Research Article

pVAXhsp65 Vaccination Primes for High IL-10 Production and Decreases Experimental Encephalomyelitis Severity

Sofia Fernanda Gonçalves Zorzella-Pezavento,1 Fernanda Chiuso-Minicucci,1 Thais Graziela Donegá França,1 Larissa Lumi Watanabe Ishikawa,1 Larissa Camargo da Rosa,1 Priscila Maria Colavite,1 Bianca Balbino,1 Camila Marques,2 Maura Rosane Valerio Ikoma,2 Ana Paula Masson,3 Célio Lopes Silva,3 and Alexandrina Sartori1

1Department of Microbiology and Immunology, Institute of Biosciences of Botucatu, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brazil
2Flow Cytometry Laboratory, Amaral Carvalho Foundation, Jau, SP, Brazil
3Department of Biochemistry and Immunology, University of São Paulo (USP), Ribeirão Preto, SP, Brazil

Correspondence should be addressed to Sofia Fernanda Gonçalves Zorzella-Pezavento; szorzella@yahoo.com.br

Received 9 November 2016; Revised 3 January 2017; Accepted 23 January 2017; Published 21 February 2017

Academic Editor: Alessandra Santos

Copyright © 2017 Sofia Fernanda Gonçalves Zorzella-Pezavento et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Experimental autoimmune encephalomyelitis (EAE) is a demyelinating pathology of the central nervous system (CNS) used as a model to study multiple sclerosis immunopathology. EAE has also been extensively employed to evaluate potentially therapeutic schemes. Considering the presence of an immune response directed to heat shock proteins (hsp) in autoimmune diseases and the immunoregulatory potential of these molecules, we evaluated the effect of a previous immunization with a genetic vaccine containing the mycobacterial hsp65 gene on EAE development. C57BL/6 mice were immunized with 4 pVAXhsp65 doses and 14 days later were submitted to EAE induction by immunization with myelin oligodendrocyte glycoprotein (MOG\textsuperscript{35–55}) emulsified in Complete Freund's Adjuvant. Vaccinated mice presented significant lower clinical scores and lost less body weight. MOG\textsuperscript{35–55} immunization also determined less inflammation in lumbar spinal cord but did not change CD4+CD25+Foxp3+ T cell frequency in spleen and CNS. Infiltrating cells from the CNS stimulated with rhs65 produced significantly higher levels of IL-10. These results suggest that the ability of pVAXhsp65 vaccination to control EAE development is associated with IL-10 induction.

1. Introduction

Multiple sclerosis (MS) is a progressive inflammatory disease that damages the brain and the spinal cord. A plethora of reports support the view that it is mediated by autoreactive T cells specific for myelin antigens [1, 2]. Once they have been activated in the periphery, these self-specific T cells cross the blood-brain barrier and destroy the myelin sheets and axons from central neurons [3–5]. This demyelination is responsible for signal conduction slowing or even signal blockage [6]. Currently available therapies for MS are primarily focused in minimizing the progression of disability and reducing the number of relapses. The standard first-line immunomodulatory therapies include IFN-β and glatiramer acetate [7, 8]. Other drugs as natalizumab and fingolimod are also effective in lowering recurrence rates and slowing disease progression [9]. Experimental autoimmune encephalomyelitis (EAE) is an induced demyelinating pathology of the central nervous system (CNS) that is commonly used as a model to investigate this disease. EAE is triggered in susceptible mouse and rat strains by immunization with myelin proteolipid protein, myelin basic protein, or myelin oligodendrocyte glycoprotein (MOG\textsuperscript{35–55}) emulsified in Complete Freund’s Adjuvant (CFA) [10–12]. These models and also human studies indicate
that many cell subsets as Th1, Th17, Tc, and Tγδ contribute to
damage of the CNS [1, 13]. More recently, subsets of T cells
endowed with immunoregulatory ability were characterized
and their contribution to restrain self-reactivity is being
established. Regulatory T cells (Treg cells) encompass both
natural and inducible (adaptive) cell types. Natural Treg
cells are identified by high CD25 expression and intracellular
presence of the forkhead box P3 (Foxp3) transcription factor,
which is required for directing regulatory function [14]. These
cells are originally identified by their ability to establish
tolerance to self-antigens [15]. Natural Treg cells develop
in the thymus after expression of Foxp3 at a relatively late
stage of thymopoiesis [16]. Adaptive Treg cells including Tr1,
Th3, and various CD8+ Treg cells subsets are triggered by
stimulation of naïve T cells by their cognate antigens (self or
nonself) in the periphery. Suppressive cytokines as IL-10 and
TGF-β contribute to both induction of these Treg cells and
also stimulation of their suppressive function. Tr1 cells exert
their suppressive function primarily through IL-10 secretion
[17] whereas Th3 cells act mainly through TGF-β release [18].

