Combination of Gene Delivery and DNA Vaccination to Protect from and Reverse Th1 Autoimmune Disease via Deviation to the Th2 Pathway

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Summary

Using a combination of local gene delivery and tolerizing DNA vaccination, we demonstrate that codelivery of the interleukin-4 (IL-4) gene and a DNA vaccine encoding the self-peptide proteolipid protein 139–151 (PLP139–151) provides protective immunity against experimental autoimmune encephalomyelitis (EAE). We provide evidence for a mechanism whereby IL-4 expressed from the naked DNA is secreted and acts locally on autoreactive T cells via activation of STAT6 to shift their cytokine profile to Th2 helper. We also show that DNA vaccines can be used to reverse established EAE by cova-ccination with the genes for myelin oligodendrocyte glycoprotein and IL-4. This treatment strategy combines the antigen-specific effects of DNA vaccination and the beneficial effects of local gene delivery.

Introduction

EAE is a model of T cell-mediated autoimmunity, with many features common to that of the human disease multiple sclerosis (MS) (Zamvil and Steinman, 1990). Many treatments have been developed for experimental autoimmune encephalomyelitis (EAE) which have subsequently been attempted in MS. One form of treatment which shows significant promise in EAE but still requires extensive modification and optimization before being put to clinical use for MS is naked DNA vaccination. DNA vaccination has proven to be a powerful method of eliciting an immune response. The utility of DNA vaccination can be divided into two broad and divergent categories, either in the elicitation of immunity or of tolerance. In diseases caused by infectious pathogens, such as hepatitis B and human immunodeficiency virus (HIV), DNA vaccines have been shown to provide effective immunity against these foreign antigens (Robinson and Torres, 1997; Tang et al., 1992). In contrast, in autoimmune diseases, such as EAE and arthritis, DNA vaccines have been able to elicit tolerance against self-antigens (Lobell et al., 1998; Garren and Steinman, 2000; Ruiz et al., 1999).

In order to modify the immune responses to DNA vaccines, several previous investigators have performed DNA covaccination with a variety of cytokine genes along with the genes for certain pathogens. Examples include DNA immunization with hepatitis B virus antigens and IL-2-encoding DNA, which enhanced T helper 1 responses, HIV antigens with IL-12-encoding DNA, which enhanced cytotoxic T cell activity, and influenza antigens with IL-6-encoding DNA, which enhanced antiviral activity (Chow et al., 1997; Kim et al., 1997; Larsen et al., 1998). In each case, there is an improvement or modulation of the immune response against the target antigen. In addition, there have been several examples of coadministration of DNA encoding the prototypical T helper 2 (Th2) cytokine IL-4 along with a pathogen-specific DNA. Examples which incorporate IL-4 DNA covaccination include HIV or simian immunodeficiency virus (SIV) surface protein-encoding DNA, a malaria protein-encoding DNA, and hepatitis C or B virus protein-encoding DNA (Chow et al., 1998; Geissler et al., 1997; Ishii et al., 1999; Kim et al., 1998, 1999). In these examples, the coadministration of the IL-4 cytokine gene caused the immune response to be driven toward a more Th2-like phenotype.

However, in the case of EAE, there has only been one example of the use of covaccination with any immunomodulatory molecule. Lobell et al. demonstrated that DNA covaccination with various cytokines along with a gene for myelin basic protein 68–85 did not improve protection against EAE induced in Lewis rats (Lobell et al., 1999). Furthermore, when IL-4 DNA was used in the covaccine, the disease actually worsened.

We nevertheless hypothesized that by delivering a functional cytokine gene along with a tolerizing DNA vaccine we should be able to obtain suppressive immunization as well as a Th2 shift. In the present work, we show that mice co-ccinated with a DNA construct encoding the entire IL-4 gene along with the gene for the self-peptide PLP139–151 were protected from EAE induced by the peptide PLP139–151. The combination of local delivery of a functional IL-4 gene along with the antigen-specific effects of the PLP139–151 DNA vaccine caused the autoreactive T cells to shift their phenotype to Th2. This Th2 phenotype in the antigen-specific T cells was achieved by STAT6 phosphorylation near the site of injection, which can only occur if functional IL-4 acts on these T cells. Additionally, we show that DNA vaccines can be used to reverse established EAE by covaccination with the genes for myelin oligodendrocyte glycoprotein (MOG) and IL-4. We have thus incorporated a modification of DNA vaccination as a treatment of EAE that is applicable to all autoimmune diseases in which a Th2 bias is beneficial.

