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Presence of CpG DNA and the Local Cytokine Milieu Determine the Efficacy of Suppressive DNA Vaccination in Experimental Autoimmune Encephalomyelitis

Anna Lobell,2* Robert Weissert,† Sana Eltayeb,‡ Cecilia Svanholm,*, Tomas Olsson,† and Hans Wigzell*

We here study the adjuvant properties of immunostimulatory DNA sequences (ISS) and coinjected cytokine-coding cDNA in suppressive vaccination with DNA encoding an autoantigenic peptide, myelin basic protein peptide 68–85, against Lewis rat experimental autoimmune encephalomyelitis (EAE). EAE is an autoimmune, T1-mediated disease of the CNS. ISS are unmethylated CpG motifs found in bacterial DNA, which can induce production of type 1 cytokines in vertebrates through the innate immune system. Because ISS in the plasmid backbone are necessary for efficient DNA vaccination, we studied the effect of one such ISS, the 5′-AACGTT-3′ motif, in our system. Treatment with a DNA vaccine encoding myelin basic protein peptide 68–85 and containing three ISS of 5′-AACGTT-3′ sequence suppressed clinical signs of EAE, while a corresponding DNA vaccine without such ISS had no effect. We further observed reduced proliferative T cell responses in rats treated with the ISS-containing DNA vaccine, compared with controls. We also studied the possible impact of coinjection of plasmid DNA encoding rat cytokines IL-4, IL-10, GM-CSF, and TNF-α with the ISS-containing DNA vaccine. Coinjection of IL-4-, IL-10-, or TNF-α-coding cDNA inhibited the suppressive effect of the DNA vaccine on EAE, whereas GM-CSF-coding cDNA had no effect. Coinjection of cytokine-coding cDNA with the ISS-deficient DNA vaccine failed to alter clinical signs of EAE. We conclude that the presence of ISS and induction of a local T1 cytokine milieu is decisive for specific protective DNA vaccination in EAE. The Journal of Immunology, 1999, 163: 4754–4762.

DNA vaccines contain two elements of interest for induction of an immune response: sequences coding for proteins and other sequences in the construct that may function as immunomodulators, such as immunostimulatory DNA sequences (ISS)1 (1). ISS are unmethylated CpG motifs within the context of certain flanking bases (2). They are found in bacterial DNA and are thought to be pattern recognition motifs recognized by the innate immune system in vertebrates (3). This recognition leads to induction of type 1 immune responses (3–5). ISS stimulation of macrophages leads to production of IL-12, IL-18, and TNF-α (5, 6), and NK cells (7) and B cells (8) are directly activated by ISS. Dendritic cells (DC), candidate APCs after DNA vaccination (9), produce large amounts of IL-12, but only low levels of TNF-α and IL-6 in response to ISS (10). ISS are necessary for effective DNA vaccination (11). In our system, we study the effect of ISS of the 5′-AACGTT-3′ sequence only, but there are other CpG motifs that can function as ISS in DNA vaccination (12). EAE is an animal model for multiple sclerosis and considered to be a T1-mediated autoimmune disease of the CNS (13, 14). We have previously reported that vaccination with DNA encoding an encephalitogenic guinea pig myelin basic protein (MBP) peptide 68–85 (MBP68–85) targeted to IgG suppresses clinical and histopathological signs of Lewis rat experimental autoimmune encephalomyelitis (EAE) (15). IFN-γ production of lymph node cells (LNC) from such DNA-vaccinated and MBP68–85-immunized rats was reduced, while there were no signs of induction of T2 immunity. In these studies, we used a vector containing three 5′-AACGTT-3′ motifs of putative importance for the vaccination effect.

In the present study, the adjuvant properties of the plasmid backbone and coinjected cytokine-coding cDNA in relation to EAE are explored. Because MBP68–85/CFA-induced EAE is a T1-mediated disease (13) and injection of the DNA vaccine containing three ISS of 5′-AACGTT-3′ sequence paradoxically suppressed EAE, we wanted to study the role of such T1-promoting DNA sequences in our system. Two new DNA vaccines were accordingly constructed. The first DNA vaccine lacked ISS (ISS-deficient). The second DNA vaccine was identical with the ISS-deficient DNA vaccine, except for a linker containing three 5′-AACGTT-3′ motifs. Both vaccines encoded MBP68–85 in tandem, fused to an IgG-binding peptide as previously described (15). We compared the ISS-positive and ISS-deficient DNA vaccines for their ability to alter the course and immune response of subsequently induced EAE.

