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Autoreactive T Cells Persist in Rats Protected against Experimental Autoimmune Encephalomyelitis and Can Be Activated through Stimulation of Innate Immunity

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Lewis rats can be rendered unresponsive to experimental autoimmune encephalomyelitis by immunization with myelin basic protein (MBP), or MBP68–86, the dominant encephalitogenic MBP epitope for this strain, administered in IFA. However, protected rats harbor potentially encephalitogenic T cells, which are maintained in an inactive state. We investigated whether these quiescent effector cells could be activated in vitro. Although these T cells respond poorly to MBP68–86, they proliferate vigorously whether cocultured with MBP68–86 and either IL-2 or IL-12, suggesting that the T cells are in a state of anergy. Moreover, we could activate these anergic T cells with peptide and cytosine-guanine dinucleotide (CpG) oligonucleotide, but not control oligonucleotide, suggesting that products of the innate immune response are capable of activating anergic autoreactive T cells. The activated T cells produced the proinflamatory cytokine, IFN-γ in response to IL-12, and IL-6 was secreted in response to CpG oligonucleotide. IL-6 has been reported to play a role in T cell activation by blocking T regulatory/suppressor (Treg) cell-mediated suppression through a Toll-like receptor-dependent pathway. However, anti-IL-6 mAb did not block CpG activation of the anergized cells. In contrast, anti-TGF-β1 Ab released the unresponsive T cells from the anergic state in the presence of MBP68–86, whereas TGF-β1 inhibited proliferation of MBP68–86 plus CpG-activated T cells. Because TGF-β1 has previously been implicated in Treg activity, this finding is consistent with a role for Treg cells in maintaining autoreactive T cells in the anergic state. The Journal of Immunology, 2004, 172: 5322–5328.

E xperimental autoimmune encephalomyelitis (EAE)3 is a T cell-mediated inflammatory disease of the CNS that can be induced in genetically susceptible animals through a single immunization with myelin basic protein (MBP), encephalitogenic MBP peptides, or other CNS Ags in CFA (1, 2). The disease is characterized by mononuclear cell infiltration into the CNS causing edema and demyelination leading to paralysis (3). In the Lewis rat model of EAE, signs of disease begin ~10–14 days after peptide immunization and full recovery is seen usually by day 18 (Ref. 4, and reviewed in Ref. 5). After spontaneous recovery, animals are no longer susceptible to EAE induced by active immunization (4, 6). T cells isolated from these animals are reactive in vitro to the immunizing peptide and produce large amounts of IFN-γ. Reactivated cells can also transfer EAE after short-term culture with Ag (7, 8). If animals are first immunized with MBP or peptide in IFA, they are protected from EAE induced with Ag and CFA at a later time point (9). This method of immunization appears to tolerate the MBP-specific T cell population and/or activates a regulatory T cell population, because protection is transferable. T cell activation is dependent on interactions with APCs that present the autoantigen and also provide costimulation to the T cells (10). Presumably the mycobacterial component of CFA activates the innate immune system by stimulation of MHC-Ag presentation and up-regulation of costimulatory molecules such as CD80, and CD86 by APCs, leading to T cell activation. APCs also present Ag after immunization in IFA, which lacks mycobacteria, but the APCs do not provide the costimulatory signals needed by the T cells for full activation. This results in tolerance or anergizing of the peptide-specific T cell population (11) and failure to elicit EAE (12, 13). Because it is a microbial component of the adjuvant that presumably induces the APC activation, it is conceivable that environmental microbial pathogens could activate the innate immune system to jump start an autoreactive response by otherwise tolerantized T cells (14). One method of APC activation is through Toll-like receptor (TLR) recognition of bacterial DNA. Cytosine-guanine dinucleotide (CpG)-containing oligodeoxynucleotides are recognized through TLR-9, and signal for maturation and production of IL-12 and IL-6, both proinflamatory cytokines, by the APC (15, 16).