Results

The IL-4 DNA Vaccine Produces IL-4 Protein

In order to construct the IL-4 DNA vaccine, the complete coding sequence for IL-4 was amplified by PCR from mouse spleen cDNA. This gene was cloned into the mammalian expression vector pTargetT under control of
the CMV promoter, and the plasmid was purified as described in Experimental Procedures. In order to demonstrate that the IL-4 cDNA plasmid can indeed produce full-length IL-4 protein, an in vitro translation system was used. When the IL-4 cDNA plasmid was transcribed and translated in vitro with [35S]methionine and resolved by polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography, a single product of the correct size for mouse IL-4 is seen (Figure 1A, lane 1). A control reaction containing vector DNA without insert (Figure 1A, lane 2) or plasmid encoding PLP139–151 produced no detectable product (Figure 1A, lane 3). The predicted molecular weight for PLP139–151 is approximately 1.5 kDa and, therefore, would be extremely difficult to visualize by electrophoresis.

**IL-4 DNA Vaccination Causes Activation of STAT6**

In order to demonstrate that a DNA vaccine can act as a gene delivery vehicle, we wanted to explore the question of whether functional IL-4 cytokine was actually expressed from the DNA vaccine administered to the animal. IL-4 is known to act through the IL-4 receptor to specifically activate STAT6, a member of the signal transducers and activators of transcription family (Paul, 1997; Quelle et al., 1995; Takeda et al., 1996). Mice were vaccinated intramuscularly on a once weekly basis with plasmid DNA encoding the IL-4 cDNA as described in Experimental Procedures. Draining lymph nodes were dissected 1 week after the last DNA vaccine. Protein lysates were isolated from the lymph node cells and probed for the presence of activated STAT6 by Western blotting using a polyclonal antibody specific for the phosphorylated form of STAT6. As controls, mice were also vaccinated with pTargeT vector alone or with no DNA. As seen in Figure 1B, activated or phosphorylated STAT6 is only seen in lymph nodes from IL-4 DNA-vaccinated mice (lane 3). The phosphorylated STAT6 identified runs at approximately 60 kDa.

Identical results were obtained in a separate experiment in which mice received three daily rather than weekly doses of the DNA vaccine. Mice were vaccinated intramuscularly with plasmid DNA on a daily basis for 3 days. At 1 day after the last DNA vaccination, protein lysates from draining lymph nodes were obtained and analyzed as above in an antiphosphorylated STAT6 Western. Results identical to those shown in Figure 1B were obtained, with a 60 kDa band seen only in the lymph node cells from IL-4 DNA-vaccinated mice (data not shown).

**Covaccination with DNA Encoding IL-4 and DNA Encoding the PLP139–151 Minigene Protects against EAE Induction**

In order to explore the possibility of modifying the protection afforded by DNA immunization with the plasmid encoding PLP139–151 alone, we covaccinated mice with the genes for IL-4 and PLP139–151 as two separate plasmids. The murine IL-4 gene was cloned into the mammalian expression vector pTargeT under control of the CMV promoter as described earlier. The gene encoding PLP139–151 was obtained as described previously (Ruiz et al., 1999).

SJL/J mice were injected with 100 μg of each plasmid, intramuscularly, twice, at 1 week intervals. Control mice were injected with PBS, vector alone, IL-4 plasmid alone, PLP139–151 plasmid alone, or both PLP139–151 and vector plasmid. At 10 days after the last injection, the mice were challenged for induction of EAE with the encephalitogenic peptide PLP139–151 emulsified in complete Freund’s adjuvant (CFA). As shown in Table 1, there is a significant decrease in the mean disease scores at several time points of mice covaccinated with both the IL-4 and PLP139–151 plasmids compared to the controls (see Table 1 for p values). There is also a decrease in the incidence of disease and mean peak disease severity with the covaccine. The onset of disease was not significantly delayed compared to the control groups (data not shown).

