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Broad Expression of Toll-Like Receptors in the Human Central Nervous System

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Abstract. The family of Toll-like receptors (TLRs) plays a key role in controlling innate immune responses to a wide variety of pathogen-associated molecules. In this study we investigated expression of TLRs in vitro by purified human microglia, astrocytes, and oligodendrocytes, and in vivo by immunohistochemical examination of brain and spinal cord sections. Cultured primary microglia were found to express mRNA encoding a wide range of different TLR family members while astrocytes and oligodendrocytes primarily express TLR2 and TLR3. Comparisons between microglia derived from a series of control subjects and neurodegenerative cases indicate distinct differences in levels of mRNA encoding the different TLRs in different microglia samples. Interestingly, expression of TLR proteins in cultured microglia as revealed by immunocytochemistry was restricted to intracellular vesicles, whereas in astrocytes they were exclusively localized on the cell surface. Finally, in vivo expression of TLR3 and TLR4 was examined by immunohistochemical analysis of brain and spinal cord sections from both control and multiple sclerosis brains, revealing enhanced expression of either TLR in inflamed CNS tissues. Together, our data reveal broad and regulated expression of TLRs both in vitro and in vivo by human glia cells.

Key Words: Astrocytes; Central nervous system; Microglia; Multiple sclerosis; Oligodendrocytes; Toll-like receptors.

INTRODUCTION

Evidence is accumulating that the family of Toll-like receptors (TLR) plays a key role in the control of innate immune responses to pathogens. TLRs recognize a wide variety of pathogen-associated molecular patterns (PAMP) such as peptidoglycan, liposaccharide (LPS), bacterial DNA, and double-stranded RNA (1). They regulate production of inflammatory mediators and other cellular functions associated with the first line of defense against pathogens. TLRs are transmembrane proteins with extracellular leucine-rich repeat domains, and cytoplasmic signaling domains that are similar to the cytoplasmic domain of the interleukin-1 receptor (IL-1R) (2). Both IL-1R and TLRs induce signal transduction pathways leading predominantly to activation of the transcription factor NF-κB (3–6), a key regulator of inflammatory responses (7). Through this pathway and others, engagement of TLRs regulate the release of inflammatory mediators such as TNF-α, IL-1β and nitric oxide and the expression of proteases and co-stimulatory molecules.

In the human genome, 10 TLR family members have been identified to date. The precise function of most of them, however, is still largely unknown. TLR1 is expressed in lymphoid organs in monocytes, polymorphonuclear leukocytes, T cells, B cells, and natural killer (NK) cells. TLR2, TLR4, and TLR5 are widely expressed in myelomonocytic elements. In contrast, TLR3 has been suggested to be uniquely restricted to dendritic cells (8). Data on ligand recognition by TLRs are rapidly accumulating: TLR2 plays an important role in the recognition of fungal, gram-positive, and mycobacterial components; TLR3 in the recognition of double-stranded RNA; TLR4 in the recognition of LPS; and TLR5 in the recognition of bacterial flagellin (9). TLR9 has recently been found associated with the cellular response against bacterial CpG DNA (10). Interestingly, several TLR family members, including TLR2 and TLR6, appear to cooperate in the recognition of PAMP in macrophages (11, 12). For example, the TLR2-mediated response to phenol-soluble modulin is enhanced by TLR6 but inhibited by TLR1, indicating functional interactions between these receptors (13). Thus, the family of TLRs appears to represent a combinatorial repertoire that allows specific recognition of large numbers of pathogen-derived products and that can discriminate between different classes of pathogens.

While the expression and functional roles of TLRs in the lymphoid system has drawn much attention over the past few years, little information has been published on TLR expression and function in CNS glial cells. Yet, it is well established that such cells do participate in innate immune responses (14). While data on TLR expression in the rodent CNS are beginning to emerge, no information is currently available on TLR expression by human glial cells. In rodent CNS, expression of TLR2 and TLR4 has been recently documented (15, 16). The TLR2 encoding gene is regulated in the murine CNS by cell wall components derived from gram-negative bacteria (17). Also, is has been shown that bacterial endotoxin sensitizes the immature rat brain to ischemic injury and that this effect is associated with altered expression of TLR4 (16). Lehnardt et al have shown that TLR4 in the mouse brain is exclusively localized in microglia and mediates lipopolysaccharide-induced oligodendrocyte injury (18).

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TABLE 1
Brain Donors

Sex	Age	Postmortem delay	Disease (hour:min)
F	96	5:30	Control
M	87	8:15	Control
F	83	3:30	Alzheimer disease
F	84	5:15	Alzheimer disease
F	87	3:55	Alzheimer disease
F	88	12:15	Alzheimer disease
F	77	4:40	Alzheimer disease
F	76	4:05	Parkinson disease
F	63	10:55	Parkinson disease
F	50	14:35	Olivopontocerebellar atrophy
M	65	10:35	Multiple sclerosis
M	88	5:00	Pick's disease
F	69	4:55	Pick's disease

In particular, activated microglia and astrocytes are able to produce many of the inflammatory mediators that are known to be regulated by TLRs in lymphoid cells. It is quite conceivable, therefore, that TLRs may also be involved in the regulation of innate immune responses in the CNS.

