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Autoreactive T cells promote post-traumatic healing in the central nervous system

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Abstract

In general, autoimmune responses are considered harmful to the host. In the best-defined model of autoimmune disease, murine experimental allergic encephalomyelitis (EAE), for example, brain-protein-specific autoimmune responses of both major classes, type-1 and type-2, have been implicated in causing brain pathology. We induced type-1 and type-2 autoimmunity to myelin oligodendrocyte protein (MOG) in C57.BL/6 mice. Instead of using pertussis toxin (PTX) to open the blood–brain barrier (BBB), which is the classic procedure, we set an aseptic cerebral injury (ACI) to see what the consequences of pre-primed, autoreactive type-1 and type-2 memory T cells gaining access to the brain in the course of sterile tissue injury would be. Neither of these autoimmune response types induced pathology; on the contrary, both accelerated re-vascularization and post-traumatic healing. The data suggest that induction of either type-1 or type-2 autoimmune responses is not inherently noxious to the host, but can have beneficial effects on tissue repair. Autoimmune pathology may develop only if molecules of microbial origin such as pertussis toxin additionally induce the ''infectious nonself/danger'' reaction in the antigen-presenting cells (APC) of the target organ itself.

Keywords: Experimental allergic encephalomyelitis; Myelin oligodendrocyte antigen; Cytokines; Th1/Th2; Central nervous system; Post-traumatic healing

1. Introduction

Clonal deletion/inactivation of autoreactive lymphocytes is a major mechanism of self-tolerance. This mechanism is leaky, however (Goverman et al., 1993; Targoni and Lehmann, 1998), and precursor cells capable of responding to most autoantigens can easily be isolated from any individual (reviewed in Steinman, 1995; Lehmann et al., 1998). Whether from the host's perspective that ''the only good autoreactive T cell is a dead autoreactive T cell'' or whether there are beneficial types of autoimmune responses and, if so, what these actually are, has been a matter of controversy over the decades (reviewed in Steinman, 1995; Lehmann et al., 1998). The prevalent thought today is basically the same as that first formulated exactly a century ago by Ehrlich (1900) as the *horror autotoxicus*, that autoimmunity is inherently noxious to the host and the immune system has evolved to avoid it.

Pro-inflammatory, Th1-type autoimmune responses have generally been considered harmful to the host. Complicating this view is the observation that the genetic disruption of the putative primary Th1 effector cytokine IFN-γ does not make mice resistant to experimental allergic encephalomyelitis (EAE) (Ferber et al., 1996). The disruption of another candidate Th1 effector cytokine, TNF-α, makes mice even more susceptible to EAE than are wild-type (wt) control (Liu et al., 1998). Also complicating this view is the observation that IFN-γ can play a protective role in EAE (Krakowski and Owens, 1996; Willenborg et al., 1996). Finally, it was reported in a landmark publication (Moalem et al., 1999) that when a pathogenic Th1 clone was injected, these T cells could exert a protective effect at the site of central nervous system (CNS) trauma (while the rats developed EAE concurrently). When autoreactive T

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cells were injected in sub-pathogenic numbers into naïve recipients, recruitment and activation of macrophages were demonstrated in parallel to its neuroprotective effect. These autoreactive Th1 cells might cause activated macrophages to be recruited into the CNS, which is an essential step in the development of autoimmune disease (Brosnan et al., 1981; Hinrichs et al., 1987). Similar reactions have been reported, but in apparent contradiction, when the implantation of activated macrophages was also observed to stimulate tissue repair (Rapalino et al., 1998). The mainstream notion that type-1 autoimmune responses are inherently harmful to the host has thus become debatable. Different types of autoimmune T cell responses have been implicated in the two opposing outcomes. Following oral administration of MBP, the size of infarct induced in the rat brain was found to be reduced, while it was increased in MBP/CFA-immunized animals (Becker et al., 1997). Can this neuroprotective effect exerted by autoreactive T cells be dissociated from their better defined neurodestructive functions, and if so, what are the mechanisms that distinguish between the two outcomes? Under what conditions is T cell-mediated autoimmunity beneficial to the host, and what makes it harmful?

As far as type-2 autoimmune responses are concerned, all data initially pointed towards them protecting the host from Th1 cell-mediated disease (Rocken et al., 1996). In subsequent studies, however, autoreactive Th2 cells were found to induce autoimmune pathology on their own (primarily when injected in large numbers into immunodeficient recipients), and even when injected in considerable excess, they did not inactivate Th1 cells injected simultaneously (Khoruts et al., 1995; Lafaille et al., 1997; Pakala et al., 1997), suggesting that bystander suppression may not be a major mechanism of Th2 cell-mediated active tolerance, if such tolerance exists at all. Moreover, it was observed that some Th2 cytokines, like IL-6, might be essential to the development of EAE (Eugster et al., 1998). These data promoted what may be regarded as the current prevalent view, that even Th2 autoreactive responses are potentially harmful to the host.

