

Sphingosine-1-phosphate (S1P)-Containing Niosomes as Vehicle to Deliver Amyloid- β in a Novel Alzheimer's Disease Vaccine

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Received July 8, 2016; Accepted July 11, 2016; Published November 30, 2016

ABSTRACT

Vaccines against amyloid-beta-peptide (A β) have been widely investigated as a potential immunotherapeutic approach for Alzheimer's disease (AD) during the past years. However, previous vaccines tested in mice and humans have failed to address the major hallmarks of AD, such as senile plaque-like A β deposits, plaque-associated dystrophic neurites and glial proinflammatory cytokines, due to massive T-cell activation that resulted in a meningoencephalitis-like reaction. We have recently developed a vaccine (EB101), based on passive immunotherapy, that delivered A β 1-42 in a novel immunogen-adjuvant consisting of sphingosine-1-phosphate (S1P)-containing niosomes, to APP/PS1 transgenic mice before and after detectable AD-like pathological effects. Quantitative analysis of amyloid burden, performed in the affected brain regions, showed a notable decrease in the mean area of A β plaques when EB101 was compared with other treatments, demonstrating that S1P plays a key role as a regenerative-neuroprotective agent during the development and consolidation periods of AD neuropathology. In the present study, we screened the efficacy of each component of our immunogenic vaccine EB101 in the APP/PS1 transgenic mouse model, in order to characterize the positive effect of S1P in the immunotherapeutic response. Complementary results demonstrated that EB101 vaccine significantly prevents and reverses the AD neuropathology hallmarks by clearing A β plaques, reducing dystrophic plaque neurites and minimizing neuroinflammation, while markedly reducing neuronal degeneration and thus prolonging lifespan in the APP/PS1 transgenic mouse model, paving the way for future relevant clinical trials.

Keywords: Alzheimer's disease, Amyloid beta peptide, APP/PS1 transgenic mice, Immunotherapy, Vaccine

Abbreviations: AD: Alzheimer's Disease; A β : β -Amyloid Protein; APP: Amyloid Precursor Protein; CA1: Field CA1 Hippocampus; CA3: Field CA3 Hippocampus; Cx: Cortex; DG: Dentate Gyrus; Ent: Entorhinal Cortex; GrDG: Granular Dentate Gyrus; IL: Interleukin; MODG: Molecular Dentate Gyrus; PoDG: Polymorph Dentate Gyrus; Tg: Transgenic; Th: T Helper.

INTRODUCTION

In Western countries, Alzheimer's disease (AD) is the most frequent form of dementia, affecting up to one-third of elderly individuals [1]. Its pathological hallmarks in the brain are characterized by the accumulation of amyloid- β (A β) peptide amyloid plaques, neurofibrillary tangles with hyperphosphorylated tau proteins, neuronal-synaptic loss affecting particularly the neocortex, hippocampus and entorhinal regions [2]. Taking advantage of the potential aspects of the APP/PS1 double-transgenic mouse line, numerous investigations have used this particular model to study emergent therapies to prevent and reduce the neurodegenerative features of AD.

The purpose of the present study is to investigate the efficacy

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Citation: Carrera I, Fernández-Novoa L, Vigo C & Cacabelos R. (2016) Sphingosine-1-phosphate (S1P)-Containing Niosomes as Vehicle to Deliver Amyloid- β in a Novel Alzheimer's Disease Vaccine. J Genomic Med Pharmacogenomics, 1(2): 225-232.