**Protection against EAE Induction is Only Effective with a Secreted Form of the IL-4 Covaccine**

In order to determine if a secreted form of IL-4, as in the native cytokine, was required in the covaccine, a DNA construct was created in which the signal sequence of full-length IL-4 was removed. Removal of the signal sequence would render the gene product unable to be packaged into appropriate secretory compartments. Using identical DNA covaccine protocols in SJL/J mice as above, we performed covaccination with this nonsecreted IL-4 construct and the PLP139–151 minigene. As shown in Figure 2, protection against disease is not obtained with the nonsecreted IL-4-encoding DNA vac-
DNA Vaccination and IL-4 Gene Delivery Treat EAE

Covaccination with DNA Encoding IL-4 Rescues the T Cell Proliferative Responses in PLP139–151 Minigene DNA-Vaccinated Animals

Mice that were vaccinated with DNA and challenged for disease induction with peptide PLP139–151 were sacrificed after recovery from the initial acute disease. Draining lymph node cells (LNC) were obtained from these mice and restimulated in vitro with the PLP139–151 peptide to determine their proliferative responses. Furthermore, antigen-specific T cell lines were maintained from these LNC in order to analyze their cytokine secretion profiles. LNC were tested for their proliferative responses to the peptide PLP139–151. As shown in Figures 3A–3C, there was no significant change in the proliferative pattern of LNC from IL-4 and PLP139–151 co-DNA-vaccinated mice (Figure 3B) compared to control mice vaccinated with vector only (Figure 3A). In contrast, LNC from mice vaccinated only with PLP139–151 DNA have a reduced proliferative capacity (Figure 3C). We have previously reported that these T cells are anergic (Ruiz et al., 1999). Therefore, the addition of IL-4 as a DNA covaccine is able to rescue the anergy imposed by the PLP139–151 DNA vaccine. Thus, a fundamentally different mechanism of protection is afforded by covaccination with IL-4 DNA as compared with vaccination with PLP139–151 DNA alone.

Covaccination with DNA Encoding IL-4 Shifts the Phenotype of T Cells into a Th2 Type

PLP139–151-specific T cell lines were isolated and maintained in culture from mice challenged for disease induction with the peptide PLP139–151, and previously vaccinated with various combinations of DNA. These T cell lines were tested for cytokine production after in vitro stimulation with the peptide PLP139–151. As shown in Figures 3D–3F, T cells from mice covaccinated with IL-4 and PLP139–151 DNA produced significantly higher amounts of IL-4 (mean of 716 ± 237 pg/ml versus 0.36 pg/ml from pTargetT-vaccinated mice, \( p < 0.0064 \)) and IL-10 (mean of 1073 ± 221 pg/ml versus 464 ± 44 pg/ml from pTargetT-vaccinated mice, \( p < 0.0151 \)) compared to T cells from control mice. In addition, T cells from the IL-4 and PLP139–151 DNA-covaccinated mice produced lower amounts of IFN-\( \gamma \) compared to control T cells (mean of 1389 ± 108 pg/ml versus 6689 ± 85 pg/ml from pTargetT-vaccinated mice, \( p < 0.0001 \)). Thus, T cells isolated from the covaccinated and protected mice produce more Th2-type cytokines compared to control T cells.

Table 1. Protection from EAE with DNA Vaccination

<table>
<thead>
<tr>
<th>DNA Vaccine</th>
<th>n</th>
<th>Incidence</th>
<th>Mean* Peak Disease Severity</th>
<th>Mean Score (Day 12)</th>
<th>Mean Score (Day 14)</th>
<th>Mean Score (Day 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>14</td>
<td>86</td>
<td>2.3 ± 0.3</td>
<td>1.6 ± 0.4</td>
<td>1.2 ± 0.2</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>pTargetT</td>
<td>15</td>
<td>93</td>
<td>2.4 ± 0.2</td>
<td>1.6 ± 0.3</td>
<td>1.7 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>IL-4</td>
<td>15</td>
<td>80</td>
<td>2.7 ± 0.3</td>
<td>1.4 ± 0.3</td>
<td>1.1 ± 0.2</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>PLP139–151</td>
<td>15</td>
<td>73</td>
<td>2.4 ± 0.3</td>
<td>1.5 ± 0.3</td>
<td>1.5 ± 0.3</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>pTargetT + PLP139–151</td>
<td>10</td>
<td>80</td>
<td>2.4 ± 0.3</td>
<td>1.8 ± 0.3</td>
<td>1.3 ± 0.3</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>IL-4 + PLP139–151</td>
<td>15</td>
<td>53</td>
<td>1.6 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>0.7 ± 0.3</td>
<td>0.5 ± 0.2</td>
</tr>
</tbody>
</table>

