Design of Novel Cyclic Altered Peptide Ligands of Myelin Basic Protein MBP_{83-99} That Modulate Immune Responses in SJL/J Mice

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The use of antagonist peptides derived from the myelin sheath constitutes a promising therapeutic approach for multiple sclerosis (MS). Cyclization of peptide analogues is of great interest, since the limited stability of linear peptides restricts their potential as therapeutic agents. Herein, we designed and synthesized a number of cyclic peptides by mutating TCR contact sites of the MBP_{83-99} epitope. A number of cyclic analogues were tested for their ability to inhibit (antagonize) Th1 (IFN-γ) responses, and cyclo(83−99)[A^{91}]MBP_{83-99} mutant peptide was found to be the most efficient inhibitor. We demonstrated that cyclo(83−99)[A^{91}]MBP_{83-99} peptide emulsified in CFA enhanced Th2 (IL-4) and antibody responses in vivo. Moreover, immunization of mice with antagonist cyclo(83−99)[A^{91}]MBP_{83-99} peptide conjugated to reduced mannan enhanced IL-4 responses compared to cyclo(83−99)MBP_{83-99} peptide. Thus, cyclized peptides, which offer greater stability and enhanced responses, are novel leads for the immunotherapy of many diseases, such as MS. In particular, cyclo(83−99)[A^{91}]MBP_{83-99} is a promising mutant peptide analogue for the potential treatment of MS.

Introduction

T cell recognition of self-myelin peptides presented by major histocompatibility complex (MHC[a]) class II is involved in autoimmune attack in the human disease multiple sclerosis (MS). MS is a chronic, demyelinating disease of the central nervous system.[1] T cells, B cells, macrophages, and microglia are infiltrated through the disrupted blood−brain barrier, migrate, and induce inflammation, demyelination, and neurodegeneration.[1] $CD4^+$ T cells have been found to be reactive with the self-antigen, myelin basic protein (MBP), residues 82−102, with the minimum T cell epitope being residues 83−99, followed by residues 87−99. These peptides are presented primarily by HLA-DR2 (DRA, DRB1*1501) in humans, which are associated with susceptibility to MS.[2−4] Autoreactive $CD4^+$ T cells in MS patients secrete proinflammatory Th1-type cytokines, IFN-γ and TNF-α.[5] T cell recognition of the region 83−99 of MBP (MBP_{83-99}) has also been identified in healthy individuals, however, at relatively lower precursor frequency.[4,6] Th1 cytokines released after therapeutic administration are associated with exacerbation of MS; however, Th2 cytokines (IL-4, IL-5, and IL-10) have anti-inflammatory properties and down-regulate Th1 responses.[7,8]

The peptide epitope MBP_{83-99} binds with high affinity to HLA-DR2 using two hydrophobic residues, V^{87} and F^{90}, as primary anchors.[2,9−12] Residues H^{88}, F^{89}, and K^{91} are the main T cell receptor (TCR) contact residues, as alanine scanning of MBP_{83-99} affected T cell recognition but not HLA-DR2 binding.[13−15] It has also been shown that residue P^{96} interacts with the TCR.[16] In the SJL/J mouse there is high correlation between the immunogenicity of MBP_{81-100} and binding affinity to H2 I-A^s, with the minimum epitope required for binding to be MBP_{83-99} followed by MBP_{87-99}.[17,18] Structure−activity studies suggested an I-A^s binding motif and that peptide anchor residues were accommodated as P1 (hydrophobic), P2 (T, S, A), and P7 (H, R).[19] We recently deduced a novel structural insight into the possible peptide binding motif of I-A^s with linear mutant MBP_{83-99} analogues in comparison to the native MBP_{83-99} peptide (Katsara et al., manuscript submitted). Molecular modeling studies with I-A^s revealed that the side chain of the mutated residue at position K^{91} is exposed to make contact with TCR (Katsara et al., manuscript submitted), similarly to HLA-DR2.

A number of studies have used mutated peptides (altered peptide ligand, APL), whereby mutations of amino acids have been made at TCR contact residues in order to alter immune responses from Th1 to Th2 or inhibit experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Two linear APL of the immunodominant epitope MBP_{83-99} were injected into MS patients in two separate phase II clinical trials; however, they were suspended because of side affects and marginal benefits of treatment.[20−22] Thus, substitutions at critical TCR contact residues play an important role as immunomodulators; however, the design of new APL with fewer side effects is required.

A number of linear compounds have been used for the treatment of infectious diseases, autoimmunity, and cancer. However, since the turn of the century, many cyclic peptides have been introduced and have made important contribution to many diseases. Penicillin, cyclosporine, the echinocandins, and bleomycin are well-known cyclic peptides, and cyclic analogues have been used as synthetic immunogens, transmembrane ion channels, antigens for herpes simplex virus, inhibitors against α-amylase, protein stabilizers, and potential therapeutic agents for diabetes and MS (reviewed in ref 23). Cyclic peptides are more stable in comparison to their linear counterparts and have improved receptor selectively and better pharmacodynamic properties. We previously demonstrated that a lysosomal fraction of an EBV-transformed B cell line containing over 50 different enzymes, in addition to cathepsin D and exopeptidases (cathe-

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[a] Abbreviations: MHC, major histocompatibility complex; MS, multiple sclerosis; MBP, myelin basic protein; TCR, T-cell receptor; HLA, human leukocyte antigen; EAE, experimental autoimmune encephalomyelitis; CFA, complete Freund's adjuvant; PLP, proteolipid protein; APL, altered peptide ligand; KLH, keyhole limpet hemocyanin.

