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Suppressive vaccination with DNA encoding a variable region gene of the T-cell receptor prevents autoimmune encephalomyelitis and activates Th2 immunity

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A variable region gene of the T-cell receptor, Vβ8.2, is rearranged, and its product is expressed on pathogenic T cells that induce experimental autoimmune encephalomyelitis (EAE) in H–2^u mice after immunization with myelin basic protein (MBP). Vaccination of these mice with naked DNA encoding Vβ8.2 protected mice from EAE. Analysis of T cells reacting to the pathogenic portion of the MBP molecule indicated that in the vaccinated mice there was a reduction in the Th1 cytokines interleukin-2 (IL-2) and interferon-γ. In parallel, there was an elevation in the production of IL-4, a Th2 cytokine associated with suppression of disease. A novel feature of DNA immunization for autoimmune disease, reversal of the autoimmune response from Th1 to Th2, may make this approach attractive for treatment of Th1-mediated diseases like multiple sclerosis, juvenile diabetes and rheumatoid arthritis.

Experimental autoimmune encephalomyelitis (EAE) is a model of T cell-mediated autoimmunity and shares many features with multiple sclerosis. Pathogenic T cells in EAE utilize a restricted repertoire of genes encoding the T-cell receptor (TCR)[1]. For example, upon immunization of H–2^u mice with either myelin basic protein (MBP) or its immunodominant fragment, peptide Ac1–20, the Vβ8.2 TCR gene product is expressed in the majority of pathogenic T cells[2–4]. Two strategies have been used to target these pathogenic T cells in a highly specific manner. Thus, the administration of monoclonal antibodies directed to pathogenic V gene products[3,5], as well as T-cell vaccination with peptides from the second or third complementarity-determining regions of the pathogenic TCR V region, have proven successful in the therapy of EAE (ref. 6, 7).

Injection of DNA promotes highly effective vaccination against microbes and tumors[8–11]. Here we report prevention of EAE by injection of DNA encoding the Vβ8.2 region of a T-cell receptor that is critical in the pathogenesis of the disease. This is a novel demonstration of "suppressive vaccination," a goal that is contrary to what is normally desired when vaccinating for immunity to microbes. In dissecting the mechanism of action of DNA vaccination against the pathogenic TCR V gene segment, a surprising result emerged. Instead of merely depleting or blocking the relevant T cells bearing the targeted V gene product, DNA vaccination promoted a shift in the pattern of cytokines produced by the pathogenic T cells. No longer were these T-cell populations producing pathogenic cytokines like interferon-γ and interleukin-2 (IL-2), cytokines that define a T helper cell (Th1)-type response[12,13], instead production was initiated for the suppressive cytokine IL-4, which characterizes a Th2 response. This shift toward Th2 immunity had not been seen previously with DNA vaccination for a microbial protein[14]. This novel mechanism of action, induced with DNA vaccination to a gene encoding a TCR variable region polypeptide, may have potential for autoimmune diseases triggered by specific Th1 T cells. Whereas immunity to microbes requires a successful Th1 immunization, treatment of autoimmunity involves induction of a Th2 response and suppressive immunization. This can be achieved with DNA vaccination to TCR V genes.

Vaccination with Vβ8.2 DNA protects from EAE

The Vβ8.2 gene of the T-cell receptor was cloned in an expression vector and injected three times at weekly intervals into the tibialis anterior muscle of PL/J female mice, beginning one week after a single intramuscular injection of cardiotoxin. Control PL/J mice were immunized once with cardiotoxin and then three times at weekly intervals with the DNA coding for the Vβ5.1 variable region of the TCR. This TCR V region is not found on pathogenic T-cell clones in H–2^u mice. After this immunization protocol, all mice were immunized with complete Freund's adjuvant containing either the myelin basic protein peptide, pAc1–20, or guinea pig (gp) MBP. The mice vaccinated with the Vβ8.2 DNA were resistant to EAE induced by the pAc1–20, whereas EAE was induced in the groups vaccinated with the control Vβ5.1 DNA, or with cardiotoxin only (Fig. 1*a* and Table 1). Only 1 mouse out of 14 vaccinated with the Vβ8.2 DNA devel-