Negative selection in the thymus is not a foolproof process and some autoreactive cells reach the periphery. To prevent damaging autoimmune responses, T regulatory/suppressor (Treg) cells maintain the unresponsiveness of autoreactive cells in the periphery. Recent interest has focused on the CD4+CD25+–contact-dependent Treg cells, and Th2 regulatory cells that produce anti-inflammatory cytokines that interfere with and/or block the inflammatory process of autoreactive cells (reviewed in Ref. 17). Our laboratory has previously identified Treg cells in Lewis rats recovering from EAE. These Treg cells produce TGF-β, which inhibits the production of IFN-γ by encephalitogenic T effector cells (18).

The present study was conducted to determine whether products of the innate immune system are able to activate unresponsive...
autoreactive T cells, and to gain insight into why these cells are unresponsive.

Materials and Methods

Peptide synthesis

Synthetic peptides were prepared using F-moc chemistry on an Applied Biosystems Synergy model 432A peptide synthesizer (PerkinElmer, Foster City, CA), according to manufacturer’s instructions. Peptide was purified by HPLC if necessary, and structure was confirmed by mass spectrometry. The “self” peptide used in these experiments was rat MBP68–86, with the amino acid sequence YGSLPQKSQRTQDENPV.

In vitro T cell assays

T cell proliferation assays were performed as previously described (19). Briefly, splenocytes were isolated from MBP68–86- plus IFA-immunized rats, adherent cells were removed by incubation on plastic culture flasks, and T cells were isolated using T cell columns (Cedarlane, Ontario, Canada). The T cells were then cultured for 96 h with irradiated (2000 rad) syngeneic thymocytes as APCs and peptide, cytokine, and/or Ab in 96-well flat-bottom microtiter plates. The cultures were pulsed with [3H]thymidine (0.5 μCi/well) for 18 h before harvest. Thymidine incorporation was measured in a Microbeta Plus liquid scintillation counter (Wallac, Gaithersburg, MD). Cultures were run in triplicate, and each experiment was repeated at least twice. Preliminary dose-response studies were run, and representative results with optimal concentrations are presented in the figures. Stimulation indices were calculated as mean cpm with peptide (plus or minus additional molecules)/background (cpm of T cells and APC only) ± SD. Stimulation was considered significant only when indices exceeded background by at least 3-fold. T cells were cultured with APCs and peptide alone or with added exogenous recombinant rat IL-2 (R&D Systems, Minneapolis, MN), rat IL-12 (BioSource International, Camarillo, CA), neutralizing chicken IgY anti-human TGF-β, Ab (R&D Systems), human TGF-β1 (BioSource International), anti-rat IL-6 mAb (R&D Systems), relevant isotype controls, or either the APC stimulator CpG oligonucleotide (ATAATCGACGTCAAGCAAG) or a control oligonucleotide (ATAATAGAGCTTCAAGCAAG) (synthesized by Qiagen, Santa Clarita, CA).

Induction of protection and active EAE

Female Lewis rats (8–12 wk old; purchased from Charles River Breeding Laboratories, Raleigh, NC) were immunized s.c. in the hind footpad with the synthetic MBP68–86 peptide emulsified in either IFA or CFA (Difco, Detroit, MI), the latter containing 8 mg/ml Mycobacterium butyricum. Previous work has indicated that 50 μg of MBP 68–86 induces optimal EAE in actively immunized animals and therefore this concentration is used in the present study. To induce unresponsiveness, rats were immunized with 200 μg of the MBP peptide in IFA. When rechallenging the animals to confirm protection, the rats were first immunized with 200 μg of peptide plus IFA s.c. in the base of the tail and then rechallenged 2 or 4 wk later with 50 μg of peptide plus CFA. The rats (four per group) were observed for clinical signs of disease graded as 0 (no disease), 1 (loss of tail toxicity), 2 (hind limb weakness), or 3 (hind limb paralysis).

Cytokine analysis

Culture supernatants from T cell cultures were evaluated for IFN-γ, IL-4, and IL-6 at 48 h using rat-specific commercial ELISA kits (BioSource International), according to the manufacturer’s instructions.

