Immunostimulatory CpG-DNA Activates Murine Microglia[1]

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Bacterial DNA containing motifs of unmethylated CpG dinucleotides (CpG-DNA) triggers innate immune cells through the pattern recognition receptor Toll-like receptor 9 (TLR-9). CpG-DNA possesses potent immunostimulatory effects on macrophages, dendritic cells, and B lymphocytes. Therefore, CpG-DNA contributes to inflammation during the course of bacterial infections. In contrast to other TLR-dependent microbial patterns, CpG-DNA is a strong inductor of IL-12. Thus, it acts as a Th1-polarizing agent that can be utilized as potent vaccine adjuvant. To assess the role of CpG-DNA in immune reactions in the CNS, we analyzed the effects of CpG-DNA on microglial cells in vitro and in vivo. Primary microglial cells as well as microglial cell lines express TLR-9 mRNA. Consequently, CpG-DNA activated microglial cells in vitro and induced TNF-α, IL-12p40, IL-12p70, and NO. Furthermore, MHC class II, B7-1, B7-2, and CD40 molecules were up-regulated. In addition, phagocytic activity of microglia was enhanced. After intracerebroventricular injection of CpG-DNA, microglial cells were activated and produced TNF-α and IL-12p40 transcripts, as shown by in situ hybridization. These results indicate that microglia is sensitive to CpG-DNA. Thus, bacterial DNA containing CpG motifs could not only play an important role during infections of the CNS, but also might trigger and sustain Th1-dominated immunopathogenic reactions. *The Journal of Immunology*, **2002, 168: 4854–4863.**

Due to the specialized blood-brain barrier and the relative lack of intraparenchymatic leukocytes, the CNS as a healthy organ is immunologically quiescent (1). Microglial cells that represent resident macrophages of the brain are especially important to guard the integrity of the brain and to initiate an immune response in case of infection. They comprise up to 20% of the total nonneuronal cell population (2). Furthermore, microglial cells play an essential role in traumatic, inflammatory, ischemic, and degenerative diseases of the CNS (3). This is due to their potent ability to produce proinflammatory cytokines and to present Ag upon activation (4).

Conserved microbial patterns such as LPS, lipoteichoic acid (LTA),[4] peptidoglycan, and bacterial DNA are recognized by pattern recognition receptors that are expressed by innate immune cells, including macrophages (5). Toll-like receptors (TLRs) play a pivotal role in pattern recognition (6). At least 10 different TLRs have been described to date, which display distinct ligand specificities (7). The respective TLRs involved in recognition of LPS, LTA, bacterial DNA, some bacterial lipoproteins, and flagellins have been identified recently (8–11). Signaling via TLRs essentially depends on the intracellular signal adapter molecule MyD88 (12–14) and results in activation of mitogen-activated protein kinases and translocation of NF-κB (15, 16). Albeit these major signaling pathways are shared by all TLRs, the biological effects of distinct TLR ligands are quite different, e.g., bacterial DNA induces high amounts of IL-12 (17), leading to a Th1-dominated immune response (18, 19), while LPS or LTA generates only minute amounts of IL-12.

Bacterial DNA activates cells of the innate immune system due to the relative abundance of unmethylated CpG-DNA motifs (20). Synthetic oligodeoxynucleotides (ODNs) with immunostimulatory CpG motifs, but not with GpC motifs, mimic these effects (21). TLR-9 is essential for activation of innate immune cells by CpG-DNA (10). CpG-DNA effects include activation, polyclonal proliferation, and Ig secretion of B cells and effects on T cells (22, 23). Macrophages and dendritic cells respond with cytokine secretion and up-regulation of costimulatory molecules (21). Inhibitory DNA sequence motifs have been described recently that antagonize the immunostimulatory capacity of CpG-DNA (24). A common inhibitory DNA motif is still elusive, yet guanosine-rich sequences seem to be important for inhibition (25).

In contrast to the peripheral immune system, the information on the effects of CpG-DNA in the CNS is limited. CpG-ODN-induced NK cell activation was utilized to successfully treat intracranial gliomas (26). After intracranial injection of CpG-DNA, a transient up-regulation of activation markers on microglia and astrocytes was reported (27). In vitro and after i.p. injection of CpG-DNA, induction of cytokines was observed (28). However, detailed information on TLR expression and phenotypical and functional consequences of CpG treatment is still lacking.

We therefore studied the effects of CpG-DNA on phenotypical and functional parameters of microglia in vitro as well as in vivo. In this study, we demonstrate that primary microglia as well as a microglial cell line express mRNA encoding TLR-9, rendering these cells sensitive to CpG-DNA. Furthermore, we show the activation of microglia through CpG-DNA in vitro as measured by induction of the proinflammatory and polarizing cytokines TNF-α, IL-12p40 and IL-12p70, NO production, phagocytosis, and Ag presentation. Guanosine-rich inhibitory ODNs blocked immunostimulation by CpG-ODN. After intracerebroventricular (i.c.v.) injection of CpG-ODN, mRNA transcripts encoding for TNF-α and

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[4] Abbreviations used in this paper: LTA, lipoteichoic acid; EAE, experimental allergic encephalomyelitis; iNOS, inducible NO synthase; ODN, oligodeoxynucleotide; TLR, Toll-like receptor; i.c.v., intracerebroventricular.

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IL-12p40 were assessed by in situ hybridization. Collectively, our results demonstrate that microglia responds to CpG-DNA in vivo and in vitro. CpG-DNA might thus play an important role during infection and during the course of autoimmune disease in the CNS.

Materials and Methods

Reagents and animals

Phosphorothioate-modified ODNs were custom synthesized by TIB Molbiol (Berlin, Germany) and MWG Biotech (Munich, Germany). The following sequences were used: 1668, TCC AT**G ACG TT**C CTG ATG CT, (the bold letters indicate the CpG motif); 1668GC, TCC AT**G AGC TT**C CTG ATG CT (control ODN with inverted CpG motif); PZ-3, CTC CTA TT**G GGG G**TT TCC TAT; PZ-2, CTC CTA GT**G GGG G**TG TCC TAT (guanosine-rich ODNs); PZ-312, CTC CTA TTG TGT GTT TCC TAT (control ODN with disrupted polyguanosine run). ODNs were negative for LPS, as measured by the *Limulus* assay (Sigma, Deisenhofen, Germany). LPS, *Escherichia coli* DNA, and calf thymus DNA were purchased from Sigma. IFN-γ was obtained from E. Adolf (Vienna, Austria). Eight- to 12-wk-old female mice (BALB/c and C57BL/6) were obtained from Harlan Winkelmann (Borchen, Germany).

Media and cells

Cells were cultured in Clicks/RPMI 1640 supplemented with 5% FCS, 50 μM 2-ME, and antibiotics (penicillin G (100 IU/ml) and streptomycin sulfate (100 IU/ml)). BV-2 cells, a murine microglial cell line immortalized with a v-*raf*/v-*myc*-carrying retrovirus (29), were obtained from A. Fontano (Zuerich, Switzerland). RAW 264.7 cells were a kind gift of R. Schuhmann (Berlin, Germany); LNC2 (a murine Th1 clone) was donated by M. Lohoff (Marburg, Germany). Bone marrow-derived dendritic cells from BALB/c mice were prepared as described (30). On day 9 of bone marrow culture, mature dendritic cells ($CD11c^+$, $GR\text{-}1^-$) were used that represented >85% of the growing cell population. Peritoneal exudate cells were prepared by peritoneal lavage of untreated BALB/c mice. Cells were seeded in culture medium and washed 2 and 4 h after plating to remove nonadherent cells. B lymphocytes were prepared from freshly isolated splenocytes by magnetic cell sorting using anti-CD45R mAb, according to the manufacturer's protocol (Miltenyi Biotec, Bergisch-Gladbach, Germany).

i.c.v. injection

Adult male BALB/c mice (>25 g body weight) were used in the study. Before surgery, all animals were anesthesized with 0.05 ml/animal of a stock solution containing 0.2 ml Rompun and 0.8 ml Ketamin 500. After placement into a stereotactic apparatus (ASI Intruments, Warren, MI), heads were fixed using a mouse nose clamp adaptor in a nose-down position (−2.8 mm) and the skulls were exposed for trepanation. A total volume of 0.5 μl CpG-DNA (250 nmol/ml), LPS (1 μg/ml), or vehicle (0.9% NaCl) was injected in the lateral ventricle. Coordinates were determined according to a mouse stereotactic atlas (31), as follows: −0.34 mm posterior, 1 mm lateral, 2.5 mm ventral to bregma.