Even though current therapies for MS are limiting the
impact of this neurodegenerative disorder, they can cause
severe side effects as cutaneous lesions [19], depression,
thyroid dysfunction, cardiotoxicity, liver enzymes abnormal-
ities, and increased susceptibility to infections [20]. The
production of anti-drug antibodies has also been described
[21]. Novel disease modifying therapies are being tested as
monoclonal antibodies, chimeric molecules, and oral
therapies [22, 23]. Prophylactic and therapeutic approaches
based on Treg cells induction would be highly useful. Even
though antigen-specific Treg cells would be preferentially
induced, other Treg cells subsets are being examined. Heat
shock proteins (hsp), for example, are being considered as
potential targets for the treatment of inflammatory diseases
due to their increased expression in inflammatory foci. This
possibility is supported by convincing evidence that hsp can
induce immunoregulatory T cell responses [24–27]. The
immunomodulatory ability of the mycobacterial hsp65 in
autoimmune diseases has been demonstrated by us and
other authors in arthritis [28, 29], diabetes [30–32], and
atherosclerosis [27, 33].

More recently, we also tested the immunomodulatory
potential of pVAXhsp65 (a genetic vaccine containing the
hsp65 mycobacterial gene) in EAE. Initially it was applied
therapeutically, that is, after disease induction. Even though
this approach was able to significantly downmodulate the
peripheral production of encephalitogenic cytokines, it was
not capable of reducing disease severity [34]. Considering
that memory and naïve T cells could present a differential
susceptibility to regulation [35], we supposed that naïve T
cells, specific for MOG35–55 and present in mice not yet
submitted to EAE induction, could be more responsive to
regulation by hsp65. We therefore tested the prophylactic
potential of pVAXhsp65 on EAE development.

2. Materials and Methods

2.1. Experimental Procedure. A preliminary experiment was
done to choose the immunization protocol with the highest
immunoregulatory potential. Mice were injected with 2, 3,
or 4 doses of pVAXhsp65 with 14 days being the interval
between doses. The animals were euthanized 14 days after
the last dose and IFN-γ and IL-10 were quantified in spleen
cell cultures stimulated with recombinant hsp65 (rHsp65).
Mice injected with saline were used as a control group.
To evaluate the prophylactic effect of pVAXhsp65 on EAE
development, mice were immunized with 4 pVAXhsp65
doses and 14 days after last dose they were submitted to EAE
induction. Disease development was evaluated by clinical
follow-up (clinical score and weight variation) and also by
histopathological analysis of the CNS. The immunomodulatory
potential of pVAXhsp65 vaccination was checked by the
profile of cytokine production by spleen and CNS infiltrating
cells stimulated with MOG35–55 or rHsp65 and also by the
presence of Foxp3+ regulatory T cells in these two organs.
Mice injected with saline (control) or with the empty vector
(pVAX) were used as control groups.

2.2. Animals. Female C57BL/6 mice (4–6 weeks old) were
purchased from CEMIB (UNICAMP, São Paulo, SP, Brazil).
The animals were fed with sterilized food and water ad
libitum and were manipulated in accordance with the eth-
ical guidelines adopted by the Brazilian College of Animal
Experimentation. Animal experiments were conducted with
the approval of the Ethics Committee for Animal Experimen-
tation, Medical School, Universidade Estadual Paulista.