TGF-β1 RT-PCR

RNA was isolated from T cells collected from immunized rats with RNeasy Mini kit (Qiagen) and treated with RNase-free DNase (Promega, Madison, WI) before reverse transcription and PCR. The purity was determined by comparing OD260/OD280 using an Ultraspec III spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ). Single-stranded cDNA was synthesized using a commercial Superscript One-Step RT-PCR kit (Invitrogen, Carlsbad, CA). cDNA was used as the template for PCR using primers (5’-3’) to amplify a 335-bp fragment of rat TGF-β1

FIGURE 1. MBP68–86 + IFA-treated Lewis rats are protected against EAE when challenged with MBP68–86 + CFA (■: clinical scores = 0, 0, 0, and 1), whereas untreated rats (□: clinical scores = 1, 3, 3, and 3) develop EAE when challenged. n = 4 rats/group; maximum severity = 3. Day 0 = day of MBP68–86 + CFA challenge.

FIGURE 2. MBP68–86 (20 μM) significantly enhances IL-2-induced proliferation of T cells from MBP68–86-unresponsive Lewis rats. Results are presented as cpm ± SD.
kit (Invitrogen, San Diego, CA), according to manufacturer’s instructions. RT-PCR was performed using rat-specific TGF-β primers (5′-CT TCAGCTCCACAGAGAAGACTG, 3′-CACGATCATGTGAGAAGACTG; Clontech Laboratories, Palo Alto, CA). β-Actin primers were used as “housekeeping” gene controls (Clontech Laboratories and BioSource International). PCR products were separated on 1.9% agarose gels in 1× Tris borate EDTA buffer containing 5 µg/ml ethidium bromide.

**Results**

It has been well documented in the Lewis rat model of EAE that disease follows a monophasic course of paralysis with complete recovery, followed by protection from reinduction of active disease (4, 6). Protection can also be induced in rats through immunization with MBP or peptide in IFA before challenge with an encephalitogenic emulsion of myelin Ag in CFA (9). The mechanism of this protection is not yet fully understood. Fig. 1 compares the disease course in untreated rats immunized with MBP68–86 and CFA to that of rats first treated with either MBP68–86, or MBP31–50 (a control peptide) administered in IFA, and challenged 2 wk later with Ag and CFA. Control rats that were either pretreated with MBP31–50 or left untreated developed EAE when challenged with MBP68–86 (Fig. 1). In contrast, rats pretreated with MBP68–86 in IFA were protected against EAE. Each group contained four rats.

It was unclear whether the unresponsiveness of the T cells to the myelin Ag in the peptide- plus IFA-treated rats was due to deletion, anergy, or active regulation. To investigate this, we immunized Lewis rats with MBP 68–86 in IFA and 10 days later isolated the T cells from the spleen for in vitro studies, with the aim of breaking the unresponsiveness to Ag. These rats did not receive peptide with CFA. It is well known that anergized T cells can be reactivated in the presence of exogenous IL-2. It has also been reported that autoreactive cells can be activated by the innate immune system. For example, bacterial CpG DNA oligonucleotides,

**FIGURE 3.** Proliferative responses of T cells from unresponsive rats cocultured with 0.2 µg/well CpG oligonucleotide or 0.5 µg/well mouse IL-12. A, Modest proliferative responses are induced by 20 µM MBP68–86 or CpG alone, or MBP68–86 plus 0.2 µg/well control oligonucleotide (CO). The response is enhanced significantly in the presence of MBP68–86 plus CpG oligonucleotide. B, T cell proliferation in the presence of IL-12 is significantly enhanced by the addition of MBP68–86 to the cultures.
which are rich in CG motifs, will activate the APCs by recognition and signaling through TLR-9 (15). Activated APCs produce IL-12, which in turn activates T cells to become inflammatory Th1 effector cells.

To test the hypothesis that the unresponsive T cells in our peptide- plus IFA-immunized rats were anergized, the isolated T cells from these animals were cultured in the presence of APCs and Ag, as well as peptide plus exogenous IL-2, IL-12, CpG oligonucleotide, or a control oligonucleotide lacking the CG motif. Anergized T cells were also cultured with IL-2, IL-12, or CpG alone, without MBP68–86. As shown in Fig. 2, T cells from rats treated with MBP68–86 and IFA show classic characteristics of anergy, in that they proliferate vigorously when exogenous IL-2 is added. Because these are bulk T cells responsive to many Ags, culture with IL-2 alone might be expected to induce proliferation. Although this was the case, responses were significantly enhanced when the T cells were activated in the presence of both IL-2 and MBP68–86 (Fig. 2), suggesting that IL-2 can rescue the unresponsive autoreactive T cells from the anergic state.

