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The Third Signal in T Cell-Mediated Autoimmune Disease?[1]

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The initial event in the pathogenesis of autoimmune disease is thought to be the priming of naive autoreactive T cells by an infection with a cross-reactive microorganism. Although such cross-reactive priming should be a common event, autoimmune disease does not frequently develop. This situation is reflected after the immunization of C57BL/6 mice with the neuroantigen myelin oligodendrocyte glycoprotein (MOG) with CFA, which primes a type 1 T cell response but does not lead to clinical or histological manifestation of experimental allergic encephalomyelitis unless pertussis toxin is injected in addition. We show in this study that, in MOG:CFA-primed mice, the autoimmune CNS pathology develops after intracerebral deposition of TLR9-activating CpG oligonucleotides, but not following non-CpG oligonucleotide injection or after aseptic cryoinjury of the brain. Thus, access of primed MOG-specific Th1 cells to the uninflamed CNS or to CNS undergoing sterile inflammation did not suffice to elicit autoimmune pathology; only if the APC in the target organ were activated in addition by the TLR9-stimulating microbial product did they exert local effector functions. The data suggest that such licensing of APC in the target organ by microbial stimuli represents a checkpoint for functional self-tolerance. Therefore, microorganisms unrelated to the cross-reactive agent that primes the autoreactive T cells could dictate the onset and exacerbation of autoimmune diseases. ***The Journal of Immunology*****, 2004, 173: 92–99.**

The mechanisms that lead to autoimmune disease are still controversial (1). These have been best studied in experimental allergic encephalomyelitis (EAE),[3] a disease caused by T cells that target autoantigens expressed in the CNS (2). EAE is the animal model for multiple sclerosis (MS) in humans (3). Neuroantigen-specific T cells that have the potential for causing EAE are not deleted in the thymus, either because they have low affinity for the autoantigen (4), or because they are specific for determinants poorly presented or absent in the thymus (5). Unless primed by environmental cross-reactive Ags or immunization, such T cells persist as naive lymphocytes in the immune periphery, apparently ignorant of the endogenous Ag (6). However, in RAG-deficient mice, where there is an absence of regulatory cells, such autoreactive T cells become spontaneously activated (7).

In normal wild-type mice, a stringent protocol needs to be followed to induce EAE. Injection of the neuroantigen itself provides the specific stimulus for the priming of the naive precursor cells (signal 1). The neuroantigen needs to be coadministered with microbial products such as heat-inactivated mycobacteria contained in CFA (8) or CpG oligonucleotides (ODN) (9, 10). By activating TLR on cells of the innate immune system (11, 12), such microbe-associated molecules engage the second signal link required for induction of a T cell response (13–16). Additionally, by triggering IL-12 production (17, 18), such TLR activators guide the induction of a proinflammatory Th1 T cell response (9, 19, 20). In normal mice, immunization with neuroantigen in the absence of TLR-activating substances induces Th2 immunity and profound protection from EAE (8). Only in RAG-deficient mice that lack regulatory cells do Th2 cells mediate neuropathology (21).

The microbial context therefore determines whether naive autoantigen-specific T cells differentiate into Th1 or Th2 cells, that is, whether potentially pathogenic or protective memory/effector T cells are engaged. As stated by Janeway (14), "The immune system evolved to discriminate between infectious nonself and noninfectious self." Although the second signal and the infectious/noninfectious context both represent important checkpoints for the control of self-tolerance at the level of the naive T cell, neither can explain why the development of autoimmune pathology is more the exception than the rule after an autoreactive Th1 response has been engaged. Is there an additional checkpoint for the memory cell?

Immunizations of EAE-susceptible mice with different neuroantigens in TLR-activating adjuvants typically induces a vigorous neuroantigen-specific Th1 response, but in many murine EAE models, histological or clinical EAE does not develop unless pertussis toxin (PTX) is coinjected (8). PTX is a potent adjuvant that promotes type 1 differentiation of T cells and promotes the clonal expansion of these cells (22–26). In addition, PTX is thought to be required to break down the blood-brain barrier (BBB), thereby providing the primed T cells access to the target organ (27). Both of these effects are likely to contribute to the disease-promoting activity of PTX. To dissect these mechanisms, we immunized mice

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[3] Abbreviations used in this paper: EAE, experimental allergic encephalomyelitis; MS, multiple sclerosis; ODN, oligonucleotide; PTX, pertussis toxin; BBB, blood-brain barrier; MOGp, MOG peptide 35–55; OVAp, OVA peptide 323–339; n, non-stimulatory; i.c., intracerebral; LN, lymph node; PLP, proteolipid protein.

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with MOG peptide 35–55 (MOGp) in CFA (without PTX) to engage peptide-specific Th1 cells, and induced aseptic cryoinjury in brains of mice that were preimmunized. Instead of PTX, therefore, the BBB was disrupted by a sterile CNS lesion, granting the autoreactive T cells access to the target organ. Strikingly, the primed T cells did not cause autoimmune pathology but promoted the repair of the CNS tissue (28). Similar effects of neuroantigen-specific Th1 cells in sterile CNS injury have been reported by others (29).

As a G protein inhibitor, PTX exerts multiple functions, including the activation of APC (30, 31). We hypothesized that APC activation in the target organ might explain the essential role of PTX in EAE models (as well as other autoimmune models that are induced by active immunization). If this is the case, then not only the differentiation of naive T cells into Th1 memory/effector cells would be under the instructive role of TLR-triggered signals, but also the effector functions of the differentiated Th1 cells would be controlled by the microbial activation of the innate immune system. To be able to distinguish these two fundamentally different PTX mechanisms, we injected CpG into the CNS of mice in which the Th1 response was already fully differentiated as induced by MOGp:CFA immunization, without using PTX. We tested whether the APC in the brain need licensing by TLR9 activation to elicit the proinflammatory effector functions of differentiated MOG-specific Th1 cells.

