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The Third Signal in T Cell-Mediated Autoimmune Disease

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This work is dedicated to my family for raising me in an environment which provided readily access to higher studies and for their endless support in all my personal and career endeavours. It is also dedicated to my teacher Mrs. Gesigora for proposing my admission to the German National Academic Foundation (Studienstiftung des deutschen Volkes), to the German National Academic Foundation for supporting me logistically and financially throughout my studies, and to the German State for offering broadly accessible university studies of highest quality.

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Abbreviations

[α 32-P]dATP – alpha 32-Phosphorus Deoxyadenosine Triphosphate
APC – Antigen Presenting Cell
BBB – Blood Brain Barrier
BSA – Bovine Serum Albumin
CD – Cluster of Differentiation
cDNA – copy Deoxyribonucleic Acid
CFA – Complete Freund's Adjuvant
CNS – Central Nervous System
CpG – Cytosine-phospho-Guanine
DMEM – Dulbecco's Modification of Eagle's Medium
DNA – Deoxyribonucleic Acid
DTH – Delayed Type Hypersensitivity
EAE – Experimental Allergic Encephalomyelitis
ELISPOT – Enzyme Linked Immuno Spot
FACS – Fluorescent Activator Cell Sorter
FITC – Fluorescein Isothiocyanate
h – hour
i.c. – intracerebral
IFA – Incomplete Freund's Adjuvant
IFN-γ – Interferon gamma
IgG – Immunoglobulin G
IL – Interleukin
IP-10 – gamma-interferon-inducible protein 10
KO – Knock Out
LN – Lymphe Node
MCP-1 – Monocyte Chemotactic Protein 1
MCP-3 – Monocyte Chemotactic Protein 3
MHC – Major Histocompatibility Complex

MIP-1α – Macrophage Inhibitory Protein 1 alpha

MIP-1β – Macrophage Inhibitory Protein 1 beta

MOG – Myelin Oligodendrocyte Glycoprotein

MOGp – Myelin Oligodendrocyte Glycoprotein peptide

mRNA – messenger Ribonucleic Acid

MS – Multiple Sclerosis

NBT/BCIP - nitro-blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate

nCpG – non Cytosine-phospho-Guanine

OD – Optical Density

ODN – Oligodeoxynucleotides

OVAp – Ovalbumin peptide

PBS – Phosphate Buffered Saline

PBST - Phosphate Buffered Saline Tween

$PDGF_A$ – Platelet Derived Growth Factor A

PLP – Proteolipoprotein

PTX – Pertussis Toxin

RAG – Recombinase Activating Gene

RNA – Ribonucleic Acid

s.c. - subcutanous

SDS – Sodium Dodecyl Sulfate

SSC – saline/sodium citrate

Streptavidin-AP – Streptavidin Alkaline Phosphatase

TCR – T Cell Receptor

TGF-β-1 – Transforming Growth Factor beta 1

Th1 – T helper 1

Th2 – T helper 2

TLR – Toll Like Receptor

TNF-α / β – Tumor Necrosis Factor alpha/beta

Introduction

The mechanisms that lead to autoimmune disease are still controversial (8). These have been best studied in experimental allergic encephalomyelitis (EAE), a disease caused by T cells that target autoantigens expressed in the central nervous system (CNS) (11). EAE is the animal model for multiple sclerosis (MS) in humans (33). 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 (13), or because they are specific for determinants poorly presented or absent in the thymus (4). Unless primed by environmental cross-reactive antigens or immunization, such T cells persist as naive lymphocytes in the immune periphery, apparently ignorant of the endogenous antigen (12). However in RAG-deficient mice, where there is an absence of regulatory cells, such autoreactive T cells become spontaneously activated (28).

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 co-administered with microbial products such as heat inactivated mycobacteria contained in CFA (14) or CpG oligonucleotides (ODN) (7;38). By activating TLR on cells of the innate immune system (1;26), such microbe-associated molecules engage the second signal link required for induction of a T cell response (21,14,29 ,9). Additionally, by triggering IL-12 production (19;25) such TLR activators guide the induction of a pro-inflammatory Th1 T cell response (7;10;42). In normal mice, immunization with neuroantigen in the absence of TLR activating substances induces Th2 immunity and profound protection from EAE (14) Only in RAG deficient mice that lack regulatory cells do Th2 cells mediate neuropathology (23).