The aim of the present study was to examine the expression of TLRs in the human CNS by glial cells. Expression was examined in cultures of purified microglia, astrocytes, and oligodendrocytes by RT-PCR and by immunocytochemical staining. Levels of TLR-encoding mRNA were examined in cultured microglia from control donors and donors that had suffered from various neurodegenerative diseases. Furthermore, in vivo expression of TLR3 and TLR4 was examined by immunohistochemical analysis of brain and spinal cord sections from both control donors and multiple sclerosis patients. The results indicate broad TLR expression on glial cells, particularly on microglia. The levels of expression of individual TLRs varied significantly in different samples, suggesting that the TLR network in the CNS is dynamically regulated. Together, our data indicate that TLRs are likely to play an important role in the regulation of innate immune responses in human CNS as well.

MATERIALS AND METHODS

Tissue Specimens

Human postmortem brain tissues were obtained from healthy donors without any neurodegenerative diseases and patients with different neurodegenerative diseases. Characteristics of the donors are listed in Table 1. From all donors, subcortical white matter or corpus callosum was used as a source of glial cells.

Isolation and Culture of Human Glial Cells

Primary human microglia, oligodendrocytes, and astrocytes used in this study were isolated from human white matter samples obtained from the patients listed in Table 1, as previously described (19–20). Briefly, brain tissues dissected from corpus callosum or subcortical white matter were collected and blood vessels were removed. After a 20-min digestion in 0.25% trypsin (Sigma, St. Louis, MO) and 0.1 mg/mL DNAase (Boehringer Mannheim, Mannheim, Germany), the cell suspension was gently triturated and washed with DMEM/HAM-F10 medium containing 10% fetal calf serum (FCS) and antibiotic supplements. After passage through a 100-μm filter, myelin was removed by Percoll gradient centrifugation. Erythrocytes were lysed by a 15-min incubation on ice with 155 mM NH_4Cl, 1 mM $KHCO_3$, 0.2% bovine serum albumin (BSA).

Primary cultures of microglia, astrocytes, and oligodendrocytes were set up in DMEM/HAM-F10 medium containing 10% FCS and antibiotic supplements. Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF, PeproTech Inc, Rocky Hill, NJ) was added to microglial cultures every 3 days at a final concentration of 20 μg/mL. Purity of the cultured glial cells was verified before RNA isolation by staining for CD68 in the case of microglia, GFAP for astrocytes, and 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNPase) for oligodendrocytes. All cultures were found to be at least 99% pure. Cells were analyzed for TLR expression after 10 to 14 days following the start of the culture.

Semi-Quantitative RT-PCR

Total cellular RNA was isolated from human glial cells using RNAZol B™ (Campro Scientific, Veenendaal, The Netherlands), and 2.5 μg RNA was reverse transcribed using reverse transcription system (Promega, Madison, WI). PCR amplifications were performed on 1 μL copy DNA and 49 μL PCR mix (40 μL water + 5 μL $MgCl_2$ (see below for concentration) + 1 μL 10 mM dNTP mix + 1 μL 20 pmol/μL primer + 1 μL 20 pmol/μL anti-sense primer and 0.2 μL taq polymerase). The primers used (from 5′ to 3′) are listed in Table 2. The amount of TLR-encoding mRNA for each TLR was calculated by comparing the yield of each amplicon after 40 cycles to that for β-actin, and by determining the amounts of β-actin encoding mRNA in each sample by competitive quantitative RT-PCR, as described previously (20, 21).

Immunostaining of Cultured Glial Cells

Immunofluorescence was performed on cells cultured on chamber slides. Cells were fixed with 4% formaldehyde for 15 min and with acetone for 5 min. Slides were incubated overnight with goat polyclonal antibodies directed against human TLR3 or TLR4 (Santa Cruz Biotechnologies, Santa Cruz, CA) diluted in 0.1% (w/v) BSA in PBS plus 1% pooled human serum. To verify the specificity of staining, specific blocking peptide for TLR3 and TLR4 were used (Santa Cruz Biotechnologies). For neutralization, TLR3 or TLR4 antibody was combined with a 5-fold excess of blocking peptide and incubated for 2 hours (h) at room temperature. The slides were incubated overnight with antibody/peptide mixture. Next, the slides were rinsed with PBS + 0.05% Tween. As secondary antibodies, biotin-labeled donkey-anti-goat antibodies were used; streptavidin-FITC was subsequently used for detection of secondary antibodies.