* All \( p \) values given as comparison of IL-4 + PLP139–151 to pTargetT by Student’s two-tailed unpaired t test.

<Figure 2. The IL-4 Gene Product Delivered in the Covaccine Must Be Secreted in Order to Provide Protection against EAE Induction>

(A) A schematic view of the predicted protein products of the two IL-4 DNA vaccine constructs shows the position of the amino acids removed in the nonsecreted construct.
(B) The calculated Kyte-Doolittle hydropathy plot of the predicted amino acid sequence of IL-4 shows the very hydrophobic first 20 amino acids constituting the signal sequence.
(C) Five mice in each group were DNA vaccinated with either a secreting IL-4 DNA construct along with the PLP139–151 minigene (●, solid line), a nonsecreting IL-4 DNA construct along with the PLP139–151 minigene (▲, solid line), or buffer only (○, dashed line). Mean EAE scores are plotted against the number of days since EAE induction with PLP139–151 peptide in CFA. The asterisks indicate a statistically significant difference (\( p = 0.05 \) by Student’s two-tailed unpaired t test) comparing the PLP139–151 minigene and secreted IL-4 versus nonsecreted IL-4 DNA vaccines.
Figure 3. Protective Th2 T Cells Are Induced by IL-4 DNA Covaccination

(A–C) Covaccination with DNA encoding IL-4 rescues the T cell proliferative response in PLP<sub>139-151</sub> DNA-vaccinated animals. After the acute phase of disease, lymph node cells were harvested from mice vaccinated with DNA for (A) vector without insert, (B) IL-4 and PLP<sub>139-151</sub> DNA, or (C) PLP<sub>139-151</sub> DNA alone. These cells were restimulated in vitro for 96 hr with a series of concentrations of either the peptide PLP<sub>139-151</sub> (solid line) or an irrelevant control peptide (dashed line). Results shown are mean counts per minute (cpm) ± SEM of triplicate samples. The experiment shown is representative of two independent experiments. The cpm of ConA-stimulated LNC were 34,206 for group (A), 14,262 for group (B), and 10,838 for group (C).

(D–F) IL-4 and PLP<sub>139-151</sub> DNA-covaccinated mice undergo a Th2 shift. T cell lines were isolated and maintained in culture from mice challenged for EAE induction with peptide PLP<sub>139-151</sub> and previously vaccinated with various combinations of DNA. These T cell lines were restimulated in vitro with peptide PLP<sub>139-151</sub> and levels of (D) IL-4, (E) IL-10, and (F) IFNγ in the supernatants after 6 days of culture were measured by sandwich ELISA. Results are expressed in pg/ml ± SD of triplicate samples. The experiment shown is representative of three independent experiments. The horizontal axis indicates the original DNA vaccine used to obtain the T cell line. ND, no data.

(G) Protection from EAE in IL-4 and PLP<sub>139-151</sub> DNA-covaccinated mice can be transferred by T cells. A T cell line was derived from mice covaccinated with both IL-4 DNA and PLP<sub>139-151</sub> DNA, which maintained proliferative capacity but underwent a Th2 shift, were then tested for the capacity to transfer protection. Mice were immunized with the encephalitogenic peptide PLP<sub>139-151</sub> emulsified in CFA, and, 8 days later, 10 million T cells were injected intravenously into each mouse. Animals were then followed for disease phenotype. T cells that are specific for PLP<sub>139-151</sub> and known to induce EAE were
Table 2. Th2 Deviation Requires STAT6

