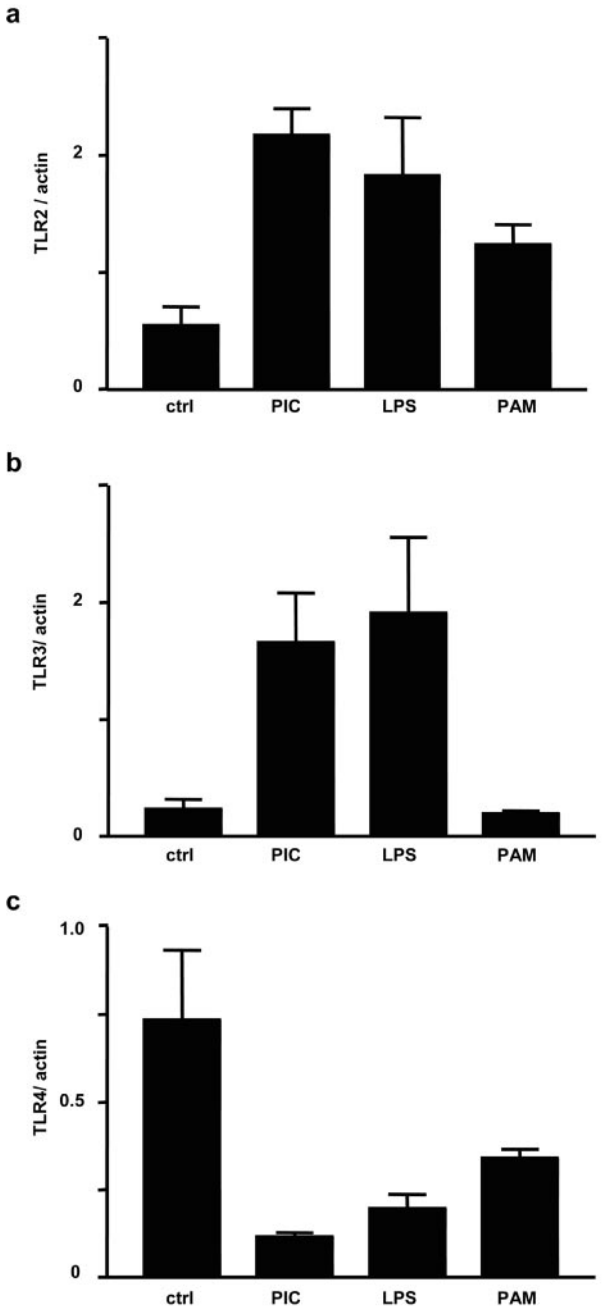

FIGURE 7. Autoregulation of mRNA expression downstream of TLR signaling in human microglia. Quantification was determined by qPCR using standard curves to extrapolate TLR gene expression levels relative to the housekeeper β-actin in cultures of human microglia treated with TLR ligands (concentrations as in Fig. 3) for 24 h. Graph shows mean ± SEM of duplicate samples from a representative of $n = 3$ experiments for each TLR. *a*, TLR2; *b*, TLR3; *c*, TLR4.

polarized, in that secretion of proinflammatory cytokines and chemokines, including those impacting the development of proinflammatory Th1 $CD4^+$ effector T cell responses, can vary according to the TLR stimulus.

Our findings concerning microglial TLR expression at the mRNA level correlate well with the report by Bsibsi et al. (22), although we add the low-level expression of TLR9 mRNA, a finding substantiated by recent work in murine microglia (21) and possibly a reflection of our use of real-time PCR. The broad range of TLRs expressed by microglia is in line with the myeloid lineage of these cells. The variable levels of TLR expression among individual donors may reflect a range of past environmental influences in a manner akin to the variation observable in human peripheral immune cells (20). As observed by Farina et al. (28), we found that human astrocytes under basal culture conditions preferentially expressed TLR3 (Fig. 1*b*). We were also able to detect a low level of expression of TLR1, 4, 5, and 9 mRNA, but determined that expression of other TLRs in astrocytes was either rare or undetectable (Fig. 1*b*).

We have established that relatively high levels of TLR2 protein are expressed on the cell surface of human microglia by using flow cytometry, a novel observation supported by findings in rodent microglia (29). TLR2 has proven to be a particularly promiscuous TLR, in that it has been found to ligate a broad array of ligands, both pathogen-associated and endogenously derived. Given this high level of expression, it is interesting to note that the cognate ligand for TLR2, peptidoglycan, was recently found in the brains of MS patients (30). In contrast to TLR2, we were unable to detect TLR4 protein on human microglia. However, peripheral $CD14^+$ mononuclear cells analyzed by the same flow cytometry protocol were found to express a low level of TLR4 in some donors. Given that human microglia express robust TLR4 mRNA and consistently respond with a robust activation program to LPS, we must affirm that TLR4 protein is expressed, at levels below detection threshold. Human immature dendritic cells have also been found to respond to the LPS ligand despite undetectable protein expression (20).

