A Suppressive Oligodeoxynucleotide Enhances the Efficacy of Myelin Cocktail/IL-4-Tolerizing DNA Vaccination and Treats Autoimmune Disease[1]

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Targeting pathogenic T cells with Ag-specific tolerizing DNA vaccines encoding autoantigens is a powerful and feasible therapeutic strategy for Th1-mediated autoimmune diseases. However, plasmid DNA contains abundant unmethylated CpG motifs, which induce a strong Th1 immune response. We describe here a novel approach to counteract this undesired side effect of plasmid DNA used for vaccination in Th1-mediated autoimmune diseases. In chronic relapsing experimental autoimmune encephalomyelitis (EAE), combining a myelin cocktail plus IL-4-tolerizing DNA vaccine with a suppressive GpG oligodeoxynucleotide (GpG-ODN) induced a shift of the autoreactive T cell response toward a protective Th2 cytokine pattern. Myelin microarrays demonstrate that tolerizing DNA vaccination plus GpG-ODN further decreased anti-myelin autoantibody epitope spreading and shifted the autoreactive B cell response to a protective IgG1 isotype. Moreover, the addition of GpG-ODN to tolerizing DNA vaccination therapy effectively reduced overall mean disease severity in both the chronic relapsing EAE and chronic progressive EAE mouse models. In conclusion, suppressive GpG-ODN effectively counteracted the undesired CpG-induced inflammatory effect of a tolerizing DNA vaccine in a Th1-mediated autoimmune disease by skewing both the autoaggressive T cell and B cell responses toward a protective Th2 phenotype. These results demonstrate that suppressive GpG-ODN is a simple and highly effective novel therapeutic adjuvant that will boost the efficacy of Ag-specific tolerizing DNA vaccines used for treating Th1-mediated autoimmune diseases. ***The Journal of Immunology,*** **2005, 175: 6226–6234.**

Vaccination using naked DNA encoding self-Ag protects and even reverses established disease in several autoimmune disease animal models for various diseases, including rheumatoid arthritis (1–3), insulin-dependent diabetes mellitus (4–9), and multiple sclerosis (10–17). Experimental autoimmune encephalomyelitis (EAE)[8] is a prototypical animal model of inflammatory T cell-mediated autoimmunity that shares both clinical and histopathologic features with the human disease, multiple sclerosis (18). DNA encoding a single encephalitogenic epitope can be used to prevent the initial onset of EAE by suppressing the autoimmune response via anergizing the autoreactive T cell (11). DNA vaccination encoding either proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG) in combination with IL-4 DNA proved to be a powerful method of modulating an immune response by causing autoimmune encephalitogenic T cells to shift to a protective Th2 phenotype (12). A DNA cocktail composed of full-length cDNAs encoding the four major components of myelin, myelin basic protein (MBP), myelin-associated glycoprotein (MAG), MOG, and PLP with or without the addition of a plasmid encoding IL-4, reduced the spread of autoantibody responses to epitopes on various myelin molecules, concomitant with a reduction in relapse rate, when given after initial disease onset characterized by hind limb paralysis (13).

One potential undesirable side effect of this approach arises from the delivery of bacterial plasmid DNA into mouse tissues, which contains proinflammatory unmethylated stimulatory CpG dinucleotides interspersed throughout the bacterial plasmid DNA. CpG motifs bind TLR9 and activate the innate immune system to generate an inflammatory milieu that is required for the induction of an Ag-specific

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[8] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; PLP, proteolipid protein; MOG, myelin oligodendrocyte glycoprotein; MBP, myelin basic protein; MAG, myelin-associated glycoprotein; ODN, oligodeoxynucleotide; CR-EAE, chronic relapsing EAE; NOGO, Nogo A; SAM, Statistical Analysis for Microarrays.

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immune response to DNA vaccines (19–21). Thus, to completely remove all CpG motifs within the plasmid backbone may actually be detrimental to DNA vaccine therapy. The efficacy of DNA vaccination depends on two criteria: 1) a plasmid backbone that delivers internal adjuvant activity via endogenous CpG motifs; and 2) a transcription unit for specific Ag synthesis (20, 22). Therefore, a more plausible scenario is to maintain a localized inflammatory response to effectively promote the effects of tolerizing DNA vaccination without enhancing a widespread uncontrolled inflammatory response that may worsen a Th1 autoimmune disease such as EAE.

We have reported previously that a suppressive GpG-oligodeoxynucleotide (ODN), with a single base switch from cytosine to guanine, can inhibit the immunostimulatory response induced by its CpG-ODN counterpart (23). Suppressive GpG-ODN specifically promotes Th2 cell proliferation and is capable of suppressing the disease severity of chronic relapsing EAE (CR-EAE) in mice when administered at the time of disease induction, and in treatment mode, mice receiving the suppressive GpG-ODN at the peak of disease exhibit improvement in overall mean disease score (23). Therefore, we hypothesized that combining a tolerizing myelin cocktail/IL-4 DNA vaccine with this suppressive GpG-ODN could potentially enhance the effectiveness of tolerizing DNA vaccines by reducing nonspecific systemic proinflammatory responses while still maintaining a localized milieu for "suppressive vaccination."

Materials and Methods

Mice

Female SJL/J and C57BL/6 (B6) mice were obtained from The Jackson Laboratory and were between 8 and 12 wk of age when experiments were initiated. All animal protocols were approved by the Division of Comparative Medicine at Stanford University and the Committee of Animal Research at the University of California, San Francisco, in accordance with the National Institutes of Health guidelines.

Reagents

Immunizing peptides in these experiments PLP p139–151 (HCLGKWL GHPDKF) and MOG p35–55 (MEVGWYRSPFSRVVHLYRNGK) were synthesized on a peptide synthesizer (model 9050; MilliGen) and purified by HPLC to >95% purity. DNA constructs encoding full-length mouse MBP, PLP, MOG, MAG, and IL-4 in the pTARGET CMV promoter-driven mammalian expression vector (Promega) have been described previously (12, 13). DNA plasmids were grown in the *Escherichia coli* strain JM-109 (Promega), and plasmid DNA purification was performed using Qiagen Endo-Free Giga Prep kits (Qiagen Operon) and confirmed as described previously (12). CpG-ODN 5′-TGACTGTGAACGTTAGAGATGA-3′ and GpG-ODN 5′-TGACTGTGAAGGTTAGAGATGA-3′ were synthesized with a phosphorothioate backbone by Qiagen Operon (the underlining indicates the CpG motif and its corresponding single base substitution).