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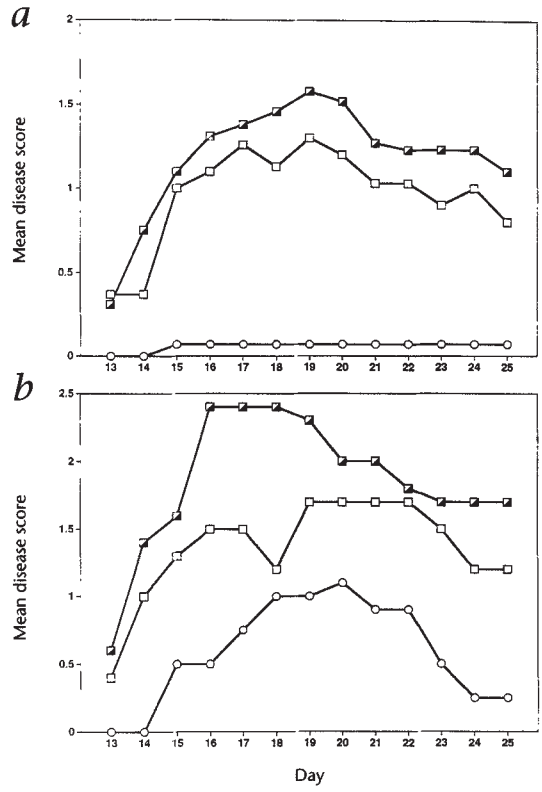

Fig. 1 Effect of DNA vaccination of mice after active immunization with pAc1–20 peptide (*a*) or gpMBP (*b*). Groups of 9–15 PL/J mice were injected s.c. in the foot pads with the peptide or protein. Mice were treated with DNA coding for Vβ5.1 (□), Vβ8.2 (○) or with PBS only (◪). Shown is the mean disease score of all the mice in each group.

oped mild paralysis of the hind limbs, whereas 11/15 and 10/13 mice were paralyzed or died in the respective control groups ($P < 0.0001$), after immunization with pAc1–20 (Table 1).

Although perivascular inflammatory infiltrates were present in the mice immunized with Vβ8.2, they were less extensive and less frequent than in mice immunized with Vβ5.1, when examined by an observer blinded to the treatment regimen. When scoring inflammatory infiltrates, significant differences were observed between mice treated with Vβ8.2 (scores 3.5 ± 1.2 for brain meningeal infiltrates, 4.4 ± 2.0 for brain parenchymal infiltrates) compared with mice treated with Vβ5.1 (28.2 ± 4.0 for brain meningeal infiltrates, and 13.5 ± 3 for brain parenchymal infiltrates) ($P < 0.02$ for comparison of meningeal infiltrates, $P < 0.03$ for comparison of parenchymal infiltrates). (Histologic infiltrates were scored separately for brain parenchyma and meninges on a scale based on infiltrate size measured by number of cells per cuff.)

Although MBP pAc1–20 is the dominant epitope in the myelin basic protein molecule in PL/J mice, there are other pathogenic epitopes within this molecule including MBP p35–47 (ref. 15). T-cell recognition of MBP p35–47 does not utilize the Vβ8.2 gene product. Therefore, we asked whether DNA vaccination with the Vβ8.2 DNA might protect mice, even when EAE was induced with the whole MBP. In this case MBP pAc1–20 would be the dominant, but not exclusive, pathogenic epitope, and other regions of MBP, which are recognized by TCR where Vβ8.2 is not rearranged, could be involved in pathogenesis. Therefore, we vaccinated PL/J mice with Vβ8.2 DNA, and then induced disease with the whole MBP. PL/J mice vaccinated with Vβ8.2 DNA developed EAE when they were immunized with gpMBP, although disease occurred at a lower frequency ($P < 0.04$, from day 15 onward) compared with the mice vaccinated with the control DNA, or cardiotoxin alone (Fig. 1*b* and Table 1).

DNA vaccination induces a Th1 to Th2 cytokine shift

We then analyzed the mechanism of action underlying the protection induced by DNA vaccination. Our initial hypothesis was that DNA vaccination with Vβ8.2 would deplete T cells expressing Vβ8.2 and would thereby mimic the effect seen when giving a cytotoxic anti-Vβ8.2 monoclonal antibody[3]. Mice were analyzed for titers of antibodies directed to Vβ8.2 TCR. We found that mice vaccinated with the DNA construct containing the Vβ8.2 DNA produced antibodies to the Vβ8.2 expressed on T cells. Sera from mice vaccinated with the Vβ8.2 DNA stained 76.9 ± 12.1% cells of a Vβ8.2$^+$ T-cell line that was reactive to MBP pAc1–20, whereas only 4.3 ± 3.0% of the cells were stained with the sera from the control mice, a figure that is similar to the background staining with normal mouse sera (3.8 ± 2.7) ($P < 0.01$). To test whether those anti-Vβ8.2 antibodies depleted the Vβ8.2-positive T cells, lymph node cells and spleen cells from the protected mice and controls were stained for the presence of this cell population. Mice vaccinated with the Vβ8.2 DNA have similar levels of Vβ8.2-

Table 1 Active EAE induction following DNA vaccination

a, Disease induction with the peptide pAc1–20[a]