2.3. DNA Vaccine Encoding hsp65 Assembly. The vaccine
pVAXhsp65 was constructed from the pVAX vector (Invit-
rogen, Carlsbad, CA, USA). This plasmid was digested
with BamHI and NotI (Gibco BRL, Gaithersburg, MD,
USA) and incubated at 30°C. The vaccine was transformed
with E. coli and incubated at 30°C. The plasmids were
purified using the Concert High Purity Maxiprep System (Gibco BRL, Gaithersburg, MD, USA). Plasmid concentrations
were determined by spectrophotometry at λ = 260 and 280 nm by using the Gene Quant II apparatus (Pharmacia
Biotech, Buckinghamshire, UK).

2.4. Recombinant hsp65 Protein (rHsp65). The rHsp65 was
obtained from Escherichia coli ER2566 previously trans-
formed with the hsp65 gene from M. leprae. The transfect ed E.
coli was cultured in LB containing ampicillin (100 μg/ml) and
the bacterial growth was monitored by spectrophotometry
at 600 nm. When the optical density reached a value of 0.6,
the culture was induced with isopropylthiogalactoside 0.1 M
(Gibco BRL, Gaithersburg, MD, USA) and incubated at 30°C
under agitation for 4 h. Details of rHsp65 production and
puriﬁcation can be found at dos Santos et al., 2010 [36].

2.5. Vaccination with pVAXhsp65. Mice were injected with 4
doses of pVAXhsp65 (100 μg/100 μl) by intramuscular route
(quadriceps muscle). Fourteen days after the last dose the
animals were submitted to EAE induction.
2.6. EAE Induction. MOG_{35-55} peptide (MEVGWYRSPFS-RVVHLYRNGK) was synthesized by Proteimax, São Paulo, Brazil. EAE was induced as previously reported [37]. Briefly, mice were immunized with 150 μg of MOG_{35-55} emulsified in CFA containing 400 μg of BCG. Two doses of 200 ng of Bordetella pertussis toxin (Sigma Aldrich, St. Louis, MO, USA) were administered by intraperitoneal route. Animals were daily checked and disease intensity was recorded as follows: (0) no symptoms, (1) limp tail, (2) hind legs weakness, (3) partially paralyzed hind legs, (4) complete hind leg paralysis, and (5) complete paralysis/death.

2.7. CNS Infiltrating Mononuclear Cells Isolation. Mononuclear cells infiltrated in the CNS were obtained as previously described by Mimura et al., 2016 [38]. Briefly, sedated (ketamine/xylazine) mice were perfused with saline solution and then brain and the whole spinal cord were collected, macerated, resuspended in RPMI medium (Sigma Aldrich) supplemented with 2.5% collagenase D (Roche Applied Science) and incubated at 37°C, 5% CO₂ incubator for 45 min. Cells were then resuspended in percoll (GE Healthcare) 37% and gently laid over percoll 70%. After centrifugation at 950 xg for 20 min the ring containing mononuclear cells was collected. Cellular suspension from percoll interface was then resuspended in supplemented RPMI medium (1% gentamicin, 2% glutamine, 1% sodium pyruvate, 1% nonessential amino acids, and 10% of fetal calf serum).

2.8. Spleen and CNS Cell Culture Conditions. Spleen and mononuclear cells isolated from the CNS were adjusted to 5 x 10⁶ cells/ml and 2 x 10⁵ cells/ml, respectively, and cultured in supplemented RPMI medium. Spleen cells were restimulated in vitro with MOG_{35-55} (20 μg/ml) or rshsp65 (10 μg/ml), while CNS-isolated cells were stimulated with MOG_{35-55} (50 μg/ml) and rshsp65 (10 μg/ml). IFN-γ, IL-10, IL-6, IL-17, and TNF-α levels were assessed in culture supernatants by ELISA (Becton Dickinson, San Jose, CA, USA) according to the manufacturer’s instructions.