EAE induction

SJL female mice were immunized s.c. with 0.1 mg of $PLP_{139-151}$ in PBS emulsified in CFA, consisting of immunofluorescence assay (Sigma-Aldrich) and 0.4 mg of heat-inactivated *Mycobacterium tuberculosis* (strain H37 RA; Difco). B6 female mice were immunized s.c. with 0.1 mg of MOG_{35-55} in PBS emulsified in CFA. On days 0 and 2, B6 mice were also injected i.v. with 400 ng of pertussis toxin in 0.1 ml of PBS. Animals were clinically scored daily using the following scale: grade 1, tail paralysis; grade 2, hind limb paraparesis; grade 3, hind limb paralysis; grade 4, complete paralysis (tetraplegy); and grade 5, death. A relapse is defined as an increase in one point or more in the EAE scale sustained for a least 2 consecutive days. Relapse rate is calculated as mean relapses of a treatment group divided by the total number of experimental days.

DNA immunization protocol

Mice were injected in both quadriceps with a total of 0.1 ml of 0.25% bupivicaine-HCl (Sigma-Aldrich) in PBS (no. 9236; Invitrogen Life Technologies). Two days later, mice were injected i.m. in the quadriceps with 0.025 mg of each myelin DNA (cocktail) and 0.05 mg of IL-4 DNA in a total volume of 0.1 ml of PBS/quadricep. At the same time as the initiation of DNA and subsequently thereafter, mice received i.p. injections of 0.05 mg of indicated ODN in 0.2 ml of PBS.

Cytokine analysis

T cell lines were established from lymph node cells derived from all groups as described previously (17). T cells were incubated with irradiated syngenic APCs in enriched RPMI-10 and 0.01 mg/ml PLP p139–151. After 72 h of culture, the supernatants were collected and assayed in triplicate for the production of IFN-γ, IL-4, and IL-10 by sandwich ELISA using standard ELISA kits (BD Pharmingen).

Array data analysis

Myelin arrays and the associated methods used in this work were previously described in detail (13, 24, 25). Ordered Ag arrays were produced using a robotic microarrayer to attach myelin and control peptides and proteins to ArrayIt SuperEpoxy microscope slides (TeleChem International). The 2304-feature myelin proteome arrays contain ~515 Ags, including proteins, and sets of overlapping peptides representing Ags relevant to EAE, including MBP, PLP, MOG, MBOP, oligodendrocyte-specific protein, αB-crystallin, cyclic nucleotide phosphodiesterase, Nogo A (NOGO), and Golli-MBP (26–28).

Arrays were probed with 1/200 dilutions of serum from individual mice. Reactive Abs were detected using Cy5-conjugated goat anti-mouse IgG1 (1/500 dilution; Southern Biotechnology Associates) in combination with either Alexa 555-conjugated goat anti-mouse IgG2a (1/300 dilution; Molecular Probes) or Cy3-conjugated goat anti-mouse IgM (1/1000 dilution; Southern Biotechnology Associates and Rockland) before scanning. GenePix Pro 5.0 software (Axon Instruments) was used to determine the net median pixel intensities for each Ag feature. Data analysis was performed using Statistical Analysis for Microarrays (SAM) software (⟨http://www.stat-class.stanford.edu/SAM/servlet/SAMServlet⟩) to identify Ag features with statistically significant differences in reactivities between the treatment groups. The reported Ag lists are SAM-identified features with q values <0.29% for Fig. 4*A*, <20% for Fig. 4*B*, and <15% for Fig. 4*C*. SAM-identified Ag features were further selected using a numerator threshold of 0.25. Cluster software was then used to hierarchically group the mice and Ag features based on a pairwise similarity function, and TreeView software was used to display the data as a heat map (⟨http://rana.lbl.gov/EisenSoftware.htm⟩; Ref. 29).

Pathology

Mice were euthanized and perfused with 10% buffered formalin. Brains and spinal cords were extracted, embedded in paraffin, sectioned, and stained with H&E or Luxol fast blue (LFB) according to standard procedures. Perivascular inflammatory foci in meninges and parenchyma and numbers of demyelinating lesions were enumerated in representative slides by a neuropathologist who was blinded to the treatment groups. Both the brain and spinal cord from representative mice were examined by our neuropathologist; however, the cerebellum was examined in isolation to maximize uniformity of what was analyzed by the LFB stain. Axonal injury was evaluated in representative brain and spinal cord tissues using Bielschowsky silver impregnation.

Results

Combination therapy treats chronic relapsing EAE in SJL mice when instituted at peak onset of paralysis

To assess whether combination therapy could modulate disease after the onset of paralytic chronic relapsing disease, SJL/J mice were immunized with PLP 139–151 in CFA. At the peak of acute EAE, when mice were paralyzed, the mice were randomized into groups, and treatment was initiated following the regimen depicted in Fig. 1*A*. The myelin cocktail/IL-4-tolerizing DNA vaccine was administered i.m. with stimulatory CpG-ODN or suppressive GpG-ODN administered i.p. Mice receiving the myelin cocktail/IL-4 DNA vaccine alone (Fig. 1*B*) or in combination with the stimulatory CpG-ODN (Fig. 1*C*) had overall mean disease scores that were not significantly different from the untreated group. Mice receiving the combination of the myelin cocktail/IL-4 DNA vaccine with suppressive GpG-ODN (Fig. 1*D*) had the most dramatic improvement in the overall mean disease score, reaching statistical significance compared with the untreated cohort during most of the course of the experiment. Mice treated with just myelin cocktail DNA plus suppressive GpG-ODN did not show any significant improvement compared with the untreated group (data not