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of each component of our immunogenic vaccine EB101 in the APP/PS1 transgenic mouse model, in order to characterize the positive effect of S1P in the immunotherapeutic response of AD-like pathology. This goal is intended to be achieved by neurohistochemical methods that will analyze the functionality of a new physiological adjuvant design, composed of naturally-occurring phospholipids that prove safe and efficacious in other types of vaccines (e.g. influenza), with an added biologically active phospholipid (S1P), known to stimulate an anti-inflammatory reaction and to act as a neuronal regenerating agent in vitro and in vivo studies [3]. Based on our previous studies, the preventive and therapeutic effect of the EB101 vaccine in APP/PS1 mice, designed to address the pitfalls of previous vaccines, conserve the immunogenicity targeted for the reduction of A β burden deposits, but avoid the massive activation of T-cell-mediated immune response that potentially causes adverse inflammatory effects. Moreover, this cost-effective vaccine was formulated to reduce A β burden by generating a robust anti-A β antibody production, in order to prevent or slow down the main AD-like pathological alterations and stimulating an anti-inflammatory T-helper (Th) 2 immune response.

MATERIALS AND METHODS

Experimental design

Two experimental treatment studies were carried out; one before AD onset, starting at 7 weeks of age (preventive treatment), and the other after AD onset at 35 weeks of age, when neuropathological AD characteristics were well established (therapeutic treatment). APP^{swe}/PS1^{dE9} double-transgenic mice (B6C3F1/J), expressing a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695^{swe}) and human presenilin 1 (PS1-^{dE9}) mutants, were randomly divided into these two experimental studies, and in each study they were divided into four treatment groups (**Figure 1**), as follows: Preventive treatment: Group A was formed by 16 mice (12 transgenic and 4 wild-type mice; 12+4) that were immunized with a cocktail of synthetic human A β ₄₂/S1P-containing niosomes (EB101); group B, formed by 16 mice (12+4), immunized with A β ₄₂/liposome without S1P; group C, formed by 16 mice (12+4) immunized with S1P-containing niosomes; and group D, formed by 10 mice (6 transgenic and 4 wild-type mice, control) inoculated with PBS. Therapeutic treatment: The same treatments were administrated in groups A, B and C, formed by 16 mice in each group, while group D, formed by 10 mice inoculated with PBS, was the control. Mice were immunized with nine injections for seven months, inoculating 100 μ l containing a cocktail of A β (100 μ g) and phospholipid mix (1mg) per injection. A representation of the model is portrayed in **Figure 1**.

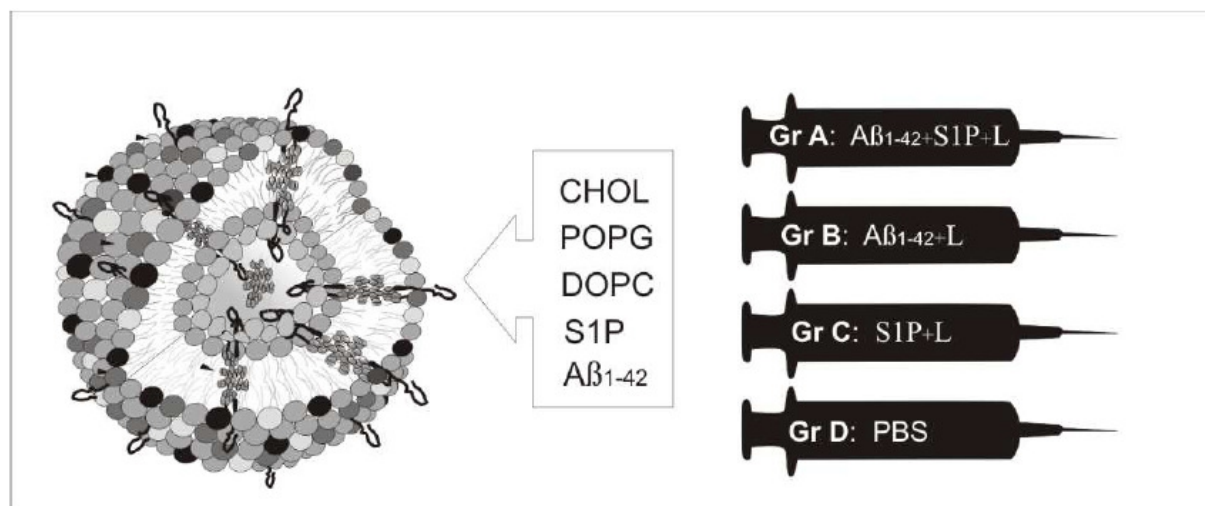


Figure 1. Structural characterization of the EB101 vaccine. Structural representation of A β ₁₋₄₂/S1P-containing liposome (EB101) and the treatment protocols of the four experimental mice groups.