2.9. Proportion of CD4+CD25+Foxp3+ T Cells. Spleen cells were collected and the red blood cells were lysed with Hank’s buffer containing NH₄Cl. Spleen and CNS infiltrating cells were adjusted to 2.5 x 10⁶ cells/100 μl and then incubated 0.5 μg of FITC labeled anti-mouse CD4 (clone GK1.5) and 0.25 μg of APC labeled anti-mouse CD25 (clone PC61.5) at room temperature during 20 min. A staining for Foxp3 was performed using the anti-mouse/rat Foxp3 Staining Set (eBioscience, San Diego, CA, USA) according to the manufacturer’s instructions. The cells were analyzed by flow cytometry using the FACSComp II (Becton Dickinson, San Jose, CA, USA) and FACSDiva software (Becton Dickinson, San Jose, CA, USA) at Amaral Carvalho Foundation (Jaú, São Paulo, Brazil).

2.10. Inflammatory Infiltration in the CNS. The histological analysis was performed in the CNS at the 30th day after EAE induction. Lumbar spinal cord samples were removed and fixed in 10% neutral buffered formalin. Paraffin slides with 5 μm were stained with hematoxylin and eosin (H&E) and analyzed with a Nikon microscope. A semiquantitative analysis of CNS inflammation was performed according to the following criteria: (0) no infiltrates; (1) partial meningeal infiltration; (2) pronounced meningeal infiltration; and (3) pronounced meningeal and some parenchymal infiltration as already adopted by us and other authors [39, 40]. This evaluation was done with a Nikon microscope by analyzing two distinct areas in the samples of each animal.

2.11. Statistical Analysis. Data were expressed as mean ± SE. Comparisons between groups were made by one-way ANOVA with post hoc Holm-Sidak test for parameters with normal distribution and by Kruskal-Wallis followed by a post hoc Dunn’s test for parameters with nonnormal distribution. Significance level was p < 0.05. Statistical analysis was accomplished with SigmaStat for Windows v 3.5 (Systat Software Inc).

3. Results

3.1. Immune Response Induced by pVAXhsp65 Immunization. Immunization with 2, 3, or 4 pVAXhsp65 doses determined the production of similar amounts of IFN-γ (Figure 1(a)) by spleen cells stimulated with rshsp65. However, only 4 pVAXhsp65 doses triggered a significant IL-10 production (Figure 1(b)) by these cells. These high IL-10 levels were not, however, associated with a higher frequency of CD4+CD25+Foxp3+ T cells in the spleen. The proportion of these cells, evaluated 14 days after DNA immunization, was similar in immunized, injected with vector or noninjected experimental groups (Figure 1(c)).

3.2. Decreased EAE Severity in Mice Previously Immunized with pVAXhsp65. Previous immunization with pVAXhsp65 significantly reduced EAE symptoms. By the 17th day of the disease, when the paralysis achieved its maximum level in the EAE control group (clinical score = 3), the average score in the previously immunized group was 1.5. Previous vaccination also delayed disease onset and determined lower clinical scores during the chronic disease phase (Figure 2(a)). The statistical significance of clinical improvement was determined by linear regression analysis (Figure 2(b)). Besides, mice previously immunized with pVAXhsp65 lost significantly less weight than the EAE control group (Figure 2(c)).