TABLE 2
Primer Sequences Used during RT-PCR

	Sense	Anti-sense	Length (bp)
β-actin	AAG ATG ACG CAG ATC ATG TTT GAC	AGG AGG AGC AAT GAT CIT GAT CTT	649
TLR1	TGC CAC CCT ACT GTG AAC CTC AAG	AAA GTC TTG AAG GCC CTC AGG GTC	233
TLR2	TCC GGA GGC TGC ATA TTC CAA AGG	CAG AGT GAG CAA AGT CTC TCC GGT	295
TLR3	TCC GTT GAG AAG AAG GTT TTC GGG	ATA TCC TCC AGC CCT CCA AGT GGA	321
TLR4	AGG ACT GGG TAA GGA ATG AGC TAG	GTA CCC ACT GGT CCT TCT GGA TTC	432
TLR5	CAC TAT AGC TGG GCC TCC TGC A	CCA GCC AAC ATC CTG GAG ATC C	684
TLR6	TGT CCA GAG TGA GTG GTG CCA TTAC	TCT CTA ACT GGC AGG CTA ACC TCAC	455
TLR7	TGT CTG GTG GGT TAA CCA TAC GGAG	TGG TAA CCA GTC CCT TTC CTC GAGA	423
TLR8	CAA CGT CAT ATC CGG AAA CGA CGCT	CTA CCA GGG ACT TGC TTT CCA GGTT	492
TLR9	GAC AGT TCT CTC TCC TGG CTG AATG	CCA GGT TGT TCC GTG ACA GAT CCAA	576

For double immunofluorescence staining for TLR and CD68, sections were processed as described above. Biotinylated donkey-anti-goat (DAKO, Glostrup, Denmark), followed by streptavidin-conjugated FITC (DAKO) and TRITC conjugated-donkey anti-mouse were used as secondary antibodies. Sections were mounted in Vectashield (Vector, Burlingame, CA).

Staining of Brain and Spinal Cord Sections

Formalin-fixed paraffin-embedded brain and spinal cord (5-μm sections) from 15 cases of clinically and histopathologically confirmed MS cases and 3 normal controls were used. The sections were stained with routine histological stains (hematoxylin-phloxine-saffron and Klüver-Barrera staining) to assess inflammation and demyelination. Active MS lesions were characterized by the presence of leukocyte infiltrates and myelin degradation products inside macrophages. Late active lesions were characterized by complete demyelination while early lesions still contained myelin. For immunohistochemistry, the 5-μm sections were deparaffinized in xylene and hydrated in ethanol. After blocking endogenous peroxidase activity (0.3% H_2O_2 in methanol) and a 15-min incubation in 1% FCS to avoid aspecific binding, sections were heated in a microwave oven for 10 min at 95°C in 10 mM sodium citrate buffer, pH 6.0. Following heat treatment, the sections were incubated overnight with anti-human TLR3 or anti-TLR4 antibodies (Santa Cruz Biotechnologies) diluted in 0.1% BSA in PBS plus 1% pooled human serum. The secondary antibody used was biotinylated rabbit anti-goat Ig (DAKO). After a 1-h incubation at room temperature, slides were incubated with ABComplex/HRP (DAKO) for 30 min, and subsequently for 10 min with NovaRED substrate kit (Vector). Counterstaining was performed using hematoxylin. Routine immunohistochemical controls included omission of primary antibodies.

RESULTS

Expression of TLR-Encoding mRNA in Primary Cultures of Human Glia Cells

As a first evaluation of TLR expression in human glial cells, the presence of TLR-encoding mRNA was examined in primary cultures of microglia, astrocytes, and oligodendrocytes derived from postmortem human control brain by RT-PCR. The results as represented in Figure 1 for a particular control donor reveal marked expression of both TLR2 and TLR3 in all 3 types of glial cells. In addition to TLR2 and TLR3, microglia were found to also express TLR1, TLR4, TLR5, TLR6, TLR7, and TLR8 at readily detectable levels and TLR9 at low but detectable levels.

Next, levels of expression for each TLR family member were examined in primary cultures of microglia isolated from normal-appearing white matter samples from a variety of donors, including control subjects and donors with neurodegenerative diseases. For microglia isolated from each donor, levels of TLR-encoding mRNA for each different TLR family member were compared to levels of β-actin-encoding mRNA, as determined by competitive quantitative RT-PCR. The result of this semi-quantitative comparison, as summarized in Figure 2, revealed remarkable differences in levels of TLR expression among different primary microglia cultures. In microglia from 2 control donors, essentially all TLRs could be detected again, except for TLR9. For each TLR family member, at least one of the donors displayed levels of expression that were markedly different from other donors, including controls. Peak expression of individual TLR was always found in cases with neurodegenerative disease rather than in control subjects. Differences in expression, however, were not always the same in donors from the same patient group.