Isolation of microglial cells

Microglia of newborn mice was prepared as described (32). Briefly, brains of newborn C57BL/6 mice were dissected and dissociated. The cells were seeded in culture medium containing 20% FCS with 200 mg material per 175-cm^2 tissue culture flask. On days 4 and 8, medium was exchanged with fresh medium containing 10% and 5% FCS, respectively. On day 14, brain cultures were agitated on a rotary shaker at 700 rpm for 1 h. Thereafter, detached cells were collected and sedimented at $1 \times g$ for 10 min, and then the supernatant was incubated in culture flasks at 37°C for 2 h. Again, cultures were agitated for 10 min at 90 rpm, and nonadherent cells were removed. Adherent cells were identified as microglia with a purity of >90% for Mac1 and F4/80 expression, as determined by flow cytometry.

RNA preparation and cDNA synthesis

For preparation of RNA from various tissues, mice were killed by CO_2 asphyxia, and samples were prepared and flash frozen in liquid nitrogen. Solid tissue was disrupted and homogenized on ice, and RNA was isolated using TRIzol (Life Technologies, Karlsruhe, Germany), according to the manufacturer's protocol. Traces of DNA were removed by incubation with DNase I (Roche, Mannheim, Germany), and finally RNA was further purified with RNeasy kit (Qiagen, Hilden, Germany). RNA was stored at −70°C before cDNA synthesis. Total RNA from cell cultures was isolated using HighPure RNA kit (Roche), which included DNase I digestion. A quantity amounting to 1 μg total RNA preparation was reverse transcribed with cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) using $oligo(dT)_{23}$.

RT-PCR

RT-PCR for β-actin and TLR-9 was performed using *Taq* polymerase (Eppendorf, Hamburg, Germany) with 2.5 μl cDNA. Primer (vide infra) and cycler conditions were the same as for quantitative RT-PCR. $MgCl_2$ concentration was 2 mM, and 33 cycles were run. PCR products were visualized on a 2% agarose gel by ethidium bromide staining.

Quantitative RT-PCR

Expression of inducible NO synthase (iNOS) was determined using SYBR-Green I as detection reagent; all other mRNAs were quantified with double-dye oligonucleotide real-time RT-PCR (Eurogentec, Brussels, Belgium). cDNA was diluted 1/4, and 2.5 μl were used as template in a 25 μl PCR mix. $MgCl_2$ was used with 5 mM (iNOS detection 1.5 mM), and cycler conditions were 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. β-Actin primer (sense, CCC TGT GCT GCT CAC CGA; antisense, ACA GTG TGG GTG ACC CCG TC); TNF-α primer (sense, AAA ATT CGA GTG ACA AGC CTG TAG; antisense, CCC TTG AAG AGA ACC TGG GAG TAG); IL-12p40 primer (sense, AAG AAG GAA AAT GGA ATT TGG TCC; antisense, ATG TCA CTG CCC GAG AGT CAG); IL-12p35 primer (sense, TGG ACC TGC CAG GTG TCT TAG; antisense, CAA TGT GCT GGT TTG GTC CC); iNOS primer (sense, CAG CTG GGC TGT ACA AAC CTT; antisense, CAT TGG AAG TGA AGC GTT TCG); and TLR-9 primer (sense, GGG CCC ATT GTG ATG AAC C; antisense, GCT GCC ACA CTT CAC ACC AT) were purchased from MWG Biotech. Fluorogenic probes (6-carboxyfluorescein) were: β-actin, CCC CTG AAC CCT AAG GCC AAC CG; TNF-α, CAC GTC GTA GCA AAC CAC CAA GTG GA; IL-12p40, AAA AAC AAG ACT TTC CTG AAG TGT GAA GCA; IL-12p35, ACA GAT GAC ATG GTG AAG ACG GCC AGA; TLR-9, CAG TTC TAG ACG TGA GAA GCA ACC CTC TGC.

Specificity of RT-PCR was controlled by no template and no reverse-transcriptase controls. SYBR-Green I detection was followed by generation of melting curves and visualization of the products to confirm specificity. Quantitative PCR results were obtained using the ΔΔCT method (33). Since PCR efficiencies for all reactions were similar (~0.95–1), threshold values were normalized to β-actin and set in reference to unstimulated control cells. Thus, the induction of mRNA was calculated as $2^{\Delta\Delta Ct}$.

Cell stimulation and determination of cytokine secretion

A total of 1×10^6 cells/well (RT-PCR) or 0.25×10^6 cells/well (ELISA) was plated in 12-well or 96-well culture plates and incubated with different stimuli at the indicated concentrations. Cytokine levels in culture supernatants were determined using commercially available ELISA kits for TNF-α, IL-12p70, and IL-12p40, according to the manufacturer's instructions (BD Biosciences, Heidelberg, Germany). Each value represents mean of duplicate values.

Determination of NO

NO accumulation was measured photometrically (550 nm). Equal parts of supernatant and Griess reagent (1:1 mixture of 1 g/100 ml sulfanilamide/5% H_3PO_4, and 0.1% naphthylethylenediamine dihydrochloride) were mixed and subsequently measured.

Phagocytosis assay

A total of 0.25×10^6 BV-2 cells was prestimulated with CpG-ODN, control ODN, DNA from *E. coli*, DNA from calf thymus, or LPS at the indicated concentration for 6 h. Cells were then incubated with 0.0025% fluorescent beads (carboxylate-modified, yellow-green fluorescent latex beads, diameter 1 μm; Sigma) for 1 h at either 37°C or 4°C. Cells were washed twice in ice-cold PBS/2% FCS, and fluorescence was determined by flow cytometry. The fraction of phagocytic active cells was calculated: % positive $cells^+_{37°C}$ − % positive $cells^+_{4°C}$.

Flow cytometry analysis

Abs used were obtained from BD PharMingen (Heidelberg, Germany). Cells were washed in PBS/2% FCS. As first step, Fc block was performed by incubating with anti-FcγRII/III mAbs (clone 2.4G2) and 10% normal mouse serum for 15 min on ice. Then cells were stained for 45 min on ice with combinations of the following Abs: FITC-conjugated anti $I\text{-}A^d/E^d$ mAbs (clone 2G9), FITC anti-CD40 (clone 3/23), FITC anti-GR-1 (clone RB6-8C5), FITC anti-F4/80 (clone CI:A3-1; Serotec, Oxford, U.K.), PE-conjugated anti-CD11c Abs (clone HL3), PE anti-CD80 (16-10A1), PE

anti-CD86 (GL1). Cells were then washed and fixed in PBS/1% paraformaldehyde. Twenty thousand cells were analyzed on a Partec PAS flow cytometer (DAKO, Hamburg, Germany). Data were analyzed using WinMDI software.

Ag presentation assay

The capacity of microglia to present Ag to T cells was determined using an OVA peptide (SIINFEKL/K^b)-specific T cell hybridoma (B3Z) transfected with a LacZ reporter under the transcriptional control of IL-2 gene promoter elements (34). Microglia was activated with different stimuli for 54 h. Thereafter, cells were serially diluted and incubated with 5×10^4 B3Z cells and 100 nM SIINFEKL (Neosystem, Strasbourg, France) in a 96-well plate overnight. Cultures were then washed with 200 μl PBS and lysed by addition of 100 μl 100 mM 2-ME, 9 mM $MgCl_2$, 0.125% Igepal, 0.15 mM chlorophenol red-β-galactoside (Calbiochem, Schwalbach, Germany) in PBS. After 5-h incubation at 37°C, 50 μl 300 mM glycine, 15 mM EDTA in water were added and the *lacZ* activity was measured photometrically (550/630 nm).

In situ hybridization

Probes. A pGEM 3 vector construct containing a 470-bp cDNA fragment of mouse TNF-α was used for riboprobe generation (gift of D. Männel, University of Regensburg, Regensburg, Germany). A 1438-bp-long cDNA fragment for mouse IL-12p40 corresponding to nt 19–1456 of the published cDNA sequence (accession M86671) was generated by RT-PCR from mouse spleen using the forward primer 5′-CTC GCA GCA AAG CAA GAT GTG TCC-3′ and the reverse primer 5′-CTA ATG TAC CTA CGC AGC CCT GAT TG-3′, and sublconed into pGEM-T (Promega). Sequence identity was confirmed by DNA sequencing (Seqlab, Göttingen, Germany). For cellular localization of C1q mRNA, a 425-bp *Bam*HI/*Pst*I subfragment of a cDNA clone encoding the β-chain of rat C1q was subcloned into pBluescript II KS(–), as described (38, 39). Riboprobes in antisense and sense orientation were generated from linearized vector constructs by in vitro transcription (35) using the appropriate RNA polymerases and [^{35}S]UTP as label. After transcription, the probes were subjected to mild alkaline hydrolysis, as described (36).

