Citrullination of Linear and Cyclic Altered Peptide Ligands from Myelin Basic Protein (MBP_{87-99}) Epitope Elicits a Th1 Polarized Response by T Cells Isolated from Multiple Sclerosis Patients: Implications in Triggering Disease

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Derangement of cellular immunity is central in the pathophysiology of multiple sclerosis (MS) and is often manifested by abnormal cytokine production. We investigated cytokine secretion in peripheral blood mononuclear cells (PBMC) of 18 MS patients and 15 controls and correlated cytokine polarization with the nature of antigenic stimulus. We synthesized two novel citrullinated peptides, linear [Cit^{91}, Ala^{96}, Cit^{97}]MBP_{87-99} and cyclo(87−99)[Cit^{91}, Ala^{96}, Cit^{97}]MBP_{87-99} that resulted from citrullination of 91,97 Arg residues in antagonists, linear [Arg^{91}, Ala^{96}]MBP_{87-99} and cyclo(87−99)[Arg^{91}, Ala^{96}]MBP_{87-99} peptides. PBMC from MS patients and controls were cultured with citrullinated peptides, and both peptides caused a Th1 polarization in all MS patients studied. In contrast, culture with noncitrullinated MBP peptides resulted in heterogeneous cytokine secretion that differed between individual patients. Thus, citrullination of self-antigens may potentially trigger disease in susceptible individuals. This finding may open new avenues in drug design of new substances that inhibit citrullination and arrest epitope spreading and worsening of MS.

Introduction

Multiple sclerosis (MS[a]) is a chronic disorder of the central nervous system that is predominantly characterized by local T cell and macrophage infiltration, leading to demyelination and loss of neurologic function.[1] MS is an autoimmune disease triggered by CD4+ T cells, although autoantibodies cannot be excluded. More recently, T helper (Th)17 cells have been shown to be involved in MS.[2] Antigens within the myelin sheath, such as myelin basic protein (MBP), proteolipid protein, and myelin oligodendrocyte glycoprotein,[3,4] have been described as the main constituents of which self-reactive CD4+ T cells and autoantibodies are directed against.

Altered peptide ligands (APL) are usually defined as analogues derived from the native, wild type peptide, which carry amino acid mutations at T cell receptor (TCR) contact residues.[5] Engagement of these APL by TCR usually impairs or drastically alters normal T cell function while retaining major histocompatibility complex (MHC) binding. Some of these APL (also known as mutant or antagonist peptides) are able to specifically antagonize and/or inhibit T cell activation induced by the wild type immunogenic (agonist) peptide.[6] Interest into the therapeutic management of MS has involved peptide analogues (mutated peptides) derived from agonist (wild type) peptides from MBP with the aim to shift T helper cell responses from the proinflammatory Th1 (IL-2, IFN-γ) to the antiinflammatory Th2 (IL-4, IL-5, or IL-10).[7−11] Peptides in the MHC−peptide−TCR complex, causing antagonism, have been shown to have fewer hydrogen bonds between peptide side chains to the complementarity-determining region 3 loops of the TCR by analysis of cocrystals.[12] A loss of hydrogen bond contact can cause wild type, agonist, or even superagonist (hyperstimulatory APL) peptides to become antagonists.[12] Similarly, structures of APL from vesicular stomatitis virus and human immunodeficiency virus in complex with MHC and/or TCR demonstrated that very minor conformational changes in the peptide side chain resulted in profound biological effects.[13,14] It has been demonstrated that APLs derived from guinea pig MBP_{74-85}, and human MBP_{83-99} and MBP_{87-99} epitopes have antagonistic effects by preventing experimental autoimmune encephalomyelitis (EAE) and diverting Th1 (IL-2, IFN-γ) cytokines toward Th2/Th3 (IL-10). Two APL derived from MBP_{83-99} epitope have entered into human clinical trials with varying responses and side effects.[15−18] We previously reported that a linear APL of the wild type MBP_{87-99} with mutations at Lys^{91} (principle TCR contact residue) and Pro^{96} (secondary TCR contact residue) resulting in [Arg^{91}, Ala^{96}]MBP_{87-99} (**P6**) was able to protect Lewis rats from development of EAE when co-injected with the encephalitogenic MBP_{74-85} peptide.[19,20] In addition, head-to-tail cyclization of **P6**−cyclo(87−99)[Arg^{91}, Ala^{96}]MBP_{87-99} (**P7**) peptide[8] completely blocked the development of EAE induced by MBP_{74-85}.[21,22] Furthermore, peripheral blood mononuclear cells (PBMC) from controls and MS patients cultured with **P6** and **P7** significantly increased the Th2/Th1 cytokine ratio.[23]

Numerous studies have implicated the role of citrullination of self-peptides in the pathogenesis of autoimmune diseases[24] including MS.[25−27] Citrullination is the conversion of Arg to citrulline (Cit), resulting in the positive charge of Arg side chain

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[a] Abbreviations: MS, multiple sclerosis; Th, T helper; MBP, myelin basic protein; APL, altered peptide ligand; TCR, T cell receptor; MHC, major histocompatibility complex; EAE, experimental autoimmune encephalomyelitis; PBMC, peripheral blood mononuclear cells; PAD, peptidylarginine deiminase; Cit, citrulline; TLC, thin layer chromatography; MD, molecular dynamics; SD, steepest descent; CG, conjugate gradient.