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. “The immune system evolved to discriminate

between infectious non-self and non-infectious self“ (20). While 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 the mice do not develop histological or clinical EAE unless PTX is co-injected (14). PTX may be required to break down the blood brain barrier (BBB) thereby providing the primed T cells access to the target organ (5). In an attempt to address this issue, we induced aseptic cryoinjury in brains of mice that were pre-immunized with MOG peptide 35-55 in CFA to engage peptide-specific Th1 cells. 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 (18). Similar effects of neuroantigen-specific Th1 cells in sterile CNS injury have been reported before (30).

As a G-protein inhibitor, PTX exerts multiple functions including the activation of APC (35;39). We hypothesized that APC activation in the target organ might explain the essential role of PTX in most 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. We tested whether the APC in the brain need “licensing“ by TLR9-activation in order to elicit the proinflammator effector functions of differentiated MOG-specific Th1 cells.

Materials and Methods

Animals, Antigens, ODN and Treatments. Female C57BL/6 mice and congenic knockout mice (Rag-$1^{-/-}$, $\mu MT^{-/-}$), 6-8 weeks 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. MOG peptide 35-55 (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: TCCATGACGTTCCTGACGTT for the immunostimulatory CpG ODN 1826, and TCCAATGAGCTTCCTGAGTCT for the non-stimulatory nCpG ODN 1745 as defined before (7). Synthetic ODN were dissolved in sterile nonpyrogenic 0.9% NaCl (Baxter, Deerfield, IL) at a concentration of 40 □g/ml. Mice were injected with 500 nl of this solution depositing 20 μg of the ODN. CFA was prepared by mixing M. tuberculosis H37RA (Difco Laboratories, Detroit, MI) at 1.0 mg/ml into IFA (Gibco BRL, Grand Island, NY). For immunizations, MOGp was mixed with CFA at a final concentration of 0.5 mg/ml and 100 μl of this emulsion (50 μg MOGp/mouse) was injected. When specified, mice were additionally injected intraperitoneally 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 = hind leg weakness, Grade 3 = full hind leg 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 (Aldrich Chemical Company, Milwaukee, WI) in t-Amyl-Alcohol (Sigma, St Louis, MO). A sagittal incision of 15 mm length was made in the skin of the scull using a scalpel blade. A liquid nitrogen-chilled steel rod (3 mm in diameter) was held for 6 seconds 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: 1.54 mm anterior, 1.5 mm lateral, and 2 mm ventral. 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 micropippetes were mounted in a micromanipulator attached to the stereotaxic apparatus, and were placed in the position indicated by the stereotaxic coordinates. After 15-20 minutes, 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). The brains were removed and immersed in PBS-4%-paraformaldehyde for 12 h, then washed in distilled water for 1-2 hours prior to 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 hematoxylin and eosin 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 hematoxylin and eosin staining were also used for immunohistochemistry. After rehydration they were subjected to antigen retrieval at 90°C for 15 minutes using Target Retrieval Solution (Dako, Carpinteria, CA). Sections were exposed to PBS-0.4% Triton containing 10% goat serum for 2h and subsequently incubated overnight at room temperature with rat anti-mouse CD40 (1C10 at 10 µg/ml, R&D Systems). In a second step, primary antibodies 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 antibody.

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, Carlsbad, CA) following 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 radiolabaled 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 x saline/sodium citrate (SSC) at 68°C, 1% SDS followed by two 20-min washes in 0.1 x SSC, 0.5% SDS), the membranes were exposed to a phosphorimaging screen for 20 h and the hybridization was analyzed with a Molecular Probes PhosphorImager (Molecular Probes, Eugene, OR). Genes were considered differentially expressed when they exhibited a 2-fold or greater increase or decrease after CpG injection, compared to the nCpG group. The relative expression of the housekeeping genes (40S ribosomal protein S29, glyceraldehyde-3-phosphate dehydrogenase, Ubiquitin and others) did not differ by more than 10% among samples.