Hybridization histochemistry. Radioactive in situ hybridization was performed as described previously (37). Briefly, following prehybridization, 14-μm-thick formaldehyde-postfixed frozen sections were incubated each for 14 h at 60°C in 50 μl hybridization buffer (3× SSC, 50 mM $NaPO_4$, 10 mM DTT, 1× Denhardt's solution, 0.25 g/L yeast tRNA, 10% dextran sulfate, and 50% formamide) containing riboprobes at a concentration of 50,000 dpm/μl. After 30-min incubation in RNase buffer (10 mM Tris (pH 8), 0.5 M NaCl, 1 mM EDTA) containing 1 U/ml RNase T1 and 20 μg/ml RNase A (Roche) at 37°C, and successive washes in SSC-decreasing salt concentrations (1×, 0.5×, and 0.2× SSC) and a final wash in H_2O, the dehydrated slides were exposed together with ^{14}C standards (ARC, St. Louis, MO) to x-ray film for 24 h to 2 days. For microscopic analysis, slides were coated with NTB-2 nuclear emulsion (Eastman Kodak, Rochester, NY). After exposure times of 14–21 days, autoradiograms were developed in D19 (Eastman Kodak), counterstained with cresyl violet, and examined under dark and bright field illumination using a Olympus AX70 microscope. Photographic documentation was performed with the MCID M4 image analysis system (Imaging Research, St. Catharines, Ontario, Canada).

Results

Microglia expresses TLR-9 mRNA

Since TLR-9 has been shown to be essential for signaling of CpG-DNA, we first determined expression of TLR-9 mRNA in microglial cells by RT-PCR. Both the microglial cell line BV-2 and primary microglia derived from murine neonatal brain cultures expressed TLR-9 transcripts (Fig. 1*A*). RAW 264.7 macrophages

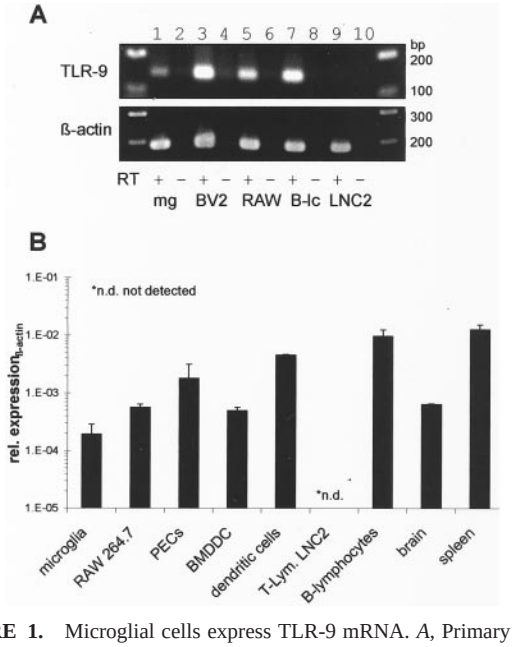

FIGURE 1. Microglial cells express TLR-9 mRNA. *A*, Primary microglia (mg), a microglial cell line (BV2), a macrophage cell line (RAW 264.7), primary B lymphocytes (B-lc), and a Th1 lymphocyte cell line (LNC2) were analyzed for expression of TLR-9 mRNA (*top*) and β-actin (*bottom*) by RT-PCR either with (+) or without (−) reverse transcriptase. *B*, TLR-9 mRNA expression in primary microglia, RAW 264.7 cells, peritoneal exudate cells (PECs), bone marrow-derived dendritic cells (BMDDC), $CD11c^+$ dendritic cells, LNC2 cells, B lymphocytes, as well as complete homogenates of brain and spleen was quantified by real-time RT-PCR. The relative (rel.) expression of TLR-9 mRNA in comparison with β-actin mRNA is given.

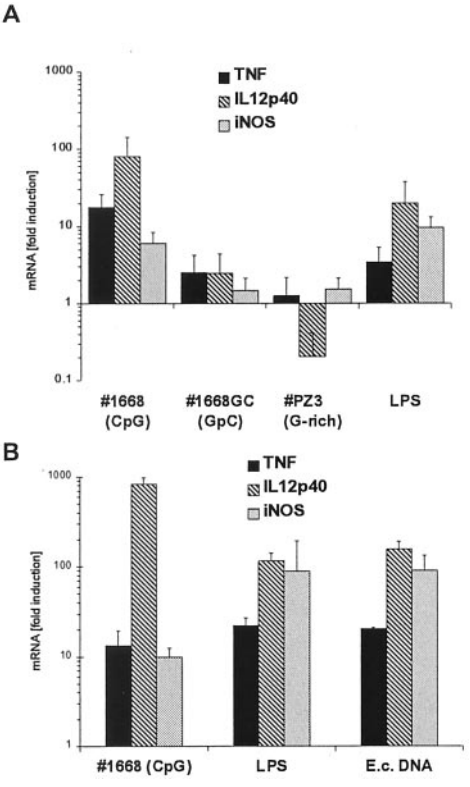

FIGURE 2. Microglial cells express TNF-α, IL-12p40, and iNOS mRNA after stimulation with CpG-ODN. *A*, BV-2 cells were stimulated with 1 μM of different ODNs containing either a CpG motif (1668), the inverted GpC sequence (1668GC), or a central guanosine-rich region (PZ3), or with 1 μg/ml LPS. After 5 h, mRNA expression of TNF-α, IL-12p40, and iNOS was quantified by quantitative real-time RT-PCR in comparison with unstimulated cells. *B*, Primary microglia was stimulated with 3 μM CpG-ODN (1668), 1 μg/ml LPS, or 30 μg/ml *E. coli* DNA for 5 h. Quantification of TNF-α, IL-12p40, and iNOS mRNA was performed as in *A*.

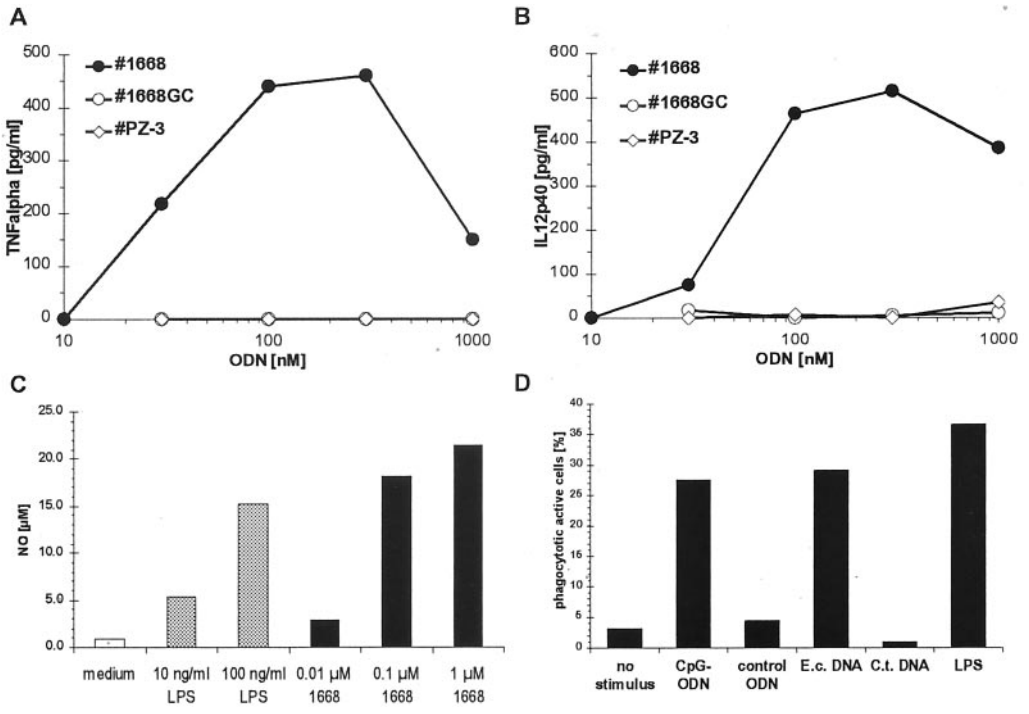

FIGURE 3. CpG-ODNs activate microglia to produce TNF-α, IL-12p40 protein, and NO as well as to enhance phagocytosis. BV-2 cells were stimulated with the indicated concentration of CpG-ODN (1668), control ODN (1668GC), and guanosine-rich ODN (PZ3). TNF-α (*A*) and IL-12p40 (*B*) were measured in the supernatant by ELISA after 24 h of stimulation. *C*, BV-2 cells were treated with LPS or CpG-ODN (1668) in the indicated concentrations, and NO was measured after 48 h in the supernatant. *D*, BV-2 cells were stimulated with 1 μM CpG-ODN 1668, 1 μM control ODN 1668GC, 30 μg/ml DNA from *E. coli* (E.c. DNA), 30 μg/ml control DNA from calf thymus (C.t. DNA), or 1 μg/ml LPS for 6 h. Cells were then incubated at either 37°C or 4°C with 0.0025% fluorescent beads (diameter 1 μm) for 1 h. Positively stained cells were determined by flow cytometry, and phagocytic uptake was determined as: $cells^{+}{}_{37°C} - cells^{+}{}_{4°C}$. Shown are the results of one of three typical experiments.

and primary B lymphocytes, which are known to be responsive to bacterial DNA, also expressed TLR-9, while no TLR-9 transcripts could be detected in the T cell clone LNC2. We next quantified TLR-9 expression by double-dye oligonucleotide real-time RT-PCR (Fig. 1*B*). Highest expression was observed in the spleen. Primary $CD11c^{+}$ dendritic cells isolated from lymph nodes showed a similar high expression. In contrast, macrophages, which are known to respond efficiently to stimulation with CpG-DNA, expressed only moderate TLR-9 mRNA (RAW 264.7, peritoneal exudate cells). Expression of TLR-9 mRNA in microglia was the lowest of all cell types examined, yet was in the range of immature murine bone marrow-derived dendritic cells and murine macrophages, known to be responsive to CpG-DNA. Interestingly, expression in total brain extract was higher than in microglia, which could be either due to passenger leukocytes, TLR-9 expression in other cell types of the brain, or down-regulation of TLR-9 in isolated microglia.