To revisit the consequences of induction of an autoimmune response, we immunized mice with a peptide of the neuroantigen myelin oligodendrocyte protein (MOG), injecting it with complete Freund's adjuvant (CFA) (Mendel Kerlero de Rosbo and Ben-Nun, 1996). Such classic immunizations with CFA typically induce the differentiation of Th1 cells (Forsthuber et al., 1996; Yip et al., 1999), but EAE does not develop in this model unless pertussis toxin (PTX) is co-injected (Lee and Olitzky, 1955; Mendel Kerlero de Rosbo and Ben-Nun, 1996). While it is not clear why PTX is a critical co-factor in the development of actual autoimmune disease, opening the blood–brain barrier (BBB), thus giving the autoreactive T cells access to the autoantigen in the central nervous system (CNS), has been identified as an essential contribution (Brabb et al., 1997). Other potential effects of PTX include direct activation of macrophages (Torre et al., 1996) in the CNS itself, mitogenic effects on the effector T cells (Grenier-Brossette et al., 1991), changes in lymphocyte recirculation/homing (Cyster and Goodnow, 1995) induction of cytokines, and expression of costimulatory molecules (Ryan et al., 1998) in the lymphatic tissues during the induction of the T cell response (affecting the differentiation of the effector cells) and/or in the target organ itself (affecting the implementation of effector cell functions). The need for PTX in this EAE model may, therefore, involve additional mechanisms required for the development of autoimmune disease that go far beyond opening the BBB.

In order to ask what the consequences for the CNS would be if the peripherally primed autoreactive Th1 cells gained access to the target organ during sterile tissue injury, we disrupted the BBB by causing severe aseptic cryoinjury to the brain instead of using PTX (Swartz et al., 2001). In similar experiments, we induced MOG peptide-specific Th2 immunity by injecting the peptide in incomplete Freund's adjuvant (IFA) (Forsthuber et al., 1996; Yip et al., 1999) before inducing ACI. Neither type of autoimmune response was found to be pathogenic in the absence of PTX injection; instead of causing additional pathology, they accelerated post-traumatic healing, primarily through re-vascularization following aseptic cerebral injury in the CNS.

2. Materials and methods

2.1. Animals, antigens, and treatments

Female C57.BL/6 mice and congenic cytokine-knockout (KO) mice (IFN-γ, IL-2, IL-4, IL-5, and IL-6) 6–8 weeks of age were purchased from The Jackson Laboratory, Bar Harbor, ME, and maintained in specific-pathogen-free animal facilities of Case Western Reserve University. MOG peptide 35–55 was synthesized and purified by Princeton Biomolecules (Columbus, OH). CFA was prepared by mixing *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI) at 1.0 mg/ml into IFA (Gibco BRL, Grand Island, NY). For immunizations, MOG peptide was mixed with either CFA or IFA at a final concentration of 1 mg/ml. One hundred microliters of this emulsion (100 μg MOGp/mouse) was injected, as specified. Starting on 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.

2.2. Aseptic cerebral injury (ACI)

We followed standard procedures, as described (Oury et al., 1993). This injury model is a well-characterized

traumatic injury model described previously by others (Cancilla et al., 1979; Cancilla and DeBault, 1980). We have chosen this injury model because the injury is readily reproducible and allows for comparison of affected (ipsilateral) and non-affected (contralateral) hemispheres (Swartz et al., 2001). Furthermore, complication of infection can be excluded and the model reflects a very common brain injury model.

Anesthesia was induced using ketamine injected intraperitoneally and verifying respirations were maintained throughout the procedure. A sagittal incision 1–2 cm in length was created in the skin using a scalpel blade. Cranial anatomy was identified. ACI was created using a liquid nitrogen-chilled steel rod (3 mm in diameter) held for 6 s against exposed but intact skull over the medial right parietal lobe, approximately 2 mm anterior to the lambdoid suture. The incision was closed using 3.0 nylon in a running whipstitch fashion, and the mice were observed until they awakened. Mice showed no evident neurological deficits or gross changes in behavior after this treatment. At the time points specified, the mice were sacrificed and their brains removed, and either divided into right and left portions prior to freezing at −70 °C or immersed in Z-Fix prior to paraffin embedding.

2.3. Tissue harvesting, hematoxylin and eosin preparation, and grading

Mice were sacrificed at days 1, 4, 7, and 14 post-injury, using a ketamine overdose for euthanasia. The prior incision was opened, and the ACI site identified. The injury was visually evident at the time of harvesting as a hemorrhagic area on the surface of the parietal cortex. Each brain was harvested by incising the brain at the level of the coronal and lambdoid sutures using a fresh scalpel blade and then immediately placed en bloc into an OCT-laden boat (Tissue Tek® OCT compound, Sakura Finetek USA, Torrance, CA, USA; Cryomold® standard disposable vinyl molds, Miles, Elkhart, IN, USA) for snap freezing over dry ice. Coronal sections were cut to 10 μm thickness using a Reichart-Jung 1800 Fridge Cut cryostat (Leica, Solms, Germany), and placed on glass slides (Fisherbrand® Superfrost®/Plus, Fisher Scientific, Pittsburgh, PA, USA) for analysis. Coronal sectioning allows for simultaneous direct (ipsilateral) and indirect (contralateral) hemispheric evaluation. Each specimen underwent either immediate standard hematoxylin and eosin staining or was placed in a −70 °C freezer until readied for immunohistologic evaluation. The day prior to immunolabeling, the slides were transferred to a −20 °C storage unit. For regular histological examination, paraffin tissue blocks were prepared, 10-μm sections were cut and placed on microscope slides. Sections were stained in hematoxylin for 1 min, washed, and counter-stained with eosin for 30 s. Slides were again washed, dehydrated, and mounted with Permount. Each slide was photographed using a CMOS-PRO digital camera mounted on an Olympus microscope and assessed by three independent investigators in a blinded fashion for the hallmarks of tissue repair following tissue injury: acellular necrosis, cellular infiltration, presence of granulation tissue and edema, and re-vascularization (Cancilla et al., 1979; Cancilla and DeBault, 1980).