Immunostaining of Cultured Glia Cells

To further confirm expression of TLR3 and TLR4 in cultured glia cells, we performed double immunofluorescent staining. As shown in Figure 3A and 3B, both TLRs were clearly detectable in CD68-positive microglia cells. It should be noted that not all microglia were uniformly stained for TLR, but rather only in a minority of cells (~15%). When expressed, TLR3 or TLR4 were exclusively localized within intracellular vesicles and were undetectable on the surface of microglia cells. This supports previous observations suggesting that TLRs in cells of the monocyte/macrophage lineage are often stored in intracellular vesicles to be recruited to elements of the endosomal pathway (22). It should be noted that TLR3 and

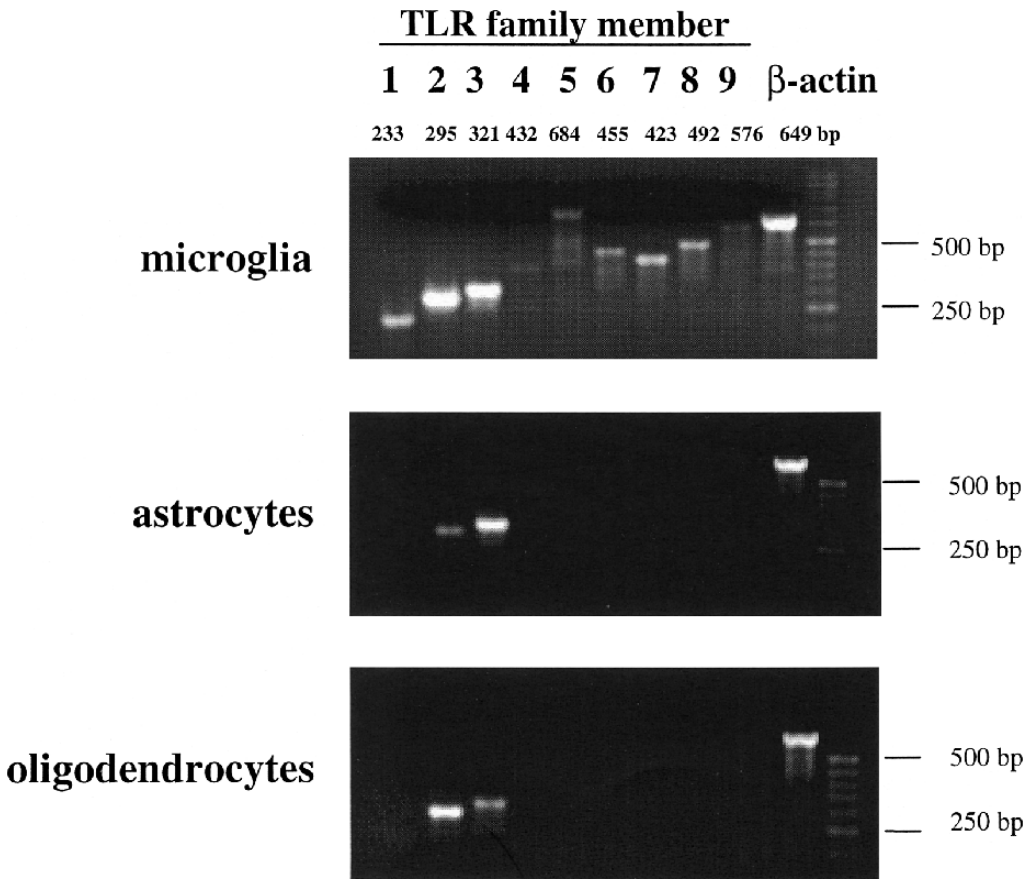

Fig. 1. Expression of 9 TLR family members in human glia cells. Primary cultures of microglia, astrocytes, and oligodendrocytes were obtained from human control white matter and TLR expression was examined by RT-PCR. Amounts of cDNA used for the amplification reactions were standardized to produce identical amounts of amplicons for β-actin. The figure shows clear expression of TLR2 and TLR3 in all 3 types of glial cells. In particular, microglia express TLR1 through TLR8 at detectable levels as well.

TLR4 expression as illustrated in Figure 3 reflects expression in a donor different from the one examined in Figure 2. GFAP-positive astrocytes were also positive for TLR3 and TLR4, as shown in Figure 3. In sharp contrast to the vesicular localization in a small minority of cultured microglia, however, TLR3 and TLR4 were exclusively found on the surface of the vast majority of astrocytes. No intracellular vesicles expressing either TLR could be detected in astrocytes.

In Vivo Expression of TLRs in MS Brains

To extend the data on TLR expression in cultured glia cells with data on in vivo expression of TLR, expression of TLR3 and TLR4 was also evaluated in brain and spinal cord sections from MS patients and control donors using immunohistochemistry. The expression patterns of TLR3 and TLR4 in the various brain and spinal cord samples are summarized in Table 3.