We have demonstrated that both human microglia and astrocytes express TLR3 protein using flow cytometry. Whereas microglial TLR3 expression is localized primarily to the intracellular compartment (Fig. 2), astrocytes express TLR3 both intracellularly and at the cell surface. Subcellular localization of TLR3 has also been reported in human dendritic cells (31); furthermore, TLR3 expression may be significant given that it has been reported to ligate not only dsRNA, a common viral intermediate, but also endogenous mRNA (32). Endogenous mRNA may act as a danger signal when its cellular localization is perturbed, as is postulated to occur following deficient clearance of apoptotic cells or following necrosis (32). Oligodendrocyte cell death, whether antecedent or consequent to inflammation during disease course, is a hallmark of MS and could potentially overwhelm the clearance capacity of the endogenous microglia, leading to the release of host-derived mRNA into the extracellular milieu.

Our functional in vitro data help define the role that TLRs play in shaping the CNS inflammatory milieu. We have established that TLR2 ligation by the triacylated lipopeptide PAM activates a limited proinflammatory response in human microglia, devoid of CXCL-10, IFN-β, and IL-12 (Figs. 4 and 5). As such, the unbalanced production of IL-10 following this activation paradigm, which also induces IL-6 secretion, may shift subsequent immune responses to the Th2-type (Figs. 3 and 5). TLR2 is unique in that it has been reported to induce Th2-polarizing cytokine secretion from peripheral innate immune cells, both in vitro and in vivo, and to play a role in allergy (33–36).

Our findings demonstrate that both TLR4 and TLR3 ligation can induce high levels of CXCL-10 as well as the production of IFN-β, a functional microglial response distinct from that downstream of TLR2 and in line with the capacity for these two TLRs to signal through the MyD88-independent pathway (Fig. 4). In particular, CXCL-10 has been found to selectively recruit Th1-polarized effector $CD4^+$ T cells (37), indicating a role for both TLR4 and TLR3 ligation in a proinflammatory context. Despite this parallel between TLR3 and TLR4 signaling, TLR3 ligation was found to polarize human microglia more strongly. For one, the production

