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# 2D immunomic approach for the study of IgG autoantibodies in the experimental model of multiple sclerosis

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ABSTRACT

2D-immunomics may be useful in the identification of autoantigens in neurological autoimmune diseases, but its application may be limited by denaturation of target proteins. Here we compared the capacity of a single or multiple antigens to elicit autoantibodies targeting multiple neural autoantigens by ELISA and 2D-immunomics. We induced experimental autoimmune encephalomyelitis (EAE) with MBP peptide$_{89-104}$, total MBP or spinal cord homogenate. Both techniques showed anti-MBP IgG only after immunization with total MBP. In addition, 2D-immunomics revealed the presence in EAE mice of autoantibodies targeting other neural proteins, some displaying partial sequence homology with MBP. The present finding by 2D-immunomics of multiple neural proteins targeted by autoantibodies generated by a single antigen may help to explain the complex autoimmune response observed in multiple sclerosis.



## 1. Introduction

Multiple sclerosis (MS) is an autoimmune disease where the pathogenic cascade probably includes a process of molecular mimicry leading to the activation of T and B cell clones, some of which cross-react with self antigens (Hohlfeld and Wekerle, 2001; Hueber et al., 2002).

A major goal of neuroimmunology is the identification of autoantigens in autoimmune diseases, which may help the identification of the foreign antigens and the design of vaccines. As far as MS is concerned, extensive efforts have been spent in the last decades, with no conclusive results (Cross and Stark, 2005; Ziemssen and Ziemssen, 2005; Fraussen et al., 2009). In most of these cases, autoreactivity to pre-selected, recombinant proteins/peptides was assessed by using ELISA or radio-immunoassay techniques; although autoreactivity to a number of neural proteins has been reported, the specificity of autoreactive IgG remains unclear (for review, see Fraussen et al., 2009). Such methodological approaches have been validated in experimental autoimmune encephalomyelitis (EAE), the animal model of MS, which can be induced upon immunization with myelin proteins or peptides (Furlan et al., 2009).

We have recently applied a 2D-immunomic approach in MS (Lovato et al., 2008) and Hashimoto's encephalopathy (HE) (Gini et al., 2008), two autoimmune conditions with production of autoantibodies towards unknown autoantigens. After 2D separation of human neural proteins and transfer to nitrocellulose membranes, sera and cerebro-spinal fluid (CSF) have been applied and the autoreactive spots identified. This approach led to the identification of few neural antigens recognized by IgG in the CSF of HE patients (Gini et al., 2008), whereas the situation in MS was highly complex, with a wide array of neural proteins recognized by autoantibodies both in serum and in CSF (Lovato et al., 2008).

A great advantage of such technique is represented by the opportunity to use the (almost complete) human neural proteome, with proteins present in all their isoforms with post-translational modifications. We have found evidence that these modifications play an important role both in MS and HE, since single isoforms were specifically recognized by autoreactive IgG, while others were targeted also by controls (Lovato et al., 2008). A major limitation of such approach may be represented by the denaturing conditions of the technique with loss of conformational structure; as a consequence, IgG directed to conformational epitopes would probably be under-estimated, when tested against denatured proteins.

Here we compared the ability of 2D-immunomic and ELISA techniques to detect the presence of autoantibodies in the serum of

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EAE mice. In addition, we assessed by 2D-immunomic approach the differential recognition of neural proteins following the induction of EAE with increasing complexity of the immunogen: a single peptide of the myelin basic protein (MBP), the total recombinant MBP or spinal cord homogenate (SCH).

## 2. Materials and methods

### 2.1. Animals and induction of EAE

Female SJL/j mice at 6–8 wk of age were purchased from Charles River (Calco, Milan, Italy) and housed in pathogen-free conditions. All procedures involving animals were performed according to the guidelines of the San Raffaele Scientific Institute Institutional Animal Care and Use Committee. For EAE induction, lyophilized antigens from SCH, total bovine MBP (Sigma-Aldrich) and bovine $MBP_{89-104}$ peptide (Espikem, Florence, Italy) were re-suspended in saline to a final concentration of 4 mg/mouse for SCH and 200 μg/mouse for MBP peptide or protein and injected subcutaneously with 1:1 CFA (Difco) (Furlan et al., 2009; Levine and Sowinski, 1973; Miller et al., 2010; Vanderlugt et al., 2000). Each immunization (5 animals per group) was followed by two intra-peritoneal injections of 500 ng of pertussis toxin (List Biological Laboratories) the day of immunization and 48 h later. As controls, immunization was performed with CFA alone (5 mice). Spinal cord homogenate has been prepared as described elsewhere (Furlan et al., 2009). Briefly, spinal cord was homogenized through a 70 μm cell strainer, suspended 4:1 (w/v) with saline, lyophilized and stored at −80 °C. Mice were weighed and scored daily. All EAE animals showed clinical signs of the disease with scores at peak between 2.5 and 3.5. Blood was collected at day 30 post-immunization (5 animals for each EAE sub-groups and control groups) and serum was stored at −20 °C until use. Before use for ELISA and 2D-immunomics, serum samples were normalized to the amount of IgG by commercial ELISA.

### 2.2. ELISA assay

$MBP\ peptide_{89-104}$ or total MBP protein (10 μg/ml of PBS) were coated onto 96-well plates (Microtest™ Flat-bottomed Plate, Falcon) overnight at 4 °C. The plates were then washed with PBS and blocked with 3% BSA solution. After washing with PBS-0.1% Tween-20 buffer, serum from each immunized and control mouse was incubated (1:100) for 2 h at 37 °C. To remove the excess of unbound serum, 4 washes with PBS-0,1% Tween-20 were performed and anti-mouse IgG HRP-conjugated (Millipore) was incubated for 1 h at 37 °C. The plates were washed with PBS-0.1% Tween-20, treated with TMB substrate solution (Sigma) for 45 min and read with a Microplate Elisa reader (Bio-Rad) at 450 nm. The OD background values were determined as the mean of OD values of wells coated with peptides or whole MBP protein without serum.

### 2.3. 2D-PAGE analysis and immunoblotting

2D immunomics was performed as previously described with minor modifications (Lovato et al., 2008). Normal SJL mice white matter brain samples were homogenized in lysis buffer (7 M Urea, 2 M Thiourea, 0.4% CHAPS, 0.1% DTT, 0.5% Triton X-100) and purified by centrifugation at 13,000 g for 5 min. Protein concentration was determined by a commercial protein assay (Sigma), with BSA as standard. Samples were solubilized in 2-D sample buffer (7 M Urea, 2 M Thiourea, 0.4% CHAPS, 0.1% DTT and 0.5% Triton X-100) to a final concentration of 120 μg/ml protein, followed by 0.5% Ampholine solution and Bromophenol blue. The 18-cm long IPG strips pH 3-11 NL (GE Healthcare) were rehydrated with 350 μl of protein solution for 1 h passively and 7 h at 30 V constant. Isoelectric focusing was carried out with a Ettan IPGphor 3 (GE Healthcare) in a sequence of constant and linear voltage gradient from 300 to 3500 V for 5 h total; the voltage was then increased to 8000 V and kept at such value until reaching 90,000 Vh total. For the second dimension, the IPGs strips were equilibrated in a solution containing 6 M Urea, 2% SDS, 30% Glycerol, 0.130 M DTT and 0.5 M Tris–HCl (pH 6.8). The alkylation reaction was performed by adding 0.136 M IAA to the solution for 5 min. The IPG strips were then loaded on an 9–16% polyacrylamide gradient SDS-PAGE with 0.8% agarose in the cathode buffer (192 mM Glycine, 0.1% SDS and 25 mM Tris). The electrophoretic run was performed with a Protean Multi-cell 2D sistem (Bio-Rad) by setting a current of 40 mA/gel. Proteins were then transferred onto 0.2 μm nitrocellulose membrane (Bio-Rad) in the transfer buffer (Tris-Glycine and 20% Methanol) at 10 V constant at 4 °C overnight. For 2D immunoblotting, membranes were blocked with 10% BSA or 10% no-fat Milk and 0.1% Tween-20 in TBS (pH 7.4) for 2 h and incubated overnight with serum from each animal (1:10,000) or antibodies anti-MBP (1:1000; Dako, Milan, Italy), myelin-oligodendrocyte glycoprotein (MOG) (1:1000; Sigma, Milan, Italy), 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (1:20,000), actin (1:25,000), α-tubulin (1:30,000; Abcam). After washing, membranes were incubated with appropriate secondary IgG HRP-conjugated (1:8000) and developed with Advanced ECL (GE Healthcare), whereas the reference 2D gel was stained with the silver or Sypro-Ruby stainings. The reference 2D-gel and autoradiographs were analyzed with the Image Master 2D Platinum v6.0 software (Amersham). By the same software, auto-reactive spots were matched with the reference gel; the threshold of intensity of each spot was arbitrarily set at 50,000.

### 2.4. In-gel digestion and peptide sequencing by nano RP-HPLC-ESI-MS/MS

In gel digestion and peptide sequencing by nano RP-HPLC-ESI-MS/MS were performed as previously described (Cecconi et al., 2009). The mass spectrometry analyses were conducted by using a nanoflow-HPLC system (Agilent 1200 series) coupled with an ion trap (Esquire 6000 Bruker-Daltonik, Germany). The scan range used was from 300 to 1800 m/z. Protein identification was performed by searching in the National Center for Biotechnology Information non-redundant database (NCBInr) using the Mascot program (Matrix Sciences, London, UK). The following parameters were adopted for database searches: complete carbamidomethyl formation of cysteines and partial oxidation of methionines, N-acetylation, peptide mass tolerance ±1 Da, fragment mass tolerance ±0.9 Da and missed cleavages of 1. For positive identification, the score of the result of $[-10\times\mathrm{Log}(P)]$ had to be over the significance threshold level ($p<0.05$). Relevant spots identified by mass spectrometry are indicated in Fig. 2 and identification parameters are summarized in Supplementary Table 1.

## 3. Results

### 3.1. Autoantibody response following different EAE immunization protocols revealed by ELISA

To characterize the immune response in EAE, immunization was performed with different encephalitogenic myelin antigens: total MBP protein, $MBP_{89-104}$ peptide and SCH; Since two-dimensional electrophoresis implicates strong denaturating conditions to separate proteins, we first evaluated anti-MBP autoantibodies in different EAE immunization protocols by ELISA, an assay in which autoreactivity is tested against non-denatured antigens, i.e. $MBP_{89-104}$ peptide and total MBP. The comparison of the reactivity by ELISA with those obtained with the 2D immunomic approach enabled us to assess whether the use of reducing agents modified the protein conformation and reduced/abolished the epitope binding. As shown in Fig. 1A, high levels of IgG recognizing total MBP protein were detected only in mice inoculated with the same antigen, but not after immunization

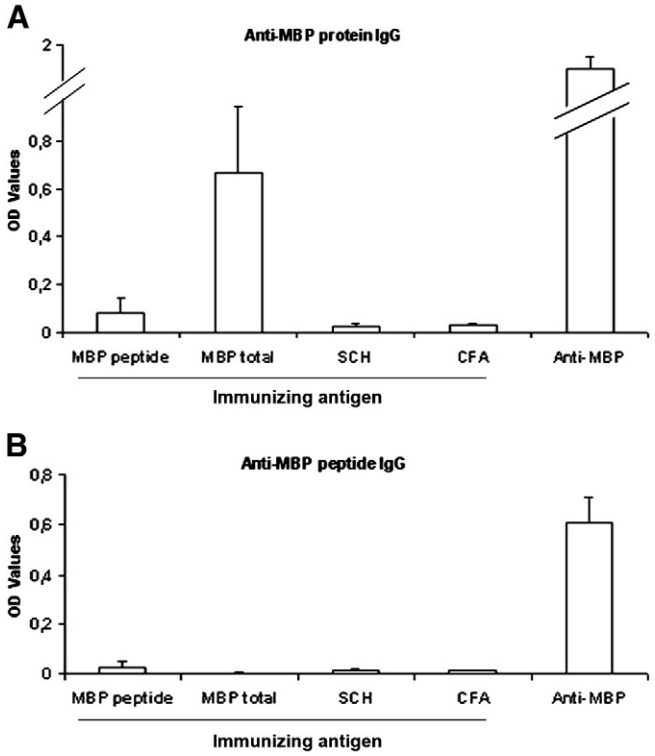


**Fig. 1.** Anti-MBP IgG reactivity by ELISA assay. IgG reactivity to total MBP protein (A) and MBP peptide (B) was assessed in serum from each animal immunized with MBP peptide, MBP total protein, SCH and CFA (5 animals per group) as indicated in *x* axis; high levels of anti-MBP were observed only in animals immunized with total MBP protein (A). In both cases, polyclonal anti-MBP antiserum (anti-MBP, last column) was used as positive control.

with MBP peptide (very low titers) or SCH (titers comparable to controls). Levels of anti-$MBP_{89-104}$ peptide IgG in all EAE animals were similar to controls (Fig. 1B).