	EAE incidence[b]							
DNA used for vaccination	Day 13	Day 15	Day 17	Day 19	Day 20	Day 21	Day 23	Day 25
Vβ5.1	2/15	7/15	10/15	11/15	10/15	9/15	9/15	8/15
Vβ8.2	0/14	1/14	1/14	1/14	1/14	1/14	1/14	1/14
No DNA	2/13	4/13	7/13	9/13	10/13	7/13	7/13	7/13

b, Disease induction with gpMBP[a]

	EAE incidence[b]							
DNA used for vaccination	Day 13	Day 15	Day 17	Day 19	Day 20	Day 21	Day 23	Day 25
Vβ5.1	2/10	7/10	8/10	9/10	9/10	9/10	8/10	7/10
Vβ8.2	0/9	2/9	5/9	5/9	5/9	5/9	4/9	3/9
No DNA	2/10	5/10	8/10	9/10	9/10	9/10	8/10	8/10

[a]Disease was induced on day 0 as described in Methods.
[b]Disease was monitored as described in Methods. Shown are mice with clinical signs of EAE and the number of mice in each group.

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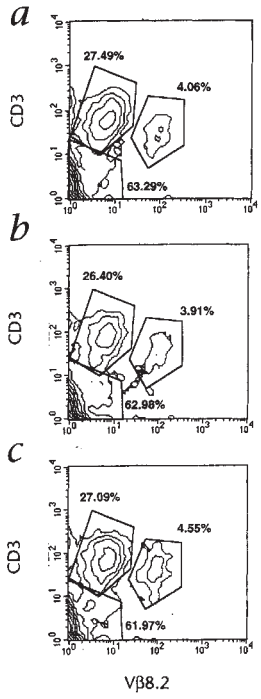

Fig. 2 Vβ8.2$^+$ T cells are not depleted in the DNA Vβ8.2-vaccinated mice. Spleen cells were taken from mice treated with Vβ5.1 (*a*), Vβ8.2 (*b*) or with PBS only (*c*) and stained with anti-CD3 antibody conjugated to PE and with the anti-Vβ8.2 mAb conjugated to FITC. Regions relating to CD3$^-$,Vβ8.2$^-$ (non-T-cells), CD3$^+$,Vβ8.2$^-$ (non-Vβ8.2 T cells) or CD3$^+$,Vβ8.2$^+$ (Vβ8.2 T cells) are indicated, with their percentage of the total population indicated.

positive T cells, as compared with the control groups, as well as when compared with normal mice, levels ranging from 3.9% to 4.6% of cells stained with anti-CD3 and anti-Vβ8 (Fig. 2).

We then asked whether DNA vaccination somehow altered the phenotype of the immune response of the Vβ8.2 T cells, rendering them unable to mount a pathogenic response to MBP pAc1–20. We analyzed whether the Vβ8.2 T cells were now anergized or whether they had altered their pattern of cytokine production after immunization. First, lymph nodes were taken from the protected animals and from the controls, in order to test whether anergy of the Vβ8.2-positive T cells to the immunizing peptide (pAc1–20) could account for the protection. As depicted in Fig. 3*a*, we found that lymph nodes cells taken from PL/J mice were able to respond by proliferation to the immunizing peptide, with a similar stimulation index to that of the cells taken from the control mice, either treated by injection with Vβ5.1 or with cardiotoxin alone. Thus, DNA vaccination to Vβ8.2 did not induce anergy. Next, when lymph node cells were tested for production of cytokines following activation with MBP pAc1–20, we found that they produce much lower levels of interferon-γ ($P < 0.002$) and IL-2 ($P < 0.0015$) in mice vaccinated with the Vβ8.2 DNA than lymph node cells taken from the control mice (Fig. 3, *b* for IL-2 and *c*, interferon-γ). Interferon-γ and IL-2 are cytokines produced by T cells of the Th1 phenotype[12,16]. In contrast, the lymph node cells taken from the mice vaccinated with Vβ8.2 DNA produced elevated levels of the Th2 cytokine, IL-4, after stimulation with MBP pAc1–20 ($P < 0.04$) (Fig. 3*d*). These data serve to indicate that the phenotype of the immune response in Vβ8.2-vaccinated mice, immunized with an antigen, MBP pAc1–20, recognized by T cells expressing Vβ8.2 TCR, had undergone a cytokine shift, from Th1 to Th2.

Interleukin-4 induces a class switch of antibody from IgG2a to IgG1 isotype. A hallmark of a Th1 to Th2 cytokine shift *in vivo* is a shift in the antibody isotype from IgG2a to IgG1 (ref. 12). We analyzed the isotype of the antibodies directed to the immunizing peptide, MBP pAc1–20, with and without DNA vaccination to Vβ8.2. As demonstrated in Fig. 4, mice vaccinated with the DNA coding for Vβ8.2 gene produced higher levels of anti-Ac1–20 antibodies of the IgG1 isotype and lower levels of antibody of the IgG2a isotype, in comparison with the control mice ($P < 0.01$). These data confirm the idea that DNA vaccination to Vβ8.2 resulted in a cytokine shift in the response of T cells to MBP pAc1-11, an antigen recognized predominately by T cells bearing Vβ8.2. This could account for the protection against the development of EAE, as EAE is known to be a Th1-type disease[17]. Moreover, injection of IL-4 protects mice from the development of EAE (ref. 18).