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Scheme 1. Conversion Reaction of Peptidyl-Arginine to Peptidyl-Citrulline by PAD II Enzyme

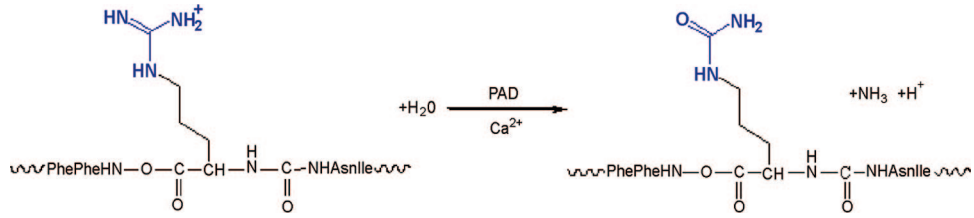
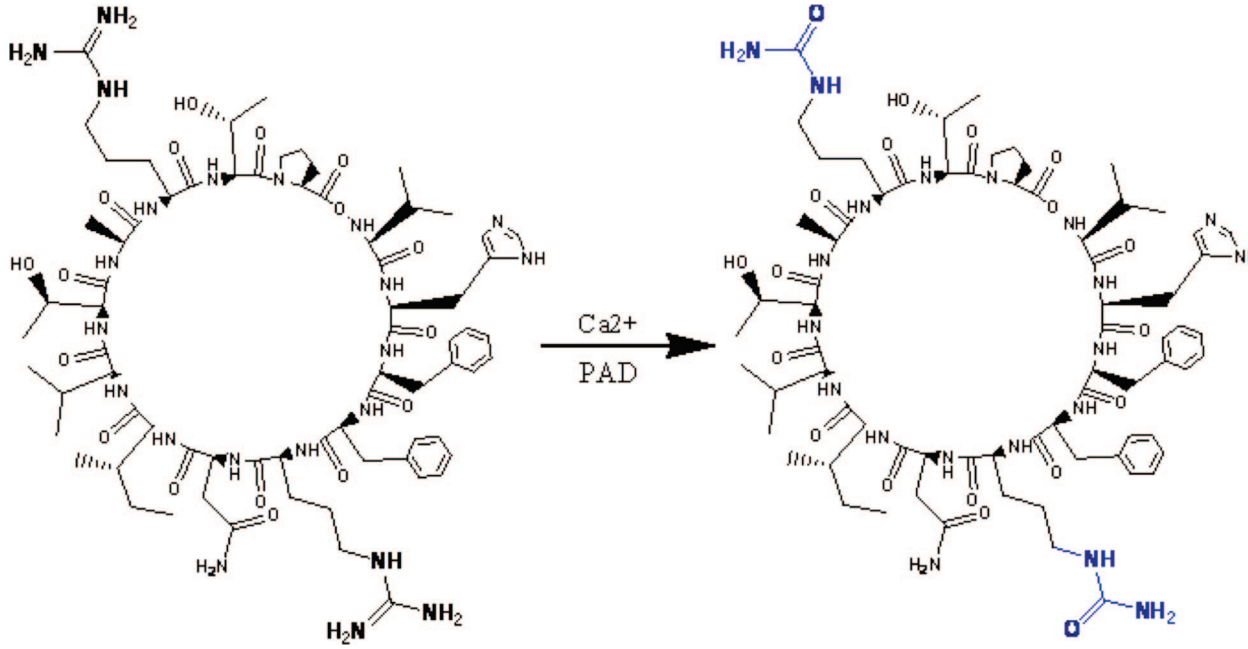


Table 1. Peptide Analogues Synthesized and Used in This Study

compound	description	amino acid sequence
wild type	linear MBP$_{87-99}$	ValHisPhePheLys^{91}AsnIleValThrPro^{96}ArgThrPro
P1	linear MBP$_{72-85}$	Glu-Lys-Ser-Glu-Arg-Ser-Glu-Asp-Glu-Asn-Pro-Val
P2	linear APL MBP$_{72-85}$	Glu-Lys-Ser-Glu-Arg-Ser-Glu-Ala-Glu-Asn-Pro-Val
P3	negative control	cyclo[(D−F)LLReKDap]
P4	cyclic MBP$_{72-85}$	Glu-Lys-Ser-Glu-Arg-Ser-Glu-Asp-Glu-Asn-Pro-Val-NH$_2$
P5	cyclic APL MBP$_{72-85}$	Glu-Lys-Ser-Glu-Arg-Ser-Glu-Ala-Glu-Asn-Pro-Val-NH$_2$
P6	linear APL, [Arg^{91}, Ala^{96}]MBP$_{87-99}$	ValHisPhePheArg^{91}AsnIleValThrAla^{96}ArgThrPro
P7	cyclo-APL, cyclo(87−99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$	ValHisPhePheArg^{91}AsnIleValThrAla^{96}ArgThrPro
cP6	linear [Cit^{91}, Ala^{96}, Cit^{97}]MBP$_{87-99}$	ValHisPhePheCit^{91}AsnIleValThrAla^{96}Cit^{97}ThrPro
cP7	cyclo(87−99)[Cit^{91}, Ala^{96}, Cit^{97}]MBP$_{87-99}$	cyclo(87−99)ValHisPhePheCit^{91}AsnIleValThrAla^{96}Cit^{97}ThrPro

Scheme 2. Conversion Reaction of Cyclo(87-99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$ (**P7**) by PAD II Enzyme



into neutral (Scheme 1). This transformation is referred to as deimination, an enzymatic reaction catalyzed by peptidylarginine deiminases (PAD). Citrullination results in a reduced positive charge of the protein, lowering its affinity to the negatively charged myelin phosphatidylserine residues which creates a noncompact structure of MBP sequence that is readily degradable by cathepsin D. Cathepsin D cleaves Phe−Phe peptide bonds, most of which are located in the internal regions of the protein, not exposed to solvent.[25,26] Therefore, citrullination of MBP results in a breakdown of the protein, leading to new citrullinated peptide epitopes that could trigger an immune response. In fact a charged isomer of MBP that is citrullinated and referred to as MBP-C8 has been shown to be recognized by CD4+ T cells from MS patients and control subjects.[28] T cell lines from MS patients also responded with higher frequency and sensitivity to MBP-C8.[27] Thus, given the high amount of citrullinated MBP protein in MS patients' brain tissue, a preferential response to MBP newly formed citrullinated peptides may be present and could contribute to disease.

Herein, we describe the conversion of linear and cyclic MBP$_{87-99}$ APLs, **P6** and **P7**, respectively, to their citrullinated analogues **cP6** ([Cit^{91}, Ala^{96}, Cit^{97}]MBP$_{87-99}$) and **cP7** (cyclo(87−99)[Cit^{91}, Ala^{96}, Cit^{97}]MBP$_{87-99}$) and their effects on cytokine stimulation by PBMC isolated from MS patients and control subjects. It is demonstrated that the APLs **P6** and **P7** in their citrullinated forms (**cP6** and **cP7**) result in the polarization of Th1 cytokines in MS patients PBMC, hence indicating that citrullination of MBP peptides may trigger disease. Furthermore, molecular modeling was used to gain insights into the actions noted by the citrullinated peptides.