Cell Isolation and Purification. Single-cell suspensions from LN or spleens were prepared. Subpopulations of T cells were isolated using commercially available murine T cell isolation columns (R&D Systems, Minneapolis, MN) 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 antibodies (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 (Becton Dickinson, San Jose, CA) and then passed through a pre-wetted nylon wool column (0.7-1.0 g 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 g/cm^3 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 were placed in 15ml 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 g at 20°C for 45 minutes. Cells at the 35%/70% interface (containing the mononuclear cells) were collected, washed twice and counted. Directly labeled antibodies (all from BD PharMingen) were used for the flow cytometric characterization of the cell populations obtained. The stained cells were analyzed with FACS® and Cell Quest software (Becton Dickinson).

ELISPOT Assays and ELISPOT Image Analysis. Assays were performed as previously described (22). ImmunoSpot M200 plates (Cellular Technology, Cleveland, OH) were coated overnight with the capture antibodies 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 (PharMingen) for IL-2; BVD4-1D11, at 2.5 μg/ml, (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, (PharMingen) was used for IL-6; JES5-2A5, at 6μg/ml, (PharMingen) was used for IL-10; 38213.11 at 5 μg/ml (R&D Systems) was used for IL-13 and polyclonal antibody at 2.5 μg/ml (Endogen, Woburn,MA) was used for TNF-α/β. The plates were blocked for 1 h with sterile PBS 1% bovine serum albumin (BSA) and washed three times with sterile PBS. Spleen cells (10^6 per well) or cells from draining LN (7 x 10^5) were plated in HL-1 medium (BioWhittaker) and antigen. In select experiments, cells and antigens were titrated. The plates were incubated at 37°C, 5% CO_2 for 24 h (for IFN-γ, TNF-α/β, 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 antibodies were added and the plates were incubated overnight. XMG1.2-biotin (PharMingen) was used at 1 μg/ml for IFN-γ; rat anti-mouse IL-2-biotin (JES6-5H4, PharMingen) was used at 2 μg/ml for IL-2; rat anti-mouse IL-4-biotin (BVD6-24G2, PharMingen) was used at 1 μg/ml for IL-4; biotinylated TRFK4 (PharMingen) was used at 2 μg/ml for IL-5; rat anti-mouse IL-6-biotin (MP5-32C11, PharMingen) was at 2 μg/ml used for IL-6; rat anti-mouse IL-10-biotin (JES5-16E3, PharMingen) was used at 2 μg/ml for IL-10; for TNF-α/β in a first step polyclonal rabbit anti-mouse TNF-α/β (Endogen) was used at 4 μg/ml then in a second step alkaline-phosphatase-labeled goat anti-rabbit IgG (HyClone Laboratories, Logan, UT) was used at 1:1000 dilution. The plates were then washed three times in PBST. Streptavidin-AP (DAKO) was added at 1:2.000 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 nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) 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.

Statistics. Microsoft Excel and SigmaPlot were used to calculate means and standard deviations.

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, while the mice that were not injected with PTX remained clinically unapparent (Fig. 1) 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. 2). The production of IL-2, IFN-γ, and TNF-α / β 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 RAG 1 KO spleen cells as APC showed that all cytokines detected were produced by T cells (Hofstetter et al., manuscript in preparation). Therefore, MOGp:CFA immunization induced a highly type 1- polarized immune response.

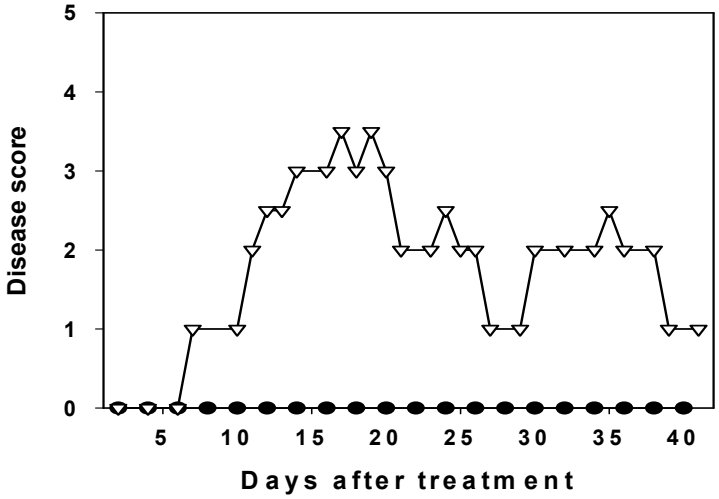

Figure 1. Immunization with MOG:35-55 in CFA does not induce EAE. C57BL/6 mice were immunized s.c with MOG:35-55 in CFA. One group of mice was additionally injected with PTX i.p. (open triangles, n=12), the second group remained uninjected (closed circles, n=12). The mice were observed daily for clinical symptoms of EAE and scored 0-5 according the standard scale. Mean disease score is shown as the function of time. The data are representative for 3 experiments performed.