Hematoxylin and eosin preparations of all sets of mice were evaluated under light microscopy for the following: cellular infiltration, necrosis, and vascularity, with the grades of minimal (1), moderate (2), evident (3), or marked (4). Four different evaluators reviewed the specimens, using a double-blinded evaluation protocol, on a scale of 0–4, with 0.5 points for intermediate findings (Swartz et al., 2001). These scores were within a margin of 0.5 (mean ± 0.5) and their means are presented in figures ($n = 12$). The individual scores were totaled to give a healing index ranging from 1 to 16 to each mouse. Representative specimens were then photographed using the CMOS-PRO image system for use with Adobe® Photoshop® 5.0.

2.4. Cell purification

Single-cell suspensions from 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 in Hank's balanced saline solution (HBSS), counted by trypan blue exclusion, and resuspended at appropriate concentrations for use in the various assays. Purity was confirmed by fluorescence-activated cell sorting (FACS) analysis using fluorescein isothiocyanate (FITC)-conjugated antibodies (Pharmingen, San Diego, CA). The columns routinely yielded >96% purity for $CD4^+$ T cells.

2.5. ELISPOT assays and ELISPOT image analysis

Assays were performed as described by Karulin et al. (2000). ImmunoSpot M200 plates (Cellular Technologies, Cleveland, OH) were coated overnight with the captured antibodies in sterile phosphate-buffered saline (PBS). R46A4, at 4 mg/ml (isolated and purified from hybridoma), was used for IFN-γ; JES6-1A12, at 3 mg/ml (Pharmingen), was used for IL-2; 11B11, at 2 mg/ml (isolated and purified from hybridoma), was used for IL-4; TRFK5, at 5 mg/ml (isolated and purified from hybridoma), was used for IL-5; MP5-20F3, at 4 mg/ml, was used for IL-6; and MP6-XT22, at 4 mg/ml, 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) were plated in HL-1 medium (BioWhittaker, Walkersville, MD) and antigen. In select experiments, cells and antigens were titrated. The plates were incubated at 37 °C, 5% CO_2 for 24 h (using IFN-γ, TNF-α/

β, IL-2, and IL-6) or for 48 h (using IL-4 and IL-5). After washing with PBS, and again with PBS plus 0.025% Tween (PBST), detection antibodies were added and the plates were incubated overnight. XMG1.2-biotin (produced in our laboratory) was used for IFN-γ, rat anti-mouse IL-2-biotin (JES6-5H4, Pharmingen) was used for IL-2, rat anti-mouse IL-4-biotin (BVD6-24G2, Pharmingen) was used for IL-4, biotinylated TRFK4 (Pharmingen) was used for IL-5, rat anti-mouse IL-6-biotin (MP5-32C11, Pharmingen) was used for IL-6, and rat anti-mouse TNF-α/β-biotin (MP6-XT3, Pharmingen) was used for TNF-α/β. The plates were then washed three times in PBST. Streptavidin-AP (DAKO, Carpenteria, CA) was added at 1:2000 dilution in PBST as a third reagent for IL-2, IL-4, and IL-5, 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, MA) that was added and left for 15–30 min. The resulting spots were counted on an ImmunoSpot Series 1 Analyzer (Cellular Technologies) specifically designed for the ELISPOT assay. Digitized images were analyzed for the presence of areas in which color density exceeds background by a factor set on the basis of the comparison of control (containing T cells and APC without antigen) and experimental wells (containing antigen). After separating spots that touch or partially overlap, additional criteria of spot size and circularity are applied to gate out speckles and noise caused by spontaneous substrate precipitation and nonspecific antibody binding. Objects that do not meet these criteria are ignored and areas that meet them are recognized as spots and counted.

3. Results

3.1. Injection of MOG peptide 35–55 in either CFA or IFA induces CD4 memory cells polarized to type-1 or type-2

While the differentiation of CD4 T cells into memory cells that are committed to produce either IL-4 or IFN-γ is well defined (Abbas et al., 1996), the extent to which they co-express all the other cytokines associated with Th1/Th2 immunity is not fully understood (Kelso, 1995). It is the joint action of these individual cytokines, however, that defines the actual effector functions of the memory T cells. T cell clones that express the full canonical set of Th1 and Th2 cytokines are rare, and there is ample evidence that these cytokines are independently expressed (reviewed in Karulin et al., 2000). What, therefore, are the cytokine signatures of the MOG: 35–55-specific T cell responses induced after the peptide is injected with Freund's classic adjuvants CFA and IFA? Because the frequencies of the antigen-specific memory cells induced after such injections are low in vivo, it has been challenging to characterize unambiguously the cytokine signatures of these responses directly ex vivo. We used an ELISPOT approach to overcome this limitation. This approach makes it possible to detect the antigen-induced cytokine produced by individual memory cells even when these cells occur in the 1:10,000–1:1,000,000 frequency range (Karulin et al., 2000). By measuring the cumulative cytokine produced over a 24–48-h period, the assay reproduces the initial effector phase when memory cells re-encounter antigen. It does not make predictions as to the cytokine that would be produced by daughter cells generated from these memory cells after renewed antigen-driven proliferation.