<table>
<thead>
<tr>
<th>Strain</th>
<th>DNA Vaccine</th>
<th>IL-4 (pg/ml)</th>
<th>IFNγ (pg/ml)</th>
<th>Th2/Th1 Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>none</td>
<td>57.3</td>
<td>573.9</td>
<td>0.1</td>
</tr>
<tr>
<td>BALB/c</td>
<td>MBP</td>
<td>11.3</td>
<td>101.1</td>
<td>0.11</td>
</tr>
<tr>
<td>BALB/c</td>
<td>IL-4</td>
<td>88.2</td>
<td>291.7</td>
<td>0.3</td>
</tr>
<tr>
<td>BALB/c</td>
<td>MBP + IL-4</td>
<td>276.4</td>
<td>145.8</td>
<td>1.9</td>
</tr>
<tr>
<td>STAT6-/-</td>
<td>MBP + IL-4</td>
<td>123.8</td>
<td>110</td>
<td>1.13</td>
</tr>
</tbody>
</table>

*a Cytokine levels were determined after stimulating the T cells for 4 days with MBP 59–76.

*b Th2/Th1 ratio calculated as IL-4 concentration divided by IFNγ concentration.

also injected as a control. As shown in Figure 3G, mice injected with T cells derived from the covaccinated mice had reduced incidence (1/5 mice compared to 4/5 mice in the controls) and reduced disease scores compared with control T cell-injected mice. These results indicate that the protective effect achieved by IL-4 and PLP139–151 DNA covaccination can be transferred to naive animals by antigen-specific Th2 cells.

STAT6 Is Necessary for the DNA Covaccination-Induced Th2 Shift

In order to determine whether STAT6 is necessary for the DNA covaccination-induced Th2 shift, comparable DNA vaccination experiments were carried out in STAT6 knockout mice. STAT6 knockout mice were obtained on a BALB/c background. BALB/c mice are generally resistant to EAE induction, but myelin basic protein (MBP)-reactive T cells have been isolated from BALB/c mice immunized with guinea pig MBP in CFA (Yoshizawa et al., 1998). STAT6 knockout mice or BALB/c controls were DNA vaccinated as previously described, with full-length mouse MBP DNA, IL-4 DNA, or a combination of MBP and IL-4 DNA. These mice were then immunized with guinea pig MBP in CFA, and draining lymph node cells were removed 10 days later. The lymph node cells were incubated with the peptide mouse MBP 59–76 in a capture ELISA assay for 4 days, and the levels of production of IL-4 and IFNγ were determined (Table 2). The wild-type BALB/c mice covaccinated with MBP and IL-4 DNA had a greater amount of deviation to Th2 cytokine production (Th2/Th1 ratio of 1.9) than in any of the other groups, including the STAT6 knockout mice. Therefore, STAT6 is required in order for the covaccine to produce a Th2 shift in cytokine production by myelin-specific T cells.

Covaccination with DNA Encoding IL-4 and DNA Encoding Full-Length MOG Can Reverse Ongoing EAE

In contrast to the prevention of initial disease, we next explored the question of whether DNA vaccination could reverse clinically established EAE. We also wanted to determine whether myelin autoantigens other than PLP could participate in EAE suppression. Therefore, we chose to attempt to treat ongoing EAE with a plasmid DNA construct encoding MOG, another myelin-specific autoantigen (Bernard et al., 1997). Some investigators have suggested that EAE relapses are caused by the spreading of T cell reactivity to additional epitopes within and among myelin proteins—epitope spreading (Lehmann et al., 1992; Vanderlugt and Miller, 1998; Yu et al., 1996). We therefore included the entire coding sequence of MOG within the DNA vaccine construct in order to suppress any intramolecular epitope spreading that might occur.