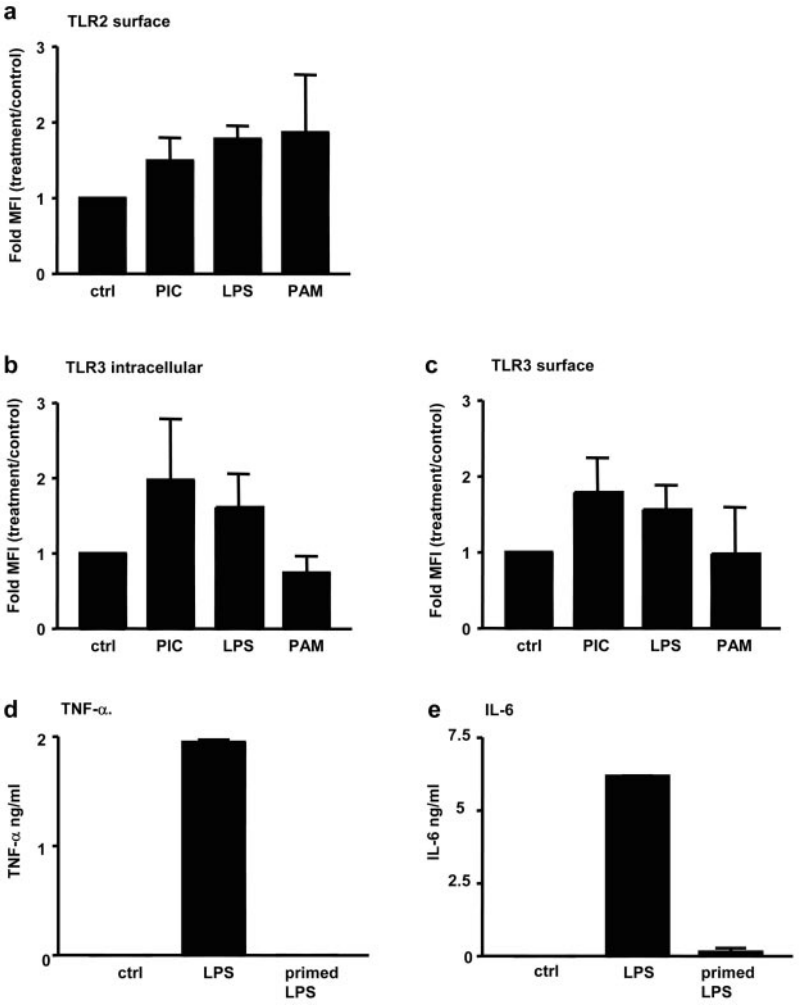

FIGURE 8. Autoregulation of TLR expression at the protein and functional level in human microglia. Cells were treated with PIC, LPS, or PAM, ligands for TLRs 3, 4, and 2, respectively, for 24 h. Flow cytometry was then used to detect ligand-induced regulation of surface TLR2 (*a*) as well as intracellular (*b*) or surface (*c*) TLR3. The MFI of each specific stain in treated cells was normalized to control (untreated) expression, and expressed as the fold increase over control. Graphs show means for up-regulation of protein expression from $n = 3$ donors $\pm$ SEM. Significant donor-to-donor variability was observed. Because TLR4 protein expression was below detection threshold, a functional assay was performed (*d* and *e*). Microglia were pretreated with LPS (10 ng/ml) or left untreated for 24 h; following washes with PBS, cells were re-stimulated (primed LPS) or stimulated (LPS) with 100 ng/ml of the TLR4 ligand for 6 h and cell supernatants were analyzed by ELISA for TNF-α (*d*)and IL-6 (*e*). Graphs show mean $\pm$ SEM of duplicates from a representative of three independent experiments.

of IFN-β was much higher following stimulation with PIC than with LPS (Fig. 4*a*). Type I IFNs have been found to play several key roles in adaptive immunity, inducing the maturation of dendritic cells, supporting the generation of memory T cells, and activating effector function in $CD8^+$ T cells (13, 38). Furthermore, TLR3 ligation induced the secretion of high levels of IL-12 (130–800 pg/ml), in contrast to TLR4 ligation, which was found to induce only very low levels of IL-12 (<20 pg/ml) even upon stimulation of microglia with very high doses of LPS (39). Microglial secretion of IL-12 following ligation with PIC may directly modulate T cells within the CNS compartment, as IL-12 is known to induce the differentiation of Th0 cells into IFN-γ-secreting Th1 cells (40). Given the primary role for Th1 effector cells specific for self-Ag in the pathology of MS, the polarizing nature of TLR3 ligation in microglia may play an important role in the local induction of inflammation.

The fact that human astrocytes respond solely to the TLR3 ligand adds further interest into the role of TLR3-mediated activation of human glial cells. We demonstrate that human astrocytes mount an activation program characterized by the secretion of IL-6 and CXCL-10 and by the expression of IFN-β following exposure to synthetic dsRNA but not in response to LPS nor to PAM. These findings are in agreement with the report by Farina et al. (28) illustrating the unresponsiveness of human astrocytes to other TLR ligands. TLR3 has also been demonstrated to induce strong Th1-polarizing responses in peripheral cells (41). Given the proinflammatory roles of these immune mediators, astrocytes may be important contributors to neuroinflammatory processes.

The distinction in TLR-mediated activation programs in human glial cells also regulates consequent receptor expression by both microglia and astrocytes. Levels of both TLR2 and TLR3 are regulated by positive feedback loops following the activation of microglia. Although mRNA and protein levels of TLR2 were up-regulated by microglia following ligation of all three TLRs, TLR3 up-regulation was only induced downstream of TLR3 and TLR4. Notably, and in contrast with findings in their rodent counterparts, human microglia down-regulated expression of TLR4 after 24-h treatment with ligands to TLR2, TLR3, or TLR4. This negative feedback may parallel that seen for the down-regulation of TLR4 during the maturation of immature dendritic cells (20) and in human peripheral blood cells (42). Human microglia exposed to LPS become refractory to a secondary stimulation with the ligand, indicative of a strict negative regulation. In astrocytes, exposure to the TLR3 ligand led to a general up-regulation in the expression of TLRs, increasing mRNA levels for TLRs 1–5 and TLR9. TLR3 expression levels (relative to untreated cells) were the most responsive to PIC up-regulation, indicative of strong positive feedback. Furthermore, regulation of TLR3 at the protein level is consistent with this observation, as intracellular expression increases following exposure to PIC.