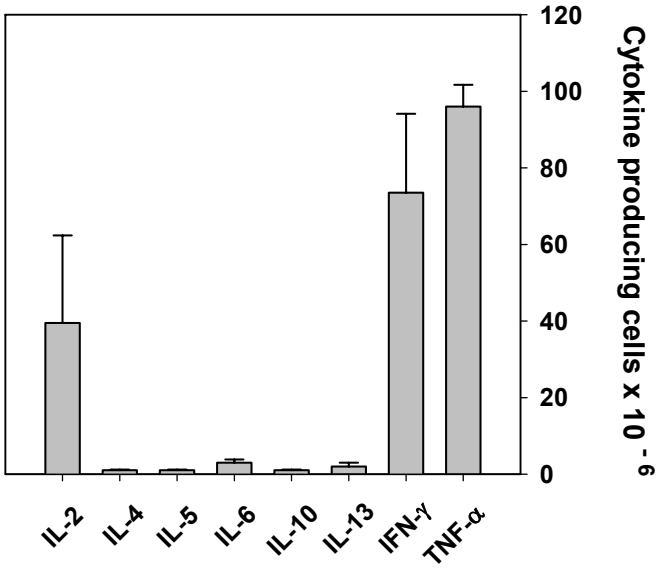

Figure 2. C57BL/6 mice were immunized s.c. with MOG:35-55 in CFA and draining LN cells were tested in ELISPOT assays for MOG:35-55 induced cytokine production. The number of cytokine producing cells per million LN cells tested is shown. The data are representative of 4 independent experiments performed.

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 antigen" ovalbumin (OVA) in CFA (42). In vitro pre-activated 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 months after immunization (data not shown), apparently ignorant of the endogenous antigen 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 to unimmunized or OVAp:CFA-injected control animals (18). 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. 3A,B & Table1), 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. 4A,B & Table1) or injection of saline (Table1). 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 (Table 1). MOGp:CFA-injected B cell deficient ($\mu MT^{-/-}$) mice also developed the inflammatory lesions (Table 1), suggesting that T cell-, rather than B cell-immunity is responsible for eliciting the inflammatory reaction in the CNS.

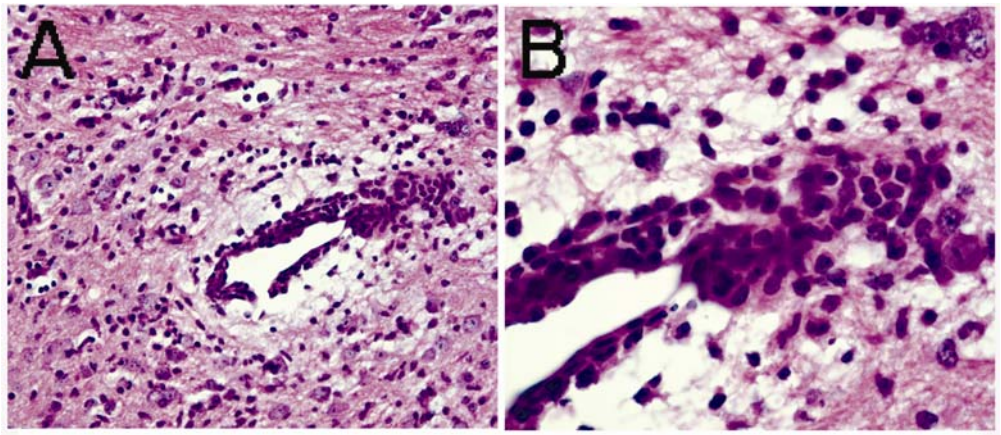

Figure 3. Mononuclear cell infiltrate develops at the site of CpG ODN injection in the frontal lobe of MOGp:CFA primed mice. C57BL/6 mice were immunized s.c. with MOG:35-55 in CFA. Twelve days later, 20 µg of CpG ODN were microinjected i.c. in 0.5 µl saline. H & E staining of the injection site was performed 5 days after the i.c. injection. A representative image of a CpG ODN-injected brain section is shown at 40 x magnification in (A) and at 100 x magnification in (B). The data are representative for 5 independent experiments performed.