CpG-DNA induces microglia to produce TNF-α, IL-12p40, and iNOS mRNA

We next examined whether microglia can be activated by CpG-DNA. Both the BV-2 cell line and primary microglial cells showed induction of mRNA for TNF-α, IL-12p40, and iNOS after stimulation with either CpG-ODN or bacterial DNA (Fig. 2), but not with eukaryotic DNA (data not shown). Neither the control CpG-ODN nor guanosine-rich ODNs activated microglia cells. LPS as positive control induced almost equal amounts of TNF-α mRNA, yet was only a poor inducer of IL-12p40. In contrast, iNOS induction was higher after stimulation with LPS.

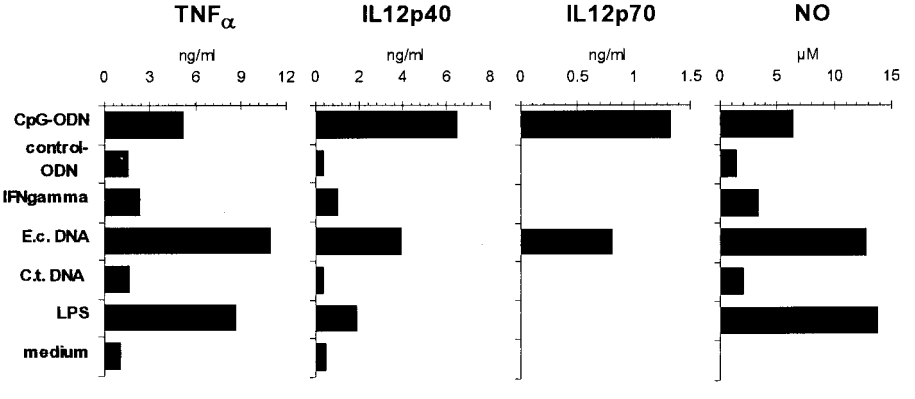

FIGURE 4. Primary microglia produces TNF-α, IL-12p40, IL-12p70, and NO in response to CpG-DNA. Neonatal derived microglial cells were incubated with 3 μM CpG-ODN (1668), 3 μM control ODN (1668GC), 10 U/ml IFN-γ, 30 μg/ml *E. coli* DNA (E.c. DNA), 30 μg/ml calf thymus DNA (C.t. DNA), or 1 μg/ml LPS. After 24 h, IL-12p40, IL-12p70, and TNF-α production as well as NO generation in the supernatant were determined.

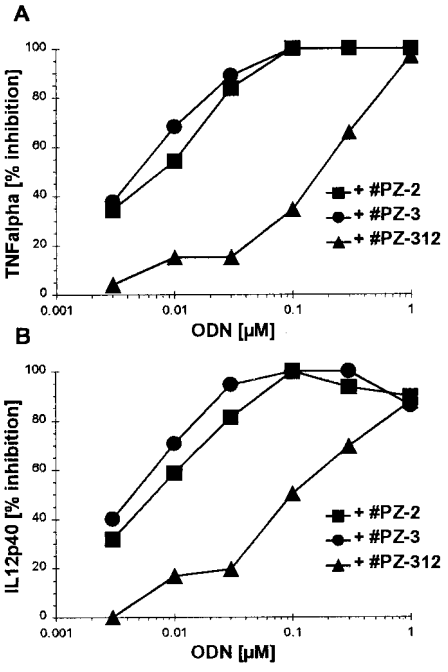

FIGURE 5. ODNs containing a central polyguanosine motif inhibit the immunostimulatory action of CpG-ODN on microglia. BV-2 cells were stimulated either with 0.3 μM CpG-ODN 1668 alone or in the presence of different concentrations of ODNs containing a central polyguanosine motif (PZ-2, PZ-3) or a disrupted polyguanosine motif (PZ-312). After 24 h, TNF-α (*A*) and IL-12p40 (*B*) were determined in the supernatant. Displayed is the inhibition of CpG-DNA stimulation.

CpG-DNA activates microglia to secrete cytokines (TNF-α, IL-12p40, IL-12p70) and effector molecules (NO) and to enhance phagocytosis

Induction of mRNA was followed by secretion of TNF-α and IL-12p40 protein after stimulation of BV-2 cells with CpG-DNA (Fig. 3). Dose-response titrations revealed that BV-2 cells required approximately 10-fold higher ODN concentrations for stimulation than those needed for stimulation of the macrophage cell line RAW 264.7 (data not shown). Microglia also was activated by CpG-ODN to produce the effector molecule NO. (Fig. 3*C*). Furthermore, CpG-ODN and *E. coli* DNA, but not control ODN and vertebrate DNA, strongly enhanced phagocytic capacity of microglia (Fig. 3*D*).

CpG-ODN as well as bacterial DNA also activated primary microglia to secrete TNF-α, IL-12p40, and IL-12p70 (Fig. 4). Again, CpG-DNA was the most potent inducer of IL-12p40. Only CpG-DNA induced measurable amounts of IL-12p70. In addition, primary microglia also generated NO after stimulation with CpG-DNA (Fig. 4).

ODNs containing runs of guanosines inhibit CpG-DNA-induced cytokine production

ODNs containing a central run of at least five guanosines (poly(G) motif) have been reported to antagonize the action of CpG-DNA, but not of LPS (25). This effect is probably due to blockade of initial CpG-specific signaling events. To test whether guanosine-rich ODNs also would antagonize CpG-DNA on microglia, we examined the effect of guanosine-rich ODNs on CpG-DNA-induced TNF-α and IL-12p40 secretion of BV-2 cells. Coincubation of CpG-ODN 1668 with different concentrations of guanosine-rich ODNs (PZ-2, PZ-3) resulted in a dose-dependent inhibition of CpG-DNA stimulation (Fig. 5). These results corroborated previous data obtained with macrophages, indicating that the mode of action of CpG-DNA on microglia cells is essentially identical to the action on peripheral macrophages.

CpG-DNA induces up-regulation of costimulatory molecules on microglia and enhances Ag presentation

Upon activation by CpG-DNA, APCs have been reported to up-regulate costimulatory molecules, leading to an enhanced Ag presentation and activation of cells of the adaptive immune system. To test whether microglia can be modulated in its ability to act as APC, we first examined the effect of CpG-DNA on the regulation of MHC class II, CD40, CD80, and CD86 in BV-2 cells (Fig. 6*A*). Cells stimulated with IFN-γ served as a positive control. IFN-γ induced a marked up-regulation of all examined molecules, as determined by flow cytometry. In contrast, stimulation with CpG-DNA led to a distinct pattern. CD40 and CD80 were up-regulated,

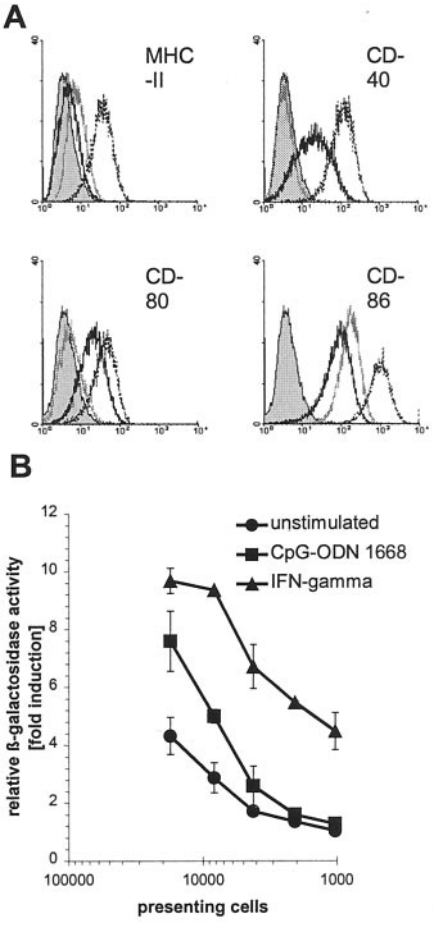

FIGURE 6. Effects of CpG-ODN and IFN-γ on Ag-presenting and costimulatory molecules. *A*, BV-2 cells were stimulated either by 1 μM CpG-ODN 1668 (black line) or 30 U/ml IFN-γ (dashed line), or were left unstimulated (gray line) for 24 h. Surface expression of MHC class II, CD40, CD80, and CD86 was determined by flow cytometry. Staining was controlled by staining with isotype-matched control Abs (filled histograms). *B*, Microglia was prepared from the brains of newborn C57BL/6 mice, as described in *Materials and Methods*. At day 14, microglial cells were stimulated by either 1 μM CpG-ODN 1668 (■) or 5 U/ml IFN-γ (▲) or were left untreated (●) for 54 h. Cells were then serially diluted and tested for presentation of the peptide SIINFEKL by overnight incubation with 5×10^4 B3Z cells (SIINFEKL/K^b complex-specific T cell hybridoma transfected with NF-AT-*lacZ* reporter) and 100 nM peptide. Induced β-galactosidase was measured, and the induction index was calculated by dividing induced activity through activity of B3Z cells without presenting cells.