Experimental procedure

Immunization procedures: 44 APP^{swe}/PS1^{dE9} transgenic mice were inoculated intraperitoneally with 100 μ l per

injection of EB101 (group A), only liposome complex EB102 (group B) or PBS (group C), during seven months (9

injections) for each treatment period. The immunization protocol was systematically used in previous similar studies [4,5,6] and was followed strictly, with 3 injections every two weeks in the first month, and then one injection each following month. This immunization regimen was followed in the preventive and therapeutical periods.

Immunohistochemistry and imaging: Immunohistochemical analysis and imaging were prepared as previously reported [5,6].

Statistical analyses. All statistical parameters were performed with the use of SPSS (version 11.0; SPSS Inc, Chicago) and a P value <0.05 indicated statistical significance. The average range of A β plaque density and burden area were analyzed by using a two-factor repeated-measures analysis of variance (ANOVA) followed by a post hoc analysis when relevant. All data were expressed as the mean $\pm$ SEM.

RESULTS

Efficacy of S1P-containing niosomes (EB101) in preventing A β burden development

EB101 vaccine prevents and reverse AD neuropathology as we recently demonstrated, by the marked reduction of A β plaques, plaque-associated dystrophic neurites and astrogliosis in affected mouse brain areas. Next, we conducted an EB101 component screening study in order to elucidate the effective role of each in the prevention of A β deposits in the APP/PS1 mouse brain. A β plaques and vascular amyloid loads were detected, counted and measured as the percentage of total surface stained by monoclonal anti-A β antibody in the hippocampal and cortical sections. At the first experimental phase (preventive treatment), the amyloid plaque load observed in the representative photomicrographs (**Figure 2**) showed a significant difference across the four experimental groups (ANOVA, $F=3.15$, $P<0.001$).