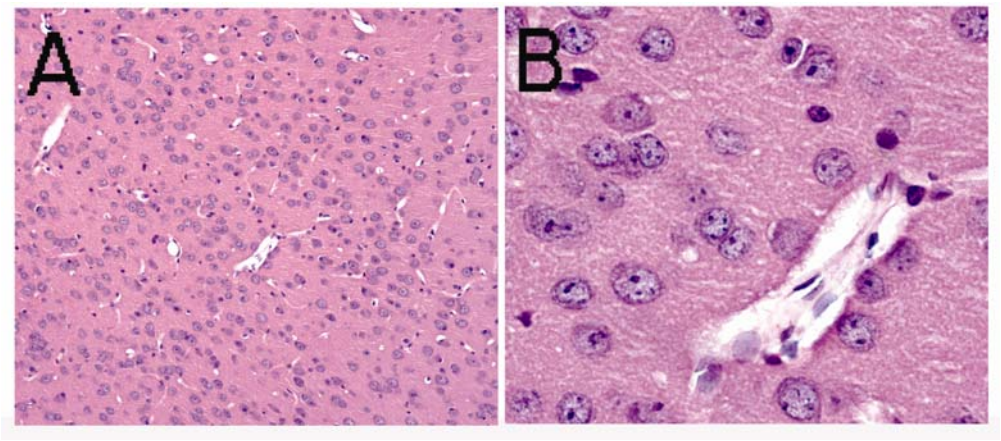

Figure 4. Mononuclear cell infiltrate does not develop at the site of nCpG ODN injection in the frontal lobe of MOGp:CFA primed mice. C57BL/6 mice were immunized s.c. with MOG:35-55 in CFA. Twelve days later, 20 µg of nCpG ODN were microinjected i.c. in 0.5 µl saline. H & E staining of the injection site was performed 5 days after the i.c. injection. A representative image of a nCpG ODN-injected brain section is shown at 40 x magnification in (A) and at 100 x magnification in (B). The data are representative for 5 independent experiments performed.

Table 1. Summary of mononuclear cell infiltrate intensity in all experimental groups.

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
B6 µMT	MOGp:CFA	3.901 ± 194	< 200	< 200	< 200

C57BL/6 wild type or antibody deficient µMT mice were immunized s.c. with MOG:35-55 in CFA, OVA:323-339 in CFA or remained without immunization. Twelve days later, 20 µg of CpG ODN, nCpG ODN or Saline were microinjected i.c. in 0.5 µl saline, respectively. Some mice were not injected i.c. at all. Mononuclear cells were counted by computerized image analysis within a 2 mm^2 area adjoining of the injection site. Each data point represents the mean number of mononuclear cells and the SD established for 4 mice per group. The data are representative for 5 independent experiments performed.

Consistent with a classic DTH response, the mononuclear cell infiltration started 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 mice of 32 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 closer define the CpG ODN's role 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. 5). The mRNA levels did not differ between nCpG ODN-injected brain (left panel) and CpG ODN-injected brain (right panel) for $PDGF_A$ (1, ODs 53 vs. 56), Pleiotrophin (2, ODs 57 vs. 53), Prothymosin-β-4 (3, ODs 49 vs. 47), TGF-β-1 (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 upregulation was detected for IP-10 (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 (34). Moreover, mRNA levels for CD40 were strongly upregulated, which encodes a potent T cell costimulatory molecule (37). The upregulation of CD40 in the CNS of i.c. CpG ODN-injected Rag-$1^{-/-}$ mice was also seen at the level of protein expression (Fig. 6).