We immunized wild-type (wt) C57.BL/6 mice with MOG: 35–55 in CFA, subcutaneously. Ten days later, the spleen cells were harvested and CD4 cells were purified and tested in serial dilutions in the presence or absence of the MOG: 35–55 peptide (MOGp). To define unambiguously the cytokines detected as being produced by these wt CD4 cells, we tested them on spleen cells of naïve, congenic cytokine-knockout (KO) C57.BL/6 mice functioning as an antigen-presenting cell (APC) layer. The splenic T cells from unimmunized or OVA-control-immunized wt C57.BL/6 mice did not produce cytokine (IFN-γ, TNF-α/β, IL-2, IL-4, IL-5 or IL-6) when cultured in such assays with MOGp (data not shown). Because naïve cells do not produce cytokine, these data are consistent with the MOGp-specific T cells being naïve prior to the immunization with this peptide. In contrast, the CD4 cells isolated from MOGp/CFA-immunized mice produced IFN-γ and IL-2 in the high-frequency range (hif) when tested on IFN-γ and IL-2 KO APC, respectively (Table 1). Serial dilutions over a wide range gave a linear function between the numbers of CD4 cells plated and the numbers of spots detected. This con-

Table 1
Cytokine response induced by MOGp in naïve C57.BL/6 mice and mice immunized with MOGp in CFA or IFA, expressed as cytokine-producing cells per million spleen cells

Immunization	Mouse number	IFN-γ	IL-2	IL-4	IL-5
MOGp 35–55/CFA	1	87	71	98	<5
	2	123	87	121	<5
	3	95	82	103	<5
	4	125	66	69	<5
MOGp 35–55/IFA	1	<5	32	41	<5
	2	<5	38	39	<5
	3	<5	23	52	<5
	4	<5	29	48	<5
Naïve	1	<5	<5	<5	<5
	2	<5	<5	<5	<5
	3	<5	<5	<5	<5
	4	<5	<5	<5	<5

Groups of four C57.BL/6 mice were either immunized with MOGp/CFA, MOGp/IFA, or remained uninjected. Fourteen days later, spleen cells were tested directly ex vivo in ELISPOT assays. The frequency of MOGp-induced cytokine-producing cells is shown for individual mice as the mean of triplicate wells (S.D. was <15% among the replicate wells) after subtracting the background spot formation (<5/million for all measurements). The data are from one representative experiment of a total of five independent experiments performed with similar results.

firms that these assays operated at single-cell resolution and permits us to establish the actual frequencies of the memory cells present in the spleens of these mice, as shown in Table 1. Identical frequency measurements were obtained when wt APC were used for IFN-γ and IL-2 measurements. MOG: 35–55-induced TNF-α/β production was also seen (the antibodies used do not distinguish between TNF-α and TNF-β), although we could not determine the exact frequencies of TNF-α/β-producing CD4 cells because TNF-α/β KO APC are not available (data not shown). When CD4 cells producing IL-4, IL-5, and IL-6 were tested on the respective cytokine-KO APC, comparably low frequencies (20–100 per million) of the cells were detected. Identical frequencies of IL-5-producing memory cells were found when the cells were tested on wt APC. In the case of IL-4 and IL-6, however, the numbers of the peptide-induced spots were up to 10 times higher than those with the cytokine-KO APC (data not shown), suggesting that considerable bystander production of these cytokines can occur in addition to the low-frequency cognate production of IL-4 and IL-6. (Supernatants of antigen-stimulated T cells induce IL-4 and IL-6 in naïve RAG2-KO APC, suggesting that this additional production of IL-4 and of IL-6 resulted from a cytokine-driven bystander reaction in non-T, non-B, non-NK1.1 cells; Karulin, manuscript in preparation.) Being IL-2-$\gamma^{\text{high-frequency range (hif)}}$, IFN^{hif}, TNF^{hif}, IL-$4^{\text{low-frequency range (lof)}}$, IL-$5^{\text{lof}}$, and IL-$6^{\text{lof}}$, the cytokine signature of the MOGp/CFA-induced CD4 memory cells corresponded to that seen in polarized Th1 immunity. Despite this autoimmune Th1 response, however, injecting MOGp with CFA did not result in the development of clinical or histologic EAE in the absence of PTX (clinical disease score, 0; number of animals with disease, 0/12); in contrast, the additional injection of PTX resulted in severe disease (clinical disease score, 3.5 ± 1; number of animals with disease, 11/12). While immunological self-tolerance has been violated without the inclusion of PTX, and a potentially pathogenic autoimmune response has been engaged (when injected as T cell blasts, in high numbers, such T cells cause passive EAE; data not shown), this alone is not enough to cause autoimmune disease in this experimental model.