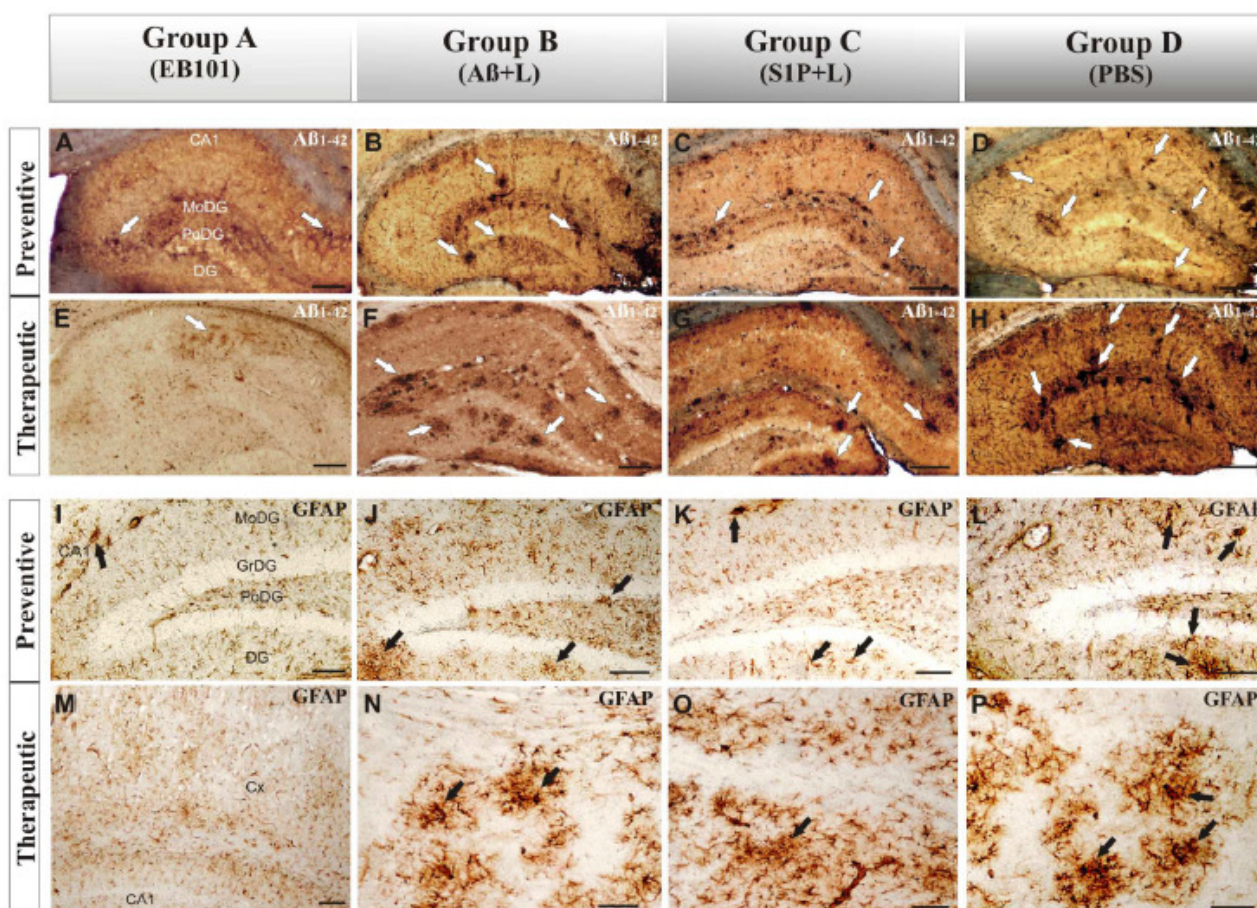


Figure 2. Effect of EB101 vaccine on beta amyloid (A β) deposits and astroglia in APP/PS1 mouse brain. Comparative photomicrographs of A β immunoreactivity were taken in the hippocampus (A-L) and cortical (M-P) brain regions of transgenic mice treated with the vaccine (Gr A) before A β plaques developed, preventive treatment (panels A-D, I-L) and after the A β plaques developed, therapeutic treatment (panels E-H, M-P). Scale bar: 100 μ m.

In this experiment we observed that EB101-immunized mice (**Group A, Figure 2A**) showed a significantly reduced hippocampal load when compared with the different vaccine components (**Groups B-C, Figure 2B, 2C**). In fact, the A β

load in these different vaccine component groups (B-C) was not significantly different from PBS-treated mice (**Group D, Figure 2D**), although there was a correlation between the presence of S1P+A β in treatment regime and the total A β burden. The total A β plaque density and size quantification also supported this data, being performed by image analysis software of anti-A β antibody-stained sections in the hippocampus (**Figure 2A-2D**). Statistical analysis of the data obtained in the present study demonstrates that the

mean A β plaque burden (**Figure 2A**) of group A (25 ± 5 p/s) was significantly different from the other treated groups (63 ± 6 p/s in 2B; 65 ± 6 p/s in 2C; 68 ± 6 p/s in 2D), which represents a notable decrease in the mean percentage of A β plaque area of 30.1-38.2% in the EB101-immunized mouse group relative to other tested groups. Therefore, these data show that in the preventive treatment period (**Figure 3**), the clearance effect of the EB101 vaccine on A β plaques per section was about 64% (**Figure 2A, Figure 3**) compared to positive controls, while 10% was detected in A β 42/Liposome without S1P (B) and 7% in Liposome with S1P-treated mice (C).