Our data thus establish that activation of innate immune responses in human glial cells is not homogenous but rather tailored according to the environmental signal. Nonetheless, the polarization of immune responses has been shown to be a complex process, and factors such as Ag dose and coreceptors may also play an important role (40, 41). TLR2 functions in association with either

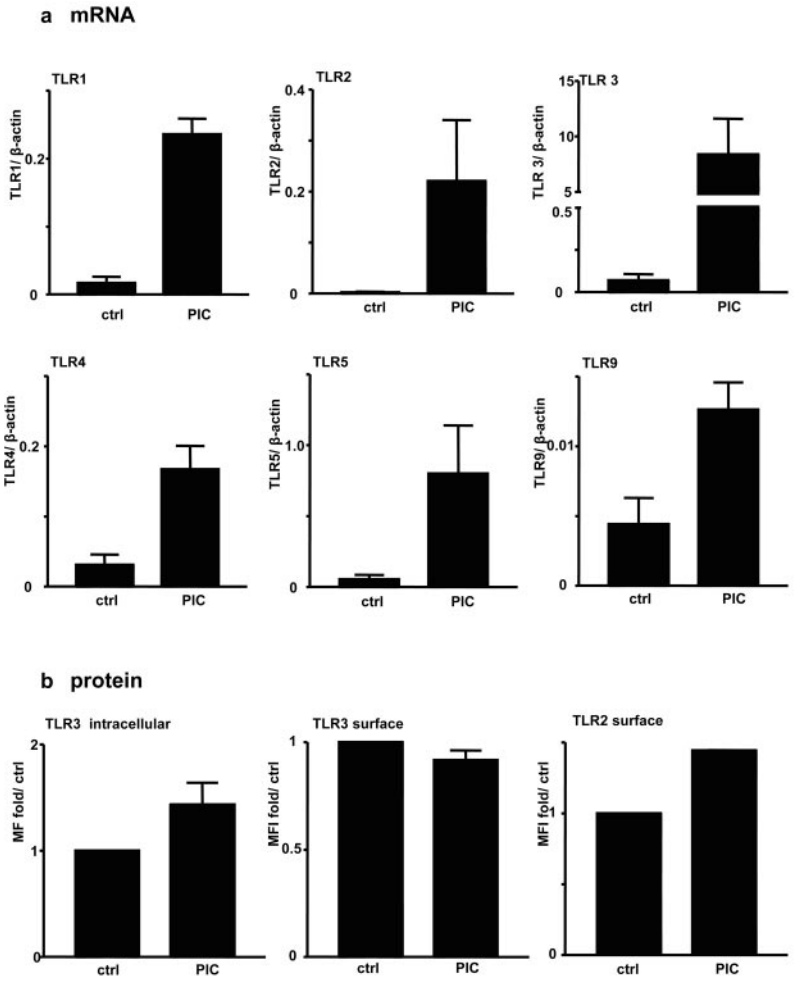

FIGURE 9. TLR3 ligation up-regulates the expression of TLRs at both the RNA and protein level in human astrocytes. Cultures of human astrocytes were exposed to PIC (50 μg/ml) for 24 h and qPCR was used to assay regulation of expression at the mRNA level for TLRs 1–5 and 9 (*a*). Graphs represent mean ± SEM for $n = 2$ individual donors. For regulation of protein expression (*b*), flow cytometry was performed. MFI of TLR staining for PIC-treated cells was normalized to control (untreated) cells; graphs show mean ± SEM for $n = 6$ (TLR3 intracellular), $n = 2$ (TLR3 surface), $n = 2$ (TLR2 surface) individual donors.

TLR1 or TLR6 as a coreceptor, a distinction which may lead to differential downstream signaling. Furthermore, peptidoglycan, a ligand for TLR2, has been shown to induce differential cytokine responses contingent on its solubility (43).

In this study, we have focused on the role of environmental "stranger" signals in the activation of innate immune responses by CNS-resident microglia and astrocytes. The potential contribution of environmental signals in triggering autoimmune responses is emphasized by the demonstration by Goverman et al. (44) that transgenic mice expressing a TCR recognizing myelin basic protein develop spontaneous experimental autoimmune encephalomyelitis only when raised in "dirty" environments. Studies have shown that TLR ligands in the periphery can lead to the activation of CNS resident cells (45). Furthermore, endogenous "danger" ligands are increasingly recognized to signal through TLRs, presenting alternate mechanisms for triggering such responses. We have shown that through TLR signaling human microglia and astrocytes can tailor their responses according to the environmental signal and thus modulate the adaptive immune response accordingly. In a disease context, multiple TLRs may be thus be engaged by multiple ligands, and the ultimate functional outcome will be dependent on coordinate stimulation and cross-regulation of TLR signaling.

Disclosures

The authors have no financial conflict of interest.

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