Injection of MOGp in IFA induced a T cell response that was IL-2^{hif}, IL-4^{hif}, and IL-6^{lof}, but negative for IFN-γ and TNF-α/β (Table 1), corresponding to Th2 immunity. In contrast to the response seen when C57.BL/6 mice were immunized with OVA/IFA and when IFA was injected with several other antigens, which always induced vigorous IL-5-producing memory cells (Yip et al., 1999), in all five repeat experiments performed, injection of this peptide in IFA did not induce IL-5-producing MOGp-specific memory cells, suggesting an antigen-specific IL-5 defect within the Th2 response. Also, the mice injected with MOGp in IFA in the absence of PTX did not develop EAE over a 12-week observation period (Heeger et al., 2000). The induction of the Th2-polarized autoimmune response was, therefore, also without any pathological consequences without PTX injection. It is possible that the autoreactive Th1 or Th2 memory cells are ignorant of the autoantigen because they did not gain access to the target antigen without the disruption of the BBB (Brabb et al., 1997). In our next experiment, we addressed the possibility that by opening the BBB via applying an aseptic cryoinjury, the MOG-specific autoimmune Th1 cells could induce EAE in the C57.BL/6 animal model.

3.2. MOG: 35–55-reactive Th1 and Th2 cells do not induce EAE in C57.BL/6 without PTX, even following aseptic cerebral trauma

The presence of primed T cells with pathogenic potential in the immune periphery of these mice in the complete absence of autoimmune pathology (split tolerance) can be readily explained by the autoreactive T cells not having access to the target organ. It seemed likely, therefore, that these cells would exert inflammatory reactions as soon as they gain access to the CNS tissue, for instance, in the course of tissue destruction. To test this hypothesis, we induced aseptic cerebral trauma in the CNS of mice at 9, 12, and 30 days after immunization with MOGp in CFA and in IFA. OVA/CFA-immunized, OVA/IFA-immunized, and unimmunized C57.BL6 mice served as specificity controls. Each type of immunization was repeated three times, including 12 experimental animals in each group. Without PTX injection, there was no detectable EAE clinical score in any of the experimental animals even when aseptic injury was applied (Table 2).

3.3. MOG: 35–55-reactive Th1 and Th2 cells promote post-traumatic healing following aseptic cerebral injury in the CNS of C57.BL/6

Four animals in each experimental group were analyzed at days 4, 7 and 14 following ACI (12 animals/time points). Our data-summarizing results, 12 days following immunization, are shown in Figs. 1–3. Similar results were obtained

Table 2
MOG: 35–55-reactive T cells do not induce EAE in C57.BL/6 without PTX even following aseptic cerebral trauma

Antigen	Adjuvant	PTX	Traumatic injury	Number of animals with disease	EAE clinical score
MOG: 35–55	CFA	Yes	No	12/12	3.5 ± 1.0
MOG: 35–55	CFA	Yes	Yes	12/12	3.5 ± 1.3
MOG: 35–55	CFA	No	Yes	0/12	0

C57.BL/6 mice were immunized with MOG: 35–55 peptide emulsified in CFA and injected with PTX according to Materials and methods or with MOG: 35–55 peptide emulsified in CFA without additional application of PTX. Nine days following immunization, aseptic cerebral trauma was initiated in the PTX-treated or non-treated animals. The mice were monitored for clinical signs of EAE. Results are presented as mean clinical score ± S.D. from three independent experiments ($n = 12$; $p < 0.0001$ as determined by paired *t*-test).

when ACI was induced 9 or 30 days following immunization (data not shown). Interestingly, 4 days after inducing ACI, the MOGp/CFA-pre-injected mice showed moderately increased mononuclear cell infiltration of the lesion itself compared to naïve and control-injected mice (asterisks in Fig. 1d, and open bars in Fig. 2). These differences were not detectable any more after day 7, the time point at which the integrity of the BBB is re-established after cryoinjury in mice (Cancilla et al., 1979) (Figs. 1 and 3). No autoimmune lesions appeared at the site of injury or at unaffected parts of the CNS (data not shown). In the spleens of these mice, MOGp-reactive Th1 cells continued to be detectable at frequencies and with cytokine signatures identical to those in the uninjured mice shown in Table 1. Contrary to expectations, neither did the destructive, EAE-like autoimmune lesions establish themselves where the autoreactive Th1 cells were exposed to the CNS autoantigen in the absence of PTX, nor did this exposure led to clonal inactivation of the autoreactive T cells.

Cellular infiltrations in CNS lesions of MOGp/IFA-injected mice on day 4 (Fig. 1c and open bars in Fig. 2), day 7 (Fig. 1h) and day 14 (data not shown) were indistinguishable from those observed in control-injected or naïve mice. In contrast to reports in which adoptive transfer of large numbers of Th2 cells were found to induce inflammatory lesions (Khoruts et al., 1995; Lafaille et al., 1997; Pakala et al., 1997), the endogenously primed MOGp-specific Th2 cells were not found to be pathogenic in this model. Also in these mice, MOGp-reactive T cells continued to be detectable in the spleen with frequencies and cytokine signatures identical to those shown in Table 1 (data not shown).