Coinjection of cytokine-coding cDNA with DNA vaccines encoding microbial or tumor Ags can act as adjuvants that modulate and/or stimulate the Ag-specific immune responses (16–19). Furthermore, the local milieu during the initiation of an immune response decisively influences the differentiation into T1 or T2 profiles of T cells (20). Induction of T2 immunity can be suppressive.
in EAE (21, 22) and is thought to be instrumental after DNA vaccination with a TCR construct (23). Thus, we tested if coinjection of IL-4- or IL-10-coding cDNA with the ISS-positive DNA vaccine encoding MBP<sub>68-85</sub> could enhance the suppressive effects of the DNA vaccine on the clinical outcome of EAE. Coinjection of GM-CSF-coding cDNA with a DNA vaccine can enhance Ag-specific immune responses against tumor and microbial Ags (18, 24–26). Mice that are deficient for the proinflammatory cytokine TNF-α contract more severe EAE after immunization with myelin Ag than wild-type mice (27). Therefore, we tested coinjected GM-CSF- or TNF-α-coding cDNA constructs for their impact on the suppressive effects of the DNA vaccine on the clinical outcome of EAE. Finally, we tested if coinjection of ISS-deficient IL-4-, IL-10-, GM-CSF-, or TNF-α-coding cDNA constructs with the ISS-deficient DNA vaccine could modulate the clinical consequences of the DNA vaccination. We demonstrate that the presence of ISS modulates immunity during DNA vaccination toward prevention of EAE. Coinjection of some cytokine-coding cDNAs can inhibit the suppressive effects of DNA vaccination in autoimmune disease.

Materials and Methods

**Immunogens and Ags**

Peptide HYGSLPQKSQRSQDENPV from guinea pig sequence MBP<sub>68-85</sub> was synthesized by the F-moc/HBTU strategy. After sequencing, the plasmid was digested with DraI and cloned into the DraI site of the bacterial plasmid pBluescript SK<sup>®</sup>. The ss/ZZ fragment was cloned into pBluescript to obtain pK0. A Kozak box (CACCC) was introduced by PCR directly upstream of the start codon to enhance translation (29). The PCR product was cloned into pCR-Script vector (Stratagene). After sequencing, two identical sequences from different PCRs, the IL-4 gene was cloned into pCI, and pK0. **pTFN-α and pK0-TNF-α**. Cloning of rat TNF-α-coding cDNA was cloned by a nested PCR strategy using outer primers 5′-CTTGGCA GCCAAAGAGGCTTCACG-3′ and 5′-GTCCAGTAGCCCGGGT GGT-3′ and subsequently the inner primers 5′-CGGAAATCCACCAT GCTTGGGCTGACGACTGAT-3′ and 5′-GCTTCAAGGCTCAGT-3′ introduced an Avai site upstream of the first codon and downstream of the stop codon of mature GM-CSF. Sequencing of the GM-CSF fragment was cloned downstream and in frame of the murine signal sequence (ss) at the Avai site. The fragment coding for ss-GM-CSF was cloned into pCI and pK0. **pGM-CSF and pK0-GM-CSF**. Cloning of rat GM-CSF-coding cDNA (encoding mature protein; protein sequence downstream of the cleavage site of its signal sequence) from Lewis rat spleen and thymus mRNA was performed by RT-PCR and PCR using primers 5′-CTCTGCA GCCACCTGTCCTCAATCTAGACG-3′ and 5′-GTCCAGTAGCCCGGGT GGT-3′ and subsequently the inner primers 5′-CGGAAATCCACCAT GCTTGGGCTGACGACTGAT-3′ and 5′-GCTTCAAGGCTCAGT-3′ introducing an Avai site upstream of the first codon and downstream of the stop codon of mature GM-CSF. Sequencing, the GM-CSF fragment was cloned downstream and in frame of the signal sequence (ss) at the Avai site. The fragment coding for ss-GM-CSF was cloned into pCI and pK0.