EAE was induced in C57BL/6 mice with the encephalitogenic peptide MOG35–55 in CFA. Mice with active EAE were randomly divided into four groups and vaccinated with vector DNA alone, MOG-encoding DNA alone, IL-4-encoding DNA alone, or with both MOG-encoding DNA and IL-4-encoding DNA. As shown in Figure 4 and Table 3, EAE is reversed only when MOG-encoding DNA is covaccinated with IL-4-encoding DNA. There is a sta-
Table 3. Reversal of EAE with DNA Vaccination

<table>
<thead>
<tr>
<th>DNA Vaccine</th>
<th>n</th>
<th>Percent Incidence Before DNA</th>
<th>After DNA</th>
<th>Mean† Peak Disease Severity after DNA Treatment</th>
<th>p Value ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTargetT</td>
<td>9</td>
<td>89</td>
<td>78</td>
<td>2.6 ± 0.4</td>
<td>0.056</td>
</tr>
<tr>
<td>MOG</td>
<td>15</td>
<td>87</td>
<td>73</td>
<td>2.6 ± 0.2</td>
<td>0.0062</td>
</tr>
<tr>
<td>IL-4</td>
<td>8</td>
<td>88</td>
<td>88</td>
<td>3.0 ± 0.3</td>
<td>0.002</td>
</tr>
<tr>
<td>MOG + IL-4</td>
<td>16</td>
<td>81</td>
<td>38</td>
<td>1.8 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

† Means given as mean ± SEM.  
‡ p values given as comparison of mean peak disease severity in each group to that of MOG + IL-4 by Student’s two-tailed unpaired t test.  
§ Percent incidence after DNA treatment given as the percentage of animals that develop relapsing disease.

Discussion

In the present study, we have applied a method of protective immunity which combines the effects of DNA vaccination and local gene delivery. We have shown that naked DNA can act as a local gene delivery vehicle to deliver functional IL-4. We demonstrate that IL-4 is produced by the naked DNA and that secreted IL-4 is required in order to suppress EAE when given as a covaccine. Further, we show that functional IL-4 is required, as implied by activation of the IL-4-specific transcription factor STAT6 only in mice receiving the IL-4 naked DNA.

When mice were immunized with both the IL-4 DNA vaccine and a separate DNA vaccine for the self-peptide PLP139–151, these mice were protected against induction of disease by the peptide PLP139–151 emulsified in CFA. When the cytokine profile of T cells from covaccinated and protected mice was examined, a shift to a Th2-type of cytokine secretion pattern was seen. Furthermore, these Th2 cells could transfer protection against disease induction in naive mice.

Additionally, we demonstrate that DNA vaccination can be used to reverse ongoing EAE. When mice with active EAE were covaccinated with separate plasmids encoding MOG and IL-4, the mice had a reduction in several disease parameters. Mice vaccinated with MOG- or IL-4-encoding DNA alone did not have significant EAE reduction. These results thus demonstrate that DNA vaccination can be used to treat established EAE in addition to the prevention of EAE onset and that IL-4-encoding DNA is required as a covaccine for this treatment.

We thus propose that the combination of the local delivery of IL-4 and vaccination with myelin-encoding DNA causes the antigen-specific autoreactive T cells to shift their phenotype to a more protective Th2-type of response. These antigen-specific, protective T cells are then directed to sites of myelin damage and attenuate the pathogenic autoimmune response. As a possible mechanism of how this protection could occur, we propose that the IL-4- and myelin-encoding DNA vaccines are both taken up by antigen-presenting cells (APCs) at the site of administration of the vaccines. The myelin-encoding gene is expressed in the APCs, and myelin epitopes are presented on MHC class II to antigen-specific T cells that are thus recruited. The APCs also express IL-4, which is secreted locally during the APC and T cell interaction. This secreted IL-4 then causes the phenotype of the antigen-specific T cell to assume a more Th2 type of phenotype. This model is compatible with earlier studies that showed that T cells grown in culture could be caused to assume a more Th2 type of phenotype by growth in the presence of IL-4 (Macatonia et al., 1993; Mocci and Coffman, 1997). We believe that it is the local microenvironment during the APC and T cell interaction that is important, since no detectable increase in serum IL-4 was seen in the IL-4 DNA-vaccinated mice (data not shown). As a method of delivery of a gene product with potential adverse systemic effects, such as a cytokine at high doses, this technique could be desirable over traditional gene therapy methods, since the gene delivered acts locally rather than systemically.