Materials and Methods

Animals, Ags, ODN, and treatments

Female C57BL/6 mice and congenic knockout mice (Rag-1$^{-/-}$, μMT$^{-/-}$), 6–8 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME), and maintained in the specific pathogen-free animal facilities at Case Western Reserve University. MOGp and OVA peptide 323–339 (OVAp) were synthesized and purified by Princeton Biomolecules (Columbus, OH). ODN were purchased from Oligos Etc. (Wilsonville, OR). ODN were phosphorothioate-modified to increase their resistance to nuclease degradation. Sequences of the ODN used were as follows: TCCATGACGTTC CTGACGTT for the immunostimulatory CpG ODN 1826, and TCCAAT GAGCTTCCTGAGTCT for the nonstimulatory (n)CpG ODN 1745 as defined before (9). Synthetic ODN were dissolved in sterile nonpyrogenic 0.9% NaCl (Baxter, Deerfield, IL) at a concentration of 40 mg/ml. Mice were injected with 500 nl of this solution depositing 20 μg of the ODN. CFA was prepared by mixing *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI) at 1.0 mg/ml into IFA (Invitrogen Life Technologies, Carlsbad, CA). For immunizations, MOGp was mixed with CFA at a final concentration of 0.5 mg/ml, and 100 μl of this emulsion (50 μg of MOGp per mouse) was injected. When specified, mice were additionally injected i.p. with PTX (List Biological Laboratories, Campbell, CA), twice with 0.2 μg, once immediately and once 24 h after immunization. Starting the second day after the injection, the mice were assessed daily for the development of paralytic symptoms and the severity of disease was recorded according to the standard scale: grade 1, floppy tail; grade 2, hindleg weakness; grade 3, full hindleg paralysis; grade 4, quadriplegia; grade 5, death. The care of mice was in accordance with institutional guidelines.

Aseptic cerebral injury

Anesthesia was induced with Avertin (Sigma-Aldrich, St. Louis, MO) in *t*-amyl-alcohol (Sigma-Aldrich). A sagittal incision of 15-mm length was made in the skin of the skull using a scalpel blade. A liquid nitrogen-chilled steel rod (3 mm in diameter) was held for 6 s against the exposed but intact skull bone over the medial right parietal lobe 2 mm anterior to the lambdoid suture. The skin incision was sutured, and the animals were returned to their cages for recovery.

Cerebral microinjections

Mice were anesthetized with Avertin and placed in a small-rodent stereotaxic apparatus. The skull bone was exposed, and a small hole was made with a dental drill using the following stereotaxic coordinates measured from the bregma: anterior, 1.54 mm; lateral, 1.5 mm; and ventral, 2 mm. Microinjection of ODN or saline was performed using a pneumatic pump (Medical Systems, Greenvale, NY) connected to a sterile glass micropipette of 30-μm diameter. The micropipettes were mounted in a micromanipulator attached to the stereotaxic apparatus, and were placed in the position indicated by the stereotaxic coordinates. After 15–20 min, a single intracerebral (i.c.) injection of ODN or saline was made. Then, the skin was sutured, and the animals were returned to their cages for recovery.

Histological examination of brain tissue

At the time points specified, the mice were transcardially perfused first with PBS/2% heparin (Pharmacia & Upjohn, Kalamazoo, MI) and thereafter with PBS/4% paraformaldehyde (Sigma-Aldrich). The brains were removed and immersed in PBS/4% paraformaldehyde for 12 h, and then washed in distilled water for 1–2 h before paraffin embedding. Coronal sections were cut at 6-μm thickness using an AO 820 microtome (American Optical, Buffalo, NY), and placed on gelatin-coated Colorfrost Microslides (VWR Scientific, Media, PA) for analysis. Sections were deparaffinized and rehydrated. They underwent standard H&E staining and were mounted with Permount (Fisher Scientific, Fair Lawn, NJ). Slides were photographed using a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI) mounted on a Nikon Eclipse E600 microscope (Nikon, Melville, NY). The numbers of mononuclear cells within a 2-mm^2 region of the injection site were counted from the digital file using Image Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD).

Immunohistochemistry

Sections from specimens examined by H&E staining were also used for immunohistochemistry. After rehydration, they were subjected to Ag retrieval at 90°C for 15 min using Target Retrieval solution (DakoCytomation, Carpinteria, CA). Sections were exposed to PBS/0.4% Triton containing 10% goat serum for 2 h and subsequently incubated overnight at room temperature with rat anti-mouse CD40 (1C10 at 10 μg/ml; R&D Systems, Minneapolis, MN). In a second step, primary Abs were captured with a biotinylated goat anti-rat IgG (Vector, Burlingame, CA). FITC-conjugated avidin-D complex (Vector) was used for immunodetection. Negative control sections received the same treatment except for using isotope control primary Ab.