**Plasmid construction**

**pZZ/MBP<sub>68-85</sub>**. Construction of pZZ/MBP<sub>68-85</sub> and pZZ has been previously described (15). Briefly, a murine H chain IgG signal sequence (ss) was ligated upstream and in frame of a fragment encoding ZZ, an IgG-binding synthetic analogue to the B domain of staphylococcal protein A (28). Seven fragments encoding MBP<sub>68-85</sub> were ligated in frame downstream of the coding sequence for ZZ (Fig. 1A). The ssZZ/MBP<sub>68-85</sub> fragment was cloned into the eukaryotic expression vector pCI (Promega, Madison, WI). pCI contains three ISS of 5′-AACGTT-3′ sequence; two ISS in the ampicillin resistance βl gene and one ISS in the βl region. The ssZZ/MBP<sub>68-85</sub> fragment contains no 5′-AACGTT-3′ sequences.

**pZZ**. A ssZZ fragment containing ss and ZZ in frame was cloned into pCI. **pK0**. The ISS-deficient pK0 vector was constructed by deleting a 27-bp StuI-ScaI fragment of the eukaryotic neomycin/kanamycin promoter of the eukaryotic expression vector pGFP-N1 (Clontech, Palo Alto, CA). The green fluorescent protein (GFP) gene was then excised by digestion with Xhol. After filling in reaction with Klenow enzyme, the vector was digested with NheI. The multiple cloning cassette of pCI was excised by digestion with NheI and Smal and ligated with the NheI-Xhol/Klenow-filled vector. **pK0-ZZ/MBP<sub>68-85</sub]**. The ssZZ/MBP<sub>68-85</sub> fragment was cloned into pK0 (Fig. 1B).

**pK0-ZZ**. The ssZZ fragment was cloned into pK0. **pK3**. The ISS-positive pK3 vector was constructed by inserting a linker containing three ISS of 5′-AACGTT-3′ sequence into the βl region of pK0. Oligodeoxynucleotides 5′-AATTCATCATGGATAGTTGAGCGTC TTTCCAAGCTTGTGATGATGATGATGATGCCATGGGTA-3′ and 5′-AATTCATCATGGATAGTTGAGCGTC TTTCCAAGCTTGTGATGATGATGATGATGCCATGGGTA-3′ were annealed, digested with <br>
in PBS emulsified in CFA, consisting of IFA (Sigma, St. Louis, MO) and 0.5 mg heat-inactivated Mycobacterium tuberculosis (strain H37 RA; Difco Laboratories, Detroit, MI). Animals were clinically scored and weighed daily. The neurological deficits were scored as follows: grade 1, tail weakness or tail paralysis; grade 2, hind leg paraparesis; grade 3, hind leg paralysis; grade 4, complete paralysis (tetraplegy), moribund state, or death.

**Determination of MBP<sub>68–85</sub>-specific IgG and IgG-isotype responses**

ELISA plates were coated with 10 μg/ml of MBP<sub>68–85</sub> in carbonate buffer, pH 9.6. Rat sera were diluted 1:50, 1:250, 1:650, or 1:1250 in PBS-M (5% milk powder, 0.05% Tween 20 in PBS). Wells were incubated 2 h with sera, washed in PBS-T (0.05% Tween 20 in PBS), and incubated 2 h with 1:1000 alkaline phosphatase (AKP)-conjugated goat-anti rat IgG (Bio-source International, Camarillo, CA) or monoclonal AKP-conjugated mouse-anti rat IgG1, IgG2a, or IgG2b (PharMingen, San Diego, CA), respectively, in PBS-M. pNPP (Sigma) was used as substrate, and OD was read at 405 nm.

**Cell preparation**

Spleens were dissected out, disrupted, and cells were suspended in DMEM (Life Technologies, Paisley, U.K.). RBC were lysed in lysing buffer, consisting of 0.15 M NH₄Cl, 1 mM KHCO₃, and 0.1 mM Na₂EDTA adjusted to pH 7.4. Mononuclear cells (MNC) were washed twice in DMEM and resuspended in complete medium consisting of DMEM supplemented with 5% newborn calf serum, 1% glutamine, 1% penicillin/streptomycin, and 50 μM 2-ME (all products from Life Technologies).