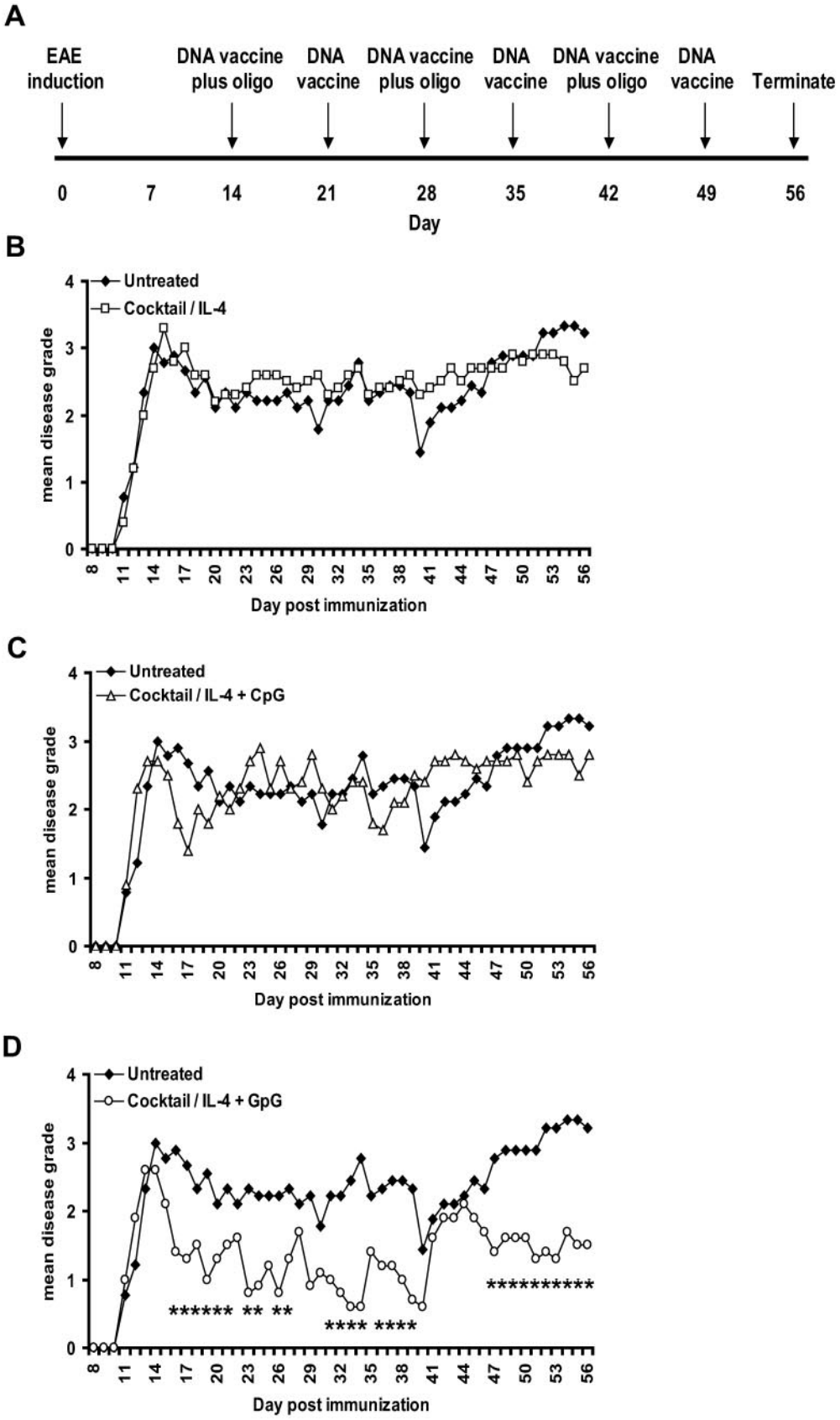

FIGURE 1. Combination therapy with myelin/IL-4-tolerizing DNA vaccine plus GpG-ODN treats chronic relapsing EAE in SJL mice. Female SJL/J mice were induced for EAE with PLP 139–151 peptide in CFA. *A*, At the peak of acute disease (day 14), mice were randomized and treated with weekly i.m. injections of myelin cocktail DNA (consisting of full-length PLP, MBP, MOG, and MAG) and IL-4 DNA and biweekly i.p. injections of oligo (CpG or GpG). Treatment groups include cocktail/IL-4 (*B*), cocktail/IL-4 + CpG-ODN (*C*), and cocktail/IL-4 + GpG-ODN (*D*). Mean EAE scores are plotted against the number of days post-EAE immunization. The asterisks indicate a statistical significant difference ($p < 0.05$ by Mann-Whitney Wilcoxon test) comparing the treatment group vs the untreated group. This experiment was performed three times with similar results.

shown), thus indicating that local gene delivery of IL-4 is still required for effective treatment with DNA vaccination (12).

The relative relapse rate in each group was also affected (Table I). Mice receiving just the myelin cocktail vaccine alone (0.4 ± 0.2 relapses, $p < 0.05$) and the myelin cocktail/IL-4 DNA vaccine alone (0.6 ± 0.3 relapses) had the lowest overall relapse rate over the 56 day time course when compared with the untreated mice (1.3 ± 0.3 relapses). These same treatment groups also had the highest percent of relapse-free mice, with 55.6% relapse-free mice in the myelin cocktail vaccine group and 50.0% relapse-free mice in the myelin cocktail/IL-4 DNA vaccine group when compared with 12.5% relapse-free mice in the untreated group. However, using the myelin cocktail vaccine alone or in combination with just the IL-4 DNA vaccine does not suppress overall disease severity, as highlighted in Table I. When mice were treated with either the myelin cocktail with the stimulatory CpG-ODN or the suppressive GpG-ODN, the relapse rates were the same (1.3 ± 0.2), and none of the mice were relapse free. Also, as highlighted in Table I, the overall mean disease severity in these two treatment groups were unchanged. In contrast, mice receiving the combination of the myelin cocktail/IL-4 DNA vaccine with stimulatory CpG-ODN had an increase in relapse rate (1.8 ± 0.3 relapses, $p < 0.05$ when compared with cocktail, cocktail/IL-4, and cocktail/IL-4 with GpG-ODN) with no relapse-free mice. However, mice receiving the combination of the myelin cocktail/IL-4 DNA vaccine with suppressive GpG-ODN achieved a significance value of $p < 0.05$ on days 24, 34, and 54 in reducing overall mean disease severity but had no statistical effect in reducing relapse rate (1.1 ± 0.2 relapses).

Combination therapy reduces overall CNS pathology in EAE

Histopathological examination of the CNS was performed to evaluate the efficacy of myelin cocktail/IL-4 DNA vaccine with GpG-ODN on inflammatory lesions. As shown in Fig. 2*A*, the mean number of meningeal and perivascular parenchymal cellular infiltrates were decreased in mice treated with the myelin cocktail/IL-4 DNA vaccine alone and further decreased with the combination of the myelin cocktail/IL-4 DNA vaccine with suppressive GpG-ODN when compared with the untreated group. The treatment group receiving the myelin cocktail/IL-4 DNA vaccine with stimulatory CpG-ODN had increased numbers of meningeal and perivascular parenchymal cellular infiltrates as compared with the untreated group. Axonal injury was also evaluated in representative brain and spinal cord tissues using Bielschowsky silver impregnation. Focal axonal injury was identified in areas with large parenchymal inflammatory foci (data not shown). There was also a decrease in the number of focal demyelinating lesions in the group receiving the combination of the myelin cocktail/IL-4 DNA with suppressive GpG-ODN (Fig. 2*B*). Thus, combination DNA vaccine therapy with GpG-ODN results in a reduction in both clinical disease and histopathological lesions in CR-EAE.