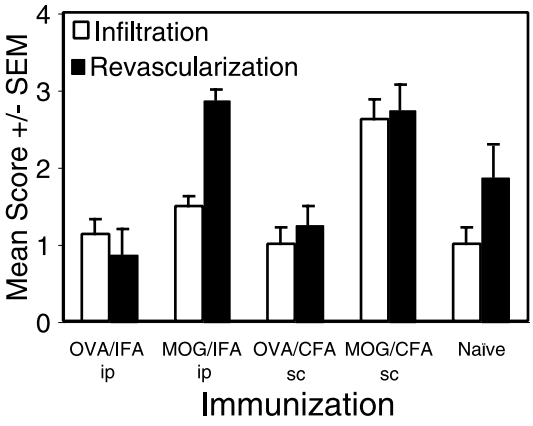

Fig. 2. Re-vascularization and cellular infiltration 4 days following ACI in MOG: 35–55-pre-immunized or control-immunized and naïve mice. Four mice in each experimental group were sacrificed after receiving ACI. Tissue sections from all of the mouse brains were prepared and graded, as described in Materials and methods. The results show mean value of infiltration and re-vascularization index scores ± S.E.M. of three independent experiments (total number of mice in each group is 12). MOG/CFA sc immunization significantly increased cellular infiltration as compared to all other groups, OVA/IFA ip, MOG/IFA ip, OVA/CFA sc immunization or naïve ($p<0.01$ as determined by paired t-test). Re-vascularization was significantly upregulated in mice immunized with MOG/CFA sc or MOG/IFA ip, compared to OVA/IFA ip or OVA/CFA sc immunization ($p<0.02$ as determined by paired t-test).

When the lesions were studied in more detail with attention to parameters of the post-traumatic healing process pertaining to the assessment of acellular necrosis, formation of granulation tissue with edema, and re-vascularization (Cancilla et al., 1979; Cancilla and DeBault, 1980; Swartz et al., 2001), significant differences were found in favor of

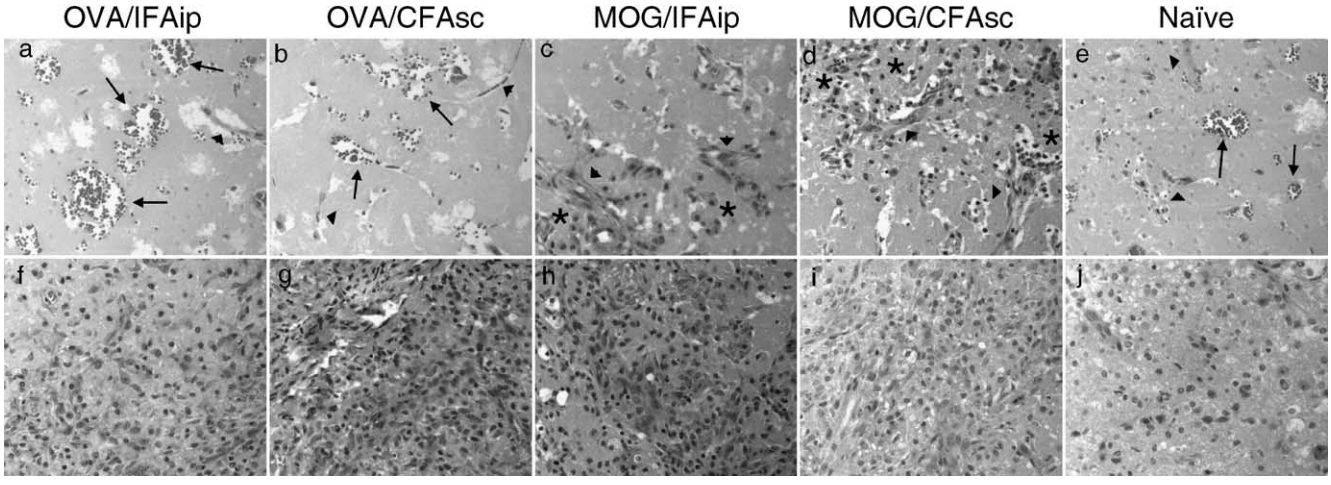

Fig. 1. Representative histologic sections of ACI lesions in MOG: 35–55- or OVA-immunized mice 4 and 7 days following injury. Hematoxylin and eosin staining of representative OVA/IFA ip (a, f), OVA/CFA sc (b, g), MOG/IFA ip (c, h), MOG/CFA sc (d, i), and naïve, non-immunized murine brains (e, j) at days 4 (a–e) and 7 (f–j) illustrate healing stages. Cellular infiltration (asterisk) and re-vascularization (arrowhead) are evident in the MOG-immunized animals, while OVA pre-immunization demonstrates a slower healing and visible tissue hemorrhage (arrow) with prominent absence of clearly formed vessels similar to the naïve animals. These differences are clearly visible at 4 days following ACI (a–e), but not at 7 days post-injury (f–j). (Magnification 400 ×, representative images of three independent experiments, using four mice per group in each experiment, $n=12$).