**In vitro stimulation of spleen cells with DNA vaccines**

Spleen MNC were cultured at 2 × 10⁶ cells/ml in 2 ml culture medium (CM). Triplicates of 1, 10, or 20 μg of either pK0-ZZ/MBP<sub>68–85</sub> or pK3-ZZ/MBP<sub>68–85</sub> was added to wells and cultured for 24 or 48 h, as previously described for human cell stimulations (30). Supernatants and cell pellets were collected for cytokine analysis.

**Assays of Ag-induced proliferation**

All proliferative experiments were performed in triplicates in 96-well round-bottom microtiter plates. A total of 2 × 10³ cells in 100 μl CM were cultured with or without relevant Ags or Con A for 60 h and subsequently pulsed with 1 μCi [³H]TdR (Amersham, Stockholm, Sweden) per well for 12 h. DNA was collected on glass fiber filters (Skatron, Sterling, VA) and [³H]TdR incorporation was measured in a beta-counter (Beckman, Palo Alto, CA).

**Enumeration of Ag-specific IFN-γ-secreting cells**

To enumerate IFN-γ MNC-secreting after Ag or Con A exposure, an enzyme-linked immunospot method was used, as previously described (31).

**ELISA to assess cytokine production in vitro**

ELISA kits for detection of secreted IFN-γ, TNF-α, and IL-10 were purchased from Biosource (Camarillo, CA). Supernatants from MNC, which had been incubated at a concentration of 2 × 10³ cells/ml with or without relevant Ags or Con A for 48 h, were analyzed. The procedure was performed as recommended by the manufacturer.

**PCR to assess cytokine production in vitro**

A quantitative PCR method was used to assess cytokine mRNA transcription, as previously described (32). Spleen MNC that had been stimulated with DNA vaccines for 24 or 48 h were analyzed.

**Statistics**

Differences between groups in EAE score were tested with Mann-Whitney’s U test. Differences in B and T cell responses were tested with Student’s t test.

**Results**

**Immunostimulatory DNA sequences are necessary for protective DNA vaccination against EAE**

First, we investigated the adjuvant properties of the plasmid backbone of a DNA vaccine encoding MBP<sub>68–85</sub>. To compare ISS-positive and ISS-deficient DNA vaccines, we constructed DNA vaccines with either three ISS of 5′-AACGTT-3′ sequence in the plasmid backbone (ISS-positive) or without ISS (ISS-deficient). The first DNA vaccine encoded MBP<sub>68–85</sub> in tandem of seven fused to a dimerized synthetic analogue Z of the IgG-binding B domain of staphylococcal protein A gene, ZZ, producing a ss/ZZ/MBP<sub>68–85</sub> fragment. The ss/ZZ/MBP<sub>68–85</sub> fragment was inserted into pCI vector. As a negative control, a corresponding plasmid coding for ZZ was constructed (pZZ/ZZ). To investigate the role of ISS in the protection from EAE, an ISS-deficient DNA vaccine, lacking 5′-AACGTT-3′ motives and encoding the ss/ZZ/MBP<sub>68–85</sub> fragment was constructed (pK0-ZZ/MBP<sub>68–85</sub>), or its negative control encoding ZZ, pK0-ZZ. The ISS-positive DNA vaccine pK3-ZZ/MBP<sub>68–85</sub> was constructed by introducing a linker containing three 5′-AACGTT-3′ motives into the fl region of pK0-ZZ/MBP<sub>68–85</sub> and pK3-ZZ was constructed as negative control. The sites of the 5′-AACGTT-3′ motives are marked in the plasmid maps.

**FIGURE 1.** A. Plasmid map of DNA vaccine pzz/MBP<sub>68–85</sub>. Seven repeats of oligonucleotides encoding autoantigen MBP<sub>68–85</sub> were cloned downstream of a murine signal sequence (ss) fused to a dimerized synthetic analogue of the IgG-binding B domain of staphylococcal protein A gene, ZZ, producing a ss/ZZ/MBP<sub>68–85</sub> fragment. The ss/ZZ/MBP<sub>68–85</sub> fragment was inserted into pCI vector. As a negative control, a corresponding plasmid coding for ZZ was constructed (pZZ/ZZ). B. To investigate the role of ISS in the protection from EAE, an ISS-deficient DNA vaccine, lacking 5′-AACGTT-3′ motives and encoding the ss/ZZ/MBP<sub>68–85</sub> fragment was constructed (pK0-ZZ/MBP<sub>68–85</sub>), or its negative control encoding ZZ, pK0-ZZ. C. The ISS-positive DNA vaccine pK3-ZZ/MBP<sub>68–85</sub> was constructed by introducing a linker containing three 5′-AACGTT-3′ motives into the fl region of pK0-ZZ/MBP<sub>68–85</sub> and pK3-ZZ was constructed as negative control. The sites of the 5′-AACGTT-3′ motives are marked in the plasmid maps.
Effect of immunostimulatory DNA sequences on clinical signs of EAE after DNA vaccination