Combination therapy produces a PLP 139–151-reactive Th2 cell phenotype

Cytokine analysis of PLP 139–151-specific T cells generated from each treatment group indicated a predominantly Th1 phenotype, with elevated IFN-γ levels, in mice treated with myelin cocktail/IL-4 DNA vaccine (Fig. 3*A*). Mice treated with the combination of the myelin cocktail/IL-4 DNA vaccine with suppressive GpG-ODN resulted in a PLP 139–151-specific T cell shift toward a Th2 phenotype, with decreased levels of IFN-γ (Fig. 3*A*) and increased levels of IL-4 (Fig. 3*B*) and IL-10 (Fig. 3*C*) production. Surprisingly, mice treated with the combination of myelin cocktail/IL-4 DNA with stimulatory CpG-ODN had equivalent levels of IFN-γ, IL-4, and IL-10 cytokine production as the untreated group, with no definitive trend toward a Th1 or Th2 phenotype.

Combination therapy further reduces autoreactive B cell diversity and shifts to PLP 139–151-reactive IgG1 autoantibody isotype

Delivery of the myelin cocktail/IL-4 DNA-tolerizing vaccine alone reduced epitope spreading of anti-myelin autoreactive B cell responses (13). To determine whether combination therapy with the myelin cocktail/IL-4 DNA vaccine plus GpG-ODN could further reduce epitope spreading of autoreactive B cell responses, sera were collected from the treatment groups at the termination of the experiment and analyzed using myelin microarrays. Myelin arrays were first used to analyze combined IgG1 (Th2) and IgG2a (Th1) autoantibody reactivities. The greatest extent of intermolecular epitope spreading of anti-myelin autoreactive B cell responses was observed in the untreated group (Fig. 4*A*). The highest B cell reactivity (red features on the heat map) was observed against the immunizing Ag, PLP 139–151. There was also intermolecular B cell spreading to target peptide sequences derived from MBP, MOG, myelin-associated oligodendrocytic basic protein, MAG, αB-crystallin, and to NOGO peptide 1–22, a Th1 epitope of NOGO-66 (28). Mice treated with myelin cocktail/IL-4 DNA exhibited multiple reductions in the epitope spreading of autoreactive B cell responses (decreased yellow feature intensity). Unexpectedly, mice receiving the combination of myelin cocktail/IL-4 DNA plus the suppressive GpG or stimulatory CpG-ODN exhibited the greatest reductions in anti-myelin IgG1 and IgG2a autoantibody production (decreased red and yellow features and increased black features). This demonstrates that the addition of a stimulatory CpG-ODN or addition of an suppressive GpG-ODN with the myelin cocktail/IL-4 DNA vaccine both reduce epitope spreading of anti-myelin B cell responses.

Table I. *Myelin cocktail/IL-4 DNA + GpG-ODN treats relapsing/remitting EAE in SJL/J mice*

Treatment	*n*	Percent Incidence	Mean Score (Day 14)[a]	Mean Score (Day 24)	Mean Score (Day 34)	Mean Score (Day 44)	Mean Score (Day 54)	Relapse Rate[a]	Percent Relapse-Free
Untreated	9	100	3.0 ± 0.4	2.2 ± 0.5	2.8 ± 0.5	2.2 ± 0.5	3.3 ± 0.4	1.3 ± 0.3	12.5
Cocktail	10	100	2.9 ± 0.3	2.9 ± 0.4	2.4 ± 0.4	2.0 ± 0.4	2.4 ± 0.3	0.4 ± 0.2[b]	55.6
Cocktail + CpG	9	100	3.0 ± 0.4	2.7 ± 0.6	2.3 ± 0.6	3.4 ± 0.3	3.4 ± 0.5	1.3 ± 0.2	0.0
Cocktail + GpG	10	100	2.7 ± 0.4	1.7 ± 0.4	2.4 ± 0.4	2.7 ± 0.4	3.1 ± 0.4	1.3 ± 0.2	0.0
Cocktail/IL-4	10	100	2.7 ± 0.4	2.6 ± 0.5	2.7 ± 0.5	2.5 ± 0.5	2.8 ± 0.5	0.6 ± 0.3	50.0
Cocktail/IL-4 + CpG	10	100	2.7 ± 0.4	2.9 ± 0.4	2.4 ± 0.5	2.7 ± 0.4	2.8 ± 0.4	1.8 ± 0.3	0.0
Cocktail/IL-4 + GpG	10	100	2.6 ± 0.2	0.9 ± 0.3[c]	0.6 ± 0.4[c]	2.1 ± 0.3	1.7 ± 0.2[c]	1.1 ± 0.2	10.0

[a] Means given as mean ± SEM.
[b] Values $p < 0.05$, given as a comparison to the untreated group by Student's *t* test.
[c] Values of $p < 0.05$, given as a comparison to the untreated group by Mann-Whitney Wilcoxon test.