To better analyze the role of the T cells in the protection mechanism, we raised short-term T-cell lines from Vβ8.2-immunized and control animals. Four T-cell lines derived from spleen and lymph nodes were tested extensively for proliferation and cytokine responses, two from mice vaccinated with Vβ8.2 DNA (T-cell lines 8.2-SP and 8.2-LN), and two from controls (T-cell lines PL-LN and PL-Sp). All T-cell lines were 85-90% Vβ8.2$^+$, 95% CD44$^+$, and CD3$^+$,CD4$^+$,CD8$^-$. As shown in Fig. 5*a*, all T-cell lines proliferated in the presence of the peptide Ac1–20 ($P < 0.003$ to $P < 0.0001$ in comparison with background). In contrast to its relatively low proliferative response, the line PL-SP produced high levels of interferon-γ upon stimulation with peptide MBP pAc1–20, similar to the levels produced by the line PL-LN, and higher than the levels produced by the two lines isolated from the mice vaccinated with Vβ8.2 DNA ($P < 0.015$) (Fig. 5*b*). The T-cell lines isolated from the mice vaccinated with Vβ8.2 DNA produced IL-4, whereas no secretion of that cytokine was ob-

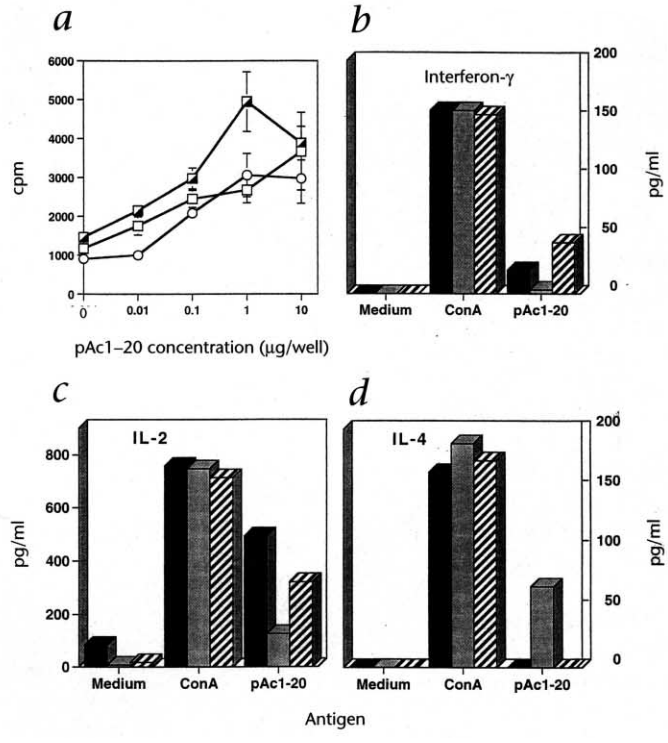

Fig. 3 Cytokine production altered by DNA vaccination. The proliferative response (*a*) in draining lymph node cells from PL/J mice immunized with peptide pAc1–20 after pretreatment with DNA coding for Vβ5.1 (□), Vβ8.2 (○) or with PBS only (◪). Supernatants of lymph node cells from the mice pretreated with DNA coding for Vβ5.1 (■), Vβ8.2 (▩) or with PBS only (▨) were activated with concanavalin A (10 μg/ml) or the peptide pAc1–20 (10 μg/ml) were tested for the presence of interferon-γ (*b*), IL-2 (*c*) and IL-4 (*d*).

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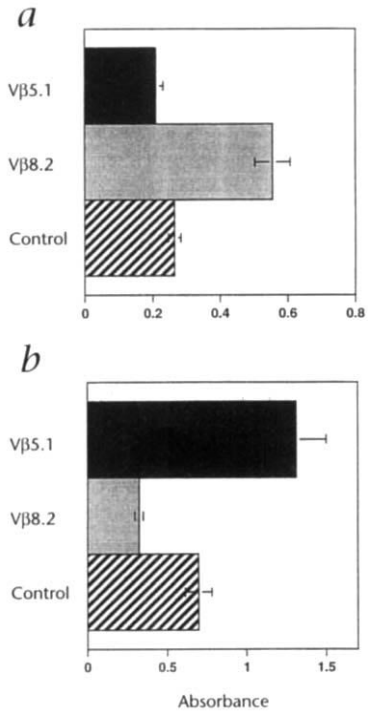