Table I. *CpG-ODN up-regulate MHC-II and costimulatory molecules on primary microglia*[a]

	MHC Class II		CD40		CD80		CD86	
	%	MFI	%	MFI	%	MFI	%	MFI
No stimulus	26.6	2.31	33.5	7.65	34.9	10.19	31.7	6.79
CpG-ODN	47.0	3.64	56.9	13.54	58.6	14.35	48.9	8.85
IFN-γ	59.7	4.69	86.2	25.76	77.5	18.93	74.9	59.3

[a] Microglial cells were prepared from brains of newborn C57BL/6 mice. Cells (2×10^5) were stimulated with 1 μM CpG-ODN 1668 or 30 U/ml IFN-γ or were left untreated for 48 h. Surface expression of MHC-II, CD40, CD80, and CD86 was determined by flow cytometry. Staining was controlled by isotype-matched control Abs. The percent of positively stained cells and mean fluorescence intensity (MFI) is given.

while no effects or even a slight down-regulation were observed for MHC class II and CD86. However, BV-2 cells exhibited a high constitutive expression of CD86, which is in accordance with published properties of microglia (4).

To test whether these results also hold true for primary cells, microglia derived from neonatal brain cultures was examined (Table I). Stimulation of these cells with CpG-DNA resulted in an increase of cells positive for MHC class II, CD40, CD80, and CD86. In parallel, the mean fluorescence intensity increased. However, in comparison with stimulation with IFN-γ, CpG-DNA was less effective. To analyze whether CpG-DNA would enhance the potency of primary microglial cells to present MHC class I-restricted peptides to T cells, primary microglia was first stimulated with CpG-DNA. The activated cells were then incubated with class I MHC (H2-K^b)-binding antigenic peptide (SIINFEKL) and B3Z cells (SIINFEKL/K^b-specific T cell hybridoma transfected with NF-AT-*lacZ* reporter). After 24 h, the induced β-galactosidase was measured by ELISA. CpG-DNA-treated microglia was able to specifically stimulate the reporter T cells, yet the efficacy was markedly lower compared with stimulation of microglia with IFN-γ (Fig. 6*B*). Together the data show that CpG-ODN not only increases expression of MHC class II and costimulatory molecules, but also enhances Ag presentation by microglia, albeit with lower efficacy compared with IFN-γ.

CpG-DNA activates microglia in vivo

We finally addressed the question as to whether CpG-DNA also activates microglia in vivo. Mice were injected i.c.v. with CpG-DNA or LPS. Brains were cut into halves. One half was subjected to quantitative mRNA analysis for TNF-α, IL-12p40, and IL-12p35 (Fig. 7*A*). In parallel, in situ hybridization analysis was performed using frozen sagittal sections of the other brain half to examine the distribution and cellular expression pattern of TNF-α and IL-12p40 mRNA-producing cells (Figs. 8 and 9).

Quantitative PCR analyses revealed induction of mRNA specific for TNF-α and IL-12p40 after injection of CpG-DNA or LPS (Fig. 7*A*). However, both stimuli failed to change the expression level of IL-12p35 (Fig. 7*A*). Induction of IL-12p40 was significantly stronger after stimulation with CpG-DNA as compared with LPS.

In situ hybridization revealed that after i.c.v. application of CpG-DNA, the expression of the IL-12p40 gene was induced in a subpopulation of cells predominantly located in the juxtaventricular regions of the brain (Fig. 8*A*). The strongest labeling was observed in the basal hypothalamus and, to a lower extent, in brain parenchyma neighboring the fourth ventricle. Cell numbers and cellular labeling intensity decreased with increasing distance from the ventricular system, suggesting a local mode of action of the injected stimuli. The distribution and cellular staining pattern of IL-12p40 mRNA-positive cells resembled that of nonneuronal cells most typical for microglia (Figs. 8 and 9, *A* and *G*). To compare the cellular distribution, adjacent sections were hybridized with a probe for C1q mRNA, a marker for microglial cells, as shown previously (38) (Fig. 8*B*). While C1q mRNA-expressing cells were randomly scattered throughout the brain parenchyma, the IL-12p40-expressing cells represented a major subpopulation of C1q-positive microglial cells in the hypothalamus with a similar, if not identical, distribution pattern. Furthermore, high power examination of bright field microscopic analysis of hybridized autoradiograms counterstained with cresyl violet (Fig. 9, *G* and *H*) showed that silver grains representing positive hybridization signals for IL-12p40 (Fig. 9*G*) and for TNF-α (Fig. 9*H*) accumulated

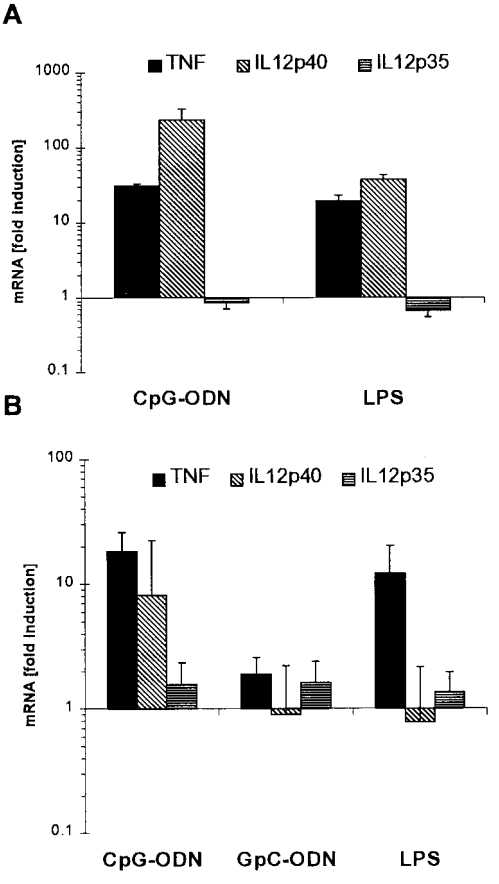

FIGURE 7. CpG-ODN induce IL-12p40, IL-12p35, and TNF-α mRNA in the brain. *A*, BALB/c mice were injected i.c.v. with either 125 pmol CpG-ODN 1668 or 500 pg LPS. After 4 h of stimulation, mice were killed, and induction of mRNA for TNF-α, IL-12p40, and IL-12p35 in the brain was determined by real-time RT-PCR in comparison with NaCl-injected controls. *B*, BALB/c mice were injected i.p. with either 10 nmol CpG-ODN 1668, 10 nmol GpC-ODN 1720 (control ODN), or 10 μg LPS. mRNA induction of IL-12p40, IL-12p35, and TNF-α in the brain was measured after 4 h, as in *A*.