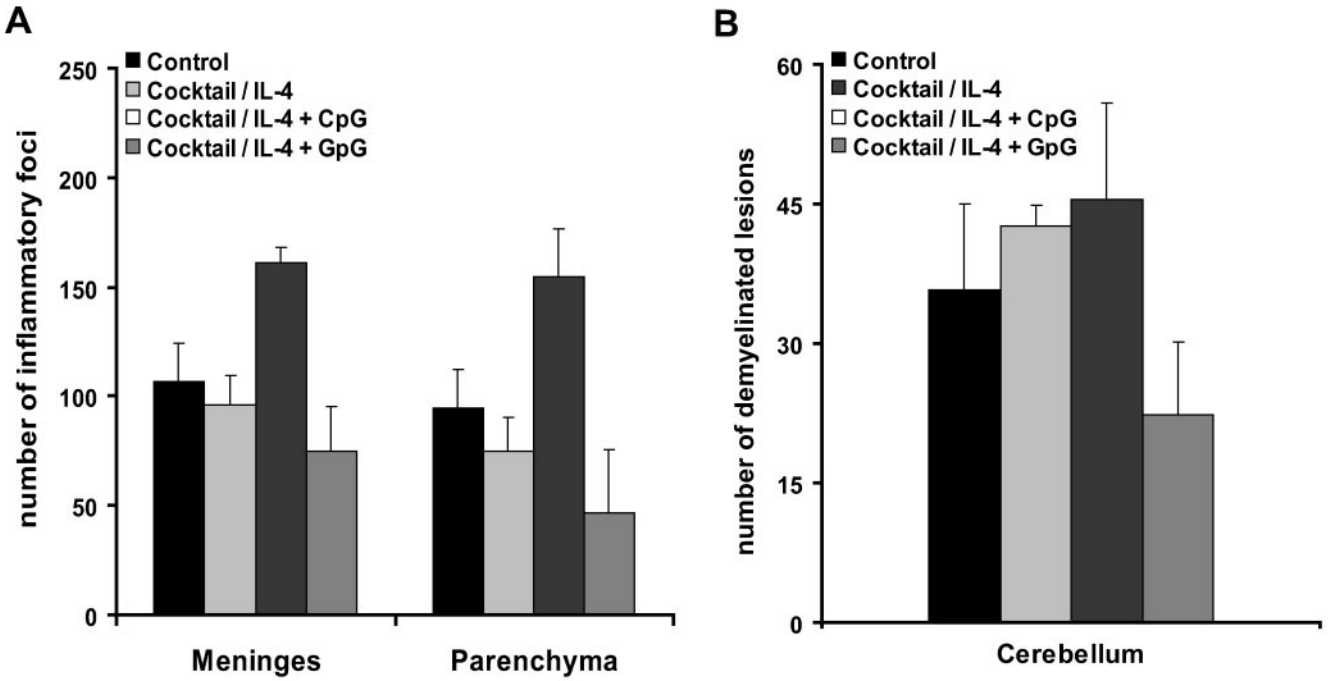

FIGURE 2. Combination therapy with myelin/IL-4-tolerizing DNA vaccine plus GpG-ODN reduces overall CNS pathology. On day 56, brains and spinal cords from representative mice described in Fig. 1 were harvested, formalin-fixed, embedded, and sectioned for H&E stain used to identify the number of inflammatory foci within the meninges and perivascular parenchyma (*A*) and LFB stain to identify the number of demyelinated lesions within the cerebellum of EAE-diseased SJL mice selected from each treatment group (*B*). The error bars are reported as the SEM within each treatment group.

The observation that mice receiving the combination of myelin cocktail/IL-4 DNA plus the suppressive GpG-ODN or stimulatory CpG-ODN exhibited the greatest reductions in anti-myelin IgG1 and IgG2a autoantibody production suggests a discordance between the extent of spreading of the autoantibody response with the relapse rate and degree of disease severity. To better understand the relationship between the reduction of autoantibody diversity observed in the myelin cocktail/IL-4 DNA with stimulatory CpG-ODN group, which had the highest overall disease grade and relapse rate, and the myelin cocktail/IL-4 DNA with suppressive GpG-ODN group, which had the lowest overall disease grade and a lowered relapse rate, the myelin array data from Fig. 4*A* were further analyzed to assess the ratio of IgG2a vs IgG1 reactivity. The heat map in Fig. 4*B* demonstrates the autoantibody response to the immunizing PLP 139–151 Ag in mice receiving myelin cocktail/IL-4 DNA with suppressive GpG-ODN resulted in production of anti-myelin Abs predominantly of the IgG1 isotype (blue features), whereas autoantibody responses to the immunizing PLP 139–151 Ag in mice receiving myelin cocktail/IL-4 DNA with stimulatory CpG-ODN were mixed or predominantly of the IgG2a isotype (black to yellow features). This suggests that a protective Th2-associated B cell response (IgG1) was induced by myelin cocktail/IL-4 DNA with suppressive GpG-ODN. In contrast, myelin cocktail/IL-4 DNA with stimulatory CpG-ODN resulted in an autoaggressive Th1-type B cell response (IgG2a), which was associated with a more severe disease course comparable to that of untreated mice (Fig. 1). Total IgM autoantibody responses between the two treatment groups were also analyzed. The heat map in Fig. 4*C* depicts an increased amount of IgM (red features) produced against the immunizing PLP 139–151 Ag in sera derived from mice receiving myelin cocktail/IL-4 DNA with suppressive GpG-ODN as compared with mice receiving myelin cocktail/IL-4 DNA with stimulatory CpG-ODN. This suggests that the uncommitted B cell response in the myelin cocktail/IL-4 DNA with suppressive GpG-ODN remained immature (more IgM) than the myelin cocktail/IL-4 DNA with stimulatory CpG-ODN (less IgM).

Combination therapy also treats chronic progressive EAE in B6 mice when instituted after the onset of paralysis

Finally, in contrast to the treatment of chronic relapsing EAE, we wanted to determine whether the same myelin cocktail/IL-4 DNA vaccine with suppressive GpG-ODN could be as effective in the MOG-induced chronic progressive EAE model in B6 mice. Female B6 mice were immunized s.c. with MOG 35–55 in CFA and given pertussis toxin i.v. at the same time and 2 days later. Twenty-three days later, at a time when the mean score was between 1 (tail paralysis) and 2 (hind limb paraparesis), the mice were randomized into groups and treatment was initiated, following the treatment regimen depicted in Fig. 5*A*. The same myelin DNA cocktail, consisting of PLP, MBP, MOG, or MAG DNA, plus IL-4 DNA was administered i.m. once a week for 10 wk. At the same time, stimulatory CpG-ODN or suppressive GpG-ODN was administered i.p. every week. As a separate control, empty pTarget plasmid was administered once a week. As shown in Fig. 5, mice receiving empty vector (Fig. 5*B*) or myelin cocktail/IL-4 DNA with the stimulatory CpG-ODN (Fig. 5*D*) had overall mean disease scores that were equal to the untreated group. Mice receiving the myelin cocktail/IL-4 DNA vaccine alone (Fig. 5*C*) had some improvement in overall mean disease score. Similarly to the chronic relapsing EAE data in SJL mice from Fig. 1, B6 mice receiving the combination of the myelin cocktail/IL-4 DNA vaccine with suppressive GpG-ODN (Fig. 5*E*) had the most dramatic improvement from chronic progressive EAE, with over 25 days showing statistically significant lower mean disease scores than the untreated group. These data indicate that Ag-specific tolerizing DNA vaccination is indeed effective in treating a second disease model of multiple sclerosis, chronic progressive EAE, and is further enhanced by the addition of a suppressive GpG-ODN.