Furthermore, T cell activation with MBP68–86 and CpG oligonucleotide, but not control oligonucleotide, results in a robust proliferative response (Fig. 3A). Thus, anergic T cells respond relatively weakly to MBP68–86 or CpG alone, but proliferation is dramatically enhanced when they are cocultured with peptide plus CpG (Fig. 3A). Additionally, there is a significant increase in proliferation in response to MBP68–86 plus IL-12 (Fig. 3B), which is consistent with the hypothesis that CpG oligonucleotide activation of APCs leads to an adaptive immune response through production of IL-12 by the APCs (20, 21). Again, and as expected of bulk cultured T cells, IL-12 induced proliferation, which was significantly enhanced in IL-12 plus MBP68–86 cocultures (Fig. 3B).

To further investigate the activation status of the T cells isolated from the protected animals, we measured IFN-γ levels in supernatants from cultures containing T cells, APCs, and MBP68–86 (Fig. 4). Lower but significant quantities of IFN-γ are secreted by T cells cultured with peptide + CpG oligonucleotide. No IFN-γ is detected in cultures containing peptide alone or peptide + control oligonucleotide. No IFN-γ is detected in cultures containing peptide alone or peptide + control oligonucleotide.

![FIGURE 4.](image) T cells cultured with peptide + IL-2 or peptide + IL-12 secrete high levels of IFN-γ. Lower but significant quantities of IFN-γ are secreted by T cells cultured with peptide + CpG oligonucleotide. No IFN-γ is detected in cultures containing peptide alone or peptide + control oligonucleotide.

![FIGURE 5.](image) IL-6 is present only in cultures containing MBP68–86 and CpG oligonucleotide. In contrast, MBP68–86 plus IL-2, IL-12, or control oligonucleotide does not elicit IL-6 production.
with or without the activating agents shown in Figs. 2 and 3. Consistent with the proliferative findings, IFN-γ was produced in cultures that contained peptide plus IL-2, IL-12, or CpG oligonucleotide (Fig. 4). No IFN-γ was detected in supernatants of cultures containing peptide alone or cocultured with the control oligonucleotide. IL-4 was not detected in any of the supernatants tested (results not shown).

Thus, we have shown that CpG oligonucleotide activation of APCs induces production of IL-12, which in turn activates anergic T cells to proliferate and secrete IFN-γ/H9253. Another molecule secreted by APCs in response to CpG oligonucleotide recognition is the cytokine IL-6 (16). IL-6 has been described as both a proinflammatory cytokine supporting a Th1 shift in T cell activation, as well as an anti-regulatory molecule that may interfere with regulatory cell function (16). To determine whether IL-6 plays a role in our protection model, we assayed IL-6 in the supernatants of activated T cell cultures by ELISA. As shown in Fig. 5, only those cultures containing MBP68–86 and CpG oligonucleotide produced high levels of IL-6. In contrast, the activated cultures stimulated with MBP68–86 plus IL-2 or IL-12, peptide alone, or peptide plus control oligonucleotide did not elicit IL-6 production (Fig. 5).

Because it has been reported that IL-6 may interfere with regulatory T cell function (16), we investigated whether anti-IL-6 treatment would block CpG oligonucleotide-driven T cell activation. In our hands, neutralizing anti-IL-6 mAb did not block T cell activation, and proliferation was still seen in response to MBP68–86 plus CpG oligonucleotide (Fig. 6).

The autoreactive T cells within the protected Lewis rats are maintained in an anergic state by an as yet to be determined regulatory mechanism. Previous work in our laboratory has defined a population of TGF-β/H9252-producing Treg cells that appear as Lewis rats spontaneously recover from EAE (18). These Treg cells inhibit IFN-γ production by encephalitogenic T cells (18). Thus, it is possible in the model under investigation, that TGF-β/H9252-producing Treg cells play a role in maintaining the MBP68–86-reactive T cells in the unresponsive state. To test this hypothesis, anergic T cells from protected rats were cultured with peptide and a neutralizing anti-TGF-β1 Ab to determine whether this cytokine was present in the cultures. Thus, if TGF-β were involved in maintaining unresponsiveness in the MBP68–86-specific T cells, then neutralization of this cytokine should abrogate suppression and result in

FIGURE 6. Neutralizing anti-IL-6 mAb (0.05–1 μg) does not abrogate proliferation of T cells stimulated in vitro with MBP68–86 + CpG oligonucleotide. Wells containing anti-IL-6 mAb also contain 20 μM MBP68–86 and 0.2 μg/well CpG oligonucleotide.