Analysis of differential gene expression by cDNA microarray

Poly(A)$^+$ RNA was isolated from the forebrain of mice injected with CpG or nCpG using the Invitrogen Fast Track 2.0 kit (Invitrogen Life Technologies) following the manufacturer's instructions. Poly(A)$^+$ RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase in the presence of [α-^{32}P]dATP for the generation of radiolabeled cDNA probes. The radiolabeled cDNA probes were purified from the unincorporated nucleotides by column chromatography (Clontech NucleoSpin; Clontech, Palo Alto, CA) and hybridized to the Atlas Mouse 1.2 cDNA microarray (Clontech) according to the manufacturer's instructions overnight at 68°C. After washing (three 20-min washes in 2× SSC at 68°C, 1% SDS, followed by two 20-min washes in 0.1× SSC/0.5% SDS), the membranes were exposed to a phosphor imaging screen for 20 h, and the hybridization was analyzed with a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Of the 1176 genes contained in the array, we analyzed those that have known immunological functions, in particular, cytokines, chemokines, and cell surface molecules with stimulatory/costimulatory functions. These genes were considered differentially expressed when they exhibited a 2-fold or greater increase or decrease after CpG injection, compared with the nCpG group (only increases were seen for these genes). The relative expression of the housekeeping genes (*40S ribosomal protein S29*, *GAPDH*, *Ubiquitin*, and others) did not differ by >10% among samples.

Cell isolation and purification

Single-cell suspensions from lymph nodes (LN) or spleens were prepared. Subpopulations of T cells were isolated using commercially available murine T cell isolation columns (R&D Systems) according to the instructions of the manufacturer. The resulting cells were washed, counted, and resuspended at appropriate concentrations for use in the various assays. Purity was confirmed by FACS analysis using FITC-conjugated Abs (BD PharMingen, San Diego, CA). The columns routinely yielded >90% purity for CD4$^+$ T cells. Cells from the CNS were prepared as follows. After sacrificing, the animals were transcardially perfused with sterile PBS/2% heparin, the brains were removed, pooled, and placed into DMEM medium (BioWhittaker, Walkersville, MD). The brains were freed of the meninges, sliced, and disrupted in DMEM (15 ml for each brain) with the back of a syringe. The homogenate was filtered through a Falcon Cell Strainer 2350 (BD Biosciences, Mountain View, CA) and then passed through a prewetted nylon wool column (0.7–1.0 g of scrubbed nylon fibers; Cellular Products, Buffalo, NY; in 20-ml syringe). Cells were isolated on a Percoll

(Pharmacia, Uppsala, Sweden) gradient using 35 and 70% Percoll solutions (densities of 1.045 and 1.085 g/cm^3, respectively). The cell effluent collected from the nylon wool column was pelleted by centrifugation and resuspended in 70% Percoll (6 ml/brain), and 2 ml of it was placed in 15-ml plastic conical tubes. This 70% Percoll-cell mixture was overlayered with 2 ml of 35% Percoll, and 2 ml of PBS was layered on the top of the gradient. The gradients were centrifuged at 1200 $\times$ *g* at 20°C for 45 min. Cells at the 35/70% interface (containing the mononuclear cells) were collected, washed twice, and counted. Directly labeled Abs (all from BD PharMingen) were used for the flow cytometric characterization of the cell populations obtained. The stained cells were analyzed with FACS and CellQuest software (BD Biosciences).

ELISPOT assays and ELISPOT image analysis

Assays were performed as previously described (32). ImmunoSpot M200 plates (Cellular Technology, Cleveland, OH) were coated overnight with the capture Abs in sterile PBS. RR46A2, at 2 μg/ml (isolated and purified from hybridoma), was used for IFN-γ; JES6-1A12, at 2.5 μg/ml (BD PharMingen), was used for IL-2; BVD4-1D11, at 2.5 μg/ml (BD PharMingen), was used for IL-4; TRFK5, at 5 μg/ml (isolated and purified from hybridoma), was used for IL-5; MP5-20F3, at 4 μg/ml (BD PharMingen), was used for IL-6; JES5-2A5, at 6 μg/ml (BD PharMingen), was used for IL-10; 38213.11, at 5 μg/ml (R&D Systems), was used for IL-13; and polyclonal Ab, at 2.5 μg/ml (Endogen, Woburn, MA), was used for TNF-$\alpha\beta$. The plates were blocked for 1 h with sterile PBS/1% BSA and washed three times with sterile PBS. Spleen cells (10^6 per well) or cells from draining LN (7×10^5) were plated in HL-1 medium (BioWhittaker) and Ag. In select experiments, cells and Ags were titrated. The plates were incubated at 37°C and 5% CO_2 for 24 h (for IFN-γ, TNF-$\alpha\beta$, IL-2, and IL-6 assays) or 48 h (for IL-4, IL-10, and IL-5). After washing first with PBS and then with PBS plus 0.025% Tween (PBST), the detection Abs were added, and the plates were incubated overnight. XMG1.2-biotin (BD PharMingen) was used at 1 μg/ml for IFN-γ; rat anti-mouse IL-2-biotin (JES6-5H4; BD PharMingen) was used at 2 μg/ml for IL-2; rat anti-mouse IL-4-biotin (BVD6-24G2; BD PharMingen) was used at 1 μg/ml for IL-4; biotinylated TRFK4 (BD PharMingen) was used at 2 μg/ml for IL-5; rat anti-mouse IL-6-biotin (MP5-32C11; BD PharMingen) was at 2 μg/ml used for IL-6; rat anti-mouse IL-10-biotin (JES5-16E3; BD PharMingen) was used at 2 μg/ml for IL-10; for TNF-$\alpha\beta$, in a first step, polyclonal rabbit anti-mouse TNF-$\alpha\beta$ (Endogen) was used at 4 μg/ml, and then in a second step, alkaline phosphatase-labeled goat anti-rabbit IgG (HyClone Laboratories, Logan, UT) was used at a 1/1000 dilution. The plates were then washed three times in PBST. Streptavidin-alkaline phosphatase (Dako-Cytomation) was added at 1/2000 dilution in PBST as a third reagent for IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-13, incubated for 2 h, and removed with three washes in PBS. The plates were developed using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (KPL, Gaithersburg, MD) that was added and left for 15–30 min. The resulting spots were counted on an ImmunoSpot Series 3 Analyzer (Cellular Technology) specifically designed for the ELISPOT assay. In addition to counting numbers of spots with the software, we also used the OD feature of the software to measure the density of the color precipitate, which reflects the amount of cytokine produced.