While in healthy white matter or normal-appearing white matter in MS patients TLR expression was barely detectable, high expression of both TLR3 and TLR4 was evident in all active MS lesions, especially in perivascular areas at the borders and in the center of lesions (Fig. 4). In early active lesions, the subcellular localization of either TLR was predominantly vesicular, indicative of microglial expression (Fig. 3). In such lesions, TLR-positive cells were primarily found around the inflamed blood vessels and at the borders of the lesion. In late active MS lesions, distinctly more cells were found with surface expression of TLR, which is typical for expression on astrocytes, and these TLR-positive cells in the tissue indeed displayed the unmistakable astroglial morphology. Some vesicular staining was usually also found in these late active MS lesions, suggestive of continued microglial expression.

It should also be noted that the vast majority of infiltrated leukocytes were negative for either TLR3 (Fig. 5) or TLR4, indicating that high expression of at least these 2 TLRs is not a constitutive feature of infiltrating monocytes. Again, this suggests that the vesicular TLR expression in microglia is the result of some form of activation.

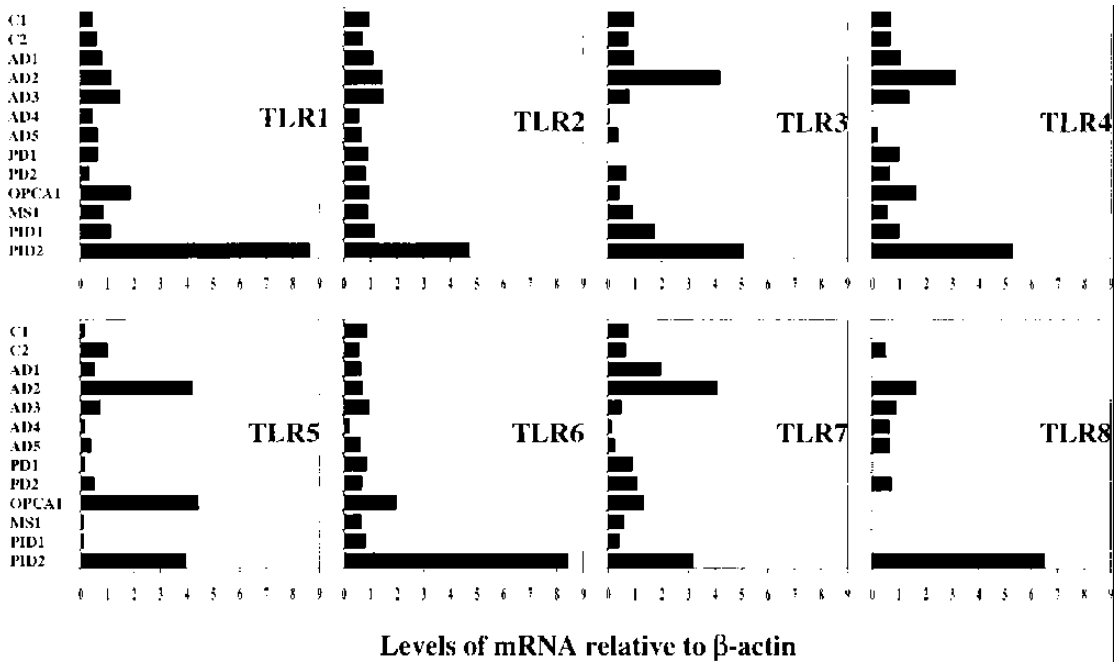

Fig. 2. Relative expression of 9 TLR family members in primary microglia from control donors and patients with different neurodegenerative diseases. Primary cultures of microglia were obtained from white matter samples of control donors and donors that represent cases of different neurodegenerative diseases (AD = Alzheimer disease, PD = Parkinson disease, OPCA = olivopontocerebellar atrophy, MS = multiple sclerosis, PID = Pick's disease). For each sample, the level of β-actin-encoding mRNA was first determined by competitive quantitative RT-PCR to allow definition of a standardized amount of cDNA that was used for analyzing TLR expression. Expression of mRNA encoding each TLR is expressed relative to that for β-actin.

DISCUSSION

This study reports the first documentation of the expression of TLRs in the human CNS. One of the major findings of our study is that human adult microglia express a wide range of TLR family members, including TLR1 through 8, at least in vitro. This strongly supports the notion that microglia are of prime importance for the regulation of immune responses in the CNS (14, 23). Expression of TLR2, TLR3, and to some extent also of TLR1 and TLR4, was found in human astrocytes and oligodendrocytes. Since previous findings indicate that murine astrocytes and oligodendrocytes are not able to express TLR4 (18), human glial cells do not necessarily display the same TLR profile as rodent glial cells.

Immunostaining of cultured microglia and astrocytes for TLR3 and TLR4 revealed 2 interesting features. First, both TLR3 and TLR4 were found exclusively localized in vesicular structures inside microglia and not on the surface of the cells. In cultured astrocytes on the other hand, TLR3 and TLR4 were only found on the cellular surface. This striking difference in subcellular localization of TLRs between microglia and astrocytes may relate to the difference in phagocytic and antigen processing properties of these cells (24–27). Secondly, only a minority of cultured microglia cells expressed sufficient levels of TLRs for them to become detectable by immunocytochemical staining, suggesting that microglial TLR expression is regulated and dependent on a certain level of activation that is reached only in a minority of cells in culture. Figure 3B shows very strong TLR4 expression on microglia cells, while Figure 1 shows only low levels of TLR4-encoding mRNA in microglia from another donor as assessed by RT-PCR. This variability illustrates the variations in TLR expression by microglia derived from different donors.