### *3.2. Autoantibody response following different EAE immunization protocols revealed by immunoproteomic approach*

IgG autoreactivity to MBP and other neural antigens was assessed by 2D immunomic approach, with white matter proteins of rodent CNS separated by 2D-PAGE and then probed with serum from EAE and control mice. The 2D map analysis showed that membranes contained all major myelin and cytoskeletal proteins of the white matter, similarly to what we have described in humans (Lovato et al., 2008). Among the isoforms identified by mass spectrometry and/or immunoblotting with specific antibodies, MBP, CNPase and α-tubulin were detected (Fig. 2 and Supplementary Table 1).

After incubation with sera from all EAE mice, several neural proteins spreading along pH 3–11 were recognized by autoreactive IgG. Although the number of mice for each group was small and the number of autoreactive spots was highly variable for each group, there was a tendency for mice immunized with MBP total protein to display more autoreactive spots than those immunized with MBP peptide or SCH (Table 1). Noteworthy and quite surprisingly, the immunization with CFA alone led to the highest number of autoreactive spots, probably due to its potent immune activation effect. The comparative analysis of the spots recognized by sera from EAE mice and control (naïve and CFA-inoculated) animals revealed that a restricted number of proteins were selectively targeted by EAE seric IgG (Table 1). Specifically, autoreactivity to MBP protein isoforms was present only in EAE mice immunized with total MBP (Fig. 3),

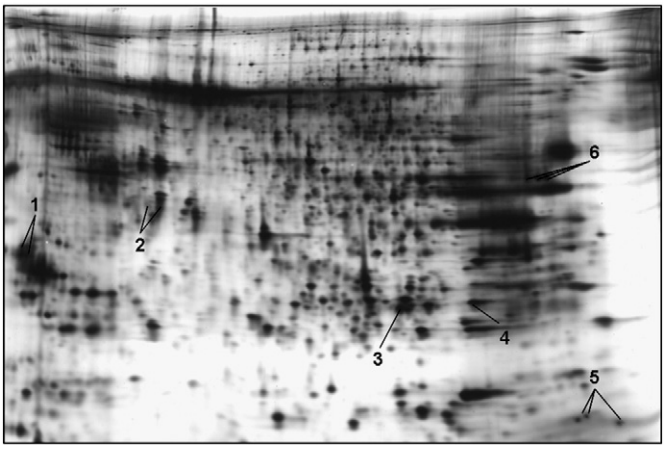


**Fig. 2.** 2D protein map of the normal rodent white matter. Normal mouse white matter has been resolved by 2D-PAGE and the gel subjected to silver staining; indicated are the spots of interest recognized by mass spectrometry and/or immunoblotting with specific antibodies. 1: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon (YWHAE); 2: α-Tubulin; 3: MOG; 4: glutathione S-transferase mu 1; 5: MBP; 6: CNPase.

whereas MBP peptide and SCH were not able to elicit any anti-MBP IgG reactivity (not shown). The present immunomic approach showed that, apart from MBP, autoreactivity to other neural proteins was variably present in all EAE mice, particularly those immunized with MBP peptide and SCH (Table 1). Among these EAE-specific autoantigens, we detected MOG, CNPase, α-tubulin and YWHAE/tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon in at least 30% of EAE mice, possibly due to molecular mimicry between epitopes of these molecules and MBP. A partial overlap has been indeed found with epitopes of these proteins (Supplementary Figure 1).