Results

Peptide Synthesis and Citrullination. The peptides used in this study are shown in Table 1. The native (wild type) linear

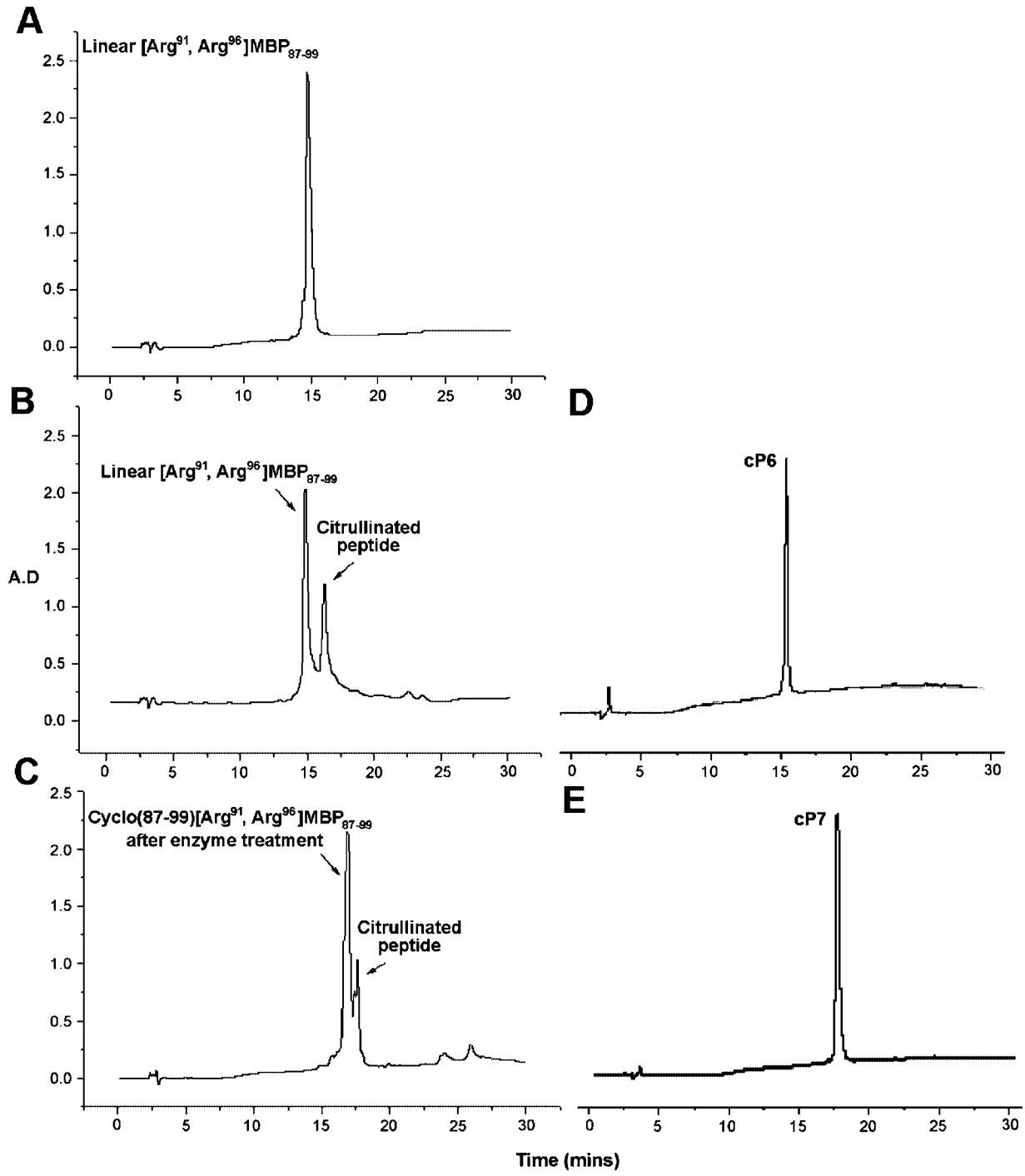


Figure 1. (A) HPLC analysis of linear [Arg^{91}, Ala^{96}]MBP$_{87-99}$ (**P6**) before treatment with enzyme PAD II and (B) after treatment with enzyme PAD II (**cP6**). (C) Cyclo(87−99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$ (**P7**) after treatment with enzyme PAD II (**cP7**). The citrullinated analogues (**cP6** and **cP7**) are eluted later than noncitrullinated **P6** and **P7**. Final purity HPLC analysis of (D) **cP6** and (E) **cP7**. Column used was Nucleosil C18, 250 mm × 4.6 mm, 5 μm. T_{Rcit} was 15.67 min (**cP6**) and 17.65 min (**cP7**). Elution conditions were as follows: 5% solvent b to 100% solvent b in 32 min; flow rate of 1 mL/min. Solvent a consisted of TFA in H_2O, 0.08% (v/v). Solvent b consisted of TFA in AcN, 0.08% (v/v).

peptide (MBP$_{87-99}$), linear APL with mutations at position Lys^{91} and Pro^{96} to result in [Arg^{91}, Ala^{96}]MBP$_{87-99}$ (**P6**) and its corresponding cyclic peptide, cyclo(87−99)[Arg^{91}, Ala^{96}]-MBP$_{87-99}$ (**P7**), were synthesized in house (Department of Chemistry, University of Patras), and were >95% pure as assessed by HPLC. In addition, control peptides (**P1**, **P2**, **P4**, **P5**) corresponding to the MBP$_{72-85}$ epitope, immunodominant guinea pig peptides, and a negative control cyclic peptide (**P3**) were also synthesized with >95% purity.

Peptides **P6** and **P7**were converted to their citrullinated forms **cP6** [Cit^{91}, Ala^{96}, Cit^{97}]MBP$_{87-99}$ and **cP7** cyclo(87−99)[Cit^{91}, Ala^{96}, Cit^{97}]MBP$_{87-99}$ (Scheme 2) by incubation with PAD II. Arginine modification was detected by thin layer chromatography (TLC) and was verified by reversed phase HPLC and mass spectroscopy. The citrullinated peptide eluted slightly later than the initial non-enzyme treated peptide (Figure 1). The converted citrullinated analogues **cP6** and **cP7** were measured to be 20.4% and 23.47%, respectively, of the final products. Citrullinated peptides were further purified and lyophilized, and the final purity HPLC of **cP6** and **cP7** (Figure 1D and Figure 1E) showed that peptides were >95% pure, the same level of purity as the parent peptide.