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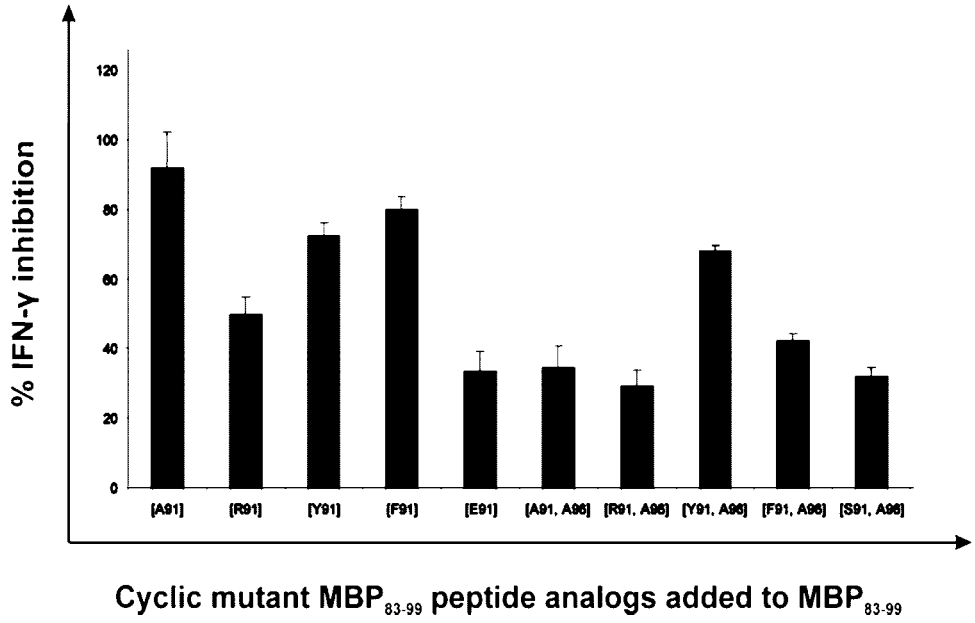


Figure 1. IFN-γ antagonism. Mice were immunized with native MBP_{83-99} peptide emulsified in CFA, and spleen cells were isolated 25 days later. The ability of cyclic mutant MBP_{83-99} peptide analogues to antagonize IFN-γ production was assessed by ELISpot analysis. The native MBP_{83-99} peptide was added together with cyclic mutated MBP_{83-99} analogues, and % IFN-γ inhibition is shown.

Table 1. Cyclic MBP_{83-99} Peptide Analogues Used in This Study

peptide analogues	sequence
cyclo(83−99)MBP_{83-99}	cyclo(83−99)E N P V V H F F K N I V T P R T P
cyclo(83−99)[A^{91}]MBP_{83-99}	cyclo(83−99)E N P V V H F F A N I V T P R T P
cyclo(83−99)[R^{91}]MBP_{83-99}	cyclo(83−99)E N P V V H F F R N I V T P R T P
cyclo(83−99)[F^{91}]MBP_{83-99}	cyclo(83−99)E N P V V H F F F N I V T P R T P
cyclo(83−99)[Y^{91}]MBP_{83-99}	cyclo(83−99)E N P V V H F F Y N I V T P R T P
cyclo(83−99)[E^{91}]MBP_{83-99}	cyclo(83−99)E N P V V H F F E N I V T P R T P
cyclo(83−99)[A^{91},A^{96}]MBP_{83-99}	cyclo(83−99)E N P V V H F F A N I V T A R T P
cyclo(83−99)[R^{91},A^{96}]MBP_{83-99}	cyclo(83−99)E N P V V H F F R N I V T A R T P
cyclo(83−99)[F^{91},A^{96}]MBP_{83-99}	cyclo(83−99)E N P V V H F F F N I V T A R T P
cyclo(83−99)[Y^{91},A^{96}]MBP_{83-99}	cyclo(83−99)E N P V V H F F Y N I V T A R T P
cyclo(83−99)[S^{91},A^{96}]MBP_{83-99}	cyclo(83−99)E N P V V H F F S N I V T A R T P

psin B and cathepsin H), was treated with linear MBP_{87-99} and [R^{91}, A^{96}]MBP_{87-99} and cyclo(87−99)MBP_{87-99}, cyclo-(91−99)[A^{91}]MBP_{87-99}, and cyclo(87−99)[R^{91}, A^{96}]MBP_{87-99} peptide analogues.[24] The linear peptides were not stable, whereas cyclic peptides clearly showed increased stability when digested with each of the enzymes.[24]

Herein, we designed and synthesized cyclic APL (head to tail) based on the longer peptide, residues 83−99, by mutating K^{91} with A^{91}, E^{91}, R^{91}, F^{91}, or Y^{91} or double mutations of K^{91}, P^{96} to [A^{91}, A^{96}], [R^{91}, A^{96}], [F^{91}, A^{96}], [Y^{91}, A^{96}], and [S^{91}, A^{96}]. Their ability to antagonize MBP_{83-99} IFN-γ responses in vitro was determined, and cyclo(83−99)[A^{91}]MBP_{83-99} antagonized IFN-γ responses up to 92%. Cyclo(83−99)[A^{91}]MBP_{83-99} peptide was tested further for its in vivo efficacy in SJL/J mice, and it was clear that cyclization together with the mutation at position 91 diverted Th1 (IFN-γ) responses to Th2 (IL-4) responses. Site directed mutagenesis studies was an important factor in these studies, which demonstrated that the cyclic peptide, cyclo(83−99)[A^{91}]MBP_{83-99}, is a novel peptide analogue for therapeutic applications against EAE.

Results

Cyclic MBP_{83-99} single and double mutant analogues are able to inhibit IFN-γ responses induced by the native MBP_{83-99} peptide. ELISpot assays are an ex vivo 18 h assay that measures IFN-γ secretion by T cells. It does not require expansion of cell cultures, as it detects specifically activated effector cells (both CD4 and CD8 cytokine producing terminal effectors). The sensitivity of the assay is higher than limiting dilution analysis, FACscan analysis, or ELISA methods and can reliably detect precursor frequencies of antigen specific effectors of 1 in every 0.5 millin cells. It is therefore an appropriate method to detect antigen specific cells. Here, we measured IFN-γ production generated by the native MBP_{83-99} peptide and assessed the % inhibition (antagonize) of IFN-γ produced in the presence of mutant peptide analogues.