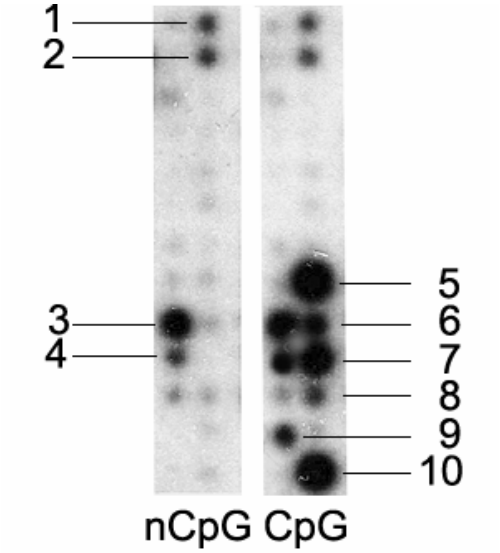

Figure 5. CpG ODN-induced mRNA expression in the CNS. C57BL/6 Rag-$1^{-/-}$ mice were microinjected i.c. with 20 µg of nCpG ODN or CpG ODN. Eight hours later, mRNA was isolated from the injected hemisphere, reverse transcribed, and hybridized with array chips. The prevalent gene products detected are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

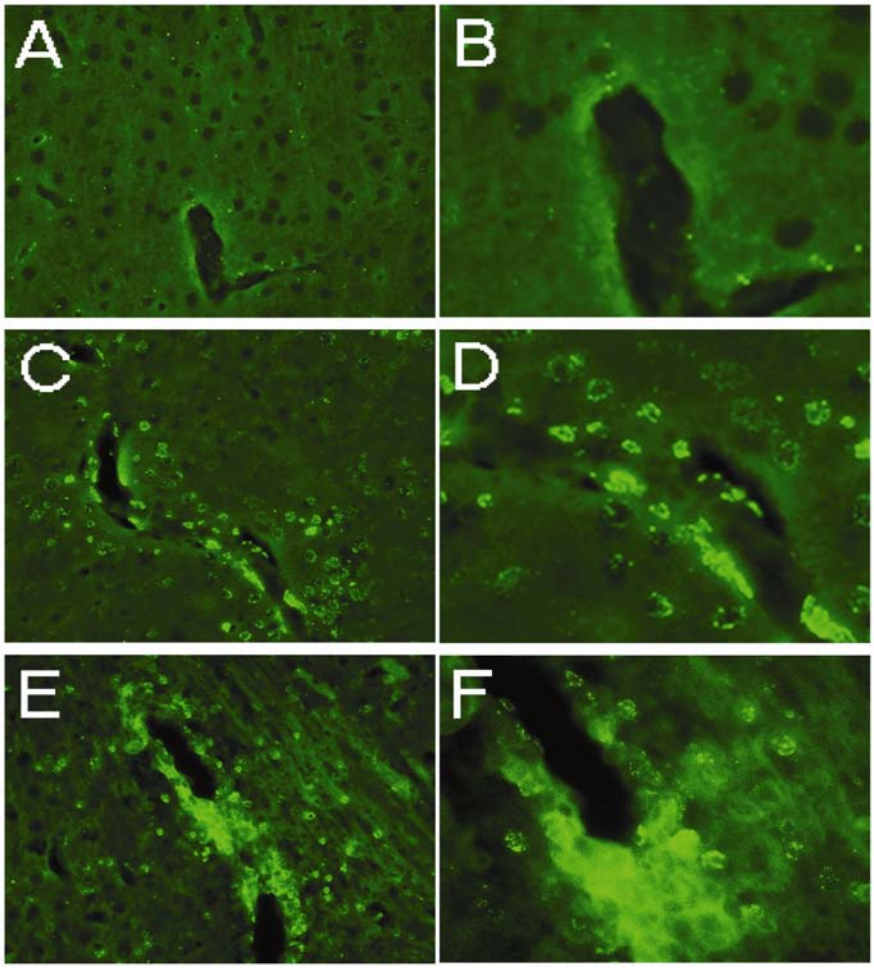

Figure 6. CD40 is upregulated at the site of CpG ODN injection in the frontal lobe. C57BL/6 Rag-$1^{-/-}$ mice were microinjected i.c. with 20 µg of nCpG ODN (A&B) or CpG ODN (C&D). CD40 staining of the injection site was performed 24h after the i.c. injection. In E & F, images are shown for MOGp:CFA immunized wild type mice 5 days after the i.c. injection of CpG ODN. Representative images are shown at 40 x (A,C,E) and 100 x (B,D,F) magnification. The data are representative for 2 independent experiments performed.