Results

Immunization with MOG$_{35–55}$:CFA results in violation of immunological self-tolerance in the absence of autoimmune pathology

C57BL/6 mice were immunized s.c. with this MOGp in CFA with and without additional injection of PTX. The mice that received PTX developed the paralytic symptoms characteristic of EAE, whereas the mice that were not injected with PTX remained clinically unapparent (Fig. 1*A*) and also did not develop histological manifestations of autoimmune pathology (see below). The mice immunized with MOGp:CFA in the absence of PTX generated a type 1-polarized T cell response specific for MOGp (Fig. 1*B*). The production of IL-2, IFN-γ, and TNF-$\alpha\beta$ was elicited in the draining LN cells in frequencies of 40–100 cytokine-producing cells per million. The frequency of MOGp-induced IL-4-, IL-5-, IL-6-, IL-10-, and IL-13-producing cells was <5 per million. Testing CD3 cells purified from such LN and using RAG1 KO spleen cells as APC showed that all cytokines detected were produced by T cells (28). Therefore, MOGp:CFA immunization induced a highly type 1-polarized immune response. The magnitude of this T cell response was in the same range as the one elicited in C57BL/6 mice by immunization with the foreign Ag OVA in CFA (20). In vitro-preactivated T cell blasts generated from the MOGp:CFA-primed LN cells adoptively transferred EAE (data not shown). Therefore, the s.c. immunization with MOGp:CFA elicited a high frequency, Th1-polarized T cell response with a potentially pathogenic phenotype. Yet this violation of immunological self-tolerance did not suffice to cause autoimmune pathology unless PTX was coadministered. There was split tolerance in these mice: the primed neuroantigen-specific Th1 cells remained detectable in the spleen for up to 2 mo after immunization (data not shown), apparently ignorant of the endogenous Ag in the CNS.

Intracerebral injection of CpG ODN evokes local autoimmune pathology in autoantigen-sensitized mice

Disruption of the BBB in MOGp-immunized mice by causing aseptic cryoinjury of the frontal lobe of the brain resulted in accelerated wound healing as compared with unimmunized or

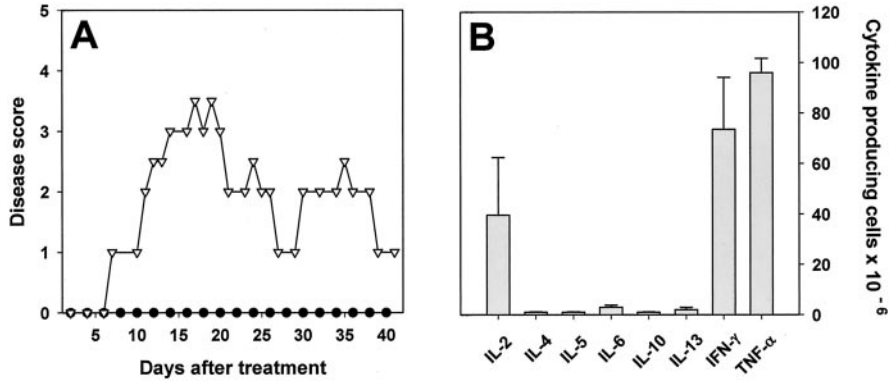
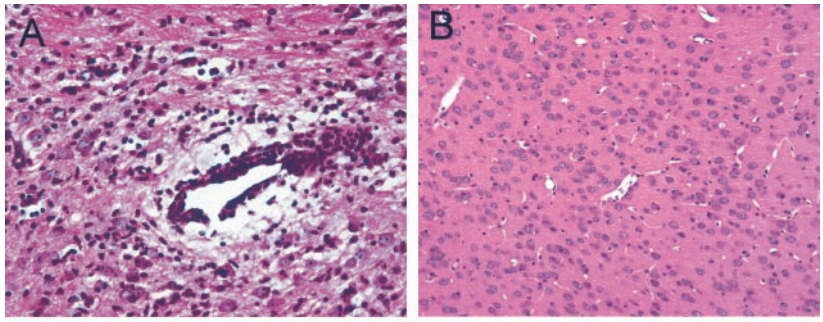


FIGURE 1. Immunization with MOG$_{35–55}$ in CFA induces a type 1 T cell response but no EAE. *A*, C57BL/6 mice were immunized s.c. with MOG$_{35–55}$ in CFA. One group of mice was additionally injected with PTX i.p. (▽; $n = 12$), the second group remained uninjected (●; $n = 12$). The mice were observed daily for clinical symptoms of EAE and scored from 0 to 5 according to the standard scale. Mean disease score is shown as a function of time. The data are representative for three experiments performed. *B*, C57BL/6 mice were immunized s.c. with MOG$_{35–55}$ in CFA, and draining LN cells were tested in ELISPOT assays for MOG$_{35–55}$-induced cytokine production. The number of cytokine-producing cells per million LN cells tested is shown. The data are representative of four independent experiments performed.