Cyclic head-to-tail MBP_{83-99} single and double mutant analogues were tested for their ability to inhibit IFN-γ responses induced by the native agonist MBP_{83-99} peptide. Mice were immunized with the native agonist MBP_{83-99} peptide emulsified in complete Freund's adjuvant (CFA). Spleen cells were isolated 25 days later, and the ability of cyclic peptides to inhibit (antagonize) IFN-γ production was assessed in in vitro antagonism ELISpot assays (Figure 1, Table 1). The results are shown as % IFN-γ inhibition of each cyclic mutant peptide analogue, in comparison to the native MBP_{83-99} peptide alone. All cyclic peptide analogues inhibited IFN-γ production between ~30% and 90%. The single mutant analogues cyclo(83−99)-[F^{91}]MBP_{83-99} and cyclo(83−99)[Y^{91}]MBP_{83-99} and the double mutant analogue cyclo(83−99)[Y^{91}, A^{96}]MBP_{83-99} were found to inhibit IFN-γ between 60% and 80%. However, the single mutant analogue cyclo(83−99)[A^{91}]MBP_{83-99} was the most

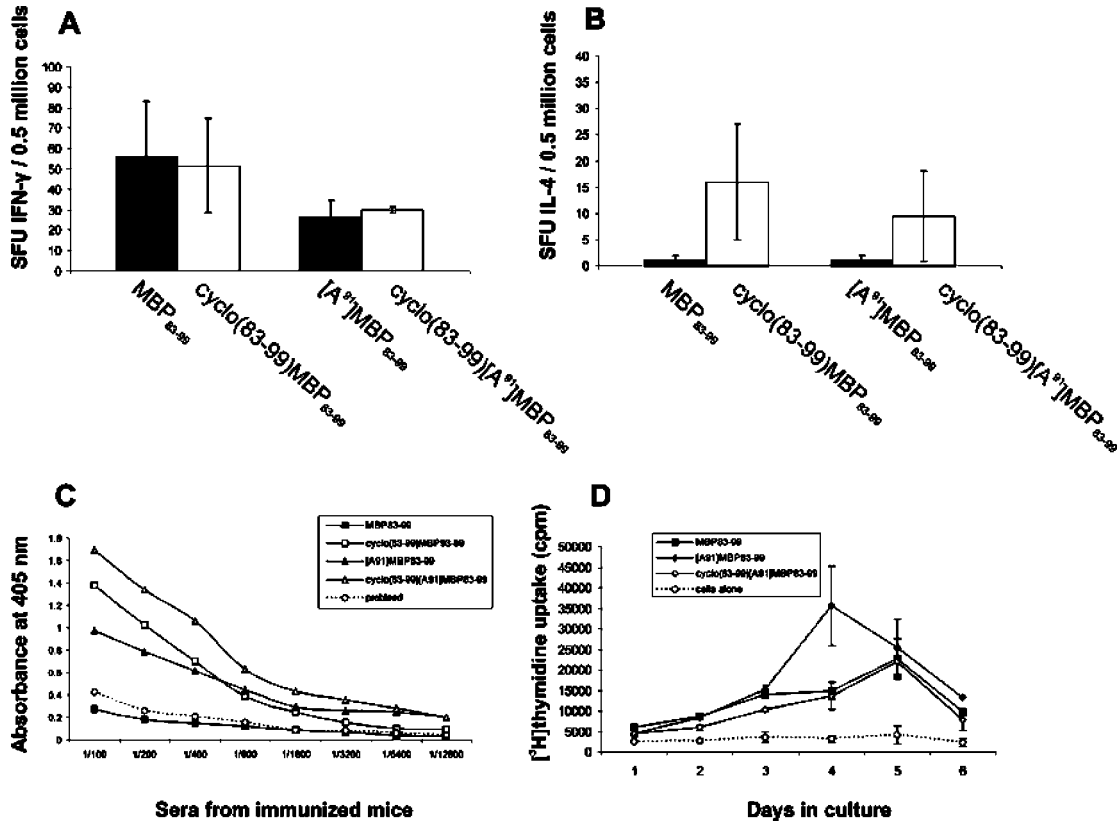


Figure 2. (A) IFN-γ responses and (B) IL-4 responses in SJL/J mice immunized with linear agonist MBP$_{83-99}$, linear antagonist [A^{91}]MBP$_{83-99}$, cyclo(83−99)MBP$_{83-99}$ and cyclo(83−99)[A^{91}]MBP$_{83-99}$ peptide analogues emulsified in CFA. IFN-γ or IL-4 responses are shown as SFU ± SEM per 0.5 million cells minus background (negative control). (C) Total IgG antibody levels were measured by ELISA coating with each respective peptide conjugated to KLH. (D) Mice were immunized with native MBP$_{83-99}$ peptide emulsified in CFA and proliferation of spleen cells determined in response to recall peptides. Data are shown as mean counts per minute (cpm) of triplicate wells ± SEM over 6 days. Results are representative of two experiments with three mice per group.

efficient in inhibiting IFN-γ production induced by the native MBP$_{83-99}$ peptide, up to 92%. On the basis of the ability of cyclo(83−99)[A^{91}]MBP$_{83-99}$ peptide to inhibit (antagonize) IFN-γ production by T cells, we chose to examine its in vivo efficacy in SJL/J mice after emulsification in CFA or conjugation to reduced mannan.