In astrocyte cultures, TLR expression was distinctly more homogeneous and comparable levels of TLRs were found on all cells. The distinct expression of TLR3 in microglia, astrocytes, as well as in oligodendrocytes is remarkable given previous reports that TLR3 would be restricted to dendritic cells only (8). These findings warrant follow-up studies, especially on the role of TLR3, a receptor for double-stranded RNA, in immune regulation in the human CNS.

The varying patterns of TLR expression in primary microglia from different donors are intriguing, but more difficult to interpret for several reasons. First, the cells were derived from white matter regions of the brain (subcortical whiter matter and corpus callosum) that show no visible signs of damage or abnormalities in many cases, despite neurodegeneration elsewhere in the brain (e.g. in gray matter). Secondly, levels of TLR-encoding mRNA in cultured microglia may have been affected to some extent by microglial activation during isolation and culturing.

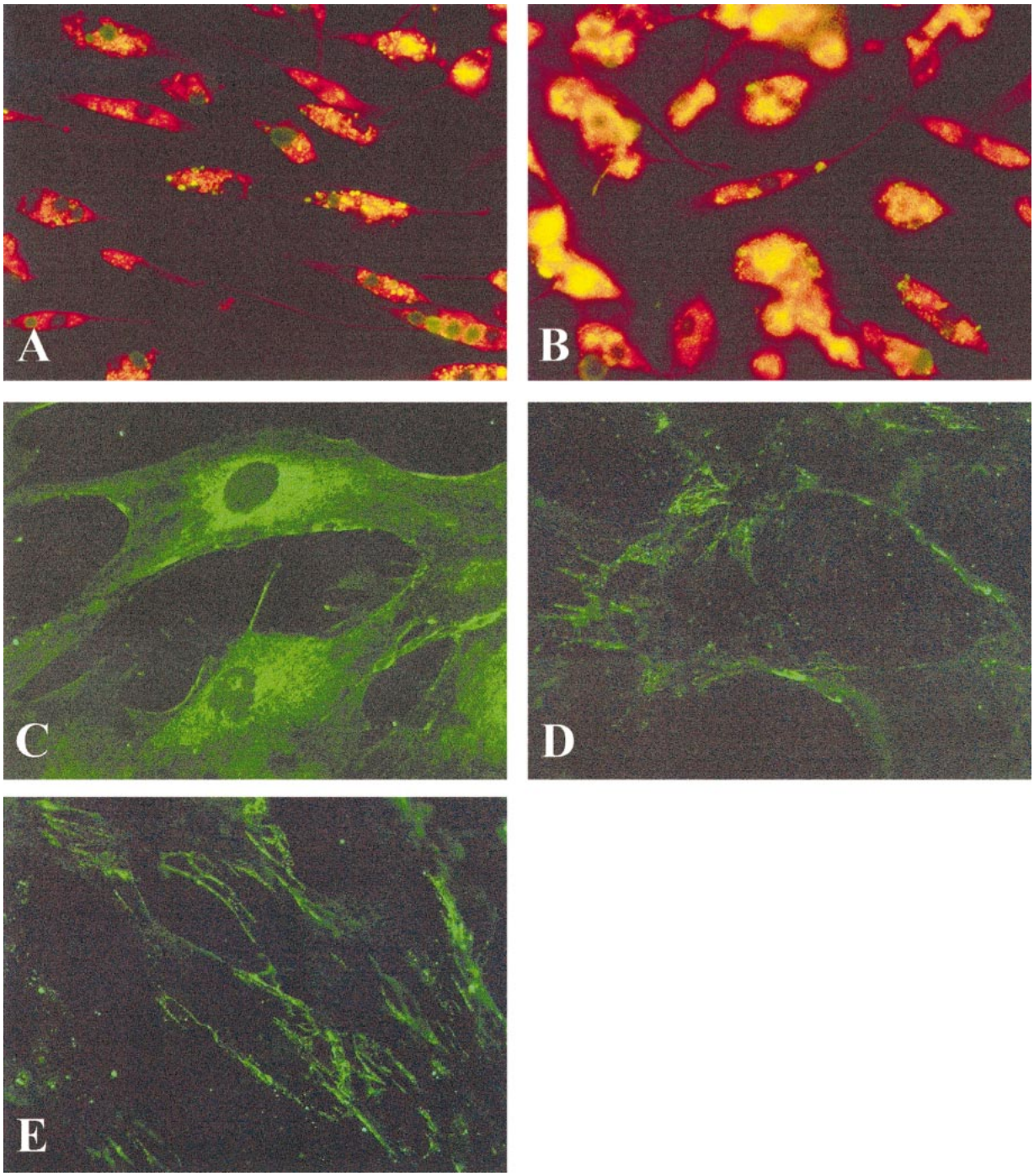

Fig. 3. Immunocytochemical localization of TLR3 and TLR4 in cultures of glia cells. Primary cultures of glial cells were obtained from white matter samples of control donors. Microglial cells were double stained for TLR3 or TLR4 (green) and CD68 (red) (A, B). Both TLRs were found exclusively localized in distinct vesicular structures inside microglia rather than on the surface of the cells. Astrocytes were stained for GFAP (C), TLR3 (D), and TLR4 (E). The subcellular localization of TLR3 and TLR4 in astrocytes was distinctly different from microglia in that TLR3 and TLR4 were found exclusively on the cell surface. Magnification: A–E, ×400.

Thirdly, TLR levels may have been affected by the agonal state during death of the donor, by drugs or treatment before death, or by the postmortem delay in sampling the brain tissue. Yet, it is difficult to envisage how the standardized isolation and culturing conditions on their own could have led to the remarkable differences observed for individual TLR family members in microglial cultures from different donors. Therefore, we think it is quite possible that different individuals, and especially those with neurodegenerative diseases, differ in in vivo patterns of