C

Mouse	Immunization	Injection (i. c.)			
		CpG	nCpG	Saline	None
B6 WT	OVAp:CFA	466±58	<200	<200	<200
B6 WT	MOGp:CFA	4,117± 222	<200	<200	<200
B6 WT	None	<200	<200	<200	<200
B6μMT	MOGp:CFA	3,901±194	<200	<200	<200

FIGURE 2. Mononuclear cell infiltrate develops at the site of CpG ODN injection in the frontal lobe of MOGp:CFA-primed mice. C57BL/6 mice were immunized s.c. with $MOG_{35–55}$ in CFA. Twelve days later, 20 μg of CpG ODN or of nCpG ODN was microinjected in 0.5 μl of saline. H&E staining of the injection site was performed 5 days after the i.c. injection. A representative image of a CpG ODN-injected brain section is shown at ×40 magnification in *A*. A representative nCpG ODN-injected brain section is shown at ×40 magnification in *B*. The data including $OVA_{323–339}$-injected controls are summarized in the table (*C*). Mononuclear cells were counted by computerized image analysis within a 2-mm^2 area adjoining the injection site. Each data point represents the mean number of mononuclear cells and the SD established for four mice per group. The data are representative for five independent experiments performed.

OVAp:CFA-injected control animals (28). In an attempt to understand what the requirements for these T cells are to induce the classic inflammatory reaction in the CNS, we microinjected CpG ODN and, as a control, nCpG ODN or saline, directly into the right frontal lobe of the brain. The characteristic mononuclear cell infiltrates developed in MOGp-immunized mice in the hemisphere of CpG ODN deposition (Fig. 2, *A* and *C*), but not in the contralateral hemisphere or the spinal cord of these mice (data not shown). The infiltrates were not elicited in MOGp:CFA-immunized mice by nCpG ODN microinjection (Fig. 2, *B* and *C*) or injection of saline (*C*). The development of the infiltrate was dependent on immunization with MOGp; the i.c. injection of CpG ODN did not elicit a marked inflammatory reaction in brains of unimmunized or OVAp:CFA control-immunized mice (Fig. 2*C*). MOGp:CFA-injected B cell-deficient (μMT) mice also developed the inflammatory lesions (Fig. 2*C*), suggesting that T cell, rather than B cell immunity is responsible for eliciting the inflammatory reaction in the CNS. Consistent with a classic delayed-type hypersensitivity response, the mononuclear cell infiltration required 2 days to establish itself in MOGp-immunized mice after the i.c. CpG ODN injection, and peaked on day 5 (data not shown). Paralleling the intensity of the mononuclear cell infiltration, the mice developed severe clinical symptoms, including weight loss, ruffled fur, hunched posture, ataxia, reduced motoric activity, and 19% lethality on day 5 (6 of 32 mice died in this group). Specific neurological deficits were not seen, consistent with the localization of the lesion in the frontal lobe. These clinical symptoms did not develop after the i.c. injection of CpG into unimmunized or OVAp:CFA-immunized mice. Therefore, to mediate inflammatory pathology, the peripherally primed MOGp-specific Th1 cells needed to encounter the autoantigen in the target organ in the context of infectious danger caused by TLR9 activation by the CpG ODN.

CpG ODN induce chemokine and CD40 expression in the CNS

To more closely define the role of CpG ODN in licensing APC, we first studied CpG effects on the brain itself. Frontal lobes of T cell/B cell-deficient (B6 Rag-$1^{-/-}$) mice were injected with CpG ODN or nCpG ODN, and mRNA levels were studied by microarray analysis (Fig. 3). The mRNA levels did not differ between nCpG ODN-injected brain (Fig. 3, *left panel*) and CpG ODN-injected brain (*right panel*) for platelet-derived growth factor-A (*1*; ODs, 53 vs 56), Pleiotrophin (*2*; ODs, 57 vs 53), Prothymosin-β4 (*3*; ODs, 49 vs 47), TGF-β1 (*4*; ODs, 63 vs 54), and housekeeping genes (not shown), which shows equal loading of cDNA from the two preparations. In the CpG ODN-injected brain, significant up-regulation was detected for IFN-inducible protein-10 (Fig. 3, *right panel*, *5*; ODs, 14 vs 2565), MCP-3 (*6*; ODs, 10 vs 2670), MIP-1β (*7*; ODs, 14 vs 2781), MIP-1α (*8*; ODs, 14 vs 2347), CD40 (*9*; ODs, 13 vs 1642), and MCP-1 (*10*; ODs, 12 vs 2937). Therefore, chemokines were induced that are produced primarily by activated macrophages and have chemotactic properties for the recruitment of inflammatory cells (33). Moreover, CD40 mRNA levels were strongly up-regulated, which encodes a potent T cell costimulatory molecule (34). The up-regulation of CD40 in the CNS of i.c. CpG ODN-injected Rag-$1^{-/-}$ mice was also seen at the level of protein expression (Fig. 4).

CpG ODN induce APC functions in brain-resident APC

To more closely characterize the CpG effects on the tissue-resident APC of the brain, we perfused unmanipulated RAG KO mice and

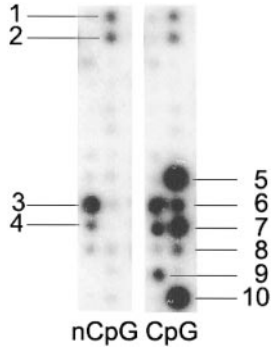


FIGURE 3. CpG ODN-induced mRNA expression in the CNS. C57BL/6 Rag-$1^{-/-}$ mice were microinjected i.c. with 20 μg of nCpG ODN or CpG ODN. Eight hours later, mRNA was isolated from the injected hemisphere, reverse transcribed, and hybridized with array chips. The prevalent gene products detected are numbered as follows: *1*, platelet-derived growth factor A; *2*, Pleiotrophin; *3*, Prothymosin-β4; *4*, TGF-β1; *5*, IFN-inducible protein-10; *6*, MCP-3; *7*, MIP-1β; *8*, MIP-1α; *9*, CD40; and *10*, MCP-1. The data were reproduced in an independent experiment.