Cyclo(83−99)MBP$_{83-99}$ and cyclo(83−99)[A^{91}]MBP$_{83-99}$ compared to their linear counterparts enhance IL-4 production when emulsified in CFA. The ability of the antagonist cyclo(83−99)[A^{91}]MBP$_{83-99}$ and the native agonist cyclo(83−99)MBP$_{83-99}$ analogues to induce T cell responses after one injection, in comparison to linear [A^{91}]MBP$_{83-99}$ and linear MBP$_{83-99}$, was determined in SJL/J mice. Spleen cells were isolated 28 days after injection, and ELISpot assay was performed to measure IFN-γ and IL-4 secretion (Figure 2A and Figure 2B). Linear and cyclic MBP$_{83-99}$ native agonist peptides induced IFN-γ responses that were lower in linear and cyclic antagonist [A^{91}]MBP$_{83-99}$ mutant analogues (Figure 2A). However, no differences in IFN-γ production were noted between linear and cyclic analogues (Figure 2A). Interestingly, linear MBP$_{83-99}$ did not induce IL-4; however, cyclo(83−99)MBP$_{83-99}$ induced IL-4 (Figure 2B). Likewise, linear [A^{91}]MBP$_{83-99}$ was not able to generate IL-4; however, cyclo(83−99)[A^{91}]MBP$_{83-99}$ did generate IL-4 (Figure 2B). It is clear that cyclization of native agonist MBP$_{83-99}$ and antagonist [A^{91}]MBP$_{83-99}$ peptides enhances IL-4 production in vivo. No peptide (cells alone) was used as negative control, and ConA was used as an internal positive control that consistently induced >1000 SFU/0.5 million cells (not shown).

Cyclo(83−99)MBP$_{83-99}$ and cyclo(83−99)[A^{91}]MBP$_{83-99}$ compared to their linear counterparts enhance antibody production when emulsified in CFA. The production of specific IgG antibody responses in mice immunized with linear MBP$_{83-99}$, linear [A^{91}]MBP$_{83-99}$, cyclo(83−99)MBP$_{83-99}$, and cyclo(83−99)[A^{91}]MBP$_{83-99}$ peptides emulsified in CFA was measured using ELISA. Linear MBP$_{83-99}$ peptide did not generate IgG antibodies in SJL/J mice; however, antibodies were generated to cyclo(83−99)MBP$_{83-99}$ (Figure 2C). The mutated linear analogue was able to induce IgG antibody responses that were enhanced by its cyclic counterpart cyclo(83−99)-[A^{91}]MBP$_{83-99}$ (Figure 2C). Thus, cyclization of agonist MBP$_{83-99}$ and antagonist [A^{91}]MBP$_{83-99}$ enhanced antibody production in SJL/J mice.

Cyclization does not affect T cell proliferation to native MBP$_{83-99}$ peptide. Proliferation assays were used to detect the level of antigen specific T cells by measuring the [^{3}H]thymidine uptake of T cells proliferating in the presence of peptides on days 1−6.

Spleen cells from mice immunized with linear agonist-MBP$_{83-99}$ peptide were isolated 28 days after immunization and assessed by T cell proliferation assay. T cells proliferated to MBP$_{83-99}$ peptide, reaching a peak by day 5 of up to 25 000 cpm (Figure 2D). In addition, the MBP$_{83-99}$ specific T cells cross-reacted with linear [A^{91}]MBP$_{83-99}$ and mutant cyclo(83−99)[A^{91}]MBP$_{83-99}$ peptide analogues (Figure 2D). The cyclo(83−99)[A^{91}]MBP$_{83-99}$ peptide proliferated similarly to linear [A^{91}]MBP$_{83-99}$ peptide, reaching a peak on day 5 (Figure 2D). ConA (internal control) yielded proliferation of