3.3. CNS Inflammation in Mice Previously Immunized with pVAXhsp65. Typical lesions mainly characterized by mononuclear cell infiltration were observed in the meningeal areas of the CNS in mice with EAE (nonimmunized) during the chronic period of the disease. Semiquantitative microscopic analysis indicated that previous DNA immunization reduced the magnitude of inflammation in the lumbar spinal cord samples in comparison to nonimmunized animals as illustrated in Figure 3.
3.4. Peripheral Immune Response in Mice Previously Immunized with pVAXhsp65. Cytokine production by cultures from different experimental groups was compared 30 days after EAE induction. IFN-γ (Figure 4(a)), IL-6 (Figure 4(b)), and IL-17 (Figure 4(c)) produced by spleen cells from EAE, pVAX/EAE, and pVAXhsp65/EAE groups stimulated with MOG35-55 reached similar levels, whereas IL-10 production was similarly downmodulated in vector and vaccine previously injected groups (Figure 4(d)). A distinct cytokine profile was observed in cultures stimulated with rhp65. In this case, only IFN-γ levels (Figure 4(a)) were significantly higher in the group previously vaccinated with pVAXhsp65. Levels of IL-6 (Figure 4(b)), IL-17 (Figure 4(c)), and IL-10 (Figure 4(d)) were similar in EAE, pVAX/EAE, and pVAXhsp65/EAE groups. EAE development was already associated with high levels of Foxp3+ regulatory T cells in peripheral lymphoid organs and pVAXhsp65 immunization did not augment the proportion of these regulatory T cells in the spleen (Figure 4(e)) when compared with the control groups (EAE and pVAX/EAE).

3.5. Immune Response at the CNS in Mice Previously Immunized with pVAXhsp65. A significant production of all checked cytokines was detected in cultures from CNS infiltrating cells. The levels of IFN-γ (Figure 5(a)), TNF-α (Figure 5(b)), IL-6 (Figure 5(c)), and IL-10 (Figure 5(d)) were similarly elevated in the three experimental groups when the cells were stimulated with MOG35-55. The amounts of TNF-α (Figure 5(b)) and IL-6 (Figure 5(c)) were also comparable in cultures from these three groups stimulated with rhp65. However, stimulation with rhp65 triggered a significantly higher production of IFN-γ (Figure 5(a)) and IL-10 (Figure 5(d)) in mice that were previously immunized with pVAXhsp65. Similarly to the peripheral findings, pVAXhsp65 immunization also did not increase the frequency of CD4+CD25+Foxp3+ T cells in the CNS (Figure 5(e)).

4. Discussion

EAE is a widely employed model to understand MS pathogenesis and also to search for prophylaxis and new
Figure 2: Effect of previous vaccination with pVAXhsp65 on EAE development. C57BL/6 mice were immunized with 4 pVAXhsp65 doses and then submitted to EAE induction. Kinetics of clinical scores (a), linear regression analysis of clinical scores (b), and body weight variation (c). Data were presented by mean ± SE of 6 mice and are representative of three independent experiments. * represents the difference between immunized and control group with EAE. *p < 0.05 and **p < 0.001.

therapeutic measures towards this pathology. In this work, we found that a genetic construction containing the mycobacterial hsp65 gene is endowed with prophylactic application against EAE development. C57BL/6 mice were initially immunized with variable pVAXhsp65 doses to choose the potentially protective schedule. Only the 4-dose scheme was able to prime these animals for a higher IL-10 production. As IL-10 producer cells have been described as being induced by rhs65 and also able to downmodulate autoimmune conditions [31], this vaccination procedure was chosen to investigate the prophylactic potential of this vaccine in EAE. C57BL/6 mice were then immunized with 4 pVAXhsp65 doses and then submitted to EAE induction. The positive control group, that is, only subjected to EAE induction, developed the classical signs of EAE as accentuated weight loss and clinical paralysis. These results were expected and very similar to what has been described by us and other authors that used the encephalomyelitis model induced by MOG\textsubscript{35–55} immunization [41–43]. Previous vaccination with pVAXhsp65 clearly modified disease development. These mice lost less body weight and also showed lower clinical scores. The onset of clinical signs was likewise delayed in this experimental group. This protective effect was also associated with decreased inflammation at the lumbar spinal cord suggesting reduced migration of peripheral encephalitogenic T cells to the CNS.

Cytokine production by spleen cells stimulated with MOG\textsubscript{35–55} highly suggested that the pVAXhsp65 is not working by decreasing the peripheral proinflammatory specific immune response, as IFN-\gamma, IL-17, and TNF-\alpha levels were similar in all experimental groups. