Published in final edited form as:

J Neuroimmunol. 2008 November 15; 204(1-2): 92–100. doi:10.1016/j.jneuroim.2008.07.005.

Targeting Myelin Proteolipid Protein to the MHC Class I Pathway by Ubiquitination Modulates the Course of Experimental Autoimmune Encephalomyelitis

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Abstract

Relapsing-remitting experimental autoimmune encephalomyelitis (EAE), a multiple sclerosis model, is induced in mice by injection of myelin proteolipid protein (PLP) encephalitogenic peptide, PLP_{139–151}, in adjuvant. In this study, prior to EAE induction, mice were vaccinated with a bacterial plasmid encoding a PLP-ubiquitin fusion (pCMVUPLP). During the relapse phase of EAE, clinical signs, histopathologic changes, *in vitro* lymphoproliferation to PLP_{139–151} and interferon- γ levels were reduced in pCMVUPLP-vaccinated mice, compared to mock-vaccinated mice (controls). Lymphocytes from pCMVUPLP-vaccinated mice produced interleukin-4, a cytokine lacking in controls. Thus, pCMVUPLP-vaccination can modulate the relapse after EAE induction.

Keywords

CNS demyelinating disease; Experimental autoimmune encephalomyelitis; DNA vaccines; Multiple sclerosis; Ubiquitin degradation pathway

1. Introduction

Relapsing-remitting experimental autoimmune encephalomyelitis (RR-EAE) is a CD4⁺ T helper (Th) type 1-mediated disease of the central nervous system (CNS) (Pender, 1995). RR-EAE mimics many of the clinical and pathological features of the human demyelinating disease multiple sclerosis (MS). RR-EAE induced in SJL/J mice using an encephalitogenic peptide of myelin proteolipid protein (PLP_{139–151}) emulsified in complete Freund's adjuvant (CFA) is characterized by an initial acute attack that lasts an average of 7 days which is then followed

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by an irregular RR phase (McRae et al., 1995; Tsunoda and Fujinami, 1996; Whitham et al., 1991).

While the pathogenesis of MS is unknown, several features, including genetics, age and environmental factors, contribute to the disease. Epidemiologic studies have demonstrated that the prevalence of MS is low around the equatorial belt of the world and as one progresses north and south from this region, the incidence of MS increases [(Kurtzke, 1993), reviewed in (Libbey and Fujinami, 2002; Libbey and Fujinami, 2003)]. Migration studies have shown that individuals moving **after** the age of 15 from an area of high-risk for MS to an area of low incidence of MS retain their high-risk for MS phenotype. Conversely, individuals migrating **before** the age of 15 acquire the low-risk phenotype of the area to which they have moved. These data have been interpreted as indicating that infections early in life in high-risk or high endemic MS areas contribute to or imprint the body's immune system to the high-risk MS phenotype. The converse of this is that infections early in life in the low-risk MS regions may actually protect individuals from developing MS (Granieri et al., 2001; Granieri and Casetta, 1997; Kurtzke, 1993). In addition, there are several reports indicating that exacerbations of MS occur in close association with microbial infections (Álvarez-Lafuente et al., 2006; Andersen et al., 1993; Berti et al., 2002; Buljevac et al., 2002; Christensen, 2007; Correale et al., 2006; De Keyser et al., 1998; Edwards et al., 1998; Gilden, 2002; Granieri et al., 2001; Kriesel et al., 2004; Kriesel and Sibley, 2005; Marrie et al., 2000; Metz et al., 1998; Panitch, 1994; Rapp et al., 1995; Sibley et al., 1985; Sospedra and Martin, 2006; Wandinger et al., 2000). Therefore from an epidemiologic standpoint, infections, whether viral, bacterial, or parasitic, probably contribute to the pathogenesis of MS.

Molecular mimicry is a sharing of immunologic determinants between a microbe, virus or bacteria, and the host. We have previously demonstrated that recombinant viruses encoding self-proteins could either prime for autoimmune CNS disease or protect from autoimmune CNS disease (Barnett et al., 1993; Barnett et al., 1996; Wang et al., 1999; Wang and Fujinami, 1997). We have also reported that vaccination with naked DNA (Tsunoda et al., 1999) and DNA encoding self-CNS antigens (whole PLP, PLP₁₃₉₋₁₅₁, PLP₁₇₈₋₁₉₁) (Tsunoda et al., 1998) enhanced the acute EAE and led to an increase in relapses. DNA immunization using naked DNA or DNA encoding a self-CNS antigen (PLP) resulted in increased proliferative responses to the sensitizing antigen. In addition, the immune response was of an enhanced Th1 type compared to mock-vaccinated RR-EAE mice (Tsunoda et al., 1999). These constructs mirror organisms which have cross-reacting epitopes with self-CNS proteins. In these instances the naked DNA (containing CpG motifs) and the DNA encoding self-antigens appear to prime mice for the development of RR-EAE.

In the present study, we tested whether ubiquitination of a self-protein, PLP, could modulate the type of priming that we had previously observed. It is known that ubiquitination improves entry into and presentation by the class I major histocompatibility complex (MHC) pathway (Rodriguez et al., 1997; Rodriguez et al., 1998) and leads to an enhanced CD8⁺ cytotoxic T lymphocyte (CTL) response. Therefore, we constructed a bacterial plasmid encoding a modified ubiquitin gene in frame with the PLP gene (pCMVUPLP). We then examined whether targeting the encoded PLP antigen, provided by the DNA vaccination, to the intracellular degradation pathway could modulate RR-EAE. We found that the ubiquitinated PLP DNA vaccine could downregulate the immune response observed during the relapse after EAE induction. Mice vaccinated with pCMVUPLP (the ubiquitinated form of PLP) had a milder clinical disease with fewer lesions in the brain and spinal cord during the relapse. Lymphoproliferative responses to PLP₁₃₉₋₁₅₁ in pCMVUPLP-vaccinated mice were also reduced, compared to mock-vaccinated mice. This was accompanied by a decrease in interferon (IFN)- γ production and an increase in interleukin (IL)-4 production. In addition, data from CTL experiments suggest that CD8⁺ T cells are activated and expanded after EAE induction

in pCMVUPLP-vaccinated mice. These CD8⁺ T cells could contribute to the modulation of disease by regulating the CD4⁺ Th1 encephalitogenic T cells.

2. Materials and Methods

2.1. Plasmid construction

Constructs used for intramuscular (i.m.) injection were based on pCMV, a plasmid that contains the widely expressed immediate-early promoter from cytomegalovirus. The PLP gene was fused to a modified ubiquitin gene [ubiquitin-A76, (Rodriguez et al., 1997)] and subcloned into the *NotI* restriction enzyme site of plasmid pCMV, which was derived by excision of the β -galactosidase gene from pCMV- β (Clontech, Palo Alto, CA). The resultant expression vector was named pCMVUPLP (Theil et al., 2001). Plasmid DNA was purified from transformed DH5 α *Escherichia coli* with the EndoFree Plasmid Maxi Kit (Qiagen, Chatsworth, CA).

2.2. DNA immunization and EAE induction

Groups of 3–5 SJL/J mice (3–4-weeks-old, female, Jackson Laboratory, Bar Harbor, ME) were vaccinated i.m. a total of 3 times at 1-week intervals with 100 μ g pCMVUPLP plasmid DNA, or were mock-vaccinated, with phosphate-buffered saline (PBS) or empty vector, pCMV. Plasmid DNA was dissolved in 1 N saline and 50 μ g were injected into each anterior tibial muscle with a 28-gauge needle. Mice were observed and weighed every other day.

One week after the last vaccination, mice received subcutaneously (at the base of the tail) 100 nM PLP_{139–151}, emulsified in CFA. After EAE induction, mice were observed and weighed daily. Mice were followed for clinical signs of disease according to the following scale (Tsunoda et al., 1998): 0 - no clinical disease; 0.5 - loss of tonicity of the distal half of the tail; 1 - complete loss of tail tonicity; 2 - mild hind leg paresis; 3 - moderate hind leg paralysis; 4 - complete paraplegia; and 5 - quadriplegia, moribund state or death. Mice were euthanized with isoflurane. All experiments were performed twice.

Previously, we have shown that pCMVPLP (lacking ubiquitin) vaccination before EAE induction resulted in the most severe EAE (Tsunoda et al., 1998), while pCMV injection before EAE induction also resulted in more severe EAE than EAE induced with PLP_{139–151}/CFA alone (Tsunoda et al., 1999). Therefore, in this study, we used pCMV-vaccinated mice followed with PLP_{139–151}/CFA-EAE induction as a control group, unless otherwise noted.

2.3. Histology

Mice sacrificed on day 26 post-EAE induction were perfused with PBS followed by 4% paraformaldehyde. Brains were embedded as 5 coronal slabs in paraffin. Spinal cords were divided into 10 to 12 transverse slabs and were embedded in paraffin by standard methods (Tsunoda and Fujinami, 1996). Sections were stained with hematoxylin and eosin (H&E) and Luxol-fast blue.

Mononuclear cell (MNC) infiltration of the meninges and parenchyma was assessed according to the following scoring system: 0 - no infiltration; 1 - meninges were slightly infiltrated; 2 - perivascular cuffing in the meninges and lateral ventricle (brain), and perivascular cuffing in the meninges comprised of up to two cell rows (spinal cord); 3 - perivascular cuffing in meninges, all ventricles, cerebellum and brain stem (brain), and severe perivascular cuffing in the meninges and ventral funiculus (spinal cord); and 4 - entire meninges and all funiculi were severely infiltrated (spinal cord). Demyelination in the spinal cord was assessed according to the following scoring system: 0 - no demyelination; 1 - white matter adjacent to the meninges was replaced with gliotic cells; 2 - less than half of the white matter was replaced with

infiltrating cells and gliotic cells; 3 - more than half of the white matter was replaced with infiltrating cells and gliotic cells.

Immunohistochemistry was performed using anti-CD3 antibody (DAKO Corporation, Carpinteria, CA) and the avidin-biotin peroxidase complex (ABC) technique with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) in 0.01% hydrogen peroxide (H₂O₂) in PBS. Sections were analyzed by Image-Pro Plus version 4.5.1 (Media Cybernetics, Inc., Silver Spring, MD).

2.4. Proliferation assay

The inguinal lymph nodes and spleens from mice in each experimental group were removed at days 23 and 28 post-EAE induction and pooled. Lymphocytes were isolated using Histopaque-1083 (Sigma). A volume of 0.2 ml containing 2×10^5 cells in RPMI 1640 media (Mediatech, Herndon, VA), supplemented with 1% glutamine (Mediatech), 1% penicillin/streptomycin (Mediatech), 5×10^{-5} M 2-mercaptoethanol (Sigma), and 10% fetal bovine serum (Invitrogen, San Diego, CA), was added to each well and PLP₁₃₉₋₁₅₁, PLP₁₇₈₋₁₉₁, myelin basic protein (MBP)₈₄₋₁₀₄ (Vanderlugt et al., 2000) and myelin oligodendrocyte glycoprotein (MOG)₉₂₋₁₀₆ (Amor et al., 1994) peptides, which are encephalitogenic in SJL/J mice, were added at 50 µg/ml. Lymphocytes prepared as above from mice at day 4 post-EAE induction were incubated with irradiated antigen presenting cells (APCs), irradiated APCs transfected with either pCMVPLP (lacks ubiquitin) or pCMVUPLP, irradiated APCs infected with a recombinant vaccinia virus (VV) that expresses PLP (VVplp) or PLP₁₃₉₋₁₅₁ peptide. PPD (tuberculin purified protein derivative) was used as a positive control. The cells were cultured for 72 hrs and each well was pulsed with 1 µCi of ³H-thymidine (PerkinElmer, Waltham, MA). Cells were cultured for another 24 hrs. Cultures were harvested onto filters using a multiwell cell harvester (Flow Laboratories, McLean, VA) and counted using standard liquid scintillation techniques. All cultures were performed in triplicate. Results are expressed as stimulation index [experimental counts per minute (cpm)/control cpm].

2.5. Cytokine assays

Lymphocytes from the same animals used in the proliferation assay were cultured at 2×10^6 cells/ml in 6-well plates and stimulated with 50 µg/ml PLP₁₃₉₋₁₅₁. Culture supernatants were harvested 24 and 48 hrs after stimulation. IL-4, IL-10 and IFN-γ were measured using the enzyme-linked immunosorbent assay (ELISA) system, OptEIA™ (BD Pharmingen, San Jose, CA), according to the manufacturer's instructions. IL-17 was measured using mouse IL-17 ELISA MAX™ Set *Deluxe* (BioLegend, San Diego, CA).

2.6. Cytotoxicity assays

2.6.1. Preparation of target cells—A simian virus 40-transformed murine fibroblast cell line, PSJLSV (PSJL) (*H-2^s*), kindly provided by Barbara Knowles (Jackson Laboratory, Bar Harbor, ME), was used as a target in the CTL assays. On the day of assay, PSJL cells were labeled with Na₂⁵¹CrO₄ (PerkinElmer) for 1 hr, and, at the same time, these cells were infected with VVplp at a multiplicity of infection (MOI) of 10 (Barnett et al., 1993). The same ⁵¹Cr loading procedure was applied to control targets: uninfected PSJL cells, PSJL cells infected with VVsc11 (recombinant VV expressing β-galactosidase), and human Jurkat cells (American Type Culture Collection, Manassas, VA).

2.6.2. Preparation of effector cells—In EAE, encephalitogenic T cells have been thought to engage in bystander killing, using the Fas-FasL interaction without MHC-T cell receptor (TCR) interaction (Thilenius et al., 1999). Available syngeneic target cells, PSJL cells, are potentially susceptible to Fas-FasL mediated killing (Tsunoda et al., 2002). Therefore, it would be difficult to analyze CTL responses if we harvest the cells from mice after induction of

encephalitogenic T cells, which are expected to be induced by day 6. Thus, in this study, we chose day 4 to examine the induction of CTL by vaccination with pCMVUPLP.

Effector cells were prepared by pooling splenocytes and inguinal lymphocytes, harvested 4 days after EAE induction with PLP_{139–151}/CFA, from 2 mice vaccinated with pCMVUPLP. These cells were treated with an NH₄Cl red blood cell-lysing solution and were used in primary and secondary CTL assays. For the secondary CTL assay, the effector cells were stimulated *in vitro* for 6 days with naïve SJL/J mouse splenocytes that had been infected with VVplp at a MOI of 1, cultured overnight and irradiated at 2000 rad (¹³⁷Cs irradiator). Effector cells were washed 3 times prior to use.

2.6.3. ⁵¹Cr release assay—Target cell lysis was assessed by a 5-hr short-term ⁵¹Cr release assay. The target cells (10⁴) were placed in wells of 96-well, round-bottomed microtiter plates. Effector cells were used at a concentration of 10⁶/100 µl, and threefold serial dilutions were made to provide effector-to-target cell (E/T) ratios of 100:1, 33:1, 11:1 and 3:1. After a 5-hr incubation, the radioactivity released from the cells was measured, as counts per minute (cpm), by means of a model 20/20 gamma counter (Iso-Data, Inc., Palatine, IL). The percentage of target cell lysis was calculated using the following equation: % lysis = [(experimental release cpm – spontaneous release cpm)/(maximal release cpm – spontaneous release cpm)] × 100.

2.7. Statistical analyses

All statistical analyses were performed using the StatView program (SAS Institute Inc., Cary, NC). Either the unpaired *t*-test (Student's *t*-test) or analysis of variance (ANOVA), followed when indicated by the Fisher's Protected Least Significant Difference (Fisher's PLSD) post hoc test, were used to determine group differences for continuous data.

3. Results

3.1. Clinical disease

In PLP_{139–151}-induced EAE, most mice develop the first attack (onset) at around day 13, the first remission at around day 18, and the second attack (the first relapse) at around day 22 post-EAE induction (Tsunoda et al., 2007). After the first relapse, the disease courses between individual mice are not synchronized (Tsunoda et al., 2007). Thus, in this study, we decided to observe mice during the first 1 month post-EAE induction. Acute clinical signs of RR-EAE were present in both pCMVUPLP-vaccinated and mock-vaccinated mice beginning on days 13–14 post-EAE induction with PLP_{139–151}/CFA. Clinical signs in RR-EAE were characterized by tail and hindlimb paresis that progressed to flaccid paralysis, accompanied by urinary incontinence. The average clinical score and variation in weight of pCMVUPLP-vaccinated and mock-vaccinated mice are shown in Fig. 1 for days 0 through 26. There was a trend for the pCMVUPLP-vaccinated mice to lose less weight, compared to mock-vaccinated mice, though the difference never reached significance (Fig. 1a). The clinical scores were significantly lower for the pCMVUPLP-vaccinated mice, compared to mock-vaccinated mice, for the days 23, 24 and 26 post-EAE induction ($p < 0.05$, $p < 0.01$ and $p = 0.0001$, respectively, *t*-test); however, the clinical score for the pCMVUPLP-vaccinated mice was significantly higher, compared to mock-vaccinated mice, for day 13 post-EAE induction ($p < 0.01$, *t*-test) (Fig. 1b). Both pCMVUPLP-vaccinated and mock-vaccinated mice recovered partially from the initial acute attack. Mock-vaccinated mice had their first relapse around day 22 after sensitization. Mice vaccinated with pCMVUPLP without PLP_{139–151}/CFA challenge or challenged with PBS/CFA had no disease (data not shown). Therefore, pCMVUPLP injections *per se* did not induce CNS disease. However, pCMVUPLP injections did downregulate the clinical disease induced with PLP_{139–151}/CFA during the relapses, while not preventing the relapses altogether.

3.2. Histological findings

Brains and spinal cords were examined histopathologically 26 days post-EAE induction. The histopathology of RR-EAE in the brain and spinal cord of mock-vaccinated mice was characterized by abundant MNC infiltration around vessels (perivascular cuffing) in the meninges and white matter (Fig. 2a). Lesions were prominent in the periventricular zone around lateral ventricles, the white matter of the cerebellar folia and the thoracolumbar segment of the spinal cord. Often present around the inflammatory cell cuffs, an abundant accumulation of macrophages and microglia was present with or without rings of demyelination. Classical plaque-like demyelination as seen in MS was not common.

The histopathology, measured as MNC infiltration in the brain, MNC infiltration in the spinal cord and demyelination, was significantly less severe ($p < 0.05$, $p < 0.01$ and $p < 0.0001$, respectively, t -test) in the mice vaccinated with pCMVUPLP and challenged with PLP_{139–151}/CFA than in the mock-vaccinated mice (Fig. 3). In general in both groups, demyelination was present in the spinal cord but was absent in the brain. In the brains of pCMVUPLP-vaccinated mice, MNC infiltration was seen around the lateral ventricles and, in only one mouse (1/5), MNC infiltration was present in the cerebellum. In contrast, the cerebellum was always affected in the mock-vaccinated mice. In the spinal cord the same anatomical region was affected, in pCMVUPLP-vaccinated and mock-vaccinated mice (Fig. 2c).

To determine the extent of T cell infiltration, immunohistochemistry on serial sections was performed. In both groups of mice, perivascular cuffing was positive for CD3⁺ T cells (Fig. 2b,d); more than half of the cells comprising a cuff stained positive using CD3 antibodies.

3.3 Proliferation assay

In general, CD8⁺ T cells recognize endogenous (intracellular) protein epitopes presented by MHC class I, and CD4⁺ T cells recognize exogenous (extracellular) protein epitopes presented by MHC class II (Leifert et al., 2004). We measured CD4⁺ T cell proliferative responses of inguinal lymph nodes and spleens, using exogenously added PPD and different CD4-specific encephalitogenic epitopes reported in SJL/J mice.

Lymphocytes, harvested on day 23 post-EAE induction, from mice vaccinated with pCMVUPLP and challenged with PLP_{139–151}/CFA, when stimulated *in vitro* with PLP_{139–151}, had a lower proliferative response than lymphocytes from mice that were mock-vaccinated ($p < 0.01$, t -test) (Fig. 4a). No significant proliferation to other known encephalitogenic peptides was detected at this time point.

The lymphoproliferative response, to the PLP_{139–151} peptide, of lymphocytes obtained from the pCMVUPLP-vaccinated mice was higher on day 28 post-EAE induction than on day 23 post-EAE induction ($p < 0.01$, t -test) (Fig. 4b). Some proliferation to another PLP epitope (PLP_{178–191}) could be seen during the relapse. This may reflect epitope spreading (Fig. 4b).

To see potential MHC class I restricted CD8⁺ T cell responses, we used APCs (spleen cells) transfected with pCMVPLP (which lacks ubiquitin), pCMVUPLP, or VVplp-infected APCs as stimulator in lymphoproliferative assays. Lymphocytes, isolated at day 4 post-EAE induction, from mice vaccinated with pCMVUPLP proliferated when incubated with irradiated APCs transfected with pCMVUPLP, but not to APCs transfected with pCMVPLP ($p < 0.001$, ANOVA, Fisher's PLSD), or to control irradiated APCs ($p < 0.001$, ANOVA, Fisher's PLSD) (Fig. 4c). This suggests that enhanced degradation and processing of PLP occurs in the APCs with the ubiquitinated vaccine. Furthermore, this finding suggests that the proliferative response might be due to an MHC class I restricted interaction, although no PLP-specific CD8⁺ T cell responses have been discovered in SJL/J mice.

3.4. Cytokines

Lymphocytes from pCMVUPLP-vaccinated mice or mock-vaccinated mice were harvested 23 days after EAE induction and were stimulated with PLP_{139–151}, as described. Then, IFN- γ and IL-4 were measured in culture fluids. Four times higher amounts of IFN- γ were detected in the mock-vaccinated mice compared to pCMVUPLP-vaccinated mice at the 48 hr time point (Fig. 5a). IL-4 was detectable only in the pCMVUPLP-vaccinated group and not in the mock-vaccinated group (Fig. 5b). IL-10 was below the detection limit (31.3 pg/ml) in all samples. These findings suggest a dampening of the Th1 response. IL-4 production contributes to the lessened Th1 response. Since IL-17 has been associated with exacerbation in some EAE models (McClain et al., 2007), we also measured IL-17 in the supernatant. We detected no or a low level of IL-17 in the pCMVUPLP-vaccinated group (exp. 1, not detectable; exp. 2, 76 pg/ml), while IL-17 was not detectable in the mock-vaccinated group (detection limit, 7.8 pg/ml). Therefore, IL-17 production was not associated with disease severity in our current EAE model.

3.5. CTL assay

We tested whether pCMVUPLP vaccination could induce PLP-specific CTL responses. In CTL assays, we used lymphocytes harvested on day 4 post-EAE induction from pCMVUPLP-vaccinated mice as effector cells; syngeneic PSJL cells infected with VVplp, control VVsc11, or uninfected PSJL cells were used as target cells. The lymphocytes showed substantial cytotoxicity against both VVplp and VVsc11 infected cell, but not uninfected cells (% lysis at E/T ratio = 100: VVplp infected, 42%; VVsc11 infected, 32%; uninfected, 0%; % lysis at E/T ratio = 33: VVplp infected, 28%; VVsc11 infected, 31%; uninfected, 0%). *In vitro* secondary stimulation with VVplp-infected APCs did not enhance the CTL responses (% lysis at E/T ratios = 100 and 33: VVplp infected, 12 and 9%, respectively). Although we did not see killing of uninfected syngeneic cells, the lymphocytes killed human Jurkat cells (% lysis at E/T ratios = 100, 33, and 11: 27%, 15%, and 12%, respectively). Blocking experiments suggested that the cytotoxicity against Jurkat cells was mediated by the Fas-FasL pathway (data not shown).

4. Discussion

The current study demonstrates that DNA vaccination with a cDNA encoding an ubiquitinated autologous protein reduces clinical disease. Studies done by Rodriguez et al. (1997) and the present work show that 1) ubiquitinated vaccines efficiently induce a CD8⁺ CTL response and 2) these types of T cells are involved in disease attenuation.

CD8⁺ T cells are present in the lesions in EAE and MS [reviewed in (Johnson et al., 2007)]. CD8⁺ suppressor or regulatory T cells have been described which are reactive to encephalitogenic T cells or to myelin protein (Kumar and Sercarz, 1996; Sun et al., 1988). Furthermore, a key feature of effective DNA vaccination is the efficient generation of long-lasting CD8⁺ T cell immunity (Riedl et al., 2006). By targeting the antigens to the proteasome pathway, we are inducing immune cells specific for epitopes presented by MHC class I, and these cells could elicit a suppressive function upon disease induction. Several mechanisms have been proposed for how CD8⁺ T cells could downregulate a destructive CD4⁺ T cell response. Sun and Klinert (1989) demonstrated that CD8⁺ T cells could modulate EAE by eliminating the CD4⁺ effector T cells through direct cytotoxicity. Kumar and Sercarz (1996) suggested that CD8⁺ T cells could recognize distinct TCR determinants in a MHC class I context displayed on the surface of effector CD4⁺ T cells. However, both authors were exploring the role of CD8⁺ T cells in EAE models that do not develop spontaneous relapses. In the RR-EAE model, we found that CD8⁺ T cells, induced by DNA vaccination, reduced clinical disease during the relapse after EAE induction. In CTL assays, we found that pCMVUPLP vaccination induced cytotoxic responses against virus infected syngeneic cells (PSJL) as well as xenogeneic cells (Jurkat). Since Jurkat is Fas positive and Fas blocking

experiments inhibited the killing (unpublished data), this xenogeneic killing could be mediated by the Fas-FasL pathway (Thilenius et al., 1999). Since CD8⁺ T cells, in theory, suppress CD4⁺ T cells by the Fas-FasL pathway, we are investigating the phenotype of the killer cells and involvement of the Fas-FasL pathway in the killing. We are also investigating whether similar cytotoxicity can be detected during the relapse phase of EAE, and if so, whether the killing is correlated with the clinical severity of EAE.

The cytokine profile of the relapsing phase in RR-EAE is of a Th1 type [IFN- γ and tumor necrosis factor (TNF)- α], and the cytokines are produced by CD4⁺ effector T cells. In the remission phase, cytokines of a Th2 type (IL-4) have been detected (Begolka et al., 1998). The source of the Th2 type cytokines is not exclusively CD4⁺ T cells; other cell types involved in an inflammatory process have been found to produce transforming growth factor (TGF)- β (Miller et al., 1992). Miller et al. (1992) demonstrated the protective role of TGF- β produced by CD8⁺ T cells in oral tolerization to MBP in monophasic EAE (Lewis rat injected in footpad with MBP/CFA). In the mice vaccinated with pCMVUPLP and challenged with PLP_{139–151}/CFA, compared to the mock-vaccinated mice, IL-4 was detectable only in the pCMVUPLP-vaccinated group. We hypothesize that the cytokine profile observed in our experiments reflects the prolonged remission phase mice were in at the time of sacrifice and not a Th2 response induced directly by the pCMVUPLP vaccine. This aspect is still under investigation, since IL-4 has been shown to play a decisive role in leading the immune response towards a Th2 type response, which is known to be protective in many EAE models (Falcone and Bloom, 1997; Karpus et al., 1992; Racke et al., 1994). IL-4 has also been shown to downregulate macrophage activity by inhibiting protein kinase C epsilon activity and reducing nitric oxide (NO) expression (Sands et al., 1994). It is still possible that suppressor cells induced by the pCMVUPLP vaccine diminish clinical and pathological disease by downregulating macrophages and glial cell activity in the brain and spinal cord.

Vaccination with naked DNA has previously been explored in EAE and has been found to be effective in some cases and not in others. DNA vaccines, with specificity for the TCR V genes commonly found on pathogenic T cells that cause EAE, can protect animals from developing EAE, as demonstrated in DA rats, PL/J mice and SJL/J mice sensitized with MBP (Buch and Waisman, 2006; Kumar et al., 2001; Matsumoto, 2005; Miyakoshi et al., 2003; Waisman et al., 1996). This protection has been found to be due to the induction of Th2 type immunity, by inducing a shift in the phenotype of the T cells responding to the antigen and utilizing the relevant V gene segment, ultimately resulting in a cytokine shift from Th1 to Th2 and thus protection from EAE (Buch and Waisman, 2006; Kumar et al., 2001; Waisman et al., 1996). This method, however, requires knowledge of the exact TCR repertoire relevant for a particular disease and individual (Waisman et al., 1996).

DNA vaccines have also been constructed which encode receptors for various chemokines known to be active in the process of EAE relapse (Matsumoto, 2005). Administration of these decoy chemokine receptor DNA vaccines encoding the binding sites of CXC chemokine receptor 3 (CXCR3) and CC chemokine receptor 2 (CCR2), receptors for IFN- γ -inducible protein-10 (IP-10) and monocyte chemoattractant protein-1 (MCP-1), respectively, was found to suppress relapse, but not the acute attack, in EAE induced in DA rats by means of immunization with purified myelin (Matsumoto, 2005).

Most DNA vaccination studies have demonstrated that DNA injection can modulate diseases/infections, if DNA is given before disease induction. Vaccination with a DNA construct encoding the entire PLP gene was shown to either suppress or exacerbate EAE in SJL/J mice depending on when the vaccine was given (Selmaj et al., 2000). Exacerbation occurred if the interval between DNA vaccination and sensitization with PLP_{139–151} was less than 10 weeks, and suppression occurred if the interval was greater than 10 weeks (Selmaj et al., 2000). In the

current study, we injected pCMVUPLP before EAE induction and found that it suppressed EAE. It would be worth testing in future experiments whether pCMVUPLP vaccination can suppress ongoing EAE when it is given after EAE induction or disease onset.

DNA vaccines with a myelin epitope alone (PLP_{139–151}) resulted in reduction of the incidence and severity of the acute phase of PLP_{139–151}-induced EAE, probably due to T cell anergy (Ruiz et al., 1999). Alternately, DNA covaccination, with a PLP_{139–151} vaccine and an IL-4 vaccine, drives the response to a Th2 type response rather than anergy (Garren et al., 2001). It is thought that both the IL-4 and myelin peptide vaccines are taken up by APCs which present the expressed myelin epitopes on MHC class II, thus recruiting antigen-specific T cells. The APCs also secrete IL-4 which influences the Th2 type phenotype of the antigen-specific T cells and thus protects from EAE (Garren et al., 2001).

Still other covaccination methods have been tested which incorporate a mixture of myelin epitopes from multiple myelin proteins and IL-4 [(Ho et al., 2005; Robinson et al., 2003), reviewed in (Fontoura et al., 2005)]. These studies demonstrated a significant reduction in relapse rate in the PLP_{139–151}-induced EAE model in SJL/J mice [(Ho et al., 2005; Robinson et al., 2003), reviewed in (Fontoura et al., 2005)].

Previously in our laboratory, vaccination with cDNAs encoding PLP (whole PLP, PLP_{139–151}, and PLP_{178–191} in pCMV) was shown to enhance RR-EAE (Tsunoda et al., 1998). Vaccination with the constructs alone induced a PLP-specific lymphoproliferative response of a CD4⁺ T cell mediated nature, but not CNS disease; whereas vaccination and subsequent peptide challenge resulted in a clinically and histologically more severe chronic EAE with enhanced lymphoproliferative responses to PLP (Tsunoda et al., 1998). The differences between the Tsunoda et al. (1998) study (enhancement of EAE) and the Ruiz et al. (1999) study (amelioration of EAE) (above) include examination of difference phases of the disease (chronic and acute, respectively), the use of older mice by Ruiz et al. (1999), the use of different expression vectors, the use of different plasmid preparation kits, the use of different immunization protocols, and the use of different EAE induction methods (Ruiz et al., 1999; Tsunoda et al., 1998).

In the current study, in which vaccination was with a bacterial plasmid, pCMVUPLP, encoding the ubiquitinated form of PLP, amelioration of EAE was observed (reduced MNC infiltration and demyelination, less proliferation to the encephalitogenic peptide PLP_{139–151} and dampening of the Th1 response). Thus, targeting the PLP antigen to the intracellular degradation pathway by ubiquitination could modulate RR-EAE in a suppressive manner.

Ubiquitin is a 76 amino-acid protein that binds covalently to target proteins, shepherding them to the proteasome for degradation (Hung et al., 2006; Pickart, 2004). Targeting proteins encoded by DNA vaccines to the proteasome has been studied as a way of increasing the immunogenicity of DNA vaccines. The fusion of antigen to ubiquitin significantly enhances the presentation of the antigen by MHC class I and, thus, the antigen-specific CD8⁺ T cell immune response to the antigen (Andersson and Barry, 2004; Hung et al., 2006). In this system, target epitope recognition by CTL can be blocked by a proteasome inhibitor, as we have previously demonstrated (Rodriguez et al., 1997). Interestingly, however, different proteasome inhibitors can not only impair but also enhance the presentation of MHC class I restricted immunodominant and subdominant epitopes in some cases (Gavioli et al., 2002). Therefore, in order to understanding the mechanism of PLP antigen presentation in greater detail, further experiments, such as analyzing the effect of different proteasome inhibitors on the generation of possible subdominant and immunodominant PLP epitopes *in vitro*, will be required.

DNA vaccines encoding an antigen fused to ubiquitin have been studied with respect to several viral, bacterial and parasitic infections, including papillomavirus (cancer), *Plasmodium*

yoelii (malaria), pseudorabies virus, *Mycobacterium tuberculosis*, hepatitis C virus, lymphocytic choriomeningitis virus, HIV, and influenza virus (Andersson and Barry, 2004; Brandsma et al., 2007; Brulet et al., 2007; Delogu et al., 2000; Delogu et al., 2002; Fu et al., 1998; Gravier et al., 2007; Leachman et al., 2002; Liu et al., 2002; Rodriguez et al., 1997; Tobery and Siliciano, 1997; Vidalin et al., 1999; Wang et al., 2004). The approach also has been exploited in studies of antitumor therapy and allergies (Bauer et al., 2006; Duan et al., 2006; Zhang et al., 2005).

Interestingly, a regulatory role of CD8⁺ T cells has been reported in the disease caused by the Theiler's murine encephalomyelitis virus (TMEV), a viral model for MS. The DA virus, a strain of TMEV, causes at first a subclinical or asymptomatic disease in the CNS, which is then followed by a chronic destructive CD4⁺ T cell response (Tsunoda and Fujinami, 1996). At first the immune system seems to cope with the infection. The first asymptomatic phase is believed to be the result of a prompt immune response; the induction of regulatory CD8⁺ T cells keeps CD4⁺ effector T cells in check (Nicholson et al., 1996; Rodriguez et al., 1991). Similar protective immune machinery might be functioning in EAE mice vaccinated with pCMVUPLP. To clarify the potential regulatory role of CD8⁺ T cells, we are planning experiments, such as *in vivo* adoptive transfer of CD8⁺ T cells (protected versus non-protected mice) to EAE mice, *in vivo* depletion of CD8⁺ T cells in vaccinated mice, and *in vitro* addition or depletion of CD8⁺ T cells in CTL as well as lymphoproliferative assays.

Relapsing in the pCMVUPLP-vaccinated mice could be the result of clonal CD8⁺ T cell exhaustion, or the result of continuous accumulation of Th1 cytokines or proinflammatory cytokine [IFN- γ , IL-12, tumor necrosis factor (TNF)- α]. The source of these cytokines is probably macrophages and activated glial cells in the brain, or newly attracted effector CD4⁺ T cells.

In this study we underscore that different methods of DNA vaccination can modulate autoimmunity. Ubiquitinated DNA vaccination represents a powerful tool in studying the pathogenesis of autoimmunity. CD8⁺ T cells may play a decisive role in regulating RR-EAE. We demonstrated that DNA vaccination modulated the onset, remission and the first relapse of RR-EAE. We are planning to determine: 1) whether DNA vaccination can decrease the number of relapses during the late chronic stage and, 2) whether DNA injection after disease onset or during the chronic stage can modulate the clinical course of EAE. These future directions will be important for potential therapeutic applications of DNA vaccination in MS.

Acknowledgements

We are grateful to Ms. Kathleen Borick for preparation of the manuscript. We thank Nikki Kirkman, BS, for technical assistance. This work was supported by NIH AI581501.

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Fig. 1.

Weight change (a) and clinical score (b) after experimental autoimmune encephalomyelitis (EAE) induction with the myelin proteolipid protein (PLP) peptide, PLP₁₃₉₋₁₅₁, emulsified in complete Freund's adjuvant (CFA). Mice were either vaccinated (pCMVUPLP), prior to EAE induction, with the bacterial plasmid, pCMVUPLP, which encodes the ubiquitinated form of PLP (5 mice), or mock-vaccinated controls (Mock) with phosphate-buffered saline (3 mice) or empty vector, pCMV (5 mice). No significant differences were found for weight change. pCMVUPLP mice were found to have a significantly higher clinical score at day 13 post-EAE induction (**, $p < 0.01$, t -test); however the control mice had significantly higher clinical scores

at days 23, 24 and 26 post-EAE induction (*, $p < 0.05$; **, $p < 0.01$ and ***, $p = 0.0001$, respectively, t -test). Values are the means $\pm$ standard error of the mean (SEM).

Fig. 2.

Spinal cord histopathology 26 days post-EAE induction with PLP that followed either mock vaccination with empty vector, pCMV (a,b), or vaccination with pCMVUPLP (c,d), which encodes the ubiquitinated form of PLP. (a) Mock-vaccinated mice had severe meningitis (arrow) with subpial demyelination (arrowhead) in the ventral root exit zone of the spinal cord white matter. (c) pCMVUPLP-vaccinated mice had mild meningitis with demyelination in regions of the spinal cord similar to mock-vaccinated mice. (b,d) In consecutive sections, both groups of mice had CD3⁺ T cells in the meninges and the parenchyma of the spinal cord white matter. (a,c) Luxol fast blue stain. (b,d) Immunohistochemistry against CD3. Magnification: a-d, $\times 100$; inset, $\times 360$.

Fig. 3.

Mononuclear cell (MNC) infiltration of the brain and spinal cord and demyelination were scored as described in the Methods section for pCMVUPLP-vaccinated mice (5 mice, pCMVUPLP) and mock-vaccinated mice (5 mice, Mock) for day 26 post-EAE induction. MNC infiltration in the brain and spinal cord and demyelination were significantly less severe (*, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.0001$, respectively, t -test) in the pCMVUPLP mice than in the mock-vaccinated mice. Values are the means $\pm$ SEM.

Fig. 4.

Lymphoproliferative responses. (a) Lymphocytes from mice vaccinated with pCMVUPLP and challenged with PLP_{139–151}/CFA (2 mice, pCMVUPLP) or mock-vaccinated (2 mice, Mock) were harvested on day 23 post-EAE induction and stimulated *in vitro* with PLP_{139–151}, PLP_{178–191}, myelin oligodendrocyte glycoprotein (MOG)_{92–106}, or myelin basic protein (MBP)_{84–104}. Lymphocyte proliferation was significantly less in response to PLP_{139–151} stimulation in the pCMVUPLP lymphocytes than the lymphocytes from mock-vaccinated mice (**, $p < 0.01$, t -test). (b) Lymphocytes from mice vaccinated with pCMVUPLP and challenged with PLP_{139–151}/CFA (2 mice each) were harvested on days 23 and 28 post-EAE induction and stimulated *in vitro* with PLP_{139–151}, PLP_{178–191}, MOG_{92–106}, MBP_{84–104}, or PPD (tuberculin purified protein derivative). Lymphocyte proliferation was significantly less in response to PLP_{139–151} stimulation on day 23 than on day 28 (**, $p < 0.01$, t -test). Some proliferation to PLP_{178–191} could be seen during the relapse phase, possibly indicating epitope spreading. (c) Lymphocytes from mice vaccinated with pCMVUPLP and challenged with PLP_{139–151}/CFA (2 mice) were harvested on day 4 post-EAE induction and stimulated *in vitro* with irradiated antigen presenting cells (APC) (CONTROL), APC transfected with pCMVUPLP (pCMVUPLP) and pCMVPLP (pCMVPLP), APC infected with a recombinant vaccinia virus that expresses PLP (VVplp), PLP_{139–151} peptide (PLP139), or PPD. Lymphocytes proliferated in response to VVplp, PLP139 and pCMVUPLP, but not to CONTROL or pCMVPLP. Lymphocyte proliferation was significantly greater in response to pCMVUPLP compared to CONTROL or pCMVPLP (§, $p < 0.001$, ANOVA). All assays were performed in triplicate. Values are the means $\pm$ SEM.

Fig. 5.

Cytokine production. Levels of interferon (IFN)- γ (a) and interleukin (IL)-4 (b) in the supernatants of lymphocytes, harvested from vaccinated mice (2 mice, pCMVUPLP), and mock-vaccinated mice (2 mice, Mock) on day 23 post-EAE induction, were measured by enzyme-linked immunosorbent assay (ELISA) at 24 and 48 hours after *in vitro* stimulation with PLP₁₃₉₋₁₅₁. (a) IFN- γ levels were 4 times higher in the mock-vaccinated mice than the pCMVUPLP mice at 48 hr. (b) IL-4 was detectable only in the pCMVUPLP mice. All assays were performed in duplicate. Values are the means $\pm$ SEM.