<table>
<thead>
<tr>
<th>Expt</th>
<th>Treatment</th>
<th>5'-AACGTT-3' CpG Motifs</th>
<th>n</th>
<th>Mean Accumulated EAE Score</th>
<th>p&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean Maximum EAE Score</th>
<th>p&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>0.03</td>
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<sup>a</sup> Rats received the DNA vaccines 4 wk before induction of EAE with MBP<sub>68–85</sub> in CFA.
<sup>b</sup> Mean accumulated EAE score is the cumulative disease score on 21 days after immunization (score obtained daily (0–4)).
<sup>c</sup> Values of p were calculated with Mann-Whitney's U test.
<sup>d</sup> Mean maximum EAE score designates the average peak score of clinical disease at any time during the disease course, here on 12–14 days after immunization.
<sup>e</sup> NS, Not significant at 0.05 level.

The three ISS are marked in Fig. 1A. The second DNA vaccine, pK0-ZZ/MBP<sub>68–85</sub> (Fig. 1B), lacked ISS, but contained the same Ag-coding sequence as pZZ/MBP<sub>68–85</sub> gene products of pZZ/MBP<sub>68–85</sub> and pK0-ZZ/MBP<sub>68–85</sub> were, af-

To study the immune mechanisms involved after vaccination with pK0-ZZ/MBP<sub>68–85</sub> or pK3-ZZ/MBP<sub>68–85</sub>, we studied the profile of T cell reactivity to MBP<sub>68–85</sub>. Ag-induced proliferative, IFN-γ, and IL-10 responses in vitro of spleen MNC from DNA-vaccinated rats (n = 8/group) were measured 3 wk after DNA vaccination or 12 days after immunization with MBP<sub>68–85</sub> in CFA. Although a low number of MBP-peptide-reactive cells were observed in rats sacrificed before MBP-peptide immunization, and that had received DNA vaccines encoding MBP<sub>68–85</sub>, the levels
did not differ statistically significant from controls. We detected 2.0 ± 1.0 (mean ± SEM) or 1.9 ± 0.9 MBP68–85-induced IFN-γ-secreting cells per 4 × 10^5 splenocytes in pK0-ZZ/MBP68–85- or pK3-ZZ/MBP68–85-treated groups, respectively, and 0.1 ± 0.1 or 0.4 ± 0.2 MBP68–85-induced IFN-γ-secreting cells per 4 × 10^5 MNC in pK0-ZZ- or pK3-ZZ-treated groups, respectively. There was no MBP-peptide-induced proliferative response or IL-10 or TNF-α secretion in any group before MBP-peptide immunization (data not shown). On day 12 after immunization with MBP68–85 in CFA, the proliferative response to MBP68–85 of splenocytes in the pK3-ZZ/MBP68–85-treated group was significantly lower than in the pK3-ZZ-treated group (p < 0.05 at Ag concentrations 5 and 10 μg/ml) (Fig. 3B). The proliferative response to MBP68–85 of splenocytes in the pK0-ZZ/MBP68–85-treated group was not significantly lower than the in pK0-ZZ-treated group (p = 0.08 at Ag concentration 5 μg/ml) (Fig. 3A). However, when comparing pK0-ZZ/MBP68–85 and pK3-ZZ/MBP68–85-treated groups, the levels of proliferation were similar (Fig. 3). Instead, the pK3-ZZ-treated group showed a tendency toward higher Ag-specific proliferative response than the pK0-ZZ-treated group (p > 0.23). We could not detect any differences in the levels of Ag-specific IFN-γ-secreting cells (Fig. 4, A and B), IFN-γ (data not shown) or IL-10 (Fig. 4, C and D) in cell-culture supernatants between rats treated with pK0-ZZ/MBP68–85 or pK0-ZZ, pK3-ZZ/MBP68–85, or pK3-ZZ, indicating no measurable alteration of the T1/T2 balance in our system. Because anti-idiotypic CD4^+ T cells can modulate the immune responses in EAE (33), and TCR BV8S2 is dominantly expressed in the rat EAE model we used (34), we studied if such anti-idiotypic T cells were induced after DNA vaccination. The proliferative T cell responses, IFN-γ and IL-10 expression in response to recombinant rat TCR BV8S2 protein (rBV8S2), were studied both at the day of and 12 days after immunization with MBP68–85 in CFA. However, we did not observe any induction of rBV8S2-specific T cells (Figs. 3 and 4).