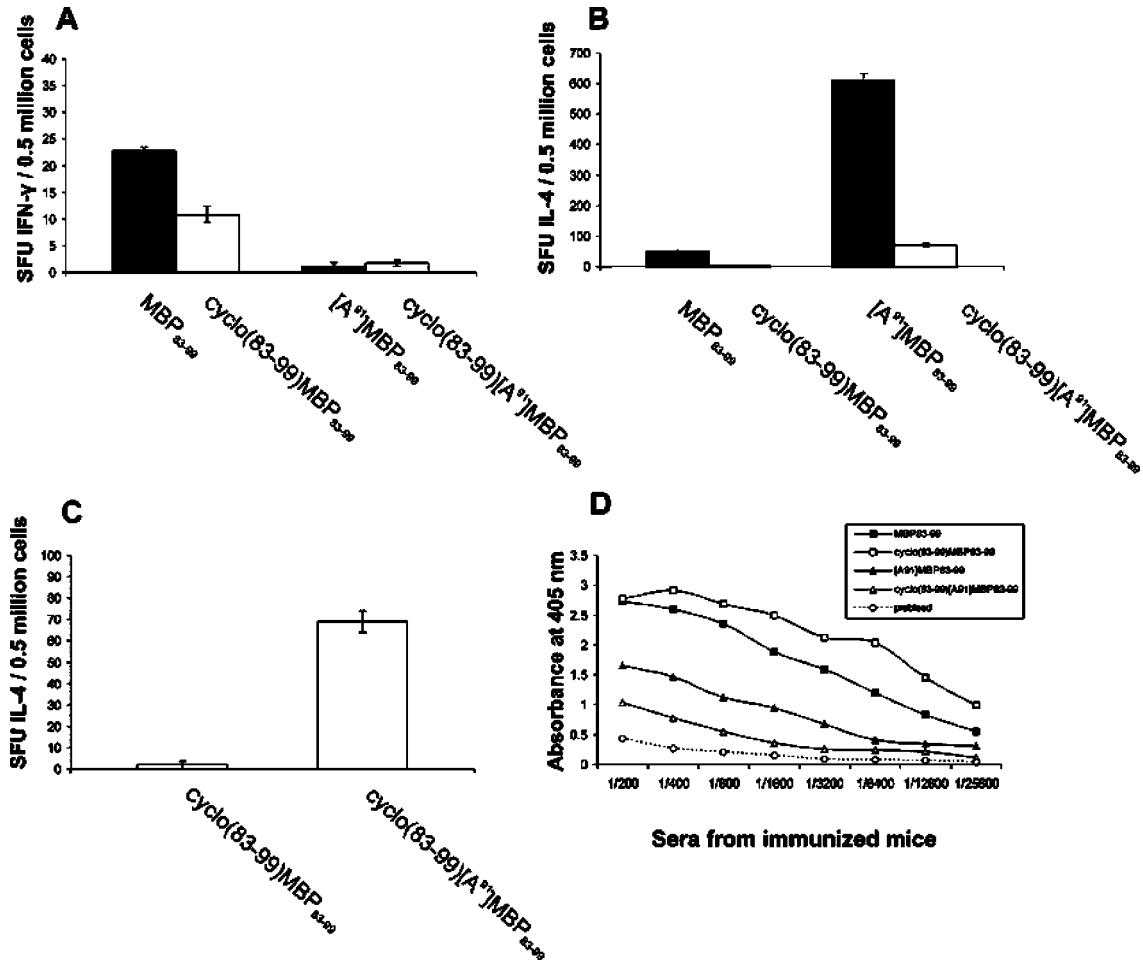


Figure 3. (A) IFN-γ responses and (B) IL-4 responses in SJL/J mice immunized with linear (agonist) MBP_{83-99}, linear mutant (antagonist) $[A^{91}]MBP_{83-99}$, cyclo(83−99)MBP_{83-99} and mutant cyclo(83−99)$[A^{91}]MBP_{83-99}$ peptide analogues conjugated to reduced mannan. IFN-γ or IL-4 responses are shown as SFU ± SEM per 0.5 million cells minus background (negative control). (C) IL-4 responses in SJL/J mice immunized with cyclo(83−99)MBP_{83-99} and cyclo(83−99)$[A^{91}]MBP_{83-99}$ peptide analogues conjugated to reduced mannan. (D) Total IgG antibody levels were measured by ELISA coating with each respective peptides conjugated to BSA. Results are representative of two experiments with three mice per group.

more than 75 000 cpm peaked on day 2 and was excluded from the figures, and no peptide (cells alone) was used as background negative control. Interestingly, although cyclization enhanced IL-4 responses and antibody production, it did not affect the proliferation of MBP_{83-99} specific T cell proliferation.

Reduced mannan conjugated to linear $[A^{91}]MBP_{83-99}$ and cyclo(83−99)$[A^{91}]MBP_{83-99}$ analogues enhances IL-4 production. The ability of linear and cyclic agonist MBP_{83-99}, and linear and cyclic antagonist $[A^{91}]MBP_{83-99}$ peptides conjugated to reduced mannan, to induce T cell responses after two injections was measured using IFN-γ and IL-4 cytokine secretion by ELISpot analysis. Mice immunized with linear and cyclic agonist MBP_{83-99} peptides generated IFN-γ secreting T cells; however, both linear and cyclic antagonist $[A^{91}]MBP_{83-99}$ peptides were negative (Figure 3A). Linear MBP_{83-99} and cyclo(83−99)MBP_{83-99} generated weak or no IL-4 cytokine secretion by T cells (Figure 3B). However, very strong IL-4 responses were induced to linear $[A^{91}]MBP_{83-99}$ peptide (up to 600 SFU/0.5 million cells) and moderate levels by cyclo(83−99)$[A^{91}]MBP_{83-99}$ (up to 70 SFU/0.5 million cells) (Figure 3B and Figure 3C). Thus, $[A^{91}]$ mutation to the agonist MBP_{83-99} peptide diverts immune responses from Th1 (IFN-γ) to Th2 (IL-4) when conjugated to reduced mannan. No peptide (cells alone) was used as negative control, and ConA was used as an internal positive control that consistently induced >1000 SFU/0.5 million cells (not shown).

Cyclization does not affect antibody responses when cyclic MBP_{83-99} peptide analogues are conjugated to reduced mannan. The production of total IgG antibody responses in mice immunized with linear agonist MBP_{83-99}, linear antagonist $[A^{91}]MBP_{83-99}$, cyclo(83−99)MBP_{83-99}, and cyclo(83−99)$[A^{91}]MBP_{83-99}$ peptide analogues, conjugated to reduced mannan, were measured using ELISA. High IgG antibody levels were generated in mice immunized with linear MBP_{83-99} and cyclo(83−99)MBP_{83-99} peptide analogues (titer up to at least $^1/_{25600}$) (Figure 3D). Lower antibody levels were induced by $[A^{91}]MBP_{83-99}$ (titers out to $^1/_{640}$) and very weak antibodies to cyclo(83−99)$[A^{91}]MBP_{83-99}$ peptide analogue (titers out to $^1/_{1600}$) (Figure 3D).

Discussion

The design and synthesis of mutated peptide analogues, APL (antagonists), from self-antigens of the myelin sheath constitute a promising approach for the treatment of MS. Many therapies use peptide analogues that could alter the immune response in patients, and peptides are valuable tools for peptide-mediated immunotherapy.[25]

We previously designed and synthesized cyclic mutated analogues based on the encephalitogenic guinea pig MBP_{74-85}

and human MBP_{87-99} epitopes in order to find effective and stable analogues to inhibit EAE. Cyclo(75−82)MBP_{74-85} was found to induce EAE in Lewis rats, while substitution of D^{91} with A^{91} (cyclo(75−82)[A^{91}]MBP_{74-85}) resulted in inhibiting EAE.[26,27] Both the linear MBP_{87-99} and cyclo(87−99)MBP_{87-99} induced weak EAE (clinical score 1) in Lewis rats. However, immunization with the double cyclic mutants, cyclo(87−99)[R^{91}, A^{96}]MBP_{87-99} or cyclo(91−99)[A^{96}]MBP_{87-99}, could not induce EAE, and when these analogues were co-injected with the encephalitogenic guinea pig MBP_{74-85}, they were found to decrease the development of EAE.[24,28] Moreover, peripheral blood mononuclear cells from healthy individuals and MS patients were pulsed with MBP_{87-99}, [R^{91}, A^{96}]MBP_{87-99}, cyclo(87−99)[R^{91}, A^{96}]MBP_{87-99}, and cyclo(91−99)[A^{96}]-MBP_{87-99} in vitro. The linear [R^{91}, A^{96}]MBP_{87-99} peptide and cyclo(91−99)[A^{96}]MBP_{87-99} peptides significantly increased the Th2/Th1 cytokine ratio, with the best effect achieved by cyclo(91−99)[A^{96}]MBP_{87-99}.[24] In addition, linear MBP_{87-99} and cyclo(87−99)MBP_{87-99} induced proliferation of a human T cell line (specific for MBP_{80-99}), whereas cyclo(87−99)[R^{91}, A^{96}]MBP_{87-99} and cyclo(91−99)[A^{96}]MBP_{87-99} inhibited the proliferation of the T cell line as did the linear [R^{91}, A^{96}]MBP_{87-99}.[24] Thus, both of the cyclic analogues behaved as altered peptide ligands of the linear native (agonist) MBP_{87-99} peptide. In addition, cyclo(87−99)MBP_{87-99}, cyclo(91−99)-[A^{96}]MBP_{87-99}, and cyclo(87−99)[R^{91}, A^{96}]MBP_{87-99} peptide analogues still maintained the biological function of the original peptide and showed greatly improved stability compared to their linear counterparts, when treated with lysosomal enzymes.[24]