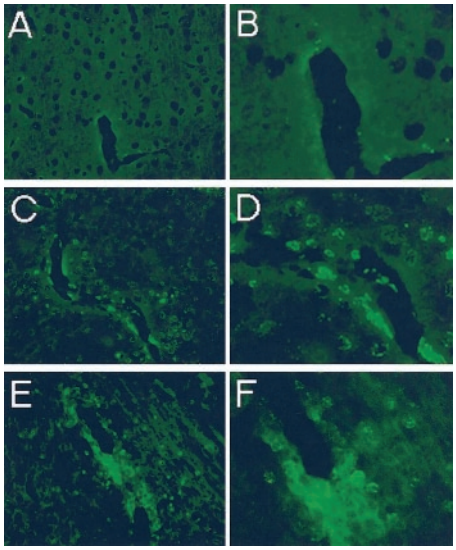


FIGURE 4. CD40 is up-regulated at the site of CpG ODN injection in the frontal lobe. C57BL/6 Rag-$1^{-/-}$ mice were microinjected i.c. with 20 μg of nCpG ODN (*A* and *B*) or CpG ODN (*C* and *D*). CD40 staining of the injection site was performed 24 h after the i.c. injection. In *E* and *F*, images are shown for MOGp:CFA-immunized wild-type mice 5 days after the i.c. injection of CpG ODN. Representative images are shown at ×40 (*A*, *C*, and *E*) and ×100 (*B*, *D*, and *F*) magnification. The data are representative for two independent experiments performed.

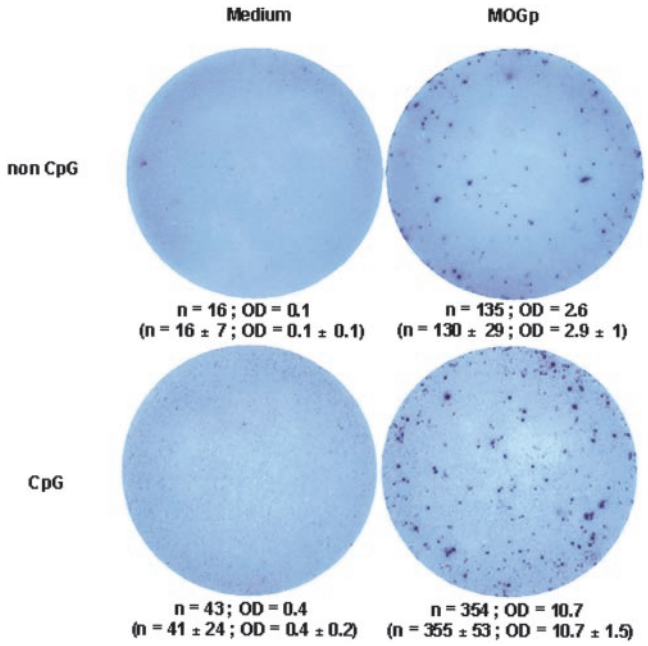


FIGURE 5. APC function of CpG- and nCpG-treated brain cell isolates. Brain-resident APC were isolated from uninjected frontal lobes of unimmunized C57BL/6 mice on Percoll gradients (as specified in *Materials and Methods*). To activate these APC, 100,000 cells per well were cultured CpG ODN or nCpG ODN (1.5 μg/ml each) for 8 h. After washing the APC four times, 300,000 CD4 cells were added (isolated from draining LNs of MOGp:CFA-immunized mice, 94% purity). IFN-γ production was measured during a 24-h ELISPOT culture in response to MOGp or medium, as indicated. Representative wells are shown, with the spot numbers and ODs of spots indicated for these wells. Underneath, in parentheses, mean spot numbers and ODs from the triplicate wells tested in this experiment are shown. The data were reproduced in four independent experiments.

isolated mononuclear cells from their frontal lobes. These cells, which contain the APC constitutively present in the uninflamed brain, were pretreated with nCpG ODN or with CpG ODN for 8 h, extensively washed, and tested for their ability to activate MOGp-specific T cells. Purified CD4 cells isolated from MOGp:CFA-immunized mice were added, and MOGp-induced production of IFN-γ was measured by ELISPOT (Fig. 5). The number of CD4 cells activated to produce IFN-γ in the presence of added MOGp was approximately three times higher in the CpG-treated brain APC, as compared with the nCpG ODN-treated APC. Additionally, the amount of cytokine produced by the individual T cells that were stimulated on CpG ODN-treated APC was approximately three times higher (Fig. 5, OD values). The combination of the increased frequency and of the augmented per-cell cytokine production rate resulted in a more vigorous IFN-γ response by the CD4 cells that recognized Ag on CpG ODN-treated brain APC.

Because under these test conditions the MOGp-specific T cells were kept constant, the increased number of the IFN-γ spots suggest that more functional APC were induced by the CpG ODN treatment. Several APC lineages in the brain express MHC class II molecules and present Ag only after activation; CpG ODN seems to increase the number of such competent APC. The per-cell cytokine production by CD4 cells is proportional to the strength of the T cell-activating signal (35). Therefore, the increased per-cell cytokine production rate of the MOGp-specific T cells suggests that, after CpG stimulation, the individual APC deliver stronger activation signals to the T cells, most likely due to the up-regulation of costimulatory molecules on these APC.

Enrichment of the autoreactive T cells in CpG ODN-activated brain tissue

To more closely define the role of the T cell in the inflammatory CNS response, we isolated cells from the injected frontal lobe and visualized their Ag-induced cytokine production (36). These isolates were tested on an APC layer of spleen cells obtained from naive congeneic mice. By providing an excess of professional APC from a lymphoid organ, this approach maximizes the assay conditions for the detection of T cells (thereby, possible limitations in APC function by the brain-resident cells are bypassed). The frequency of IFN-γ-secreting, MOGp-reactive cells was ~14,100 per million in the CNS isolates obtained from MOGp:CFA-immunized mice that received i.c. CpG ODN injection (Table I). In contrast, no OVAp- or MOGp-induced IFN-γ-producing cells were detected in isolates of unimmunized mice injected with CpG ODN, or in OVAp- or MOGp-immunized mice injected with nCpG ODN i.c. OVAp-reactive T cells were present in CNS isolates of OVAp:

Table I. *ELISPOT detection of Ag-induced IFN-γ-producing cells in CNS isolates*[a]

Immunization	Injection (i.c.)	IFN-γ Recall	
		MOGp	OVAp
MOGp:CFA	CpG	14,133 ± 533	<100
MOGp:CFA	nCpG	<100	<100
OVAp:CFA	CpG	<100	1,267 ± 133
OVAp:CFA	nCpG	<100	<100
None	CpG	<100	<100
None	nCpG	<100	<100

[a] Groups of eight C57BL/6 mice were immunized s.c. with MOGp or OVAp, or remained unimmunized. Twelve days later, four mice in each group were injected with 20 μg of CpG ODN or nCpG ODN as indicated. Five days later, mononuclear cells were isolated from the injected lobe, and 15,000 of these cells were tested in each well on a monolayer of 250,000 APC per well (spleen cells from naive mice). The mean number and SD of spots in three replicate wells are shown. The data were reproduced in three experiments.

CFA-immunized, i.c. CpG ODN-injected mice; they occurred in the 1300 per million frequency range. The data provide evidence for the accumulation of functional neuroantigen-specific T cells in the CpG-primed target organ, with the enrichment being ~200 times that for MOGp-reactive cells in the draining LN (Fig. 1, 70 per million).

Discussion

Our data suggest the CpG ODN strongly augment the Ag-presenting functions of the APC that are constitutively present in the brain. It seems that the brain APC have to be preactivated by local TLR signaling to trigger a cytokine response of sufficient magnitude for the induction of the T cell-mediated inflammatory cascade. Autoreactive T cells will induce delayed-type hypersensitivity-type inflammation in the target organ (that is, mediate autoimmune pathology) only if they re-encounter the autoantigen on APC that have been presensitized/licensed by microbial stimuli. We used CpG ODN to engage the site-specific infectious nonself signal in brain APC, because these ODN are small-molecular-mass substances with a highly defined mode of action: they activate APC by TLR9 signaling (37). Cells of the innate immune system use the TLR9 pathway for pattern recognition of bacterial DNA that is rich in CpG motifs. Because PTX is also a potent activator of APC, it seems likely that it also exerts its critical role in experimental autoimmune diseases via the APC licensing mechanism. An increasing number of TLR have been discovered, each rendering the innate immune system with different pattern recognition capabilities for common features of foreign on microorganisms, including viruses. TLR3, for example, recognizes dsRNA (38). The common function of TLRs is to alert the innate and the adaptive immune system to the presence of infectious nonself. Therefore, it is likely that the effects that we describe in this study for the well-defined TLR-9:CpG pathway extend to most TLR-induced APC activation. Bacterial infections are valid candidates for the cross-reactive priming of autoreactive T cells, but they are unlikely to mediate the third signal in the target organs of human autoimmune disease, including in the brains of MS patients. More likely, virus replication in the target organ itself engages the third signal in the human autoimmune disease.

The initial step in developing T cell-mediated inflammation in the CNS might be the induction of chemokines and of costimulatory molecules on tissue-resident APC (Fig. 3). CD40 up-regulation induced by CpG ODN was clearly detectable after 24 h in Rag-$1^{-/-}$ mice (Fig. 4, *C* and *D*), but returned to background levels by day 5 (data not shown). Therefore, it seems that Ag recognition and the subsequent local release of proinflammatory cytokines by primed autoantigen-specific T cells are required to perpetuate and amplify the initial inflammatory reaction induced by TLR activation. Consistent with this notion, CD40 up-regulation was maintained and increased through day 5 in MOGp-primed wild-type mice (Fig. 4, *E* and *F*), in which strong inflammatory infiltrates also established themselves (Fig. 2). In OVAp:CFA control-immunized mice or naive wild-type mice, CD40 up-regulation was not detected on day 5 (data not shown) and a mononuclear infiltrate also did not establish itself in these mice (Fig. 2*C*). Therefore, although the mild inflammation induced by the CpG ODN was critical for the priming of the brain-resident cells to render them competent APC for the first wave of Th1 cells that are recruited from the blood, eventually, the T cells will take over. The cytokines that the first wave of T cells release will recruit professional APC and further T cells from the blood: these cells jointly constitute the classical mononuclear cell infiltrate. The staining indicated perivascular aggregations with single cells in the adjacent parenchyma. Because CpG induces chemokine production in the CNS-resident cells, these chemokines are likely to attract the infiltrating cells. Consistent with the notion that these cells entered from the blood vessel, the aggregates occurred in perivascular space. The recruited APC and second-wave autoreactive T cells will amplify the local inflammatory response, and will maintain the immune competence of the local APC (as evidenced by CD40 expression) beyond the time point when the initiating CpG ODN effect faded. Under these conditions, T cells perpetuate the third signal in the target organ. Nonspecific microbial stimuli initiated a transient state of activation in the innate immune system that the specific and adaptive T cell system amplifies. Our data provide clear evidence for the accumulation of functional neuroantigen-specific T cells in the CpG-primed target organ. With frequencies of ~70 per million in draining LN (Fig. 1) vs ~14,000 per million in the CpG-primed CNS (Table I), there is a ~200-fold enrichment of MOGp-reactive cells in the target organ. Although the frequency of the OVAp- and MOGp-reactive T cells was comparable in draining LN, the higher frequency of the MOGp-specific IFN-γ-producing cells in the CNS argues for the selective retention and possibly local proliferation of the autoreactive cells caused by the endogenous autoantigen (39).

We would like to propose that the requirement for TLR-mediated licensing of APC in the target organ is the rule for developing T cell-mediated autoimmune disease. Autoreactive CD4 cells might be able to bootstrap autoimmune pathology only when their frequency is unusually high. This is the case for transgenic mice that express neuroantigen-specific TCR. It is also the case for proteolipid protein (PLP)-induced EAE in SJL mice, because in this mouse strain, the PLP-specific T cell repertoire does not undergo negative selection in the thymus, resulting in atypically high numbers of PLP-specific precursor T cells (5, 40). But, even in these models, it is unclear to what extent mycobacterial Ags leak from the massive s.c. CFA deposits and exert direct TLR activation in the CNS, or whether peripheral cytokine storms activate the APC in the brain. Finally, passive EAE can be induced without PTX. However, these models typically require the injection of a high number of neuroantigen-specific T cells that, importantly, can mediate disease only when they have been preactivated in vitro 3–5 days before the injection. It is conceivable that, in passive EAE, the constitutive cytokine production by the injected T cell blasts activates the APC providing the third signal. In EAE models that do not require PTX, it has been shown that neuroantigen-specific T cells are attracted to sites of sterile CNS injury, and augment the severity of EAE (41). As discussed above, in these models, the licensing of the brain APC might result from CFA or from cytokine storms, whereas the higher number of neuroantigen-specific cells in the brain (recognizing the autoantigen on licensed APC) might explain the increased pathogenic effect. In our model, autoantigen recognition on TLR-activated CNS APC leads to autoimmune pathology, whereas T cell entry into sites of sterile tissue damage (cryoinjury) promotes wound healing (28). T cells are recruited under both conditions, yet autoantigen recognition on APC activated by sterile injury or by microbial stimuli seems to result in fundamentally different consequences. Recently, it became evident that APC activated by different signals have fundamentally different immune functions (42).

Our data suggest a new checkpoint in the control of autoimmune pathology. Clonal elimination of autoreactive T cells is the first level of control over self/nonself discrimination. If self-reactive T cells evade negative selection in the thymus or the immune periphery, and persist as naive T cells, they constitute precursor cells for the development of a pathogenic autoimmune response. The encounter of the autoantigen, or of a cross-reactive Ag by such

naive T cells in the presence or absence of TLR signaling represents the second level of control. Only in the presence of TLR-activating microbial stimuli do pathogenic Th1 cells develop. This level avoids harmful autoimmune T cell response after common sterile tissue injury. Our data introduce a third level of control showing that effector functions of already differentiated Th1 cells are also governed by the infectious nonself context. Cross-reactive priming of autoreactive T cells by an infectious agent in the immune periphery will not result in autoimmune disease unless the target organ itself is also infected. An exception to this rule might be that APC-activating cytokines (such as IL-12) are massively produced in an acutely infected peripheral organ (for example, the intestine) and reach high enough serum concentrations to activate the APC in a distant organ (for example, the brain).

A major implication of the third-signal model is that it dissociates in space, time, and molecular nature the infectious nonself signals that lead to the priming of the naive autoreactive T cells in secondary lymphoid tissues, and that sensitize the target organ for the immune attack. The model predicts that different microbial agents can be involved in mediating the second signal and the third signal. For example, an infection with cross-reactive microorganism 1 might prime the neuroantigen-specific T cells, and TLR4 engagement by LPS might cause these T cells to differentiate into Th1 cells. If microorganism 1 does not infect the target organ (and does not induce a massive cytokine storm), the violation of immunologic self-tolerance will not result in autoimmune disease: the Th1 memory cells will persist, ignorant of the autoantigen. Years later, the infection of the CNS with an unrelated microorganism, for example, a virus that activates TLR3 (but not sterile tissue injury!), or a massive infection in the immune periphery leading to a cytokine storm might engage the third signal in the brain, triggering the actual onset of the autoimmune disease. In humans, there is evidence that the infection that leads to the cross-reactive priming can occur many years before the first actual exacerbation of MS (43). Moreover, relapses of MS are frequently associated with common infections (44–46) that, according to our model, would not need to be immunologically related to microorganism 1, which caused the initial violation of self-tolerance. In addition to acute peripheral infections, chronic infections of the brain itself could also mediate the third signal. Common viruses such as HSV, varicella-zoster virus, EBV, CMV, and human herpes virus 6 are frequently found in the CNS of MS patients, but also of healthy individuals (47–49). Any event that leads to viral replication in the CNS might trigger via TLR3 activation the third signal soliciting an autoimmune T cell attack. As our data clearly show, such cytokine storms or viral replication in the CNS will elicit full-blown inflammation only in individuals in whom primed neuroantigen-specific Th1 cells are present. The focal activation of the TLR-triggering virus in the CNS might define the actual site of the autoimmune attack (defining the site of the T cell attack within the organ) and explain the variable focal lesions that characterize MS. Therefore, the instructive role of the innate immune system within the target organ itself on autoreactive T cells may help in closing the gap in our understanding between violation of immunological self-tolerance and the actual manifestation of autoimmune disease.

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