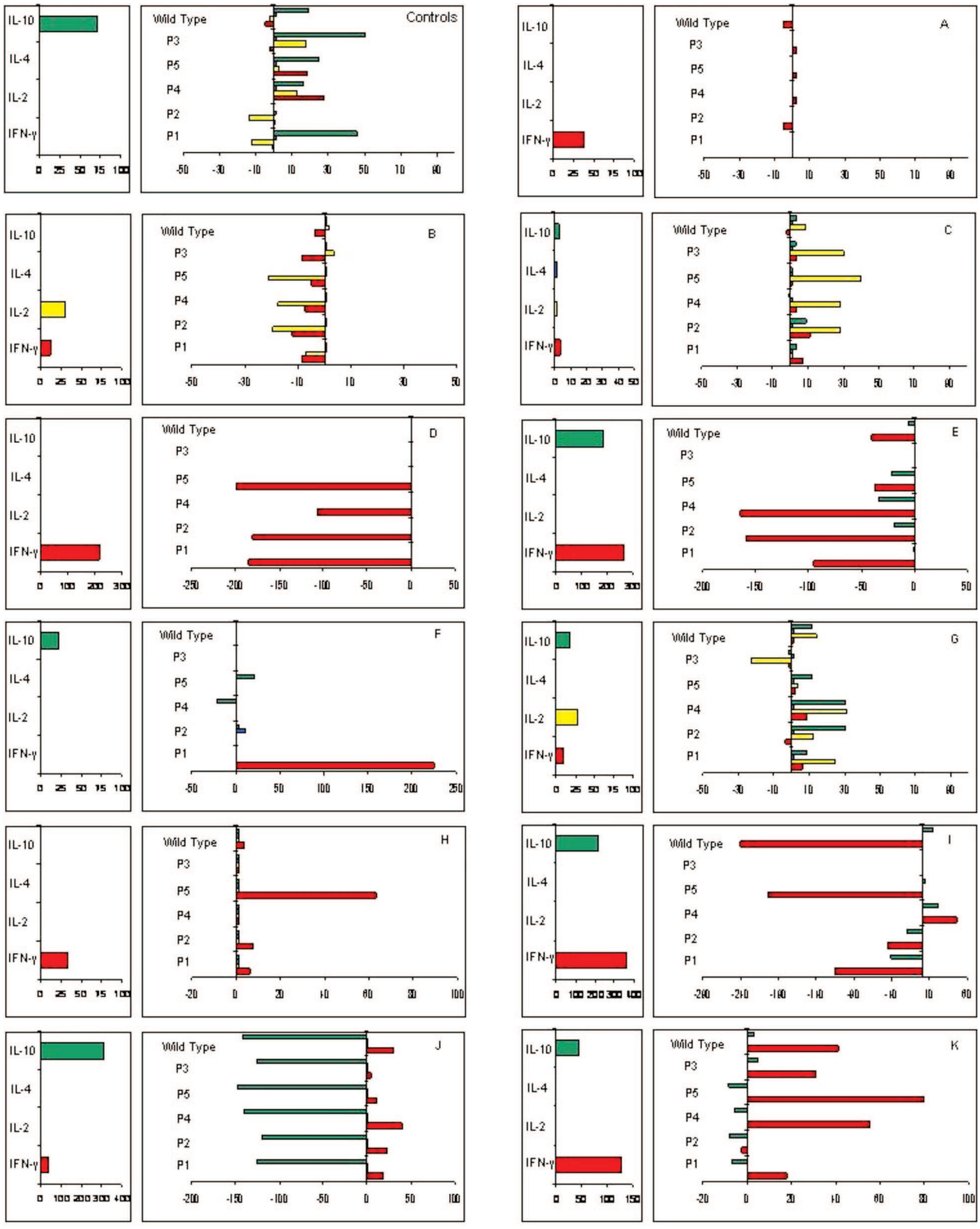


Figure 2. Net change in cytokine production over placebo values (cultures in plain culture media) of PBMC cultures from MS patients and controls cultured in the presence of 10 pg/mL per 10^6 cells of control peptides (**P1**−**P5**) and wild type peptide. Cytokines are measured in pg/mL per 10^6 cells: (A−K) results from individual MS patients. Color code for cytokines is as follows: IL-10 = green; IL-4 = blue; IL-2 = yellow; IFN-γ = red. Left-hand side panels show cytokine production by control and patient PBMC cultured in plain culture media for 72 h without the addition of peptides (placebo values).

Citrullination of APL Results in Th1 Cytokine Polarization. PBMC from MS patients and control subjects cultured in the presence of wild type and control peptides (**P1**−**P5**) clearly demonstrate the heterogeneity of the responses of MS patients PBMC to the peptides (Figure 2). The results shown are grouped together for control subjects and plotted separately for 11 individual MS patients. It was of interest to note that PBMC cultured with citrullinated analogues, **cP6** and **cP7**, resulted in an increase in the secretion of Th1 cytokines IL-2 and IFN-γ in all MS patients PBMC (n = 18) (Figure 3). Furthermore,