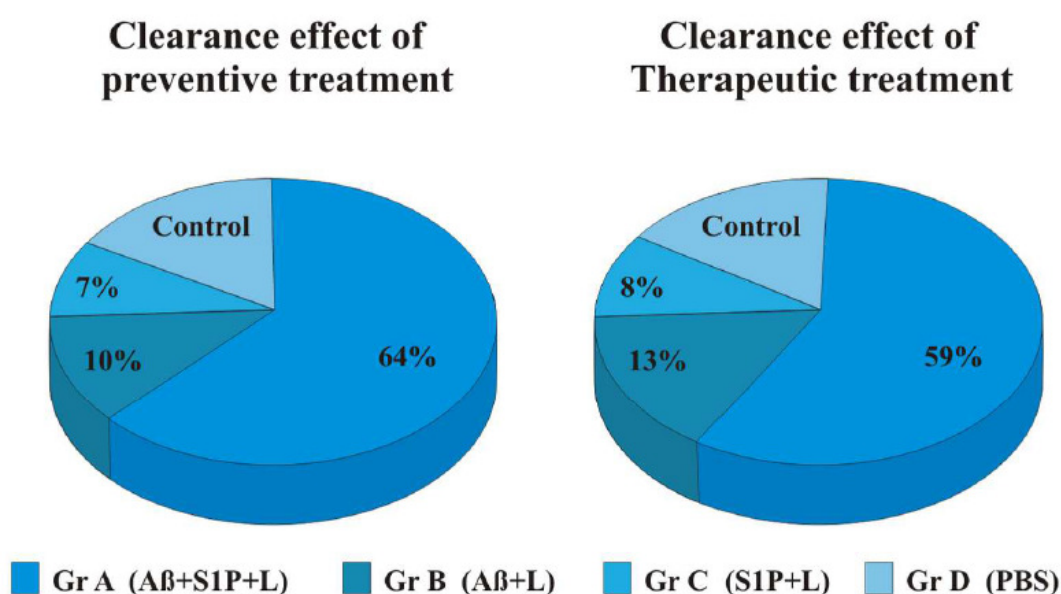


Figure 3. Comparative A β clearance effect. Pie charts representing the different effects of the treatment in the clearance of A β plaques in the transgenic mouse hippocampus. Both preventive (64%) and therapeutic (59%) treatment of EB101 showed a notable percentage of A β clearance compared to the other treatments in mouse brains.

Efficacy of S1P-containing niosomes (EB101) in clearing A β plaque burden

At the second experimental phase, (therapeutic treatment), we compared the effect of EB101 vaccine with different vaccine component groups in the reduction of A β in the brain after the plaques were established in the 35-week-old APP/PS1 transgenic mice (**Figure 2E-2H**). The results obtained showed that A β deposits were significantly reduced in the hippocampal brain sections of the EB101-treated mice (A), markedly different from the A β burden density observed in the different vaccine component groups (B-C). Photomicrographs of EB101-treated mice showed a few compacted A β plaques located at the CA1 layer of the hippocampus (**Figure 2E**), while in the other experimental groups no reduction in plaque density was observed (**Figure**

2F-2H), numerous large compacted A β plaques being located in almost every layer of each brain section. The data obtained showed that the treatment groups without A β 1-42, C (**Figure 2G**) and D (**Figure 2H**), were the section most densely populated with A β plaques, while a slight reduction in this hallmark was observed in mouse group B (**Figure 2F**). As observed in the preventive treatment, the mean burden of A β plaques in group A (35 ± 4 ; **Figure 3**) was significantly different from the other treated groups (65 ± 5 in B; 70 ± 5 in C; 80 ± 6 in D), while the A β plaque area in the EB101-immunized mouse group was reduced by 29.7-34.5%, relative to the other groups tested (**Figure 3**). In both preventive and therapeutic treatments, a reduced A β plaque staining intensity was detected in the EB101-immunized mouse group, as shown in the photomicrographs of **figure**

2A,2E, contrasting with the intense A β -immunoreactivity of A β plaques in the other experimental groups.

Immune response effects of S1P-containing niosomes (EB101) in APP/PS1 mouse models

In order to analyze astrocyte activation in the hippocampal regions of APP/PS1 mice, which is known to be implicated in neuroinflammation, amyloidogenesis and neuronal cell death in AD, we used immunohistochemical analysis to quantify and compare the GFAP-reactive cells in the affected regions after each treatment period (**Figure 2I-P**). At the end of the preventive treatment (**Figure 2I-L**), EB101 significantly reduced the density of GFAP-reactive cell clusters in the hippocampal and cortical sections as compared with other treatments, such as A β /Lip without S1P (B), Lip with S1P (C) and PBS (D). Only a few scattered

GFAP-reactive clusters, mainly at the CA1 hippocampal layers, were observed in the transverse section of the mouse brains treated with EB101 (**Figure 2I**), markedly contrasting with the numerous dystrophic reactive astrocytes observed in different hippocampal areas of mouse brains in groups B-D (**Figure 2J-2L**). After therapeutic treatment, mouse brains treated with EB101 were similar to those of control mice, mostly devoid of reactive GFAP clusters, except a few scattered ones in the CA1 hippocampal layers (**Figure 2M**). In contrast, there was a moderate density in group B (**Figure 2N**) and extensive density in group C (**Figure 2O**), and group D mice (**Figure 2P**). No astrogliosis was observed in control mice of each group during preventive or therapeutic treatment periods.

Table 1: Antibodies used for immunohistochemistry

Antibody	Antigen	Type	Source	Dilution	Ref.
β-amyloid	A β ₁₋₄₂ (mouse)	Mouse monoclonal	Millipore	1:1000	5,6,38
Neurofibrillary tangles	NT (rabbit)	Rabbit polyclonal	Millipore	1:300	5,6,39
Glial fibrillary acidic p	GFAP (mouse)	Mouse monoclonal	Sigma	1:400	5,6,40

DISCUSSION

Liposome-based vaccines have demonstrated great effective potential in preventing and treating AD [7]. Based on previous effectiveness of active immunotherapy in reducing A β plaque accumulation [8], we have developed a new vaccine cocktail to circumvent the previous vaccine failures, due to an extensive T-cell-mediated immune response [9,10], by judiciously selecting an adjuvant that addresses all the above targets while avoiding the massive autoimmune activation of T-cells. This novel immunogen-adjuvant configuration consists of a physiological matrix, niosomes composed of naturally-occurring phospholipids (phosphatidylglycerol, phosphatidylcholine, and cholesterol), that has proven to be safe and efficacious in other types of vaccines. In order to increase the immunogenic potential we added a biologically active sphingolipid, sphingosine-1-phosphate (S1P) to this phospholipidic configuration. S1P is a phosphorylated product of sphingosine, catalyzed by sphingosine kinase, which has been reported to be an important lipid mediator acting both inside and outside the cell membrane [11,12]. Due to its role in the regulation of neuronal function, S1P facilitates glutamate secretion in hippocampal neurons by secretion actions, being involved in regulatory mechanisms of synaptic transmission. In particular, S1P itself causes glutamate secretion from presynaptic sites and potentiates glutamate-induced transmitter secretion in primary hippocampal neurons [13], possibly facilitating the

formation of a positive activation cycle in excitatory neurons such as glutamergic neurons. As extracellular effects, S1P binds to members of GTP-binding protein (G-protein)-coupled S1P receptor family (S1P1-5), triggering active immunity, angiogenesis, cell motility, and neurite growth [14,15]. As intracellular effects, S1P modulates cellular calcium mobilization, cell growth, and the inhibition of apoptosis [16,17].

Recently, the potential effect of S1P in neuronal regeneration was demonstrated in detail, since intracellular S1P enhances nerve growth factor-induced excitability in the sensory neurons of rats [18,19], controls migration of neuronal stem cells toward a site of spinal cord injury [20], and induces cytoskeletal rearrangements through small G-protein Rac activation [21], which seems to facilitate synaptic vesicle fusion to plasma membranes, enhancing transmitter secretion. Taking advantage of its important role in neuronal regeneration, cell growth, suppression of apoptosis and glutamate secretion from presynaptic sites that are affected in AD neuropathology, we incorporated the S1P into the phospholipid liposome to form a phospholipid-S1P-liposome matrix used as an immunogen-adjuvant to deliver the active antigen, A β ₁₋₄₂ [5]. Thus, this combination added a regenerative and anti-inflammatory component to the vaccine, key elements to increase neuronal activity and prevent inflammation in the brains of APPswe/PS1dE9 transgenic mice, improving the immunotherapeutic results previously reported by the studies based on A β -

immunization [22,23], while minimizing potential side effects.