Recently, we investigated the use of linear mutated analogues by mutating principal TCR contact residues emulsified in CFA to alter immune responses in SJL/J mice (Katsara et al., manuscript submitted). In particular, the double mutant [R^{91}, A^{96}]MBP_{83-99} peptide analogue was able to antagonize IFN-γ production in vitro by T cells against the native MBP_{83-99} peptide. Mannan binds to C-type lectins, such as the mannose receptor on antigen presenting cells (dendritic cells). In addition, mannan matures dendritic cells via TLR4.[29] Mannan stimulates Th1 response (IL-2, IFN-γ, IL-12, TNF-α, and IgG2a antibodies) or Th2 responses (not IFN-γ or IL-12 but significant amounts of IL-4, IL-10, and TGF-β and IgG1 antibodies) depending on mode of conjugation, oxidized or reduced mannan, respectively.[30−32] On the basis of these observations, we conjugated linear MBP_{83-99} peptide and mutant analogues to reduced mannan in order to examine if they could alter the immune response from Th1 to Th2 (Katsara et al., manuscript submitted). Interestingly, the linear single mutant [A^{91}]-MBP_{83-99}, [E^{91}]MBP_{83-99}, and [Y^{91}]MBP_{83-99} peptide analogues gave the best cytokine and antibody reactivity profile, with [Y^{91}]MBP_{83-99} being the most promising candidate peptide for immunotherapy of MS (Katsara et al., manuscript submitted). The use of reduced mannan to further divert immune responses to Th2 when conjugated to MBP peptides constitutes a novel strategy for immunotherapy of the disease.

Herein, we designed and synthesized cyclic mutant peptide analogues based on the epitope MBP_{83-99} in order to examine if these analogues were able to induce a Th2 cytokine profile. Current therapy of MS using interferon β-1a (IFN-β) can down-regulate IFN-γ secretion, and patients benefit from early subcutaneous injections.[33,34] Thus, we tested the cyclic analogues with single and double mutations in antagonism experiments in vitro to examine their ability to antagonize IFN-γ responses, and then we chose the most efficient for in vivo testing. All selected mutations were able to antagonize IFN-γ production induced by the native MBP_{83-99} peptide; however, the simplest aliphatic alanine substitution gave the best antagonism result. Thus, cyclo(83−99)[A^{91}]MBP_{83-99} peptide analogue was examined for its cytokine and antibody profile in vivo in SJL/J mice.

Cyclization of peptides MBP_{83-99} and mutant [A^{91}]MBP_{83-99} emulsified in CFA was able to enhance IL-4 production by T cells and antibody production in SJL/J mice. Furthermore, conjugation of peptide analogues to reduced mannan demonstrated that IL-4 was greatly enhanced with cyclo(83−99)-[A^{91}]MBP_{83-99} but more so with linear [A^{91}]MBP_{83-99} peptide.

We previously demonstrated that cyclic peptides bind to HLA-DR4,[24] and herein it is likely that the cyclic peptides bind to MHC class II I-A^s in cyclic form (Scheme 3); however, this needs to be further investigated. The enhanced IL-4 secretion and antibody production give promise for the use of cyclic peptides in the immunotherapy of diseases such as MS. The structures of these peptides, in particular cyclo83−99-[A^{91}]MBP_{83-99}, and their biological effects in SJL/J mice offer a template to progress to the development of synthetic compounds.