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 isolated mononuclear cells from their frontal lobes. These cells, which entail the APC constitutively present in the uninflamed brain, were pre-treated with nCpG ODN or with CpG ODN for 8 h, extensively washed, and were 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. 7).

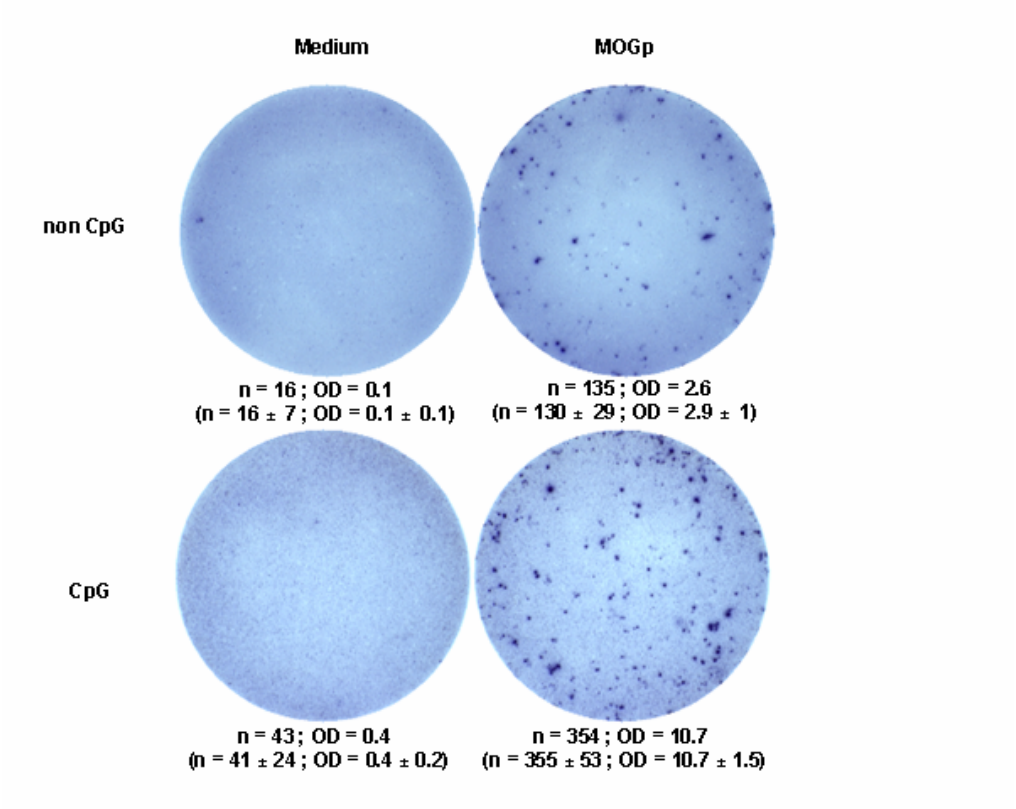

Figure 7. APC function of CpG and nCpG treated brain cell isolates. Cells were isolated from the frontal lobes of unimmunized C57BL/6 mice and plated at 100,000 cells per well with CpG ODN or nCpG ODN (1.5 µg/ml) for 8 hours. After washing the APC 4 times, 300,000 CD4 cells were added (isolated from draining LNs of MOGp:CFA immunized mice, 94% purity). IFN-γ production was measured by ELISPOT 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 parenthesis, mean spot numbers and ODs from the triplicate wells tested in this experiment are shown. The data were reproduced in 4 independent experiments.

The number of CD4 cells activated to produce IFN-γ in the presence of added MOGp was ~3 times higher in the CpG-treated brain APC, as compared to 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 ~3 times higher (the OD values in Fig. 7). 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 antigen 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 antigen 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 (16). 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 upregulation of costimulatory molecules on these APC.

*Enrichment of the Autoreactive T Cells in CpG ODN-Activated Brain Tissue***.**

To closer define the role of the T cell in the inflammatory CNS response, we isolated cells from the injected frontal lobe and visualized their antigen-induced cytokine production (41). 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 2). 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:CFA-immunized, i.c. CpG ODN-injected mice; they occurred in the 1.300 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 (70/million, Fig. 2).

Table 2. ELISPOT detection of antigen-induced IFN-γ producing cells in CNS isolates.