We then measured MBP68–85-specific serum Abs to study if the ISS content of the DNA vaccines would affect the IgG isotype profile, as ISS are T1-promoting adjuvants. On 12 days after immunization with MBP68–85 in CFA, Ag-specific IgG1 responses were higher in the DNA vaccinated groups compared with their controls, irrespective of whether the DNA vaccine contained ISS or not. Ag-specific IgG, IgG2a, or IgG2b responses did not differ between the DNA vaccinated and control groups (Fig. 5, A and B). On day 24 after immunization, likewise no differences in Ag-specific IgG or IgG isotype responses were observed between DNA-vaccinated rats and controls (data not shown).

**Coinjection of plasmid DNA-encoding cytokines with DNA vaccines encoding MBP68–85**

Next, we studied the adjuvant properties of coinjection of cytokine-coding cDNA constructs with pZZ/MBP68–85 or pZZ. Coinjection of cytokine-coding cDNA can modulate the immune responses to DNA vaccines (17–19). We constructed plasmids encoding rat cytokines; pIL-4, pIL-10, pGM-CSF, and pTNF-α (see Material and Methods). Lewis rats were injected with 800 μg of pZZ/MBP68–85 or pZZ or 800 μg of pZZ/MBP68–85 or pZZ mixed with 200 μg of either pIL-4, pIL-10, pGM-CSF, or pTNF-α. Four to 5 wk later, the rats were immunized with MBP68–85 in CFA.

A survey of the effects of coinjection of cytokine-coding cDNA with pZZ/MBP68–85 on clinical signs of EAE is presented in Table II. All rats that received pZZ with or without cytokine DNA displayed a classical monophasic EAE course. The pZZ/MBP68–85-treated rats were protected from EAE (Fig. 6A and Table II). In contrast, coinjection of pIL-4, pIL-10, or pTNF-α inhibited the protective effect of pZZ/MBP68–85 (Fig. 6, B, C, and E, and Table II). However, coinjection of pGM-CSF did not alter the effect of pZZ/MBP68–85 on clinical signs of EAE (Fig. 6D).
We then studied the adjuvant properties of coinjected plasmid DNA-encoding cytokines with the ISS-deficient pK0-ZZ/MBP_{68–85} or pK0-ZZ. We argued that expression of certain cytokines might compensate for the ISS deficiency in pK0-ZZ/MBP_{68–85}. If a cytokine could compensate for the lack of ISS, it would give insight into the mechanism by which ISS work in our system. A summary of the effects of coinjection of cytokine DNA with pK0-ZZ/MBP_{68–85} on clinical signs of EAE is presented in Table II. Lewis rats were injected with 800 mg of pK0-ZZ/MBP_{68–85} or pK0-ZZ or 800 mg of pK0-ZZ/MBP_{68–85} or pK0-ZZ mixed with 200 mg of either pK0-IL-4, pK0-IL-10, pK0-GM-CSF, or pK0-TNF-α. Five weeks later, the rats were immunized with MBP_{68–85} in CFA. All rats displayed a classical monophasic EAE course (Fig. 7, A–E, and Table II).