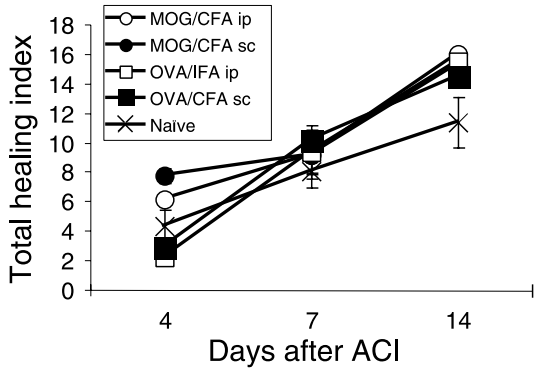

Fig. 3. Time course of the healing index after cryoinjury in MOG: 35–55-immunized or control-immunized mice. In each experiment, four mice per experimental group were sacrificed 4, 7, and 14 days following ACI. Four different evaluators reviewed the specimens, using a double-blinded evaluation protocol, on a scale of 0–4, with 0.5 points for intermediate findings. These scores were within a margin of 0.5 (mean ± 0.5) and their means are presented in the figure. The total healing index was calculated, as specified in Materials and methods. The results show mean values ± S.E.M. of three independent experiments involving a total of 12 mice per group. Four days following ACI, significant differences were detected between MOG- and OVA-pre-immunized mice, irrespective of the application of IFA or CFA adjuvants or the localization of immunization (intraperitoneal vs. subcutaneous) ($p < 0.001$ as determined by paired t-test). Fourteen days following ACI, the total healing index of all combined immunized groups was determined to be significantly different from that of naïve mice ($p < 0.02$ as determined by paired t-test).

the mice with preexisting MOGp-specific autoimmunity (Fig. 3).

4. Discussion

The priming of naïve CNS-antigen-reactive T cells is the first critical step in the induction of autoimmune disease. We induced a CNS autoimmune disease by injecting MOGp in CFA, a protocol commonly used in conjunction with PTX administration to induce EAE. The CFA-induced, MOGp-specific T cells proved to be highly Th1 polarized. When studied at single-cell resolution, the frequencies of IFN-γ- and IL-2-producing T cells was approximately 10-fold higher than those producing the type-2 cytokines IL-4, IL-5 and IL-6 (Table 1). In spite of the peripheral priming of autoreactive type-1 cells, histological or clinical EAE did not develop (Table 2). The classic interpretation of this finding would be that without disrupting the BBB by PTX, the Th1 effector cells have no access to the brain, and stay ignorant of the endogenous antigen. Notably, injection of MOGp with IFA also induced a T cell response, although this protocol has been widely used in the past to induce tolerance (for the prevention of EAE). The IFA-induced response was type-2 polarized: the frequency of MOGp-specific IL-4-producing T cells was approximately 40-fold higher than those producing IFN-γ (Table 1). It has been suggested that type-2 autoimmunity to MOG is pathogenic. In mice, our data do not support this notion. Like after CFA immunization, one might argue that the primed Th2 cells do not gain access to the CNS after such peripheral priming without PTX disrupting the BBB.

Challenging such classic interpretations, we show here that if the primed T cells gain access to the CNS after sterile tissue injury caused by cryoinjury, both the autoreactive Th1 or Th2 cells promoted post-traumatic healing in the CNS rather than inducing autoimmunity (Figs. 2 and 3).

We provide data in our paper that activation of cellular and humoral Th1 or Th2 immunity, under conditions of sterile tissue injury, can be beneficial for tissue repair rather than inducing EAE pathology (Figs. 1 and 3). Injection of MOG in IFA induces specific IgG1 antibodies; MOG immunization with CFA triggers the production of IgG1 and IgG2a antibodies that specifically deposit in situ in the CNS (Heeger et al., 2000). The relative contribution of these autoantibodies vs. the specific T cells awaits further clarification. Our preliminary data studying the healing process in congenic, MOG-immunized, immunoglobulin-deficient (μ KO) mice showed no significant differences from the wild-type mice on which we report here. This finding suggests that T cells mediate the effect. In the mouse model we applied, access of autoreactive cells to the CNS was provided by inducing an aseptic cerebral injury known to open the BBB (Swartz et al., 2001), instead of PTX. The number of these autoreactive T cells would be most likely numerically optimal to induce EAE, as it was shown that the clinical manifestations of MOG: 35–55-induced EAE do not correlate quantitatively with the perivascular infiltration of inflammatory cells (Kerlero de Rosbo et al., 1995). Both in mice injected with MOGp/CFA and with MOGp/IFA, re-vascularization, a critical initial step in tissue post-traumatic healing process (Fukushi et al., 1998), occurred more quickly than it did in unimmunized or OVA-control-immunized mice (arrows in Fig. 1c–d and closed bars in Fig. 2). These differences faded by day 7 (Fig. 1f–j), indicating that although the autoimmune response was apparently the result of preestablished autoimmune memory, the beneficial effects it had on post-traumatic healing were self-limiting. As IL-4 has been implicated in promoting angiogenesis (Woiciechowsky et al., 1998), the presence of this cytokine in the T cell response induced by MOGp/CFA or MOGp/IFA could be responsible for the observed effect. Whereas, based on the very nature of immune responses, one would expect renewed access of the peripherally primed memory cells to the autoantigen to boost the autoimmune T cell response and thereby increase the clonal sizes of the cytokine-producing memory cells and cause excessive vascularization, we did not find evidence for this in either the CNS or in the immune periphery.