## 4. Discussion

Autoantibodies are known to be present in MS since many years. Unfortunately, in most cases the target of such autoantibodies is not known, despite much efforts spent over last decades. The studies so far performed testing autoreactivity to recombinant peptide or proteins failed to convincingly demonstrate any MS-specific autoantibody (Cross and Stark, 2005; Ziemssen and Ziemssen, 2005; Fraussen et al., 2009). The use of large numbers of antigens (protein and lipid arrays) has provided important information about the prognostic value of autoantibodies in experimental models (Robinson et al., 2002; Quintana et al., 2008); however, the pre-selection of the putative autoantigens hampers the discovery of new molecules. We have recently performed an immunomic study to assess IgG autoreactivity in the CSF from MS and HE patients, describing that transketolase and dimethylargininase I may be the main targets of the autoimmune response in these conditions, respectively (Lovato et al., 2008; Gini et al., 2008). In addition to transketolase, we found that the autoimmune response in MS targeted a complex array of neural proteins with different cellular localization (myelin, axons, energy-related enzymes), suggesting that multiple B cell clones were activated or, alternatively, that a process of molecular mimicry occurred between the triggering autoantigen(s) and other neural proteins.

To test this latter hypothesis, we verified whether immunization with a single autoantigen (MBP) can generate IgG autoantibodies targeting multiple neural proteins, adopting the same technique for the detection of autoantibodies in EAE. In parallel, the presence of anti-MBP IgG was tested also with ELISA technique, a standard assay performed in minimal denaturing conditions (Shields et al., 1991), to assess whether the high degree denaturation of 2D-PAGE prevent the

**Table 1**
Autoreactivity to MBP and other neural proteins in EAE mice revealed by 2D-immunomics.

| Immunizing antigen | MBP peptide | Total MBP | SCH | CFA | None (naïve) |
|---|---|---|---|---|---|
| Number of autoreactive spots (mean ± SD) | 44 ± 23.1 | 27 ± 9.8 | 32 ± 15.7 | 55 ± 13.1 | 20.3 ± 7.1 |
| EAE-specific spots (mean ± SD)[a] | 8.4 ± 6.1 | 13.5 ± 7.1 | 12.2 ± 7.8 | 0 | 0 |
| MBP[b] | 0/5 | 5/5 | 0/5 | 0/5 | 0/5 |
| MOG | 3/5 | 0/5 | 2/5 | 0/5 | 0/5 |
| CNPase | 0/5 | 2/5 | 0/5 | 0/5 | 0/5 |
| α-Tubulin | 3/5 | 0/5 | 1/5 | 0/5 | 0/5 |
| YWHAE | 2/5 | 1/5 | 2/5 | 0/5 | 0/5 |
| Glutathione S-transferase mu 1 | 3/5 | 0/5 | 3/5 | 0/5 | 0/5 |

[a] Spots absent in control (naïve and CFA-immunized) animals.
[b] Number of animals with autoreactivity to a given neural protein.

recognition of neural antigens by autoantibodies. Both techniques showed concordant results, with high levels of IgG against MBP being detected only in mice immunized with total MBP protein. This result clearly confirms that proteins in denatured condition are still detectable by autoantibodies, as observed in a different experimental model (Mouquet et al., 2006). On the contrary, no anti-MBP reactivity was detected with both ELISA or 2D immunomics techniques in mice immunized with MBP peptide or SCH. This is not surprising, since different results have been reported regarding the capacity of SCH as well as of MBP peptides to elicit autoantibodies in this experimental model (Gould and Swanborg, 1993; Figueiredo et al., 1999; Lorentzen et al., 1995). This expected finding is probably related to the preferential activation of T, rather than B cells and/or to different protocols of immunization in other studies (Paterson et al., 1981; Rivero et al., 1999). Apart from these technical considerations, a good concordance has been observed between 2D immunomics and ELISA in detecting the presence or absence of anti-MBP antibodies in EAE following different immunization protocols. In addition to MBP, the present immunomic approach after 2D-PAGE separation of neural proteins allowed the identification of additional autoreactive neural proteins in all EAE animals. This result confirms previous studies where immunization with a different antigen [proteolipid protein (PLP)] induced the production of IgG to PLP, but also to other neural proteins evidenced by mono- and bi-dimensional PAGE (Zephir et al., 2006; El Behi et al., 2007). Among EAE-specific molecules, the present study showed IgG reactivity to myelin proteins such as MOG and CNPase, which might contribute to myelin damage. Interestingly, most of the autoreactive molecules have some epitopes displaying a partial sequence homology with MBP, suggesting the possibility that inter-molecular mimicry may occur as a consequence of cross-recognition. Although the back-validation with ELISA assay for these EAE-specific molecules has not been performed in the present study, anti-MOG autoreactivity has been already reported in EAE following immunization with SCH (Lorentzen et al., 1995). The analysis of EAE-specific autoreactive neural proteins recognized by IgG after SCH vs. recombinant immunizations showed no quantitative and qualitative difference. This finding is apparently surprising considering that SCH contains several thousands of different proteins; however, similar observations have been already reported (Whitham et al., 1991; Mor and Cohen, 2006) and may reflect a sort of hierarchy between immunogenic proteins, with PLP, MBP and MOG being dominant molecules (Whitham et al., 1991). A plausible alternative explanation for the low autoreactive IgG after SCH immunization may be related to the insufficient concentration of single proteins to induce a strong humoral response. Obviously, we cannot exclude the possibility that the lack of additional autoreactivities by 2D immunomics may reflect the loss of conformational epitopes of natural autoantigens in our sample separated in denaturing conditions.