FIGURE 7. MBP68–86-unresponsive T cells are released from the anergic state by the addition of neutralizing anti-TGF-β1 Ab. This Ab contained <10 ng endotoxin/mg.

FIGURE 8. TGF-β1 inhibits proliferation of T cells activated with CpG (0.2 ng/ml) and MBP68–86 (20 μM) in a dosage-dependent fashion.

FIGURE 9. T cells isolated ex vivo from MBP68–86- plus IFA-immunized unresponsive Lewis rats, or MBP68–86- plus CFA-immunized rats express TGF-β mRNA ex vivo, as determined by RT-PCR. a = β-actin; b = TGF-β. Lane 1, m.w. markers; lanes 2 and 3, β-actin and TGF-β positive controls, respectively; lanes 4 and 5, PCR product from T cells of MBP68–86- plus IFA-immunized unresponsive rat; lanes 6 and 7, PCR product from MBP- plus CFA-immunized rat.
proliferation to MBP68–86 alone. As shown in Fig. 7, there was a dose-dependent response of the T cells to MBP68–86 in the presence of increasing concentrations of anti-TGF-β1 Ab. Furthermore, TGF-β1 inhibited proliferation when added to cultures activated with MBP68–86 and CpG (Fig. 8).

To further support the role of TGF-β in the regulation of T cell activation, we evaluated RNA isolated from T cells of rats immunized with either MBP68–86 plus IFA, or MBP68–86 plus CFA. mRNA was isolated from T cells collected directly ex vivo and assayed by RT-PCR for TGF-β1 message (Fig. 9). TGF-β1 message is seen in T cells isolated from both groups, which is not surprising because these are heterogeneous populations of T cells, which may include Treg cells. It will be important to identify the population of cells that produce TGF-β1 in response to activation with MBP68–86.

Thus, restored proliferation following neutralization of TGF-β1 with Ab, inhibition of proliferation in the presence of TGF-β1, and TGF-β1 mRNA in T cells isolated ex vivo from MBP68–86–plus IFA-immunized Lewis rats support a role for this cytokine in the regulation of MBP68–86-specific T cell responsiveness.

**Discussion**

The objective of the present study was to determine whether products of the innate immune system are capable of activating unresponsive autoreactive T cells. MBP-specific T cells are present in healthy humans without inducing multiple sclerosis (22). Our system mimics this because potentially autoreactive T cells are present in Lewis rats rendered unresponsive by pretreatment with either intact MBP or MBP68–86 in IFA, although EAE does not develop (Ref. 9, and this report). Thus, unresponsive Lewis rats provide an ideal model to study triggering events that lead to the activation of self-reactive T cells, and the regulatory processes that maintain these potentially pathogenic lymphocytes in the unresponsive state.

It has been known for decades that the induction of EAE in most susceptible species requires the use of CFA (23, 24), which contains mycobacteria. More recent studies have revealed that microbial products, including bacterial LPS or CpG oligonucleotides, in the presence of Ag, can activate MBP-primed T cells from B10.S mice, a normally resistant strain, to transfer EAE (21). The ability of the microbial products to potentiate transfer of EAE is IL-12 dependent, and involves TLRs on APCs, with which the microbial products interact (15, 25). For example, CpG oligonucleotide is recognized by TLR-9, and this interaction leads to the production of IL-12 and up-regulated expression of CD80 and CD86 by the APC (15). Thus, the innate immune system can prime the adaptive immune system when an infectious agent is present.