Fig. 4 Antibody isotype altered by DNA vaccination. IgG1 (*a*) and IgG2a (*b*) production in PL/J mice immunized with peptide pAc1–20 after pretreatment with DNA coding for Vβ5.1 (■), Vβ8.2 (▩) or with PBS only (▨).

served from the T-cell lines isolated from the control mice (Fig. 6*a*). As depicted in Fig. 6*b*, IL-10 levels are elevated in the T-cell lines isolated from the mice vaccinated with Vβ8.2 DNA, whereas no secretion of this cytokine, which is characteristic of Th2 cells, was observed from the T-cell lines isolated from the control mice ($P < 0.005$). As seen in Fig. 6*c*, the two T-cell lines isolated from control mice with EAE, produced significantly higher levels of tumor necrosis factor-α (TNF-α) in comparison with the lines isolated from the protected mice ($P < 0.02$). The latter is in agreement with the findings that encephalitogenic T-cell lines produce high levels of TNF-α (ref. 17). These findings all serve to indicate that DNA vaccination to the TCR Vβ8.2 induced a shift in the phenotype of T cells responding to the antigen MBP pAc1–20, and induced a shift from Th1 to Th2.

In order to see whether T-cell lines reactive to MBP pAc1–20 from vaccinated animals could induce EAE, we raised T-cell lines in an identical manner from mice immunized with MBP pAc1–20 and then vaccinated with either Vβ8.2 or Vβ5.1. Five million cells were injected intravenously, and the clinical course of disease was monitored. In Fig. 7 it can be seen that T-cell lines reactive to MBP pAc1–20 from Vβ8.2-immunized mice failed to induce EAE, whereas T-cell lines from Vβ5.1-vaccinated mice developed classic EAE, beginning on day 12.

We next checked to see whether the Th2 T-cell lines directed to MBP pAc1–20 derived from mice immunized with Vβ8.2 DNA would suppress EAE. In Fig. 7*b* it can be seen that five mice receiving 5 million Th2 T cells did not acquire EAE after immunization on the same day with MBP pAc1–20 in complete Freund's adjuvant, whereas five mice receiving MBP pAc1–20 in complete Freund's adjuvant developed grade 2 EAE. Thus, these Th2 cells from mice immunized with Vβ8.2 DNA could suppress EAE.

Discussion

The search for specific immunotherapy for autoimmune diseases mediated by pathogenic T cells has focused on the development of strategies that target the T-cell receptor[19]. In 1981 Ben-Nun, Wekerle and Cohen established the utility of vaccinating animals with pathogenic T cells capable of causing

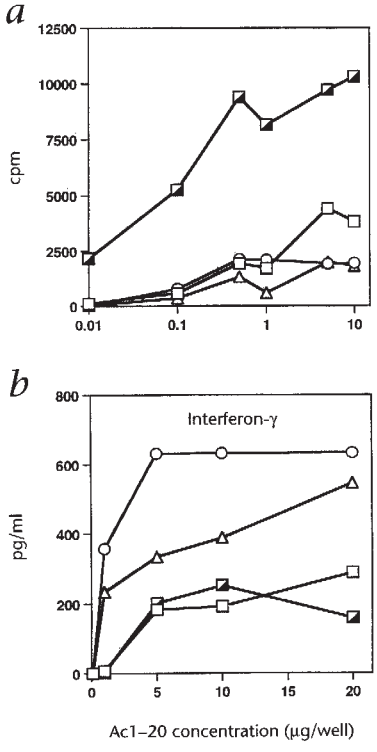

Fig. 5 Proliferation and interferon-γ in T-cell lines. The proliferative response (*a*) and interferon-γ (*b*) production of four pAc1–20-specific T-cell lines isolated from PL/J mice, after immunization with peptide pAc1–20. Two lines, PL-LN (○) and PL-SP (△) were isolated from sick control PL/J mice (vaccinated with Vβ5.1 DNA), whereas two other lines, 8.2-LN (□) and 8.2-SP (◪) were isolated from protected PL/J mice.

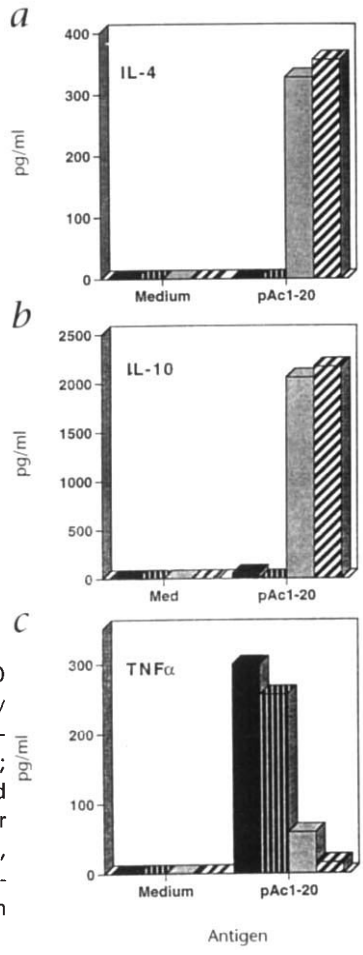

Fig. 6 Interleukin-4, IL-10 and TNF-α production by T-cell lines. The lines described in Fig. 5, PL-LN (■); PL-SP (▥); 8.2-LN (▩) and 8.2-SP (▨) were tested for the production of IL-4 (*a*), IL-10 (*b*) and TNF-α (*c*) following stimulation with 10 μg/ml of peptide Ac1–20.