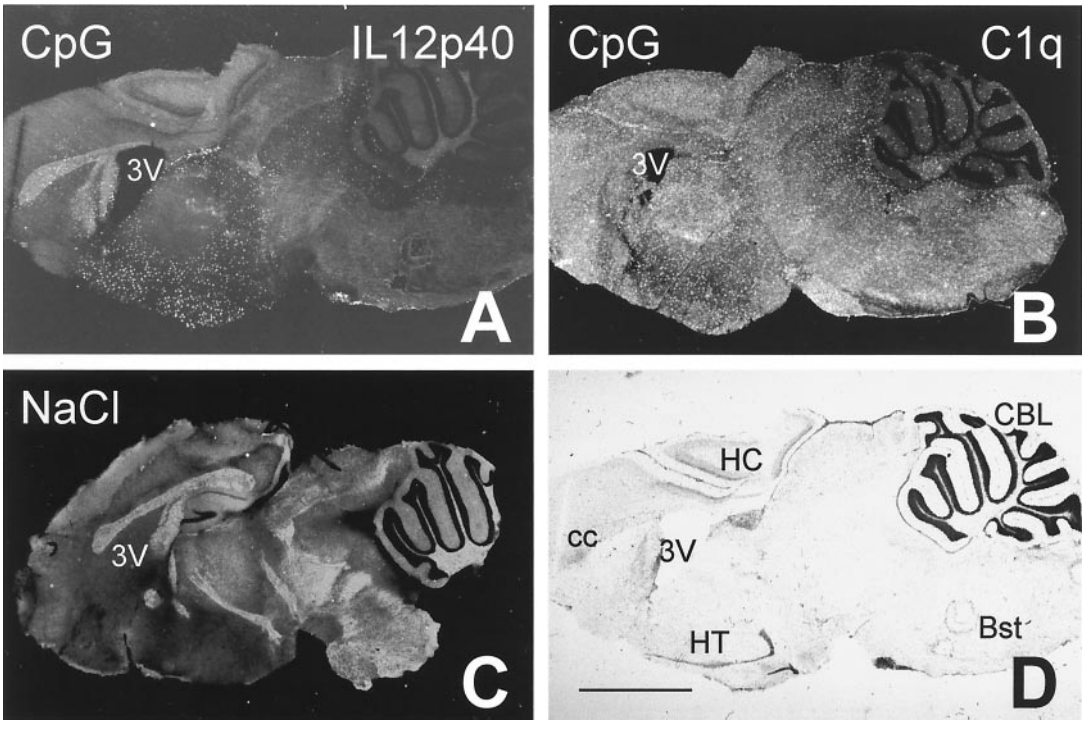

FIGURE 8. Dark field micrographs illustrating the comparative distribution of cells expressing IL-12p40 mRNA (*A*) and the microglial marker C1q (*B*) in mouse brain after i.c.v. application of 125 pmol CpG-ODN 1668. IL-12p40 mRNA is induced after CpG-ODN application as compared with NaCl (*C*) in regions in close proximity to the third ventricle (3V), especially in the hypothalamus (HT). Note that cells exhibiting IL-12p40 mRNA have a similar distribution pattern and appearance as cells expressing the microglial marker C1q in these regions (*B*). In contrast to the restricted distribution of IL-12 C1q mRNA-positive cells appear randomly distributed throughout the brain. Bright field photograph in *D* represents the cresyl violet-counterstained section shown in *A*. CBL, cerebellum; cc, corpus callosum; HC, hippocampus; Bst, brain stem. Size bar (*A–D*) = 5 mm.

over small darker stained perikarya typical for microglia, but not over the lighter stained neuronal perikarya, thus corroborating the in vitro results that microglia can be stimulated by CpG-DNA. CpG-DNA also induced TNF-α mRNA expression in nonneuronal cells restricted to the juxtaventricular regions and with an identical distribution pattern to that of IL-12p40 mRNA (Fig. 9).

In accordance with the quantitative mRNA data, both cell number and cellular labeling intensity for IL-12p40 mRNA were more pronounced in CpG-ODN-treated mice (Fig. 9*A*) as compared with mice receiving LPS (Fig. 9*C*). TNF-α mRNA expression in response to both stimuli was comparable (Fig. 9, *B* and *D*). In mice receiving control injections with NaCl, no positive labeled cells either for IL-12p40 or for TNF-α mRNA were observed (Fig. 9, *E* and *F*).

Intraperitoneal injection of CpG-DNA induces cytokine mRNA in the brain

Next we asked whether peripheral injection of CpG-DNA would also result in mRNA expression in the brain. Mice were injected with CpG-DNA i.p. (Fig. 7*B*), and brain homogenates were examined for induction of mRNA of TNF-α, IL-12p40, and IL-12p35. Again, a strong induction of TNF-α and IL-12p40 was observed after stimulation with CpG-DNA, but not with control ODN. In contrast, LPS did not show any increase in IL-12p40 mRNA, yet induced TNF-α mRNA. Again, IL-12p35 mRNA was not regulated after stimulation with either compound. In situ hybridization after peripheral injection of CpG-DNA or LPS revealed only weak hybridization signals, in particular in circumventricular regions and meninges, and slightly above background in cells disseminated throughout the brain (data not shown).

Discussion

Conserved microbial patterns interact with the host's peripheral immune system via pattern recognition receptors (5). Molecular bacterial patterns comprise different cell wall components, lipoproteins, and bacterial CpG-DNA. A set of at least 10 TLRs plays a pivotal role in recognition of these bacterial patterns. Natural mutants or gene-targeted mice have revealed that certain TLRs govern a distinct class of bacterial pattern, e.g., mice lacking a functional TLR-4 have been shown to be hyporesponsive to LPS (8, 9). Recently, TLR-9 has been identified as the receptor-mediating signaling by CpG-DNA (10, 40). Murine TLR-9 is expressed on cells of hemopoietic origin, such as dendritic cells, macrophages, and B cells (40). To date, only a limited number of reports have addressed the question of TLR distribution in the brain. TLR-4 transcripts were found by in situ hybridization in the circumventricular organs, as well as in few well-defined nuclei and scattered small cells throughout the brain parenchyma (41), which probably represents the microglia compartment. Furthermore, TLR-4 is expressed in the pituitary, which results in LPS responsiveness, as determined by LPS-induced mitogen-activated protein kinase and NF-κB activation (42). We show in this study that microglia that arises from $CD45^+$ bone marrow precursors expresses TLR-9 mRNA transcripts and is sensitive to CpG-DNA. This was shown for the microglial cell line BV-2 as well as for primary microglia. Surprisingly, TLR-9 mRNA expression in a whole brain homogenate was higher than in isolated microglia, leaving the possibility that cells in the CNS different from microglia also express TLR-9. However, another explanation could be a down-regulation of TLR-9 during the isolation and culture period of microglia. Decreasing TLR-4 and TLR-9 expression has been described in