EB101 vaccine was generated by using the hydration-rehydration method to effectively combine phospholipids, S1P and liposomes, forming niosomes against A β 1-42 oligomers. This was used in other vaccines for an efficient liposomal-protein configuration, overcoming the immune pathological response encountered when using other types of adjuvants such as Freund's adjuvant [24,25], Quil-A [26], QS-21 and the detergent polysorbate 80 to solubilize A β , which are believed to induce a proinflammatory Th1 response [10,27]. Over the last decade, A β immunotherapy studies in APP-tg mice have reported different A β antibody levels, depending on the mouse model used, the immunization methodology, and the type of adjuvant for the vaccine formulation, emphasizing the significant impact of the adjuvants on the immune response evoked [28]. Considering this, we strategically planned a novel adjuvant-liposomal vaccine formulation in which the phospholipid-S1P-liposomes represent the pivotal structure that provides an immunotherapeutic response markedly different from previous vaccine formulations tested in AD-like mice. Moreover, the significant effect of EB101, obtained in mice after the establishment of A β plaques in the hippocampus/neocortex and subsequent neuropathological changes, shows that EB101 vaccine presents a tremendous potential as a therapeutic agent to reverse neuropathological hallmarks in the AD-like brain, which represent an effective immunotherapeutic approach [29,5,6]. The results obtained in the preventive and therapeutic treatments showed that EB101 vaccine is efficacious not only in preventing the development of AD-like pathology but also in reducing it once established. The APP^{swe}/PS1^{dE9} transgenic mice showed tiny and compact nonfibrillary amyloid plaque accumulation in the initial deposition period mainly at the hippocampus/neocortex, consistent with previous reported studies [30,31], whereas at later stages sparse A β -fibrillar plaques are progressively observed also at nearby cortical areas. After the preventive immunization period with EB101 vaccine, a significant reduction in density of A β plaque burden was observed in APP/PS1 transgenic mouse regions, as well as reduced burden areas when compared with APP/PS1 mice treated with different EB101 vaccine components or PBS. Schenk and colleagues [4] reported similar preventive immunization results in PDAPP mice, although differences in immunization efficiency and vaccine conformation have been improved in the present study. During this preventive period, EB101 immunization also prevented the progressive development of other AD pathological effects such as dystrophic plaque neurites, astrogliosis and motor coordination deficits. The hindrance of massive glial activation in response to early development of A β deposits has been accepted by the scientific community as the main key in efficient preventive immunization [32-35], this being the case of our EB101

vaccine, which has demonstrated, when compared with different EB101 vaccine components or PBS-treated mouse groups, that it prevents the development of astrocyte activation. Since the activation of astrocytes and microglia has been reported to induce the degradation of A β [36,37], the near-absence of A β -related astrogliosis in cortex and hippocampus of mice treated with EB101 vaccine confirmed the preventive effect against the development of AD-like pathology in this mouse model.

CONCLUSION

We have shown above the beneficial effect of combining the liposomal/sphingosine-1-phosphate adjuvant with an A β antigen (EB101 vaccine) to induce an effective immunological response with a wide anti-inflammatory spectrum in the APP/PS1 mouse model of AD. Based on the present results, the EB101 vaccine strongly prevented or reduced the main AD-like hallmarks (beta amyloid plaques and plaque-associated dystrophic neurites), induced the inhibition of astrocyte activation and neuroinflammatory response that naturally develops in APP/PS1 transgenic mice.

ACKNOWLEDGEMENTS

This work was supported by the III Research Grant Program of the Camilo José Cela University 2014-15 (TransBioModel – 2014/36).

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