Bearing in mind these limitations, the finding by 2D immunomics of multiple neural proteins targeted by autoantibodies generated by a single antigen confirms previous studies (Zephir et al., 2006; El Behi et al., 2007) and provides interesting information for MS, where we have recently observed a complex autoimmune response to several targets with different cellular localization (Lovato et al., 2008). Such diversity may indicate that multiple B cell clones are activated in a given patient; a plausible alternative suggested by the present study

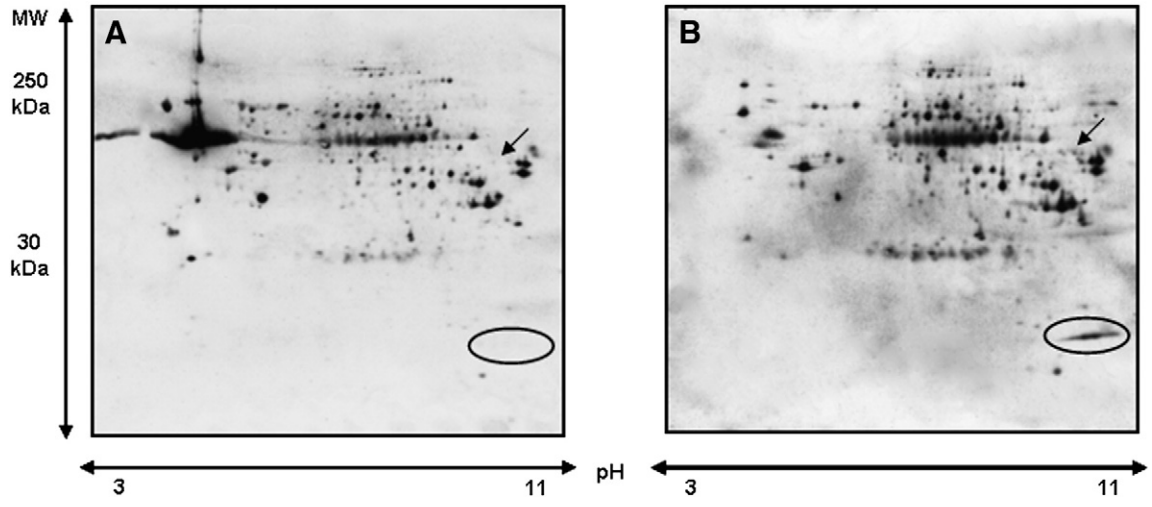


**Fig. 3.** Anti-neural autoantibodies by 2D immunomics. Representative membranes showing autoreactivity to neural proteins in sera from mice immunized with CFA (A) and MBP total protein (B). Note the presence of anti-MBP and -CNPase autoreactivities only in the group of mice immunized with total protein MBP (oval and arrow in B, respectively).

indicates that such a complex array of autoreactivities may largely depends on a process of inter-molecular mimicry triggered by a very restricted number of autoantigens.

Supplementary materials related to this article can be found online at doi:10.1016/j.jneuroim.2010.10.004.

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