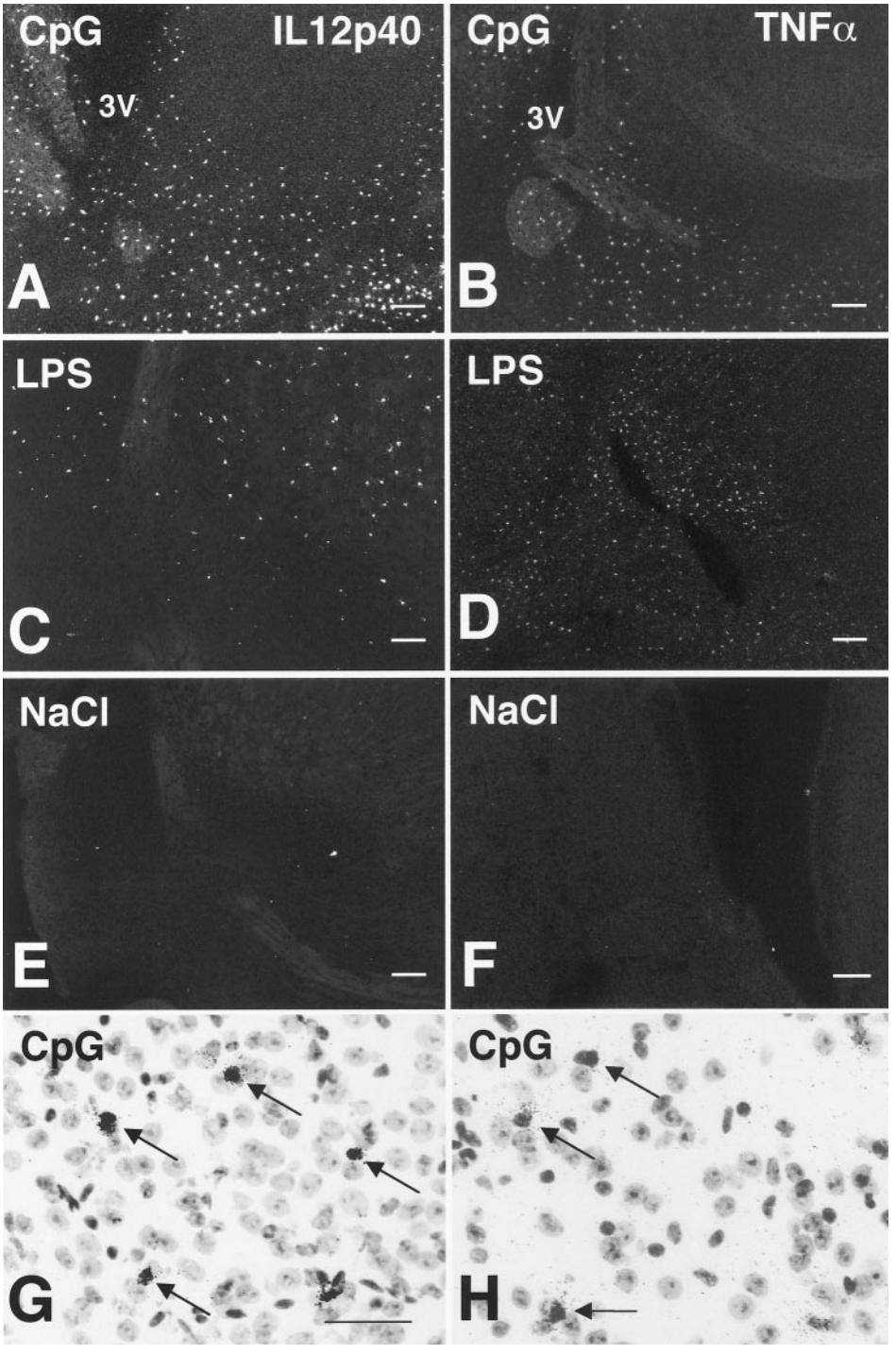

FIGURE 9. Comparison of IL-12p40 mRNA and TNF-α mRNA expression in mouse brain after i.c.v. application of CpG-DNA or LPS. Dark field autoradiograms illustrating the distribution of cells expressing IL-12p40 mRNA (*A*, *C*, *E*) and TNF-α mRNA (*B*, *D*, and *F*) in mouse hypothalamus after i.c.v. application of 125 pmol CpG-DNA 1668 (*A* and *B*), 500 pg LPS (*C* and *D*), or NaCl (*E* and *F*). CpG treatment induces a dramatic increase of IL-12p40 mRNA expression both in cell number and signal intensity (*A*) as compared with LPS treatment (*C*). In comparison, TNF-α mRNA-positive cells observed after CpG or LPS treatment appeared to be similar (*B* and *D*). In animals injected with NaCl as vehicle, no positive hybridization signals were observed for IL-12p40 mRNA (*E*) nor for TNF-α mRNA (*F*). Examination of the hybridized sections counterstained with cresyl violet with high resolution bright field microscopy revealed that both IL-12p40 mRNA (arrows in *G*) and TNF-α mRNA-expressing cells (arrows in *H*) were of nonneuronal presumed microglial origin. Size bars 200 μm (*A–F*); 50 μm (*G* and *H*).

human dendritic cells in the course of maturation (43, 44). Further detailed studies examining the distribution of different TLRs will be needed to establish a TLR expression profile of the various cell types of the CNS to correlate TLR-dependent responsiveness to the array of microbial patterns.

Interestingly, TLR-4 expression was mainly found in organs outside the blood-brain barrier (41), where microbial stimuli could exert central effects even after peripheral infectious challenge. Whereas it has been demonstrated repeatedly that microbial stimuli can induce central effects such as fever indirectly via induction of cytokines like IL-1, IL-6, and TNF-α (45, 46), the presence of TLR transcripts in circumventricular organs strongly argues for the possibility of a direct influence of microbial patterns on central nervous cells (47, 48). We were able to show in this study that i.p. injection of CpG-DNA induced central production of cytokine mRNA (Fig. 7*B*). Similar results have been described after administration of LPS (49). Since CpG-DNA cannot pass the blood-brain barrier due to its polarity (50), direct interactions of bacterial DNA with CNS cells might require a disrupted blood-brain barrier or local infection within the CNS. Alternatively, peripheral infection might lead to CNS activation via immune mediators that are induced in the periphery.

Injection of CpG-DNA i.c.v. activated microglia to produce TNF-α and high amounts of IL-12p40. IL-12p35 expression could also be observed, yet was not regulated. It has been shown previously that LPS stimulates the production of IL-1β, IL-6, TNF-α,

and IL-18 in microglia (49, 51, 52). Both stimuli resulted in a marked induction of costimulatory molecules (Table I) (45). However, in contrast to LPS, the propensity of CpG-DNA to induce IL-12 (Figs. 7, 8, and 9) could be of decisive importance in those pathophysiological processes of the CNS in which T cells are critically involved. Furthermore, activated T lymphocytes might amplify microglia activation via IFN-γ (53). Activated microglia not only enhances T cell responses, but also induces direct bactericidal effector molecules such as NO. We have shown in this study direct induction of iNOS, and subsequently NO by CpG-DNA, corroborating similar results described for iNOS induction by LPS (54).

It has been described recently that adult murine microglia has some unique features compared with other macrophages. Microglial cells could be induced to express CD11c after stimulation with the lineage growth factor GM-CSF. In parallel, $CD11c^+$ cells showed increased production of IL-12p70, changes in the regulation of MHC class II complexes, as well as in morphology, and enhanced the activation of naive T cells (55, 56). The phenotype of these cells resembled peripheral myeloid dendritic cells. Thus, microglia displays considerable plasticity and embodies the potential to respond to specific environmental requirements in the course of an infection. Obviously, the cytokine milieu critically influences the cell's state of differentiation and activity. CpG-DNA has been described to influence maturation and activation of peripheral dendritic cells (57, 58); thus, it will be intriguing to clarify whether CpG-DNA or other microbial stimuli can influence plasticity of microglia too.

Besides potential beneficial effects of microglia activation in the course of infections, activated microglia is also thought to cause detrimental reactions in autoimmune and neurodegenerative diseases. In this context, the strong IL-12 production induced by CpG-DNA is of particular importance. While in the periphery this unique capacity of CpG-DNA to induce Th1-biased immune responses (19) is utilized in vaccination protocols (18, 59), excessive IL-12 levels induced by CpG-DNA might also give reasons for severe concerns. It has been shown that CpG-DNA can lead to the exacerbation of Theiler's murine encephalomyelitis virus infection and relapsing-remitting experimental allergic encephalomyelitis (EAE) (60), as well as induction of EAE in a myelin basic protein-based EAE model (61, 62). Our results show that extensive IL-12 production can be induced by CpG-DNA also in microglia. These results could point to an important role of CpG-DNA in those pathophysiological autoimmune processes.

Besides the activation of microglia by CpG-DNA, we also show in this study that ODNs containing a central guanosine-rich region specifically inhibit the effects of CpG-DNA on microglia. Inhibitory DNA sequences have been described in adenoviral DNA (24), yet the mechanisms of action are still elusive. Thus, the overall effects of bacterial DNA are influenced by stimulatory as well as inhibitory DNA motifs. It is at present unclear whether the balance of these motifs contributes to the different pathogenic potential of distinct bacteria species. On the other side, inhibitory DNA motifs such as guanosine-rich ODNs could be of use in therapeutic strategies aiming to reduce the pathophysiological potential of bacterial CpG-DNA.

Taken together, our results show that not only peripheral innate immune cells, but also microglia express TLR-9. Accordingly, CpG-DNA activates microglia in vitro and in vivo to produce TNF-α, IL-12p40, and IL-12p70, and to enhance Ag-presenting functions. In situ hybridization clearly identified microglia as CpG-responsive cells. Hence, CpG-DNA might play an important role in infectious as well as pathophysiological processes of the CNS.

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References

1. Barker, C. F., and R. E. Billingham. 1977. Immunologically privileged sites. *Adv. Immunol. 25:1.*
2. Lawson, L. J., V. H. Perry, P. Dri, and S. Gordon. 1990. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience 39:151.*
3. Stoll, G., and S. Jander. 1999. The role of microglia and macrophages in the pathophysiology of the CNS. *Prog. Neurobiol. 58:233.*
4. Shrikant, P., and E. N. Benveniste. 1996. The central nervous system as an immunocompetent organ. *J. Immunol. 157:1819.*
5. Medzhitov, R., and C. A. Janeway. 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell 91:295.*
6. Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, et al. 1999. Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. *Science 285:732.*
7. Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immun. 2:675.*
8. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. V. Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science 282:2085.*
9. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the LPS gene product. *J. Immunol. 162:3749.*
10. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature 408:740.*
11. Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature 410: 1099.*
12. Medzhitov, R., P. Preston-Hurlburt, E. Kopp, A. Stadlen, C. Chen, S. Ghosh, and C. A. Janeway, Jr. 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol. Cell 2:253.*
13. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity 11:115.*
14. Schnare, M., A. C. Holtdagger, K. Takeda, S. Akira, and R. Medzhitov. 2000. Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. *Curr. Biol. 10:1139.*
15. Muzio, M., G. Natoli, S. Saccani, M. Levrero, and A. Mantovani. 1998. The human Toll signaling pathway: divergence of nuclear factor κB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). *J. Exp. Med. 187:2097.*
16. Häcker, H., R. M. Vabulas, O. Takeuchi, K. Hoshino, S. Akira, and H. Wagner. 2000. Immune cell activation by bacterial CpG-DNA through MyD88 and TRAF6. *J. Exp. Med. 192:595.*
17. Cowdery, J. S., N. J. Boerth, L. A. Norian, P. S. Myung, and G. A. Koretzky. 1999. Differential regulation of the IL-12 p40 promoter and of p40 secretion by CpG DNA and lipopolysaccharide. *J. Immunol. 162:6770.*
18. Chu, R. S., O. S. Targoni, A. M. Krieg, P. V. Lehmann, and C. V. Harding. 1997. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J. Exp. Med. 186:1623.*
19. Zimmermann, S., O. Egeter, S. Hausmann, G. B. Lipford, M. Roecken, H. Wagner, and K. Heeg. 1998. Cutting edge: CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. *J. Immunol. 160:3627.*
20. Krieg, A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature 374:546.*
21. Wagner, H. 1999. Bacterial CpG DNA activates immune cells to signal infectious danger. *Adv. Immunol. 73:329.*
22. Krieg, A. M. 2000. The role of CpG motifs in innate immunity. *Curr. Opin. Immunol. 12:35.*
23. Bendigs, S., U. Salzer, G. B. Lipford, H. Wagner, and K. Heeg. 1999. CpG-oligodeoxynucleotides co-stimulate primary T cells in the absence of antigen-presenting cells. *Eur. J. Immunol. 29:1209.*
24. Krieg, A. M., T. Wu, R. Weeranta, S. M. Efler, L. Love-Homan, L. Yang, A. K. Yi, D. Short, and H. L. Davis. 1998. Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. *Proc. Natl. Acad. Sci. USA 95:12631.*
25. Häcker, H., H. Mischak, T. Miethke, S. Liptay, R. Schmid, T. Sparwasser, K. Heeg, G. B. Lipford, and H. Wagner. 1998. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J. 17:101.*
26. Carpentier, A. F., J. Xie, K. Mokhtari, and J. Y. Delattre. 2000. Successful treatment of intracranial gliomas in rat by oligodeoxynucleotides containing CpG motifs. *Clin. Cancer Res. 6:2469.*