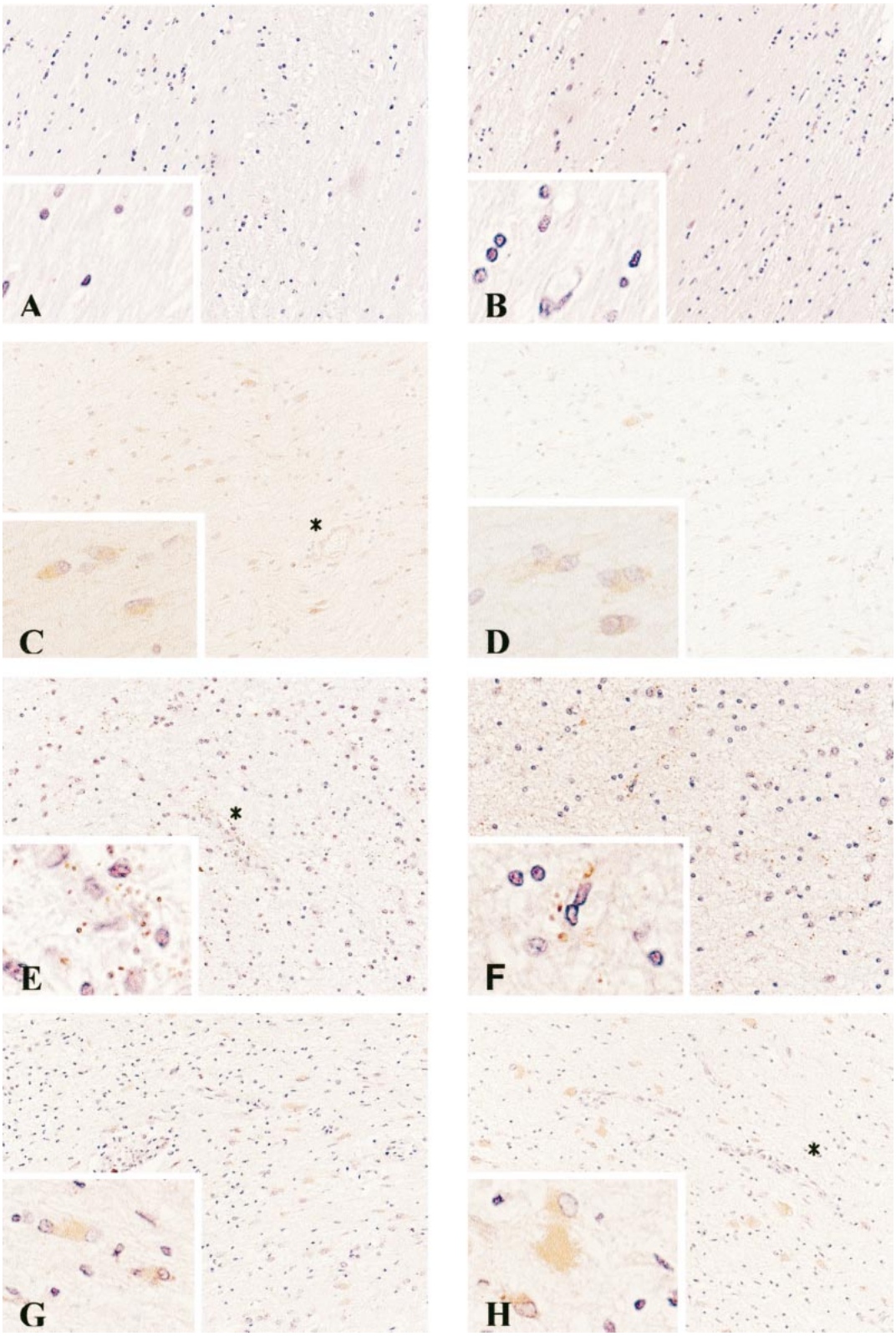

Fig. 4. Elevated expression of TLR3 and TLR4 in multiple sclerosis lesions. Expression of TL3 and TLR4 in healthy control white matter and sections representing MS lesions. Low-power photomicrographs (×100) of white matter from MS stained for either TLR3 (C, E, G) or TLR4 (D, F, H). Numbers of TLR-expression cells are strongly increased in MS lesions, with relatively high expression of TLR3 and TLR4 observed particularly in perivascular areas (*). TLR3 (A) and TLR4 (B) were almost absent from control brains. Inserts show TLR-positive cells at high magnification (×400).

TABLE 3
Expression of TLR3 and TLR4 in Brain and Spinal Cord Sections from MS Patients and Controls as Evaluated by Immunohistochemical Staining*

Diagnosis	Sample type	Type of lesion	TLR3	TLR4	Vesicular	Cell surface
MS	Brain	Late active	+/−	+/−	+	+/−
MS	Brain	Late active	+/−	++	+	+
MS	Brain	Late active	++	++	+	+
MS	Brain	Early active	++	++	+	−
MS	Spinal cord	Late active	++	++	+	+
MS	Brain	Late active	+/−	+/−	+	+
MS	Brain	Late active	+/−	+/−	+	+
MS	Brain	Late active	+/−	+/−	+	+
MS	Brain	Late active	++	++	+	+
MS	Spinal cord	Late active	++	++	+	+/−
MS	Spinal cord	Early active	++	++	+	+/−
MS	Spinal cord	Late active	+/−	+/−	+	+
MS	Brain	Late active	+/−	+/−	+	+
Control	Brain		−	−	−	−
Control	Brain		+/−	+/−	+	−
Control	Brain		−	−	−	−

* Staining intensities are summarized as follows: ++, many positive cells; + several positive cells; +/−, only few positive cells; −, absent.

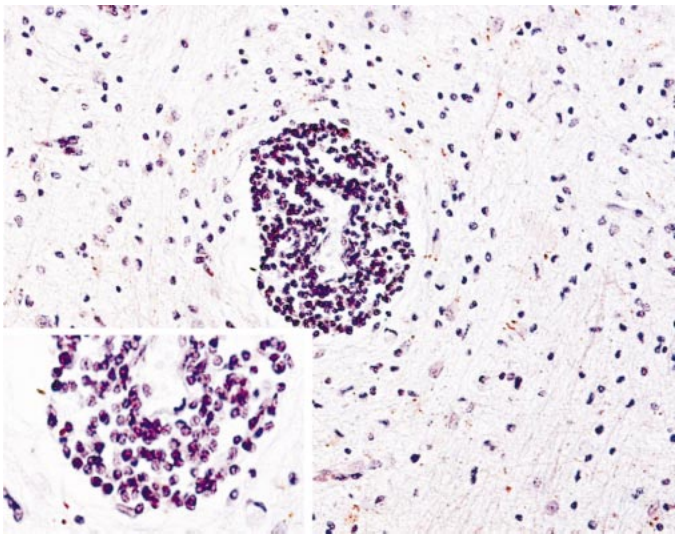

Fig. 5. Lack of TLR3 expression in infiltrating leukocytes. While TLR3 expression is prominent in resident microglia, no TLR3 expression can be detected by immunohistochemistry in leukocytes infiltrating the CNS parenchyma in MS. Low-power photomicrograph (×100) shows inflamed blood vessel in MS. The insert shows a high magnification detail (×400).

TLR expression and that these differences persist in primary cultures of microglia. Also, the differences persist when the microglia are derived from regions in the CNS that are not visibly affected by certain diseases. Alternatively, microglia from different donors somehow differ in their predisposition to upregulate individual TLR family members upon in vitro activation.

The data in Table 3 and Figure 4 reveal the almost complete absence of TLR3 and TLR4 from control brains and their upregulation during the development of MS lesions. Again, a clear distinction was observed between vesicular expression of either TLR in some cells (based on the above data taken to represent microglial expression) and surface expression on others (taken to represent astroglial expression). While more extensive studies are required to fully document the relationship between TLR expression and lesional development in MS, these first observations suggest that elevated vesicular expression in microglia is more prominent in early lesions whereas membrane-associated TLR expression on astrocytes typifies older active lesions. We are currently extending these initial observations by more closely comparing TLR expression patterns in MS lesions of varying stages, including all necessary staging markers for such lesions.

In summary, our data provide evidence for broad and regulated expression of several different TLRs in the human CNS. These findings warrant further investigations on the role of TLRs in controlling immune and neurodegenerative processes involving microglia and astrocytes.

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