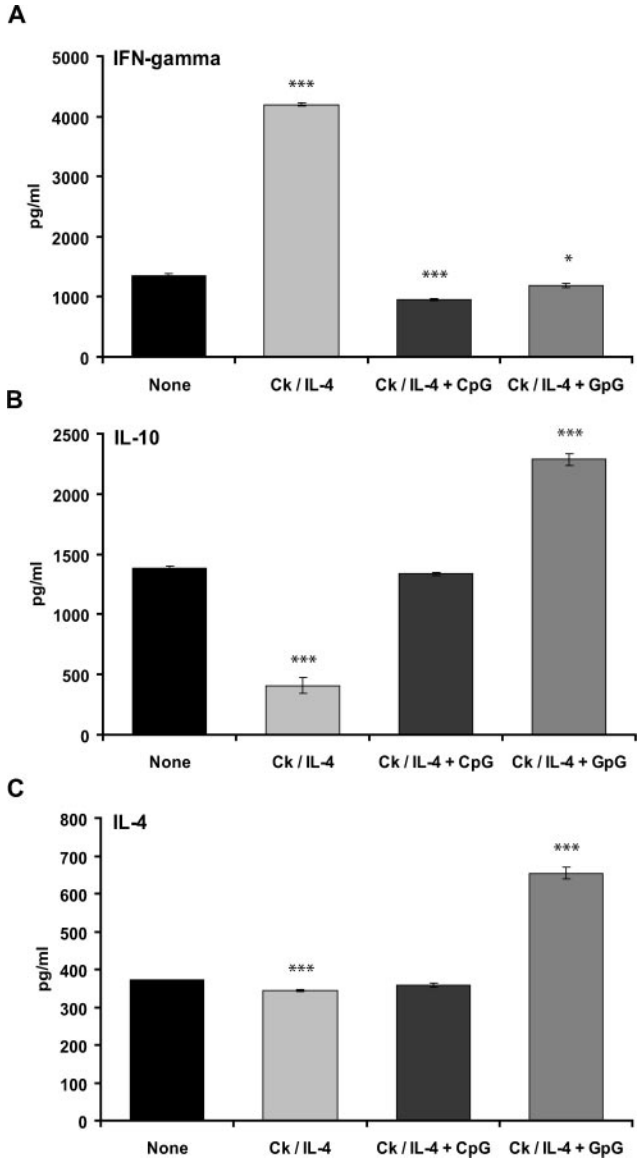

FIGURE 3. Combination therapy with myelin/IL-4-tolerizing DNA vaccine plus GpG-ODN promotes PLP 139–151-specific Th2 cells. On day 56, lymphocytes harvested from mice described in Fig. 1 were stimulated with PLP 139–151. Ag stimulation was performed twice more, every 14 days, before supernatants were harvested and assayed for IFN-γ production (*A*), IL-4 production (*B*), and IL-10 production (*C*). The asterisks indicate a statistically significant difference by Student's two-tailed unpaired *t* test comparing the treatment groups vs the untreated group. (*, $p < 0.05$; ***, $p < 0.0005$).

Discussion

The basis for Ag-specific immune therapy against autoimmune diseases is to develop a therapeutic with predetermined specificity that will down-regulate the body's ongoing destructive autoimmune response to self-Ags. Initially, vaccination with naked DNA was directed against infectious agents and oncologic diseases to evoke a robust cellular and humoral response. More recently, DNA vaccination was demonstrated to protect against autoimmunity by selectively tolerizing autoreactive immune cells. For use in the clinic, protective strategies are far less important than strategies capable of reversing ongoing disease, because for the foreseeable future, it is unlikely that preventive strategies will be used to treat autoimmune diseases.

The etiopathogenesis of autoimmune diseases is attributed to the combination of both genetic and environmental factors. Bacterial infections are potential triggers in the initiation of autoimmune diseases. The current theory is that certain microbial molecular structures resembling molecules of self can provoke autoimmunity through molecular mimicry (30, 31). Alternatively, bacterial products, such as cell wall components and CpG DNA, potently stimulate nonspecific inflammatory responses through the innate immune system that could also evolve into autoimmune responses in predisposed individuals. Recent animal model studies have shown that suppressive ODN therapy may inhibit the development of inflammatory organ-specific autoimmune diseases such as arthritis and multiple sclerosis and delay the onset of the systemic autoimmune disease, systemic lupus erythematosus (23, 32–34). The direct mechanism by which suppressive ODN treats autoimmune disease still remains to be elucidated; however, we reported that our suppressive GpG-ODN inhibited CpG-ODN-driven stimulation and promoted the proliferation of a PLP 139–151 Th2 cell line but not a PLP 139–151 Th1 cell line (23).

With DNA vaccination alone, CpG motifs within the plasmid backbone work as an adjuvant, triggering an uncontrolled proinflammatory response. However, DNA vaccination in combination with local IL-4 gene delivery and the administration of a suppressive GpG-ODN reduces the proinflammatory effects generated by the CpG motifs. In this study, the efficacy of myelin cocktail/IL-4-tolerizing DNA vaccination is dramatically improved with the administration of a suppressive GpG-ODN, as indicated by a shift of both autoreactive T and B cells toward a Th2 bias and a significant reduction in CR-EAE disease severity. By using large-scale myelin proteome microarrays, the data not only show that autoantibody diversity can be predictive of disease severity and further relapses, but more importantly, the actual autoantibody isotype may be the key to evaluating disease activity and treatment efficacy.

Lobell et al. (21) reported the importance of CpG DNA in the backbone of the plasmid in producing a "protective" T1-inducing immunity with a MOG peptide DNA vaccine. We, in contrast, believe that the presence of CpG DNA in the plasmid backbone hinders the full potential of our tolerizing myelin cocktail/IL-4 DNA vaccine by creating a continuous nonspecific inflammatory response in vivo when administered weekly for 6–10 wk. Thus, when treating with the tolerizing myelin cocktail/IL-4 DNA, we only observed a reduction in relapse rate and decreased autoantibody diversity (13). However, by mixing five separate plasmids together, the CpG-DNA load is increased dramatically, which we believe is the underlying reason why overall disease severity remained unchanged (Ref. 13; Fig. 1*B*). Therefore, even though SJL mice receiving myelin cocktail/IL-4 DNA + CpG-ODN exhibited severe clinical and histopathological EAE (Figs. 1*C* and 2) and unchanged relapse rate (Table I), the observed decrease in autoantibody diversity (Fig. 4*A*) may be an indication that the tolerizing myelin cocktail/IL-4 DNA vaccine is still effective in modulating the immune response.