**Discussion**

We here demonstrate that ISS in the plasmid backbone of the DNA vaccine are crucial for the suppressive vaccination effect with DNA encoding MBP_{68–85} against EAE. pK0-ZZ/MBP_{68–85} failed to reduce clinical signs of EAE, while pK3-ZZ/MBP_{68–85} suppressed clinical signs of EAE. We tested one particular ISS—of 5′-AACGTT-3′ sequence—and it is likely that other CpG motifs

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**Table II. Effect of coinjection of cytokine-coding cDNA on clinical signs of EAE after DNA vaccination**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Treatmenta</th>
<th>Cytokine-Coding cDNA</th>
<th>5′-AACGTT-3′ CpG Motifs</th>
<th>n</th>
<th>Mean Accumulated EAE Scoreb</th>
<th>pb</th>
<th>Mean Maximum EAE Scorec</th>
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a Rats received the DNA vaccines with or without cytokine DNA 4 wk before induction of EAE with MBP_{68–85} in CFA.

b Mean accumulated EAE score is the cumulative disease score 21 days after immunization (score obtained daily (0–4)).

c Mean maximum EAE score designates the average peak score of clinical disease at any time during the disease course, here on 12–15 days after immunization.

d NS, Not significant at 0.05 level.
ISS function as T1-promoting adjuvants and stimulate innate immunity (3–5). It is likely that the ISS effect described here depends on these abilities. This may seem paradoxical, because actively induced EAE in the Lewis rat is a T1-mediated autoimmune disease. However, there also seems to be a need for a proinflammatory milieu promoted by ISS during initiation of protective immune responses in EAE after DNA vaccination. Indeed, we observed an increased, early secretion of TNF-α of spleen MNC after in vitro stimulation with pK3-ZZ/MBP_68–85, but not after stimulation with pK0-ZZ/MBP_68–85. This is also supported by the present covaccinations with IL-4- and IL-10-coding cDNA, which abrogated the protective effects. Gilkeson et al. reported a similar paradoxical suppression of lupus in lupus-prone NZB/NZW mice, but increased production of anti-DNA autoantibodies, after treatment with E. coli DNA (35). Previous and present observations from our laboratory (15) and others (36), demonstrate that potential EAE effector cells become partially anergic after protective DNA vaccination. It is possible that the DNA vaccination leads to this attenuated T cell function by primarily affecting APC function. ISS-positive oligodeoxynucleotides affect APCs to promote T1-biased vaccination effects against exogenous pathogens (37). Indeed, we have observed an IgG isotype pattern after DNA vaccination, before EAE challenge, consistent with a vaccination-induced T1 bias (data not shown). Furthermore, Ruiz et al. demonstrated altered expression of CD80 and CD86 on APCs in the spleen after vaccination with DNA-encoding myelin proteolipid protein-peptide 139–151, another myelin autoantigen, against EAE (36). Treatment with ISS itself is not sufficient in our system to protect from EAE; expression of Ag is required during the initiation phase of the immune response, as the control plasmid pZZ, which does not encode MBP_68–85, failed to protect from EAE. In contrast, Boccaccio et al. reported suppression of EAE after treatment with noncoding plasmid DNA (38). No p values of the suppression of EAE was given and the control rats exhibited very mild EAE. For most of their experiments, they used a plasmid DNA purification method, in which considerable amounts of LPS is coprecipitated with the plasmid DNA (39, 40). Because LPS can be suppressive in EAE (41), the suppressive effects on EAE after administration of plasmid DNA could have been caused by LPS contamination. Indeed, no suppression of EAE after injection of highly purified genomic E. coli DNA was observed.

In the current study, we observed a relative reduction of Ag-specific proliferative T cell responses during the peak of disease, after treatment with ISS-positive DNA vaccine, compared with treatment with ISS-positive control plasmid. However, the Ag-specific proliferative response tended to be reduced also after treatment with the ISS-deficient DNA vaccine, compared with controls. Such reduced proliferative response could be caused by either partial anergy, deletion, or migration away from spleen of T cells. We could not detect an altered Ag-induced expression of the T1-cytokine IFN-γ or of the T2-cytokine IL-10. This stands in marked