In this study, we have confirmed that T cells from Lewis rats immunized with rat MBP68–86 and IFA do not proliferate in response to the priming peptide (i.e., MBP68–86). However, these animals harbor MBP68–86-specific T cells that can be activated to proliferate when CpG oligonucleotide is included in culture with the Ag (Fig. 3A). Moreover, these unresponsive cells can also be activated when cocultured with peptide and IL-12, which is consistent with a mechanism where an exogenous agent (in this case, CpG oligonucleotide) induces secretion of IL-12 by APCs, and leads to activation of the unresponsive T cells. Indeed, it has been shown that administration of IL-12 to Lewis rats that have spontaneously recovered from acute EAE induces relapses of disease, implicating this inducer of IFN-γ production by Th1 cells in the initiation of EAE (26).

We have also shown that peptide plus IL-2 induces vigorous proliferation of the unresponsive T cells, which suggests that these lymphocytes are maintained in vivo in an anergic state. It is well established that exogenous IL-2 activates anergic T cells (27, 28).

Unresponsive T cells activated with peptide plus IL-2 or IL-12 produced large quantities of IFN-γ, whereas peptide plus CpG oligonucleotide-activated lymphocytes secreted detectable, but lower amounts of this Th1 cytokine (Fig. 4). In contrast, MBP68–86 plus CpG oligonucleotide-activated cells produced significant quantities of IL-6, whereas this cytokine was undetectable in supernatants of cultures activated in the presence of either IL-2 or IL-12 (Fig. 5).

Ichikawa et al. (29) recently reported that autoreactive proteolipid-protein-specific T cells could be activated from the lymph nodes of proteolipid-protein-tolerized SJL mice with CpG oligonucleotide or IL-12. However, their findings in mice differ from the present study in Lewis rats in that the unresponsive murine T cells could not be activated with IL-2 (29). Thus, differences exist with respect to the mechanism of Ag– plus IFA-induced tolerance to encephalitogenic peptide in mice and rats.

To investigate the mechanism responsible for maintaining the unresponsive rat T cells in the anergic state, we considered the possibility that Treg cells might be involved. Early work from our laboratory supports this notion (9). Moreover, it was recently reported that CpG oligonucleotide activation via TLR-9 on APCs blocks the suppressive effect of CD4+CD25+ Treg cells, and that this effect is dependent, in part, on IL-6 that was produced by APCs upon recognition of CpG oligonucleotide (16). Thus, the induction of T cell responses can be regulated by the innate immune system. In support of this possibility, we observed that the CpG oligonucleotide plus peptide-activated rat T cells secreted significant quantities of IL-6. However, our attempts to block activation with a neutralizing anti-IL-6 Ab were unsuccessful.

We (18) and others (30) previously reported that Treg cells secrete TGF-β, which inhibits pathogenic autoreactive Th1 cells. Thus, we considered the possibility that TGF-β also plays a role in maintaining MBP68–86–specific T cells in the anergic state. This hypothesis is supported by our present findings that: 1) unresponsive T cells proliferated vigorously when a neutralizing anti-TGF-β Ab was included with MBP68–86 in microtiter wells (Fig. 7), and 2) TGF-β1 inhibited proliferation of T cells cultured with peptide plus CpG oligonucleotide. This is consistent with the hypothesis that Treg cells that secrete TGF-β inhibit the activation of autoreactive T cells.

It has been reported that CTLA-4 engagement leads to the secretion of TGF-β by mouse T cells, thus inhibiting T cell activation (31), and we have found that anti-TGF-β Ab overcomes CTLA-4-induced inhibition of rat T cell proliferative responses (32). It has also been determined that murine CD4+CD25+ Treg cells express CTLA-4 (33), and a very recent report implicates Treg cells with the CD4+CD25+ phenotype in remission from diabetes in nonobese diabetic mice treated with anti-CD3 Ab to restore self-tolerance (34). Remission from diabetes was TGF-β dependent, and could be prevented by treatment with anti-CTLA-4 Ab (34).

Studies are in progress to determine whether TGF-β-mediated effects on costimulatory pathways are involved in the maintenance of T cell anergy. It will also be of interest to determine the effect of CpG oligonucleotide on TGF-β production in the rat EAE model, which would support the hypothesis that activators of the innate immune response can override regulatory mechanisms that normally prevent autoreactivity.

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