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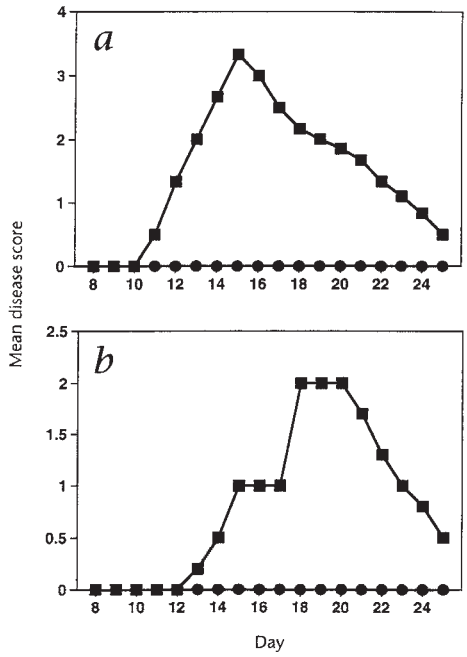

Fig. 7 Disease induction by T-cell lines. *a*, T-cell lines isolated from control PL/J (vaccinated with Vβ5.1) with EAE (■) or from mice protected with the Vβ8.2 DNA, 8.2-LN (●) were inoculated into female PL/J mice intravenously, 5 million cells per mouse. Shown is the mean disease score of all the mice ($n = 5$) in each group ± s.d. *b*, Five million Th2 T cells directed to MBP pAc1–20 derived from mice immunized with Vβ8.2 DNA were able to suppress EAE (●), whereas mice receiving MBP pAc1–20 in complete Freund's adjuvant develop EAE (■).

EAE in order to elicit anti-T cell immunity and to treat clinical disease[20,21]. In 1989 Vandenbark and colleagues[6] and Brostoff and colleagues[7] showed that immunization with peptides spanning either the CDR2 or CDR3 regions of the beta chain of the T-cell receptor, which recognizes the dominant epitope of myelin basic protein, would protect animals from EAE. These approaches, T-cell vaccination[22,23] and immunization with CDR peptides[24,25], have been attempted in humans with multiple sclerosis, in early-stage clinical trials, with some indications of success. For example, vaccination with MBP p84–102 T cells reduced clinical relapses and decreased lesion area on magnetic resonance scan, while reducing the frequency of T cells reactive to MBP (ref. 23).

A novel method for targeting the T cell is explored here, which appears to have unexpected advantages. Immunization with DNA has proven successful in obtaining immunity to microbes and to tumor antigens[8–11]. Here we demonstrate the feasibility of DNA vaccination to a T-cell receptor V gene that is commonly found on pathogenic T cells that cause EAE in H-2^u mice. This approach prevents disease when it is elicited with MBP pAc1–20. T cells with specificity for MBP pAc1–20 predominately rearrange and express the Vβ8.2 product in their TCR. This approach also prevented disease, although to a weaker extent, when EAE was induced with the entire MBP molecule. The response to MBP is more diverse and involves immunity to several epitopes, where Vβ8.2 is not involved in T-cell recognition. Nonetheless, as we have previously shown the response to MBP pAc1–11 dominates the immune response to MBP. Thus when Vβ8.2-bearing T cells are deleted with a monoclonal antibody targeting the Vβ8.2 gene product, EAE can be prevented and even reversed when disease is induced by the entire MBP molecule[3].

Dividing T-cell phenotypes according to the amount and type of cytokine produced after stimulation has proven to be a useful paradigm in cellular immunology[12,13]. Th1 cytokines like TNF-α are involved in the development of EAE, whereas Th2 cytokines like IL-4 and IL-10 suppress the development of EAE (ref. 16, 26). We have demonstrated here that DNA vaccination with a TCR V gene induces a shift in the pattern of cytokine production in T cells that utilize the relevant V gene segment. Thus, after TCR V gene vaccination the immune response to the dominant epitope on MBP shifts from Th1 to Th2. This observation is corroborated by a shift *in vivo* in the isotype of the antibodies produced against the immunodominant antigen MBP pAc1–20 (Fig. 4). The development of Th2 responses toward MBP pAc1–20 may allow the prevention of EAE without the need for depletion of Vβ8.2-bearing T cells. Moreover, these Th2 cytokines may prevent EAE when whole MBP is used as the immunogen, and determinant spreading to other epitopes other than MBP pAc1–20 has occurred[27].