In Robinson et al. (13), we demonstrated that greater diversity of autoreactive B cell responses at the time of recovery from acute EAE predicted increased subsequent disease activity. There are several potential explanations for the apparent discordance of autoantibody diversity with disease severity when comparing these experiments. First, there are clear differences in the methodologies: here, we used the myelin microarrays to detect specific autoantibody isotypes (IgG1, IgG2a, and IgM), whereas previously we looked at total IgG/M reactivity (13). Second, due to the nature of SAM analysis, identified Ag features with statistically significant

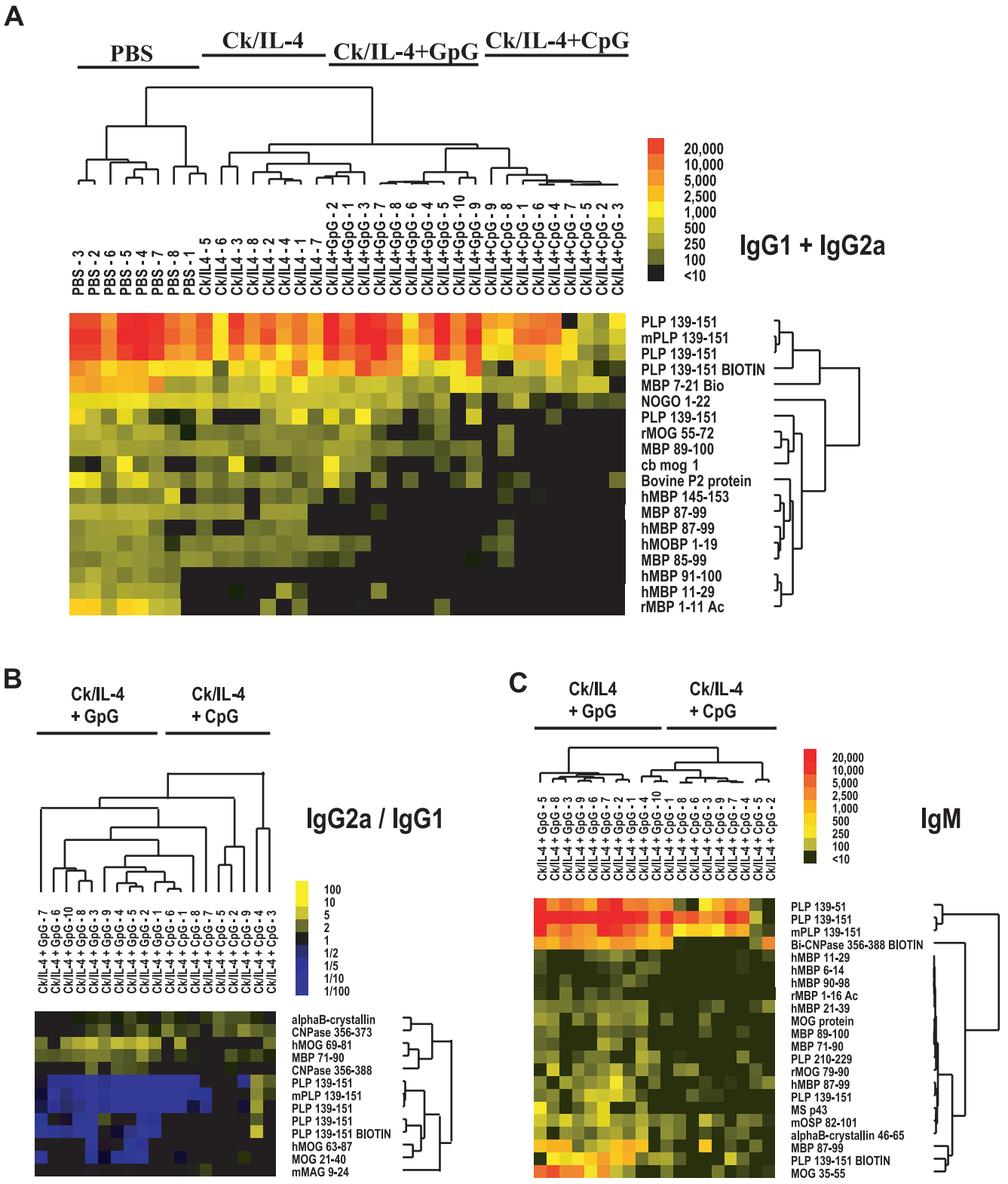

FIGURE 4. Combination therapy with myelin/IL-4-tolerizing DNA vaccine plus GpG-ODN further reduced myelin-specific autoreactive B cell diversity and shifts toward a Th2 isotype. On day 56, sera from the mice described in Fig. 1 were collected and analyzed using myelin proteome arrays. Multiclass SAM analysis of total IgG1 + IgG2a autoantibody reactivities between all four groups (*A*), two-class SAM analysis of the ratios of IgG2a (yellow) to IgG1 (blue) autoantibody reactivities in samples derived from mice treated with either myelin cocktail/IL-4 + CpG-ODN or myelin cocktail/IL-4 + GpG-ODN (*B*), and two-class SAM analysis of total IgM anti-myelin Abs in serum samples derived from mice treated with either myelin cocktail/IL4 + CpG-ODN or myelin cocktail/IL4 + GpG-ODN (*C*). Ab reactivity was consistently detected against the immunizing PLP 139–151 peptide and to several other myelin peptides, including peptides derived from MBP, MOG, MAG, myelin-associated oligodendrocytic basic protein, αB-crystallin, and NOGO. Each column represents results from a single animal from each group; each row, fluorescent reactivity against a myelin peptide or protein based on the displayed color scale. Represented are only Ags with differences identified by the SAM algorithm. Prefixes denote the species from which each peptide was taken (h, human; r, rat; m, mouse); peptide abbreviations are as described in the text.