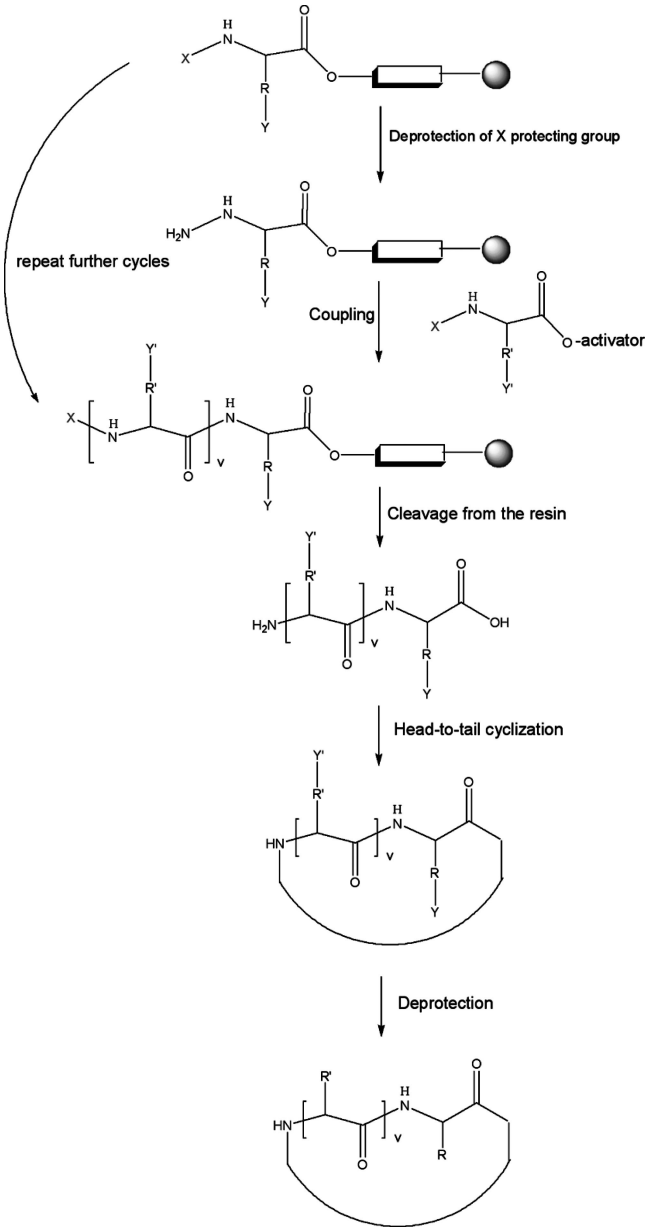
Experimental Procedures

(1) Solid-Phase Peptide Synthesis of Linear and Cyclic Analogues. Peptides (Table 1) were prepared on 2-chlorotrityl chloride resin (CLTR-Cl) using Fmoc/tBu methodology.[35,36] Head-to-tail cyclization was achieved with TBTU/HOAt and 2,4,6-collidine as base, as previously described (Scheme 1).[24,26−28,37] Preparative HPLC for peptide analogues were performed using a Lichrosorb RP-18 reversed phase semipreparative column with 7 μm packing material. The peptides were >95% pure as analyzed by mass spectrometry.

(2) Conjugation of Reduced Mannan to Linear and Cyclic MBP_{83-99} Peptide Analogues. Linear and cyclic MBP_{83-99} and [A^{91}]MBP_{83-99} peptide analogues (Table 1) were conjugated to keyhole limpet hemocyanin (KLH) via glutaraldehyde, which acts as a linker between mannan and peptide.[38] The 14 mg mannan (from *Saccharomyces cerevisiae*, Sigma, St Louis, MO) was dissolved in 1 mL of sodium phosphate buffer, pH 6.0, followed by the addition of 100 μL of 0.1 M sodium periodate (dissolved in pH 6.0 phosphate buffer) and incubation on ice for 1 h in the dark (Scheme 2). An amount of 10 μL of ethanediol was added to the mixture and incubated for a further 30 min on ice. The resultant mixture (oxidized mannan) was passed through a PD-10 column (Sephadex G-25 M column, Amersham Biosciences, Sweden) pre-equilibrated in pH 9.0 phosphate buffer to exclude sodium periodate and ethanediol. Oxidized mannan (7.0 mg/mL) was eluted with 2.0 mL of pH 9.0 phosphate buffer, to which 1.0 mg of MBP_{83-99}-KLH analogues were added and allowed to react overnight (O/N) at room temperature (RT) in the dark. Conjugation occurs via Schiff base formation between free amino groups of KLH and oxidized mannan. Reduced mannan-KLH-MBP_{83-99} complexes were prepared by adding 1.0 mg of sodium borohydride to each mixture for 6−8 h at RT in the dark (Scheme 2) and were used without further purification as previously described.[31] Samples were aliquoted and stored at −20 °C until used. MBP peptide analogues were previously characterized by capillary electropheresis for conjugation to mannan[39] and by SDS−PAGE, staining with Coomassie, silver, or Schiff's reagent (data not shown). Peptides were 100% conjugated to reduced mannan.

(3) Mice and Immunizations. Female 6−8 week old SJL/J mice used in all experiments were purchased from Walter and Eliza Hall Institute (Victoria, Australia) and housed at the Biological Research Laboratory at Burnet Institute (Austin Campus), Heidelberg, Australia. Linear and cyclic MBP_{83-99} peptide analogues were dissolved in PBS and emulsified in an equal volume of CFA containing 1.0 mg/mL of heat-killed *Mycobacterium tuberculosis* H37RA (Sigma, Victoria, Australia) and were given one subcutaneous injection containing 50 μg of peptide into the base of tail. In

Scheme 1. Schematic Representation for the Synthesis of Cyclic Analogues

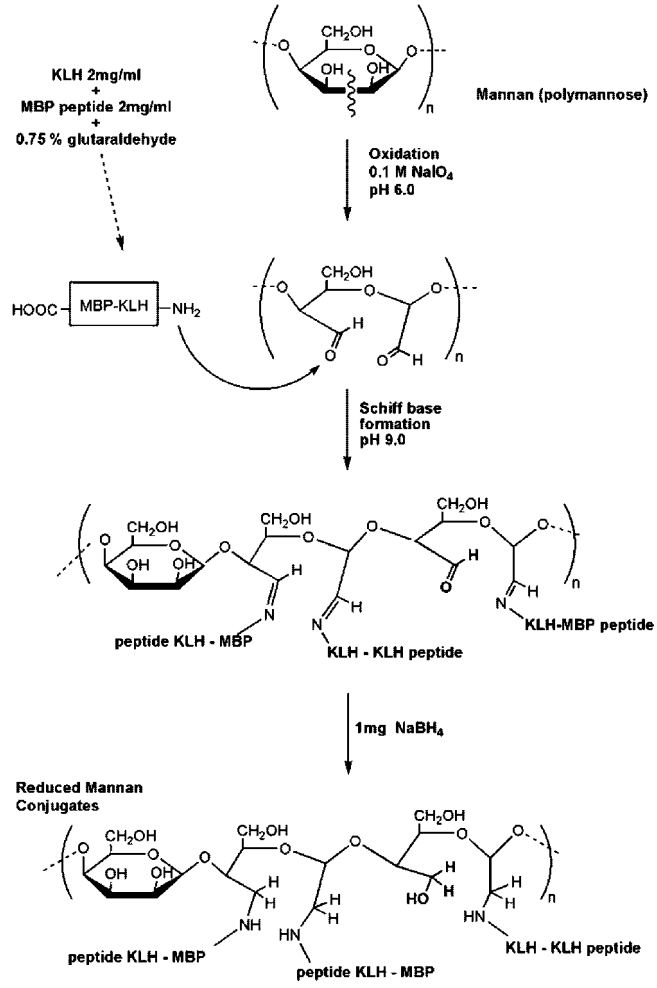


addition, SJL/J mice were immunized twice on days 0 and 14 intradermally (base of tail) with 50 μg of linear and cyclic MBP_{83-99} peptide analogues conjugated to reduced mannan.