![FIGURE 6. Effect of coinjection of cytokine-coding cDNA with DNA vaccine pZZ/MBP_68–85 or pZZ. Mean clinical EAE score after treatment with DNA vaccines or control constructs (A) pZZ/MBP_68–85 or pZZ without coinjection of cytokine-coding cDNA, (B) pIL-4 plus pZZ/MBP_68–85 or pZZ, (C) pIL-10 plus pZZ/MBP_68–85 or pZZ, (D) pGM-CSF plus pZZ/MBP_68–85 or pZZ, or (E) pTNF-α plus pZZ/MBP_68–85 or pZZ 4–5 wk before induction of EAE with MBP_68–85 in CFA.](http://www.jimmunol.org/)

![FIGURE 7. Effect of coinjection of cytokine-coding cDNA with ISS-deficient DNA vaccine pK0-ZZ/MBP_68–85 or pK0-ZZ. Mean clinical EAE score after treatment with DNA vaccines or control constructs (A) pZZ/MBP_68–85 or pZZ without coinjection of cytokine DNA, (B) pK0-IL-4 plus pZZ/MBP_68–85 or pZZ, (C) pK0-IL-10 plus pZZ/MBP_68–85 or pZZ, (D) pK0-GM-CSF plus pZZ/MBP_68–85 or pZZ, or (E) pK0-TNF-α plus pZZ/MBP_68–85 or pZZ 5 wk before induction of EAE with MBP_68–85 in CFA.](http://www.jimmunol.org/)
contrast to our earlier findings, where we measured reduced MBP-peptide-specific IFN-γ secretion of LNC after pZZ/MBP68–85 vaccination and MBP-peptide immunization, compared with controls. A possible explanation for these differing results could be that we studied T cells from different lymphoid organs: lymph nodes and spleen. In an earlier study, we measured unaltered expression of the T3-cytokine TGF-β after vaccination with pZZ/MBP68–85 (data not shown). We looked for, but did not observe, any induction of anti-idiotype T cells in the current study, but these cells may be too rare for detection. Furthermore, early Ag-specific IgGl Ab responses are greater in the DNA-vaccinated groups, regardless of whether they received ISS-positive or ISS-deficient DNA, compared with controls. Thus, the effects on proliferative, cytokine, and Ab responses are similar in the ISS-positive and ISS-deficient DNA vaccine groups, whereas the effects protecting against clinical signs of EAE differ. From this, we can only conclude that the protective mechanisms do not involve these autoantibody responses in any simple way and do not suggest a classical T1/T2 or T1/T3 shift as instrumental. The Ag-specific T cells are affected by the DNA vaccination, as observed by reduced Ag-specific proliferative responses, but the mechanism behind this altered T cell function is not elucidated in this study.

Coinjection of cytokine-coding cDNA with pZZ/MBP68–85 altered in some combinations the clinical signs of subsequently induced EAE, compared with treatment with the DNA vaccine alone. As discussed above, the disease-enhancing effect of IL-4- and IL-10-coding cDNA suggest that an initial T1 cytokine milieu is necessary for the peptide-specific protective effect after vaccination with pZZ/MBP68–85. In contrast, cDNA coding for GM-CSF failed to abrogate the protective effect. Covaccination with this cytokine is relevant, because GM-CSF is involved in recruitment of APCs (42). However, GM-CSF addition could not compensate for lack of ISS in the construct in the present autoimmune protection. In contrast, TNF-α-coding cDNA inhibited the protective effect of pZZ/MBP68–85 like the IL-4- and IL-10-coding cDNAs, even though we observed increased secretion of TNF-α after 24 h or 48 h exposure of naive spleen cells to ISS-positive DNA vaccine. However, the directly opposite outcomes might be due to timing and dose effects. The effects of covaccination with TNF-α-coding cDNA can thus not be interpreted in any simple way. Although TNF-α in most cases is proinflammatory and contributes to tissue damage, it can have paradoxical opposite effects also in autoimmune disease (27, 43, 44). The expressed TNF-α may act by inducing programmed cell death (45) of downregulatory cells induced by the DNA vaccination.

In this study, we demonstrate that 1) the presence of ISS during DNA vaccination is necessary for prevention of autoimmune disease, 2) ISS-positive plasmids require simultaneous expression of autoantigen to achieve this prevention, and 3) coinjection of certain, but not other, cytokine-coding cDNA can inhibit the suppressive effect of DNA vaccination in autoimmune disease. These effects strongly suggest that induction of a T1 cytokine milieu is decisive for the success of attempts to achieve protective DNA vaccination in EAE.

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References