A shift from Th1 to Th2 immunity has not been seen with DNA immunization towards microbial antigens. For example, immunization with pCMV/gp63 DNA, encoding the *Leishmania major* surface glycoprotein) produced T cells making significant amounts of IL-2, interferon-γ, but not IL-4 in response to *L. major*[14]. Clearly immunization with DNA encoding TCR V genes induces alterations in the immune response that are not seen following DNA immunization with other antigens. Whether the shift from Th1 to Th2 immunity is uniquely seen with DNA vaccination to TCR genes is under investigation. Preliminary experiments indicate that vaccination with TCR Vβ8.2 changes the cytokine profile of T cells recognizing sperm whale myoglobin peptide 110–121, recognized predominately by Vβ8.2 T cells, from a Th1 to a Th2 response (data not shown). Thus, DNA vaccination with TCR constructs may induce changes in T cells responding to foreign antigens and not merely in T cells responding to autoantigens like MBP. In contrast the cytokines produced by T cells recognizing purified protein derivative of mycobacterium tuberculosis are not changed (data not shown). Purified protein derivative is not recognized predominately by T cells expressing Vβ8.2 (ref. 3). Thus, the effect may be restricted to T cells utilizing the TCR Vβ gene used in the vaccination.

DNA vaccination to a dominant T-cell receptor V gene found on T cells recognizing a critical pathogenic determinant on a myelin protein may be useful in treating MS. Certain TCR V genes are expanded clonally in the T-cell response to the immunodominant portion of the MBP molecule[22,23,28–32]. It would be possible to target the TCR V genes expressed in these clonally expanded T cells with DNA vaccination. This strategy has the added advantage that the response to the target antigen may then modulate from Th1 to Th2 and thus provide a suppressive environment for the responses to other myelin components that occur in the MS plaque[33,34].

Methods

Plasmid construction. RNA was isolated from normal PL/J mouse spleen cells, as described[35], and used as template for reverse transcriptase using poly(dT) (Boehringer Mannheim) as primer. This cDNA was then used for PCR using *Taq* polymerase (Stratagene, La Jolla,

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California) with primers specific for Vβ5.1 (5'–CCGGAATTCATGAATTCTGGGGTTGTCCAGTCTCCAAGA–3' and 5'–TGCTCTAGATTAGCTGGCACAGAAGTACACGGCAGA–3') or Vβ8.2 (5'–CCGGAATTCATGGAGGCTGCAGTCACCCAAAGC–3' and 5'–TGCTCTAGATTAGCTGGCACAGAAGTACACTGATGT–3'). These primers cover the complete V region (about 310 bp) and include *Eco*RI and *Xba*I sites used for cloning. The PCR products were then cloned into pcDNA3 plasmid (Invitrogen, San Diego, California), that was then used for transfection of competent cells as described[35]. Colonies were picked and used for miniprep (Qiagen, Chatsworth, California). DNA from colonies with an insert in the right length were amplified and sequenced, to verify the insertion of the right gene with an appropriate open reading frame.

DNA injection and preparation. Large-scale preparation of plasmid DNA was conducted using Maxi or Mega prep (Qiagen). Cardiotoxin (Sigma) was prepared as described[9,36]. The latter was found to enhance the efficiency of DNA vaccination[9]. Cardiotoxin (50 ml) was injected into the tibialis anterior muscle of female, 6- to 8-old-week PL/J mice (Jackson Laboratory, Bar Harbor, Maine). One week after injection, mice were injected with 100 μg DNA in PBS (1 mg/ml), three times, with intervals of 6–7 days between each injection. To verify expression, muscle was removed from an injected mouse, and RNA was prepared[35]. This RNA was used as a template for cDNA that was used for PCR, as described. We found PCR products of Vβ5.1 and Vβ8.2 only in the mice injected with the DNA coding for Vβ5.1 and Vβ8.2 genes, respectively (data not shown).

Disease induction. Peptide Ac1–20 of rat MBP (Ac-ASQKRPSQRHGSKYLATAST) or gpMBP (ref. 37) were mixed 1:1 in incomplete Freund's adjuvant (Difco, Detroit, Michigan) supplemented with killed *Mycobacterium tuberculosis*, strain H37 RA (Difco), to a final concentration of 1 mg/ml antigen and 2 mg/ml bacteria. This mixture was then used for intradermal immunization of the treated PL/J mice, 50 μl in each foot pad. Thereafter, mice were immunized with *Bordetella pertussis* toxin (200 ng, List Biological Laboratories, Campbell, California), i.v., on the day of antigen inoculation and 48 h later. Adoptive transfer of EAE was accomplished by i.v. inoculation of 5 million cells of T-cell line per animal. All animals were followed for clinical signs of disease according to the following scale: 0, no clinical disease; 1, tail weakness; 2, paraparesis; 3, paraplegia; 4, paraplegia with forelimb weakness or paralysis; 5, moribund or dead animals.