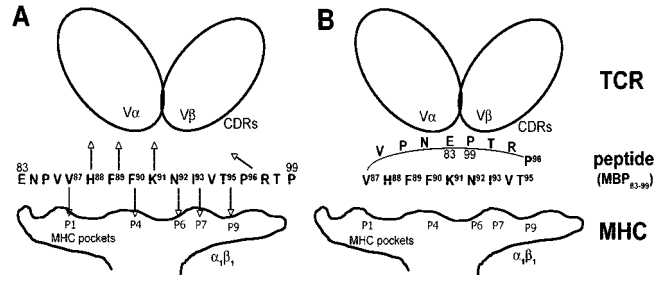
(4) Antagonism. Spleen cells from SJL/J mice immunized with agonist (native) MBP_{83-99} peptide were isolated 25 days after immunization and assessed by ELISpot for IFN-γ secretion by T cells to detect inhibition (antagonism) using cyclic MBP_{83-99} analogues. Assay was set up as ELISpot assay with the following modifications. An amount of 0.5 μg/mL of native MBP_{83-99} peptide was added together with 5 μg/mL ($\times$10) cyclic mutated MBP_{83-99} substituted analogues and incubated O/N.

(5) Immunological Assays. ELISpot. Spleen cells from immunized SJL/J mice were isolated 28 days after immunization and assessed by ELISpot for IFN-γ or IL-4 secretion by T cells. IFN-γ ELISpot assay was performed on MultiScreen-IP filter plate (MAIP S4510) with hydrophobic PVDF filters (Millipore, U.K.), while IL-4 ELISpot assays were performed on MultiScreen-HA filter plate (MAHA S4510) with mixed cellulose esters filters (Millipore, U.K.). MAIP S4510 plates were prewetted with 50 μL of 70% ethanol, washed 5 times with 200 μL of sterile phosphate buffered saline (PBS), and coated with 70 μL of 5 μg/mL anti-IFN-γ capture

Scheme 2. Conjugation of Reduced Mannan to Peptide Analogues



Scheme 3. Cartoon Model of (A) Linear and (B) Cyclic MBP_{83-99} Peptides in Complex with Generic MHC and TCR



antibody, AN18 (Mabtech, Australia) in PBS, and incubated at 4 °C O/N. An amount of 70 μL of 5 μg/mL anti-IL-4 capture antibody (Mabtech, Australia) was added directly to MAHA S4510 plates and incubated at 4 °C O/N without 70% ethanol treatment. Following five washes with PBS, plates were blocked by adding 200 μL of culture media (supplemented with 2.5% FCS) and incubated for 2 h at 37 °C. The blocking medium was discarded, and an amount of 10 μg/mL recall peptides was added to each defined well. ConA (1.0 μg/mL) was used as internal positive control and no peptide (cells alone) as negative control. Triplicate wells were set up for each condition. The 5×10^5 spleen cells in 100 μL of culture medium were seeded into each well and incubated at 37 °C for 18 h (IFN-γ) or 24 h (IL-4). Plates were washed five times with PBS/0.05% Tween-20 followed by five times with PBS and incubated for 2 h at RT with antimurine IFN-γ or IL-4 monoclonal antibody biotin. Plates were washed, and streptavidin-

ALP was added at 1.0 μg/mL and incubated for 2 h at RT. Spots of activity were detected using a colorimetric AP kit (Biorad, Hercules, CA) and counted using an AID ELISpot plate reader (Autoimmun Diagnostika GmbH, Germany). Data are presented as mean spot forming units (SFU) per 0.5 million cells ± standard error of the mean (SEM).

ELISA. Blood was collected and sera were isolated from mice prior to and after the first immunization. Linear and cyclic MBP_{83-99} peptide analogues conjugated to bovine serum albumin (BSA) or KLH were coated (10 μg/ml) onto polyvinyl chloride microtiter plates in 0.2 M $NaHCO_3$ buffer, pH 9.6, O/N at 4 °C, and nonspecific binding was blocked with 2% (w/v) BSA for 1 h at 37 °C. After washing (0.05% Tween-20/PBS), serial dilutions of sera were added and incubated for a further 2 h at RT. The plates were washed, and bound antibody was detected using HRP-conjugated sheep antimouse antibody (total IgG) (1:1000 in PBS) (Amersham, U.K.) and developed using 2,2′-azino-di(3-ethylbenzthiazoline)6-sulfonic acid (ABTS) (Sigma, U.K.). Absorbance at 405 nm was recorded using an ELISA microplate reader.

Proliferation. Spleen cells from agonist (native) MBP_{83-99} peptide immunized SJL/J mice were isolated 28 days after immunization and assessed by T cell proliferation assay. The 1×10^5 spleen cells in 100 μL of culture medium were seeded into 96-well U-bottom plates and incubated for 1−6 days at 37 °C in the presence of recall peptide (10 μg/ml), ConA (internal control), or no peptide (negative control). ConA (internal positive control) yielded proliferation of more than 75 000 cpm and was excluded from the figures, and no peptide (cells alone) was used as background negative control. Proliferation was assessed by adding 1 μCi of [^{3}H]thymidine per well to one plate per time point (days 1−6). Cells were incubated for further 6 h before harvesting onto glass fiber filters. [^{3}H]Thymidine uptake was measured using a β-scintillation counter (Top Count γ counter, Packard).

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Supporting Information Available: Purity data and mass specturm of the target compound (peptide) cyclo(83−99)-[A^{91}]MBP_{83-99}. This material is available free of charge via the Internet at http://pubs.acs.org.

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