DNA vaccines have proven to be effective in protecting against some animal models of autoimmune disease. One of the many advantages of DNA vaccines over traditional treatments of autoimmune disease is the ability to easily modify the treatment vehicle. We have shown here that, with the addition of a genetically delivered IL-4 cytokine to a myelin-encoding DNA vaccine, we can protect against and reverse established EAE and, further, drive the protective response to a more Th2 type. We have previously reported that vaccination with DNA encoding the Vp8.2 variable region of a T cell receptor (TCR) that is important in the pathogenesis of EAE is effective in protecting against EAE and causes the myelin-reactive T cells to become more Th2-like (Waisman et al., 1996). However, such a strategy would be prohibitive in widespread clinical trials or in other autoimmune diseases because it requires the knowledge of the exact TCR repertoire relevant for a particular disease and for a particular individual. The obvious advantage of an IL-4 DNA covaccine is that it can be easily included along with the antigen-specific DNA vaccine for a particular autoimmune disease.

We have also previously reported that, after DNA vaccination with a myelin epitope alone, T cells are anergic (Ruiz et al., 1999). In the current report, the addition of IL-4 as a DNA covaccine rescues the anergy imposed by the PLP139–151 DNA vaccine and drives the response to a Th2 phenotype. This distinctly different mechanism of protection, afforded by covaccination with IL-4 DNA compared with vaccination with PLP139–151 DNA alone, may have particular advantages. This technique could
prove beneficial in the treatment of other autoimmune diseases. Immunization against the antigens that trigger those autoimmune diseases caused by Th1 autoreactive cells, diseases such as multiple sclerosis, juvenile diabetes, and rheumatoid arthritis, would be conditions in which covaccination with DNA encoding IL-4 might prove beneficial (Nicholson and Kuchroo, 1996; Steinman, 1995).

Experimental Procedures

Animals
Female SJL/J, BALB/c, and C57BL/6 mice (6- to 8-week-old) were purchased from the Jackson Laboratory (Bar Harbor, ME). STA76 null mice (C129S2-Stat6<sup>−/−</sup>) in a BALB/c background were obtained from Jackson Laboratory.

Peptides
Peptides were synthesized on a peptide synthesizer (model 9050; MilliGen, Burlington, MA) by standard 9-fluorenylemethoxycarbonyl chemistry. Peptides were purified by HPLC. Structures were confirmed by amino acid analysis and mass spectroscopy. Peptides used in these experiments were plasmid DNA (at a concentration of 1 mg/ml in PBS) 2 and 9 days later in the same muscles. Animals receiving a covaccine received two separate injections of each plasmid DNA.

DNA Vaccines
A minigenine encoding PLP<sub>139–151</sub> was cloned by PCR from brain cDNA (Clontech, Palo Alto, CA) by use of the following PCR primers: 5'-CGAGGTACTACGAGTAATCCATTTGCATGATGC-3' and 5'-CCTTGATGGGTCTCAACCCCCAGCTAGTTGTC-3'. This pair of primers would result in the truncation of the first 20 amino acids of full-length IL-4.

DNA constructs used for DNA vaccination were tested for the production of the correctly sized product by an in vitro translation assay. Approximately 1 µg of plasmid DNA was incubated for 2 hr at 30°C in a 50 µl volume containing the following: 25 µl of TNT rabbit reticulocyte lysate (Promega Corp., Madison, WI), 2 µl of TNT reaction buffer (Promega Corp.), 1 µl of TNT T7 RNA polymerase (Promega Corp.), 1 µl of a 1 mM amino acid mixture minus methionine (Promega Corp.), 4 µl of [<sup>35</sup>S]methionine at 10 µCi/ml (Amersham Life Sciences, Inc., Arlington Heights, IL), and 1 µl of RNasin ribonuclease inhibitor at 40 U/µl (Promega Corp.). A 3 µl volume of the products of this reaction was mixed with SDS-sample buffer and run on an 18% SDS polyacrylamide gel. After drying, the gel was then exposed to autoradiography film.