27. Schluesener, H. J., K. Seid, M. Deininger, and J. Schwab. 2001. Transient in vivo activation of rat brain macrophages/microglial cells and astrocytes by immunostimulatory multiple CpG oligonucleotides. *J. Neuroimmunol. 113:89.*
28. Takeshita, S., F. Takeshita, D. E. Haddad, N. Janabi, and D. M. Klinman. 2001. Activation of microglia and astrocytes by CpG oligodeoxynucleotides. *NeuroReport 12:3029.*
29. Blasi, E., R. Barluzzi, V. Bocchini, R. Mazzolla, and F. Bistoni. 1990. Immortalization of murine microglial cells by a v-*raf*/v-*myc* carrying retrovirus. *J. Neuroimmunol. 27:229.*
30. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med. 176:1693.*
31. Paxinos, G., and K. B. J. Franklin. 2000. *The Mouse Brain in Stereotactic Coordinates*. Academic Press, San Diego.
32. Frei, K., S. Bodmer, C. Schwerdel, and A. Fontana. 1986. Astrocyte-derived interleukin 3 as a growth factor for microglia cells and peritoneal macrophages. *J. Immunol. 137:3521.*
33. Dickensheets, H. L., and R. P. Donnelly. 1999. Inhibition of IL-4-inducible gene expression in human monocytes by type I and type II interferons. *J. Leukocyte Biol. 65:307.*
34. Karttunen, J., S. Sanderson, and N. Shastri. 1992. Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. *Proc. Natl. Acad. Sci. USA 89:6020.*
35. Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res. 12:7035.*
36. Angerer, L. M., K. H. Cox, and R. C. Angerer. 1987. Demonstration of tissue-specific gene expression by in situ hybridization. *Methods Enzymol. 152:649.*
37. Schaefer, M. K. H., J. P. Herman, and S. J. J. Watson. 1993. In situ hybridization histochemistry. In *Imaging Drug Action in the Brain*. E. D. London, ed. CRC Press, Boca Raton, p. 337.
38. Schafer, M. K., W. J. Schwaeble, C. Post, P. Salvati, M. Calabresi, R. B. Sim, F. Petry, M. Loos, and E. Weihe. 2000. Complement C1q is dramatically up-regulated in brain microglia in response to transient global cerebral ischemia. *J. Immunol. 164:5446.*
39. Schwaeble, W., M. K. Schafer, F. Petry, T. Fink, D. Knebel, E. Weihe, and M. Loos. 1995. Follicular dendritic cells, interdigitating cells, and cells of the monocyte-macrophage lineage are the C1q-producing sources in the spleen: identification of specific cell types by in situ hybridization and immunohistochemical analysis. *J. Immunol. 155:4971.*
40. Bauer, S., C. J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, and G. B. Lipford. 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc. Natl. Acad. Sci. USA 98:9237.*
41. Laflamme, N., and S. Rivest. 2001. Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating Gram-negative bacterial cell wall components. *FASEB J. 15:155.*
42. Lohrer, P., J. Gloddek, A. C. Nagashima, Z. Korali, U. Hopfner, M. P. Pereda, E. Arzt, G. K. Stalla, and U. Renner. 2000. Lipopolysaccharide directly stimulates the intrapituitary interleukin-6 production by folliculostellate cells via specific receptors and the p38α mitogen-activated protein kinase/nuclear factor-κB pathway. *Endocrinology 141:4457.*
43. Visintin, A., A. Mazzoni, J. H. Spitzer, D. H. Wyllie, S. K. Dower, and D. M. Segal. 2001. Regulation of Toll-like receptors in human monocytes and dendritic cells. *J. Immunol. 166:249.*
44. Kadowaki, N., S. Ho, S. Antonenko, M. R. de Waal, R. A. Kastelein, F. Bazan, and Y. J. Liu. 2001. Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. *J. Exp. Med. 194: 863.*
45. Buttini, M., S. Limonta, and H. W. Boddecke. 1996. Peripheral administration of lipopolysaccharide induces activation of microglial cells in rat brain. *Neurochem. Int. 29:25.*
46. Ghezzi, P., S. Sacco, D. Agnello, A. Marullo, G. Caselli, and R. Bertini. 2000. LPS induces IL-6 in the brain and in serum largely through TNF production. *Cytokine 12:1205.*
47. Netea, M. G., B. J. Kullberg, and J. W. Van der Meer. 2000. Circulating cytokines as mediators of fever. *Clin. Infect. Dis. 31:S178.*
48. Dinarello, C. A., S. Gatti, and T. Bartfai. 1999. Fever: links with an ancient receptor. *Curr. Biol. 9:R147.*
49. Buttini, M., A. Mir, K. Appel, K. H. Wiederhold, S. Limonta, P. J. Gebicke-Haerter, and H. W. Boddecke. 1997. Lipopolysaccharide induces expression of tumor necrosis factor α in rat brain: inhibition by methylprednisolone and by rolipram. *Br. J. Pharmacol. 122:1483.*
50. Crooke, R. M. 1991. In vitro toxicology and pharmacokinetics of antisense oligonucleotides. *Anticancer Drug Des. 6:609.*
51. Hopkins, S. J., and N. J. Rothwell. 1995. Cytokines and the nervous system. I. Expression and recognition. *Trends Neurosci. 18:83.*
52. Prinz, M., and U. K. Hanisch. 1999. Murine microglial cells produce and respond to interleukin-18. *J. Neurochem. 72:2215.*
53. Benveniste, E. N. 1992. Inflammatory cytokines within the central nervous system: sources, function, and mechanism of action. *Am. J. Physiol. 263:C1.*
54. Kitamura, Y., H. Takahashi, Y. Matsuoka, I. Tooyama, H. Kimura, Y. Nomura, and T. Taniguchi. 1996. In vivo induction of inducible nitric oxide synthase by microinjection with interferon-γ and lipopolysaccharide in rat hippocampus. *Glia 18:233.*
55. Fischer, H. G., and G. Reichmann. 2001. Brain dendritic cells and macrophages/microglia in central nervous system inflammation. *J. Immunol. 166:2717.*
56. Santambrogio, L., S. L. Belyanskaya, F. R. Fischer, B. Cipriani, C. F. Brosnan, P. Ricciardi-Castagnoli, L. J. Stern, J. L. Strominger, and R. Riese. 2001. Developmental plasticity of CNS microglia. *Proc. Natl. Acad. Sci. USA 98:6295.*
57. Brown, W. C., C. E. Suarez, L. K. Shoda, and D. M. Estes. 1999. Modulation of host immune responses by protozoal DNA. *Vet. Immunol. Immunopathol. 72:87.*
58. Behboudi, S., D. Chao, P. Klenerman, and J. Austyn. 2000. The effects of DNA containing CpG motif on dendritic cells. *Immunology 99:361.*
59. Van Uden, J., and E. Raz. 1999. Immunostimulatory DNA and applications to allergic disease. *J. Allergy Clin. Immunol. 104:902.*
60. Tsunoda, I., N. D. Tolley, D. J. Theil, J. L. Whitton, H. Kobayashi, and R. S. Fujinami. 1999. Exacerbation of viral and autoimmune animal models for multiple sclerosis by bacterial DNA. *Brain Pathol. 9:481.*
61. Segal, B. M., D. M. Klinman, and E. M. Shevach. 1997. Microbial products induce autoimmune disease by an IL-12-dependent pathway. *J. Immunol. 158: 5087.*
62. Segal, B. M., J. T. Chang, and E. M. Shevach. 2000. CpG oligonucleotides are potent adjuvants for the activation of autoreactive encephalitogenic T cells in vivo. *J. Immunol. 164:5683.*