Fluorescence-activated cell sorting (FACS) after staining. Cells were incubated (5×10^5 cells per tube) with anti-Vβ5, anti-Vβ8.2 or, as control, with anti-Vβ17a antibodies conjugated to fluorescein isothiocyanate (FITC; PharMingen, San Diego, California), as described previously[2]. The cells were also coincubated with either anti-CD3, anti-CD4 or anti-CD8 antibodies (PharMingen) conjugated to phycoerythrin (PE). The cells were then analyzed for the presence of $CD3^+$, $CD4^+$ or $CD8^+$ populations that express Vβ5 or Vβ8.2 TCR. Antibodies to the Vβ8.2 TCR in the sera of the vaccinated mice were analyzed as above, only that an additional step was included, incubation with goat anti-mouse conjugated to FITC (Jackson ImmunoResearch Laboratories, West Grove, Prince Edward Island). In addition, T-cell lines were analyzed for their expression of anti-CD3, CD4, CD8, CD44 surface markers, using antibodies conjugated to FITC (PharMingen).

Lymph node cell proliferation. Lymph node cells and spleen cells of immunized mice (2×10^5 per well) were cultured in the presence of different antigens. Cultures were set up in 200 ml RPMI 1640 medium supplemented with 2 mM glutamine, nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml fungizone (BioLab, Jerusalem, Israel), 5×10^{-5} M β-mercaptoethanol (Fluka AG, Buchs, Switzerland) and 10 mM HEPES buffer (Sigma), referred to as enriched RPMI, containing 1% syngeneic normal mouse serum in round-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark). Following five days' incubation, [^{3}H]thymidine (0.5 mCi of 5 Ci/mmol, Nuclear Research Center, Negev, Israel) was added. Sixteen hours later, cells were harvested, and radioactivity was counted.

T-cell lines. Lymph node cells ($5–10 \times 10^6$/ml) were incubated in enriched RPMI supplement with 1% syngeneic sera with 10 μg/ml peptide Ac1–20 for 3 days. Thereafter cells were washed and resuspended in rest medium (enriched RPMI, 10% FCS and 10% supernatant of spleen cells activated with concanavalin A) for 10 days. The cells were then activated in the presence of syngeneic irradiated spleen cells (10^6/ml) and 10 μg/ml pAc1–20 for 3 days, washed and incubated in resting medium for 3 days. The cells were continuously grown in the above 2-week cycles. They were used for analysis, typically, 1 week after stimulation.

T-cell line proliferation assay. Proliferation assays were conducted as previously described above for lymph node cells with minor changes: T-cell lines (10^4) were incubated in round-bottomed plates (Corning, Corning, New York) with 2×10^5 irradiated syngeneic APC in a total volume of 250 μl enriched RPMI and 10% FCS, and different concentrations of the peptide. After 24 h, 100 μl was removed from each well for cytokine secretion analysis. The remaining cells were incubated for an additional 24 h, pulsed and harvested as in the lymph node cell proliferation assay.

Cytokines from lymph node cells and T-cell lines. Lymph node cells or spleen cells (1×10^7) were incubated in enriched RPMI with 1% syngeneic sera with 10 μg of the indicated peptides. T-cell lines were incubated in the same antigen concentration, but the medium was supplemented with 10% FCS. Medium was collected 24 h later. The levels of IL-2 and interferon-γ were measured using the antibody pairs purchased from PharMingen, according to the manufacturer's instructions. The levels of IL-4, IL-10 and TNF-α were measured using kits purchased from Genzyme (Cambridge, Massachusetts).

Anti-pAc1–20 antibodies. Mice were bled at the peak of disease, and antibodies against pAc1–20 were measured. Maxisorb microtiter plates (Nunc) were coated with 50 ml per well of 10 mg/ml pAc1–20 for 90 min. Thereafter, the plates were washed and blocked for overnight with 10% FCS in PBS. Thereafter, the sera of the mice were incubated for 90 min. Plates were then washed and incubated for 75 min with goat anti-mouse IgG1 or IgG2a (Fc fragment-specific) conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, Alabama). After washing, plates were incubated with the substrate, ABTS (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland), and read at 405 nm using an ELISA reader.

Statistical analysis. Significance of differences was examined using Student's *t*-test for proliferation and cytokine secretion assays. For disease severity the Mann-Whitney test was utilized. For analysis of FACS data the chi-square test was used. A value of $P < 0.05$ was considered significant.

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