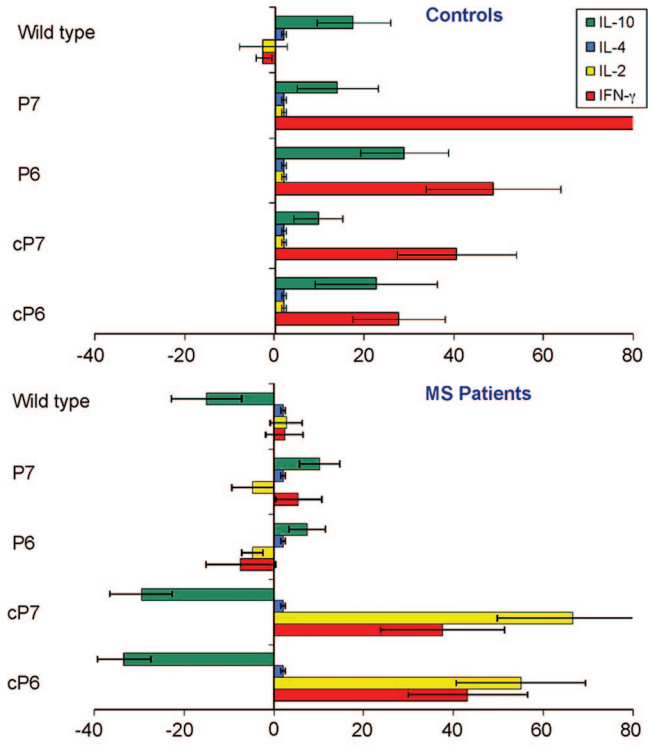


Figure 3. Net change in cytokine production over placebo values of PBMC cultures of MS patients and control subjects cultured in the presence of 10 pg/mL per 10^6 cells of peptide analogues **P6**, **P7**, **cP6**, **cP7**, and wild type. Grouped results from $n = 15$ controls and $n = 18$ MS patients are shown ± standard deviation.

the increase in Th1 cytokines was accompanied by a decrease in the Th2 cytokine, IL-10, creating a clear Th1 polarization. In contrast, culture with the noncitrullinated linear APL peptide **P6** resulted in a moderate induction of IL-10 and a moderate reduction of IL-2 and IFN-γ, demonstrating a Th2 dominance, as previously demonstrated.[23] Moreover, culture with the noncitrullinated cyclic peptide **P7** resulted in a reduction in IL-2 cytokine and induction of IFN-γ and IL-10, demonstrating a mixed Th1/Th2 response (Figure 3). Finally, culture with the wild type native peptide, MBP_{83-99}, resulted in a reduction of IL-10 cytokine, as expected. There was no effect in the secretion of the Th2 cytokine IL-4 by any of the peptides tested. By contrast, culture of PBMC from control donors ($n = 15$) with peptides **P6**, **P7**, **cP6**, and **cP7** resulted in the stimulation of IFN-γ and IL-10 cytokines whereas wild type peptide stimulated only the production of IL-10. Similar to MS PBMC, peptides **P6**, **P7**, **cP6**, and **cP7** had no effect on IL-4 production by control PBMC.

Structural Insights of cP6 and cP7 Peptides. In order to gain insights into the structures of **cP6** and **cP7** peptides in their free state used in this study, molecular dynamics (MD) simulations and minimization protocols were used. The six lowest energy frames for **cP6** (Figure 4A) and **cP7** (Figure 4B) peptides and the global energy minimum structures (Figure 4C) demonstrate some differences and similarities. The linear analogue (**cP6**) is extended compared to its cyclic form (**cP7**). Hence, the substitution of Arg^{91} and Arg^{97} to citrullines in its linear conformation allows a more extended conformation compared to the linear noncitrullinated analog (**P6**). The primary TCR contact amino acids His^{88}, Phe^{89}, Cit^{91} in the linear analogue (**cP6**) point up toward the TCR, and the major MHC anchor amino acid Phe^{90} points down into the MHC. This is in line with the crystal structure of MBP_{83-99} in complex with human MHC class II (HLA-DR2) and the deduced model for binding to murine MHC class II ($H2\text{-}I\text{-}A^s$).[9,10,29−31] In addition, His^{88} and Phe^{89} are in proximity to the cyclic citrullinated analogue (**cP7**), much closer to that of its linear counterpart, **cP6**. Furthermore, in both models **cP6** and **cP7**, Cit^{97} points up toward the TCR (Figure 4C); thus, it is quite possible that Cit^{97} may make novel contacts with the TCR, leading to T cell stimulation. Likewise, Cit^{91} could also make novel contacts with the TCR leading to altered responses. Furthermore, Figure 5 shows the superimposition of His^{88}, Phe^{89}, and Phe^{90} counterparts of linear (**cP6**) and cyclic (**cP7**) analogues, with the same region obtained from that of the X-ray crystal structure of the native MBP_{83-99} peptide (PDB code 1ymm).[32]

Discussion

Posttranslational modifications (citrullination, phosphorylation, deamidation, deimination, methylation, glycosylation) are common biological processes that alter specific parts of a protein after synthesis. Most proteins undergo some form of posttranslational modification, and almost all amino acids can be altered by one or more of these processes. The modified protein thus contains new or rare amino acids or new specific side groups that can have critical influence on the structure and function of the protein. Furthermore, degradation of the protein results in several new peptide fragments that may elicit immune responses. The conversion of Arg to citrulline[33] may result in the induction of an altered immune response compared to the native noncitrullinated counterpart. Such responses have been implicated in the pathogenesis of autoimmune/inflammatory diseases, such as MS and rheumatoid arthritis.[25,26,34,35] In rheumatoid arthritis detection of antibodies against cyclic citrullinated peptides (anti-CPP antibodies) is an indicator and diagnosis of the disease.[36,37] In addition, T cells have been shown to be stimulated to citrullinated hen egg-white lysozyme peptides bound to MHC.[38] Furthermore, citrullinated peptides bind with higher affinity to HLA-DR4 and lead to activation of CD4+ T cells in HLA-DR4-IE transgenic mice.[39] In the adult human brain 15−20% of MBP is citrullinated, whereas in the brain of MS patients the amount of citrullinated MBP is about 3-fold higher (45−60%).[40] CD4+ T cells from MS patients recognize citrullinated MBP (MBP-C8)[28] and respond with higher sensitivity.[27] An increased proportion of citrullinated MBP (MBP-C8) is present in the brains of MS patients. Isolation of MBP-C8 from guinea pig brains was shown to be encephalitogenic in Lewis rats. MBP-C8 was also able to reinduce EAE in Lewis rats that had been previously recovered from unmodified MBP induced EAE.[41] Different MBP charged isomers (C1−C8) isolated from normal and MS white matter tissue indicated that deimination was found at R^{43}, R^{49}, R^{54}, R^{65}, R^{97}, R^{122}, R^{130}, and R^{162}. The citrullinated residues varied between MS samples, and partial deimination at several residues was common.[42] Other reports also suggest that R^{25}, R^{33}, R^{159}, and R^{170} are also citrullinated.[40,43,44] Hence, R^{97} studied herein has been found to be citrullinated in MS patients, and the **cP6** and **cP7** studied herein reflect the natural state of citrullinated arginine residues. Given the high amount of citrullinated MBP protein in MS patients brain tissue and areas of spinal cord,[45] it is highly likely that preferential T cell responses to MBP newly formed citrullinated peptides may be present and could contribute to disease.

We previously noted that double mutations of the linear wild type peptide MBP_{87-99} at positions 91 and 96 with Arg and Ala, respectively, **P6**, suppressed guinea pig MBP_{72-85} induced EAE in Lewis rats. Head-to-tail cyclization of linear APL **P6** led to cyclic **P7**, which retained its antagonistic effect in an EAE model. In addition, **P6** and **P7** have been shown to bind

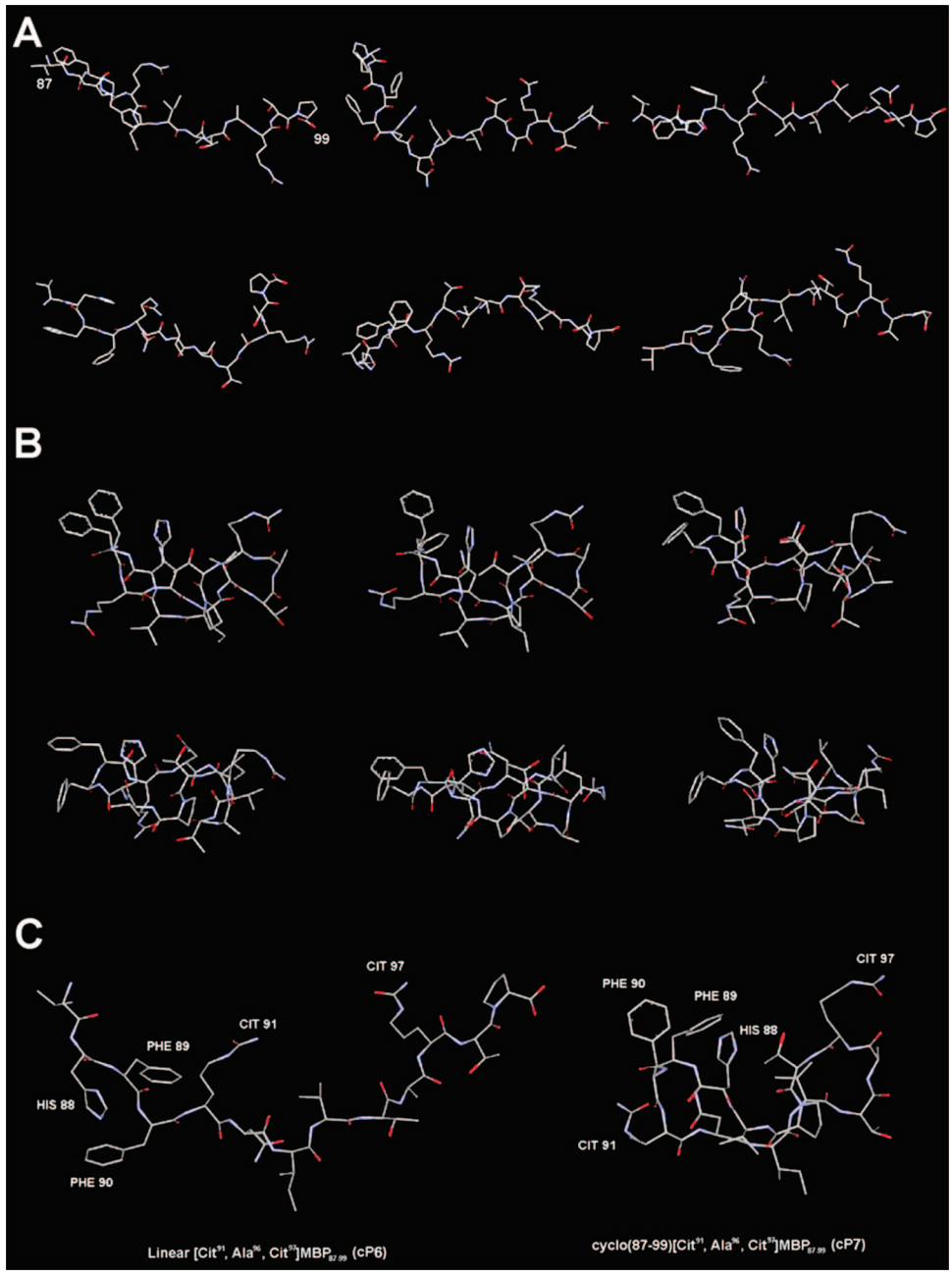


Figure 4. Representative lowest energy conformations of (A) linear [Cit^{91}, Ala^{96}, Cit^{97}]MBP$_{87-99}$ (**cP6**) and (B) cyclo(87−99)[Cit^{91}, Ala^{96}, Cit^{97}]MBP$_{87-99}$ (**cP7**) after MD simulations and minimizations with SD and CG algorithms. (C) Global energy minima for citrullinated **cP6** and**cP7** peptides after MD simulations.

to HLA-DR4, increase the Th2/Th1 cytokine ratio of PBMC from MS patients, and were able to suppress the proliferation of a CD4+ T cell clone specific to wild type MBP$_{87-99}$.[23] Changing cytokine secretion patterns is an important approach in regulating disease. In MS patients, the majority of MBP$_{87-99}$ specific CD4+ T cells, isolated during active disease, secrete Th1 proinflammatory cytokines whereas during remission the cytokine profile shifts to Th2/Th3 antiinflammatory cytokines.[46] Hence, the induction of Th2 responses during active disease should reverse disease. In addition, the role of IL-10 in the induction and function of natural and antigen induced regulatory T cells in autoimmune diseases has been demonstrated.[47] Cytokine secretion investigations were included in the evaluation study herein of linear and cyclic APL (**P6** and **P7**) and were compared to their citrullinated converted analogues (**cP6** and **cP7**). The results of the present study strongly support the long suspected importance of citrullination as a major mechanism in triggering autoimmune/inflammatory diseases such as MS. In particular, culture of PBMC from MS patients with citrullinated analogues **cP6** and **cP7** increased the secretion of IL-2 and IFN-γ and simultaneously decreased the secretion of IL-10, characteristic of Th1 polarization. On the contrary, the noncitrullinated linear APL peptide **P6** induced the production of IL-10 and reduced the production of IL-2 and IFN-γ characteristic of Th2 polarization. Cyclic APL peptide **P7**, however, stimulated IFN-γ, but simultaneously IL-10 was also induced. These findings indicate that citrullination of APL (antagonistic) peptides from MBP$_{87-99}$ immunodominant peptide, leading to Th1 dominance, may have implications in exacerbations or triggering disease. It would be of interest to study the effects of the citrullinated peptides on the numbers and function of T regulatory cell populations[48] from MS patients.

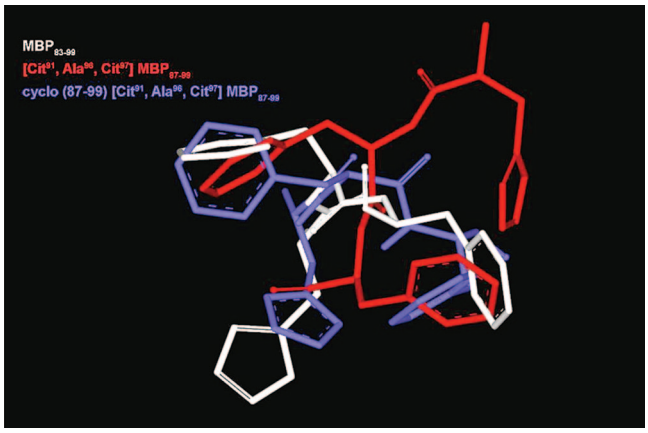


Figure 5. Superimposition of His^{88}, Phe^{89}, and Phe^{90} obtained from the X-ray structure of MBP_{83-99} peptide with the lowest energy conformations of linear [Cit^{91}, Ala^{96}, Cit^{97}]MBP_{87-99} (**cP6**) and cyclo(87−99)[Cit^{91}, Ala^{96}, Cit^{97}]MBP_{87-99} (**cP7**) analogues.

Furthermore, these studies may open new avenues in drug design of citrullination inhibitors.

Moreover, previous conformations of the cyclic APL **P7**[49] revealed that the proximity of residues at positions 91 and 97 were not favorable. Charge repulsion between Arg^{91} and Arg^{97} due to the positive charges at these position, seemed to account for the opposite orientations of Arg^{91} and Arg^{97} side chains. A β II turn present in the region 92−96 is a conformational feature in **P7**.[50] This conformational characteristic is responsible for a more compact conformation of the APL, which may be responsible for its altered effect. This conformational pharmacophore model defines the MHC (Phe^{90}) anchor and TCR (His^{88}, Phe^{89}) contact sites and the overall volume of the target molecules resulting in weak interactions with the TCR and, hence, possibly its antagonistic outcomes.[49,51,52] In order to gain insights of the structures of their citrullinated analogues **cP6** and **cP7** in possibly inducing disease, MD simulations of **cP6** and **cP7** were performed. The primary TCR contact amino acids His^{88}, Phe^{89}, Cit^{91} in the linear analogue (**cP6**) point up toward the TCR with Phe^{90} pointing down into the MHC. Of interest, in both models **cP6** and **cP7**, Cit^{91} and Cit^{97} point up toward the TCR, thus implicating that both these noncanonical amino acids may play a role in the activation of T cells leading to altered or enhanced responses. Furthermore, it is clear that there is overlap of His^{88}, Phe^{89}, and Phe^{90} residues between the models and with that of the crystal structure in complex with MHC, in particular cyclo(87−99)[Cit^{91}, Ala^{96}, Cit^{97}]MBP_{87-99} (**cP7**) with the native MBP_{83-99} peptide.

Conclusion

We synthesized citrullinated analogues of a linear and cyclic APL from MBP_{87-99}. The results presented here demonstrate that culture of PBMC from MS patients with citrullinated MBP_{87-99} APL results in a strong and uniform Th1 polarization. In contrast, culture with noncitrullinated MBP_{87-99} or other MBP peptides resulted in heterogeneous cytokine secretion patterns, with each patient's PBMC responding differently when cultured with each peptide. Molecular modeling suggested that both Cit^{91} and Cit^{97} residues point up toward the TCR, thus implicating the possible interaction with the CDR3 loops of the TCR and inducing an altered cytokine response. This study provides direct experimental evidence that citrullination is essential for antigenic recongition of self-epitopes by MS T cells and may open new avenues in drug design of citrullination inhibitors to suppress disease.

Experimental Procedures

1. Synthesis of Linear and Cyclic Analogues. Control peptides **P1**−**P5** (Table 1) were synthesized and used in this study. In addition, the native (wild type) linear peptide (MBP_{87-99}) and its mutant analogues, linear [Arg^{91}, Ala^{96}]MBP_{87-99} (**P6**) and cyclo(87−99)[Arg^{91}, Ala^{96}]MBP_{87-99} (**P7**), were synthesized as previously described.[19,21,22] Briefly, the syntheses of linear peptides were carried out in solid phase using the Fmoc/tBu methodology, utilizing the 2-chlorotrityl chloride resin. For the total synthesis N^{a}-Fmoc protected L-amino acids were used. The protected final products were further used for the synthesis of the cyclic analogues. To a mixture of the linear protected peptide in dry DMF and 2,4,6-collidine was added 1-hydroxy-7-azabenzotriazol. The solution was then added dropwise to a solution of *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate in dry dimethylformamide for 2 h and stirred for 4 h. The reaction was followed by the ninhydrin test on TLC. The solvent was removed from the reaction mixture under reduced pressure, affording a light-yellow oily residue. The cyclic protected peptides were precipitated from H_2O and were dried in vacuo for 12 h. The protected cyclic peptides were treated with the deprotection mixture DCM/TFA/ethanedithiol/anisole/H_2O (32/65/2/1) for 4 h at room temperature. The resulting solution was concentrated under vacuum to a small volume. The final free cyclic peptides were precipitated as a light-yellow amorphous solid by the addition of diethyl ether, filtered, and dried in vacuo for 12 h. The purity of peptides were >80%. The final crude products were further purified using RP-HPLC, and the resultant peptide purities were >95% (Figure 1). All peptides used in this study are in Table 1.

2. Citrullination of Linear and Cyclic Analogues Using Peptidylarginine Deiminase. Linear [Arg^{91}, Ala^{96}]MBP_{87-99} (**P6**) and cyclo(87−99)[Arg^{91}, Ala^{96}]MBP_{87-99} (**P2**) peptides were treated with PAD II (Sigma Chemical Co.) to substitute all arginine residues with citrullines.[33,53] Usually, an amount of 4.9 mg of peptide (about 267 nmol for **P6** and 318 nmol for **P7**) was dissolved in 0.1 mL of 10 mM HEPES buffer, pH 7.6, containing 5 mM $CaCl_2$ and 2 mM dithiothreitol. This was followed by the addition of 2 units of PAD II, and the mixture was incubated at 52 °C for 48 h. The enzyme treatment was stopped by heating at 100 °C for 5 min, and the solutions were clarified by centrifugation at 14000*g* for 5 min. Arg modifications were assessed by TLC, using 5% *p*-dimethylaminobenzaldehyde in HCl as developer, where citrulline residues produced a yellow color. Arg modifications were also verified by reversed phase HPLC. Modified peptides were further purified/lyophilized, and the resultant peptide purities were >95% (Figure 1). **P6** in its citrullinated form is designated **cP6** (linear [Cit^{91}, Ala^{96}, Cit^{97}]MBP_{87-99}), and **P7** in its citrullinated form is designated as **cP7** (cyclo(87−99)[Cit^{91}, Ala^{96}, Cit^{97}]MBP_{87-99}). All peptides used are shown in Table 1.

Table 2. Demographic Details of MS Patients and Control Subjects[a]

subjects	sex	age	type of disease	phase of disease	treatment	EDSS	disease duration
MS patients, n = 18	12F, 6M	35.1 (17−66)	RR	acute	no rx (n = 12), IFN-β (n = 6)	2.58 (1−6.5)	5.01 (0−19)
controls, n = 15	8F, 7M	28.5 (20−55)	N/A	N/A	N/A	N/A	N/A

[a] Age (years), duration of disease (years), and EDSS scores are given as median with (range); n, sample size; F, female; M, male; RR, remitting−relapsing MS; no rx, no treatment; IFN-β, under continuous prophylactic therapy with IFN-β (IFN-β1β, Betaferon, Schering A.G., 0.25 mg subcutaneously every second day or IFN-β1α, Avonex, Biogen Inc., 3 μg intramuscularly once a week or Rebif, Serono A.G., 44 μg subcutaneously every second day). N/A, not applicable.

3. Biological Evaluation. 3.1. Study Subjects. Eighteen adult patients with definite MS of the remitting relapsing type (RR-MS) were studied. The patients were treated and followed up in the Neurology Clinic of Patras University Hospital (PUH). All patients were in the acute phase of the disease at the time of blood sample collection and did not suffer from any additional ailment. The blood samples were drawn before administration of treatment. A control group consisted of 15 adult healthy volunteers. An amount of 10 mL of heparinized venous blood was drawn from MS patients and control subjects once. Demographic details of all study subjects are shown in Table 2. Informed consent was obtained from each participating patient. PUH abides by the Helsinki declaration on ethical principles for medical research involving human subjects.

3.2. Cell Cultures and Measurement of Cytokines. PBMC were isolated from MS patients and control subjects and cultured for 72 h in culture medium as described previously in the presence or absence of peptides (wild type, **P6**, **P7**, **cP6**, or **cP7**) at 10 pg/mL of culture per 10^6 cells.[21] This stimulation peptide dose was chosen after titration of peptides from 1 pg/mL to 1 μg/mL and found to be the most effective (not shown). At the end of the culture period culture supernatants were collected by centrifugation at 12 000 rpm at 4 °C for 1 min and stored at −30 °C. ELISA assays for human IL-2, IFN-γ, IL-4, and IL-10 were performed according to the manufacturers instructions (Diaclone, Besançon, France).

4. Molecular Modeling. All computer calculations were performed on Pentium IV 2.14 GHz and Silicon Graphics O2 workstations using Discovery Studio, version 1.7, Quanta 2005 molecular modeling systems by Accelrys Software Inc. (San Diego, CA). The representative conformers of linear and cyclic analogues were generated using backbone unrestrained molecular dynamics simulation. Thus, populations of the various conformers that represent local minima at the potential energy surface were identified.

4.1. Generating the Initial Conformations. The starting conformations of linear [Cit^{91}, Ala^{96}, Cit^{97}]MBP_{87-99} (**cP6**) and cyclo(87−99)[Cit^{91}, Ala^{96}, Cit^{97}]MBP_{87-99} (**cP7**) peptide analogues were extendedly built, consisting of L-amino acids, on the CHARMm force field.[54] The dielectric constant was set to 45, as DMSO, which is a polar, aprotic solvent that simulates better the receptor environment during all the experiments. The generalized Born solvent model with a simple switching[55] was used, as well as the SHAKE algorithm, which serves bond geometry constraints during the MD simulations.[56] First, the structures of linear [Cit^{91}, Ala^{96}, Cit^{97}]MBP_{87-99} (**cP6**) and cyclo(87−99)[Cit^{91}, Ala^{96}, Cit^{97}]MBP_{87-99} (**cP7**) peptides in their free state were energy-minimized using steepest descent (SD) and conjugate gradient (CG) algorithms, with 10 000 steps and rmsd of 0.001 Å as energy convergence criterion.

4.2. Molecular Dynamics (MD) Simulations. MD runs were performed using the CHARMm force field as follows: Heating, from 0 to 300 K gradually, and equilibration were set with a time step of 0.002 ps while the time step of production was 0.002 ps for a total time of 1 ns. Parameters on saving the resulting frequencies were set in such a way in order to extract 500 conformations for each molecule. Six local energy minimum conformations were extracted from each molecule according to the potential energy surface. After the dynamics simulations, minimization protocols were performed on the chosen structures as follows: SD and CG algorithms, with 10 000 steps using an rmsd of 0.001 Å as the energy convergence criterion.

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