The recruitment and activation of macrophages has also been shown to stimulate tissue repair in the CNS (Rapalino et al., 1998). The IFN-γ produced by the CFA-induced type-1 T cells specific for MOGp apparently induced recruitment of leukocytes in the CNS (Fig. 1d), and this reaction might be part of the beneficial effect of type-1 autoimmunity. The

IFA-induced, autoreactive, type-2 T cells, however, did not initiate a strong infiltration of small mononuclear cells (Fig. 1c and open bars in Fig. 2). Cellular infiltration is therefore unlikely to be the only mechanism that accounts for the protective effect seen. Finally, the down-regulation of monocyte/macrophage activation through the release of the type-2 cytokine IL-10 has been linked to the control of tissue damage after brain trauma (Woiciechowsky et al., 1998). In addition, as mentioned above, by producing IL-4, the autoreactive type-2 cells could exert beneficial effects on the course of wound healing by promoting angiogenesis (Fukushi et al., 1998). Moreover, T cells were shown to secrete neural growth factors (Kerschensteiner et al., 1999) that might induce a ''survival'' response in the CNS involving the induction of heat-shock proteins, anti-apoptotic proteins (Hancock et al., 1998), protective chemokines (Ransohoff and Tani, 1998), or cytokines (Motro et al., 1990; Kumar et al., 1998). While several mechanisms could be involved, there could have been strong evolutionary pressure to render local cytokine production in T cells, a common event, beneficial rather than pathogenic to the host, irrespective of whether the cytokine produced is type-1 or type-2. Consequently, autoreactive T cells might protect tissue as a rule, with autoimmune destruction being the exception. The view that the physiological role of autoimmune T cell responses is to contribute to tissue repair is consistent with the long-standing observation that wound healing in the skin is delayed if T cells are deleted from the organism (Efron et al., 1990).

The ability of autoreactive T cells to cause inflammatory reactions in the target organ has recently been shown to depend upon the APC in the target organ being activated (Limmer et al., 1998). Thus, hepatocytes transgenic for an alloantigen were ignored by TCR-transgenic T cells specific for this alloantigen, unless the liver was infected. In the EAE model, this requirement can be apparently bypassed by injecting activated T cell blasts, as has been customary for the induction of passive disease. The ability of neuroantigen-specific T cells to induce passive EAE seems (at least at the first sight) to contradict the hypothesis that T cells need to encounter the autoantigen in the CNS on APC that signal 'danger'. However, it is a prerequisite of adoptive transfer models that activated T cells are injected, typically 3–10 days after antigen/mitogen stimulation. In addition to gaining the ability to migrate trough the BBB, such activated T cells constitutively secrete cytokines and express high levels of APC-(co)stimulatory cell surface molecules. Therefore, such pre-activated T cells seem to be able to activate the APC in the CNS, bypassing the requirement that the APC be first licensed by microbial 'danger' signals. Also, in some EAE models, the frequency of neuroantigen-reactive T cells is very high (Anderson et al., 2000), enabling the specific T cells to enhance the activation of APC in the brain. In most EAE models that are dependent on PTX, however, it is likely that PTX's role is not only the opening of the BBB, but also the activation of the APC (Torre et al., 1996) in the CNS, including the induction of cytokine production and the expression of the co-stimulatory molecules B7-1, B7-2, and CD28 (Ryan et al., 1998) in these APC, and down-regulating inhibitory IL-10 cytokine production (Arimoto et al., 2000). Without APC being activated by microbial products that signal ''infectious nonself'' (Janeway, 1992) or ''danger'' (Matzinger, 1994) in the course of sterile tissue injury, the peripherally primed Th1 or Th2 cells do not seem to be able to engage co-stimulation-dependent cell functions such as the CD40–CD40L/IL-12/IFN-γ cascade, and the local antigen recognition does not result in the development of an inflammatory lesion. Instead, self-limiting production of cytokines occurs, after which the T cells might undergo apoptosis in the CNS (Pender et al., 1991; Bauer et al., 1998; Lehmann et al., 1998). Because of the lack of, or different type of, co-stimulation provided by the CNS APC in sterile inflammation, a different cytokine expression pattern, or patterns, may even be engaged in the autoreactive T cells as seen in the periphery (Table 1), similar to stimulation of T cells by altered peptide ligands (Brocke et al., 1996). Recently, it has also been demonstrated that PTX is capable of circumventing the genetically determined checkpoints associated with EAE susceptibility (Blankenhorn et al., 2000). However, it is also demonstrated that the induction of EAE in DAB/1 and C57.BL/10 (B10)-H2^{q} mice or DA and LEW.AV1 rats is possible in the absence of PTX (Storch et al., 1998; Abdul-Majid et al., 2000; Blankenhorn et al., 2000). This later observation brings up the complexity of EAE disease susceptibility associated with MHC genotype. Interestingly, PTX susceptibility has been suggested to be genetically controlled by the Bphs locus mapped to mouse chromosome 6, together with a susceptibility allele to the induction of EAE (Sudweeks et al., 1993).

Our model suggests that the induction of Th1- or Th2-type immune responses is not always sufficient to induce destructive autoimmunity. Destructive tissue damage might develop only if it coincides with the activation of APC in the target organ. The priming of autoreactive T cells in the course of a cross-reactive infection will result in auto-aggression only if the respective target organ is also infected or otherwise exposed to microbial products. The induction of an autoimmune response will either have no effects, or it will have beneficial effects on post-traumatic healing after sterile tissue injury.

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