STAT6 Westerns
After dissection of draining lymph nodes from DNA-vaccinated mice, the tissues were mechanically homogenized in 1 ml of the following buffer: 0.1 M NaCl, 0.01 M Tris-HCL (pH7.4), 0.001 M EDTA, 1 µg/ml aprotinin, and 1.6 µg/Petabloc SC (Boehringer Mannheim, Indianapolis, IN). Half of the resultant lysate was used in a BCA protein assay (Pierce, Rockford, IL) in order to determine the total protein concentration. The remaining 0.5 ml was added to 0.25 ml of 3× SDS loading buffer (New England Biolabs, Beverly, MA) containing DTT at a final concentration of 0.04 M. The products were resolved on a 4%–15% gradient SDS-PAGE gel (Bio-Rad, Hercules, CA). Prestained markers were used to determine the molecular weights (Bio-Rad). After electrophoresis, the gels were blotted to PVDF membranes (Amersham Life Sciences Inc.) at constant voltage of 100V in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol as the transfer buffer. The membranes were blocked for 1 hr at room temperature with Tris-buffered saline (TBS), 0.1% Tween 20, and 20% nonfat dry milk. After washing the membranes with TBS and 0.1% Tween 20, the membranes were hybridized overnight at 4°C with anti-phospho STAT6 antibody (New England Biolabs) diluted 1:1000 in TBS, 0.1% Tween 20, and 5% BSA. The membranes were then processed as in the ECL Plus protocol (Amersham Life Sciences Inc.) for visualization of the bands by chemiluminescence. The membranes were stripped by incubation in 100 mM β-mercaptoethanol, 2% (v/v) SDS, and 62.5 mM Tris-HCL (pH 7.4) for 30 min at 60°C. These same membranes were then probed with an antibody against mouse CD3; (PharMingen, San Diego, CA) as a control to verify equal loading of the lanes.

DNA Immunization Protocol
Animals were injected in both quadriceps with a total of 0.1 ml of 0.25% bupivicaine-HCL (Sigma, St. Louis, MO) in PBS. Unless otherwise indicated, mice were injected with a total of 100 µg of plasmid DNA (at a concentration of 1 mg/ml in PBS) 2 and 9 days later in the same muscles. Animals receiving a covaccine received two separate injections of each plasmid DNA.

EAE Induction
At 7–10 days after the final DNA vaccine, EAE was induced in SJL/J mice with 100 µg of PLP<sub>139–151</sub> peptide. The peptide was dissolved in PBS at a concentration of 2 mg/ml and an equal volume of CFA, which consists of incomplete Freund’s adjuvant supplemented with 4 mg/ml heat-killed mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI). Mice were injected subcutaneously with 0.1 ml of the peptide emulsion. EAE was induced in C57BL/6 mice with 100 µg of MOG<sub>35–55</sub> peptide in CFA. On the day of peptide immunization and 48 hr later, the C57BL/6 mice were also injected intravenously with 0.1 ml of 4 µg/ml Bordetella pertussis toxin in PBS. BALB/c and STAT6 null mice were immunized with 5 mg of guinea pig MBP in CFA. Experimental animals were scored as follows: 1, tail weakness or paralysis; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb weakness or paralysis; and 5, moribund or dead animals.

Lymph Node Cell Proliferation Assays
After the acute phase of disease, draining lymph nodes were dissected, and LNC were cultured in vitro for specific proliferative response to the PLP<sub>139–151</sub> peptide. LNCs were prepared in 96-well microtiter plates in a volume of 0.2 ml/well at a concentration of 2.5 × 10<sup>5</sup> cells/ml. The culture medium consisted of enriched RPMI (RPMI 1640 supplemented with L-glutamine [2 mM], sodium pyruvate [1 mM], nonessential amino acids [0.1 mM], penicillin [100 U/ml], streptomycin [0.1 mg/ml], 2-ME [5 × 10<sup>−4</sup>] supplemented with 1% autologous fresh normal mouse serum. Cultures were incubated at 37°C, and, after 72 hr, cells were pulsed for 18 hr with 1 µCi/well of [3H]thymidine. The cells were then harvested and counted in a β counter.

Cytokine Profile Determination
T cell lines were established from LNCs derived from DNA-vaccinated mice as previously described (Waisman et al., 1996). These T cells were then tested for the production of various cytokines. T cells (50 × 10<sup>5</sup>/ml) were incubated with 2.5 × 10<sup>5</sup> irradiated syngeneic APCs/ml in enriched RPMI and 10% FCS. After 6 days of culture, the supernatants were collected and tested by sandwich ELISA using standard ELISA kits (PharMingen).