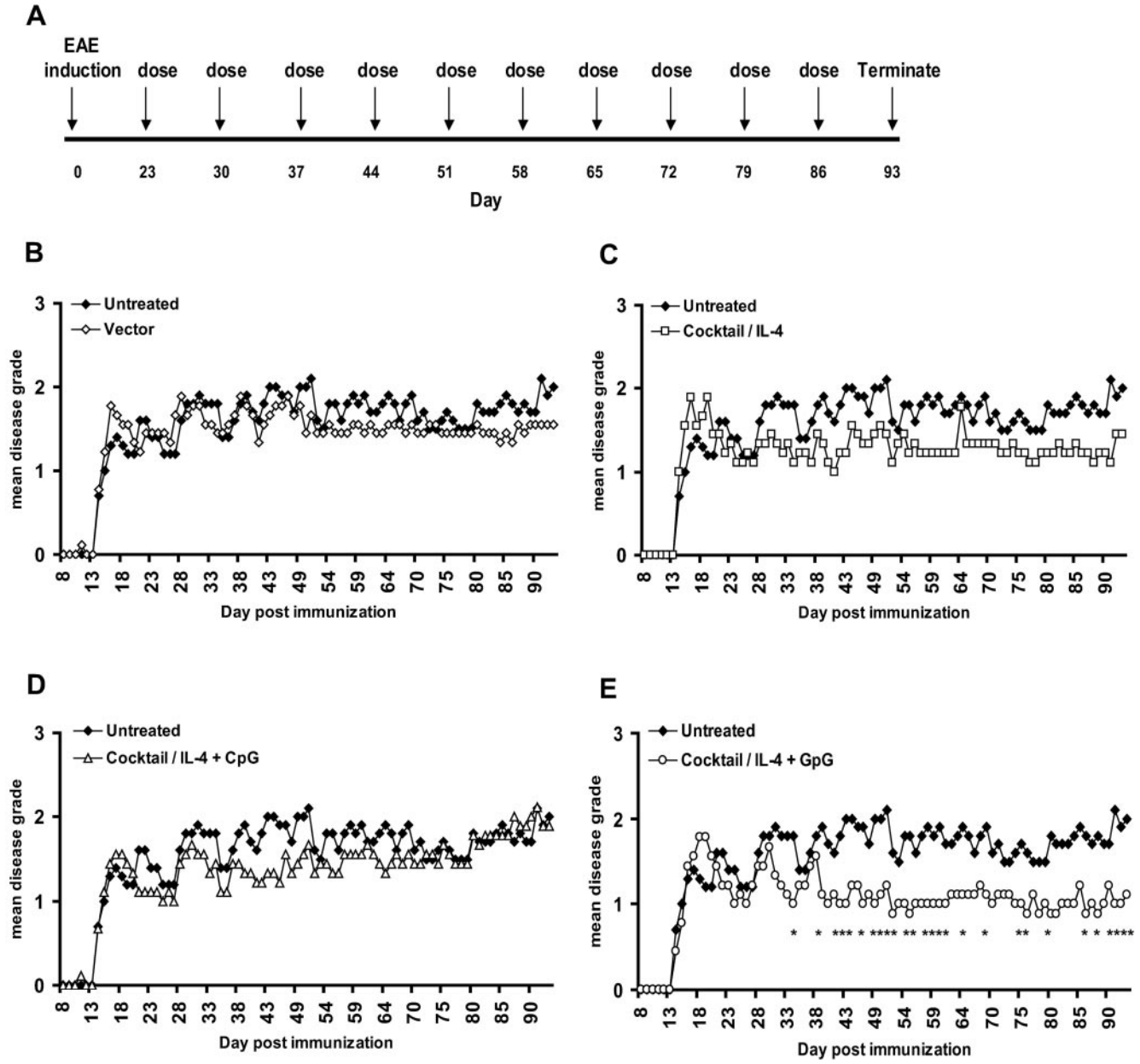

FIGURE 5. Combination therapy with myelin/IL-4-tolerizing DNA vaccine plus GpG-ODN treats chronic progressive EAE in B6 mice. Female B6 mice were induced for EAE with MOG 35–55 peptide in CFA followed by pertussis toxin at the same time and 2 days later. *A*, At the peak of acute disease (day 23), mice were randomized and treated with weekly i.m. injections of myelin cocktail DNA and IL-4 DNA and/or weekly i.p. injections of oligo (CpG or GpG). Treatment groups include empty pTarget vector alone (*B*), myelin cocktail/IL-4 (*C*), myelin cocktail/IL-4 + CpG-ODN (*D*), and myelin cocktail/IL-4 + GpG-ODN (*E*). Mean EAE scores are plotted against the number of days post-EAE immunization. The asterisks indicate a statistical significant difference ($p < 0.05$ by Mann-Whitney Wilcoxon test) comparing the treatment group vs the untreated group.

differences in reactivities between the treatment groups are different between each experiment. Our prior observation that increased diversity of the autoantibody response can predict more severe EAE is consistent with observations in humans demonstrating that increased autoantibody diversity predicts progression to systemic lupus erythematosus (35) and autoimmune diabetes (36). It is likely that autoantibody targeting of certain epitopes might contribute to pathogenesis more than targeting of other epitopes, providing a basis for discordance between autoantibody diversity and disease severity. In support of this is the observation in EAE that autoantibody targeting of epitopes in the extracellular Ig-like domain of MOG facilitates demyelination, while autoantibody targeting of other epitopes does not (26). Although we did not observe clear differences in autoantibody targeting of MOG in Fig. 4, it is possible that differences in epitope specificity and/or autoantibody isotypes could result in more severe EAE in the context of less diversity.

In autoimmune diseases, autoantibodies can contribute to pathogenesis either directly, with or without complement fixation, or indirectly through the activation of innate immune effector cells (37, 38). Studies have shown that IFN-γ acts as a costimulatory factor that augments B cell IL-6 and IgM secretion and promotes B cell differentiation to autoantibody-secreting IgG2a cells, even in the absence of T cells (39–45). IL-4 induces dividing B cells to switch to IgG1 and IgE isotypes, an effect that can be antagonized by IFN-γ (46–49). We show that the combination therapy of the

myelin cocktail/IL-4-tolerizing DNA vaccine plus suppressive GpG-ODN effectively reduced PLP 139–151 T cell-specific IFN-γ production while enhancing IL-4 and IL-10 production. We hypothesize that this shift toward a Th2 bias in T cells enabled PLP 139–151-specific autoantibodies to switch to a protective IgG1 isotype. As a result, the efficacy of tolerizing DNA vaccination is improved dramatically as indicated by the significant reduction in CR-EAE disease severity. Therefore, these results substantiate the promising outlook of Ag-specific immune therapy, through the combination of tolerizing DNA vaccination and suppressive ODN therapy, for the treatment of Th1-mediated autoimmune diseases.

Disclosures

The authors have no financial conflict of interest.

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