		IFN-γ recall	
Immunization	Injection (i.c.)	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

Groups of eight C57BL/6 mice were immunized s.c. with MOGp, OVAp or remained unimmunized. Twelve days later, 4 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 3 replicate wells are shown. The data were reproduced in 3 experiments.

Discussion

Our data suggest that the CpG ODN strongly augment the antigen presenting functions of the APC that are constitutively present in the brain. It seems that the brain APC have to be pre-activated 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 DTH-type inflammation in the target organ (that is, mediate autoimmune pathology) only if they reencounter the autoantigen on APC that have been presensitized/licensed by microbial stimuli. We used CpG ODN to engage the site-specific “infectious non-self“ signal in brain APC because these ODN are small molecular weight substances with a highly defined mode of action: they activate APC by TLR9 signaling (15). Cells of the innate immune system utilize the TLR9 pathway for pattern recognition of bacterial DNA that is rich in CpG motifs. Since 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. TLR 3, for example recognizes double stranded RNA (2). The common function of TLRs is to alert the innate and the adaptive immune system to the presence of “infectious non-self“. Therefore, it is likely that the effects that we here describe 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. 7). CD40 upregulation induced by CpG ODN was clearly detectable after 24 h in

Rag-1 $^{-/-}$ mice (Fig. 6 C&D), but returned to background levels by day 5 (data not shown). It seems therefore that antigen recognition and the subsequent local release of proinflammatory cytokines by primed autoantigen-specific T cells is required to perpetuate and amplify the initial inflammatory reaction induced by TLR activation. Consistent with this notion, CD40 upregulation was maintained and increased through day 5 in MOGp-primed wild-type mice (Fig. 6E&F, in whom also strong inflammatory infiltrates established themselves, Fig. 3). In OVAp:CFA control-immunized mice or naive wild-type mice CD40 upregulation was not detected on day 5 (data not shown, and a mononuclear infiltrate did also not establish itself in these mice, Table 1). Therefore, while 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 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. Non-specific 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 ~ 70/million in draining LN (Fig. 2) vs. ~ 14,000/million in the CpG-primed CNS (Table 2) there is a ~200 fold enrichment of MOGp-reactive cells in the target organ. While 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 (17).

Because models in which EAE can be induced without PTX are rare exceptions, 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 “boot strap“ 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 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 (4;24). But even in these models it is unclear to what extent mycobacterial antigens 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.

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/non-self 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 antigen 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 non-self“ context. Crossreactive 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 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 non-self 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 TLR 4 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 TLR 3 (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 (27). Moreover, relapses of MS are frequently associated with common infections (40,31,3) that, according to our model, would not need to be immunologically related to microorganism 1 which caused the initial violation of self-tolerance. While these infections could be acute peripheral infections, also chronic infections of the brain itself could mediate the third signal. Common viruses such as Herpes-Simplex-Virus, Varicella-Zoster-Virus, Epstein-Barr-Virus, Cytomegalovirus and Human-Herpes-Virus-6 are frequently found in the CNS of MS patients, but also of healthy individuals (32,6,36). Any event that leads to viral replication in the CNS might trigger via TLR-3 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 only elicit full blown inflammation 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 explaining the variable focal lesions that characterize MS. The instructive role of the innate immune system within the target organ itself on autoreactive T cells therefore may help closing the gap in our understanding between violation of immunological self-tolerance, and the actual manifestation of autoimmune disease.

Abstract

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. While 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 MOG (Myelin Oligodendrocyte Glycoprotein) with CFA (Complete Freund's Adjuvant) which primes a type 1 T cell response but does not lead to clinical or histological manifestation of EAE (Experimental Allergic Encephalomyelitis) unless pertussis toxin is injected in addition. We show here that in MOG:CFA-primed mice the autoimmune CNS (Central Nervous System) pathology develops after intracerebral deposition of Toll-like receptor (TLR) 9 activating CpG (Cytosine-phospho-Guanine) 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 uninflammed CNS or to CNS undergoing sterile inflammation did not suffice to elicit autoimmune pathology; only if the APC (Antigen Presenting Cell) 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.

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2002 Paul V. Lehmann, Kamruz Darabi, Harald H. Hofstetter, Bernhard O. Boehm, Zsuzsa Fabry, Joseph C. LaManna, Juan C. Chavez, Alexey Y. Karulin.
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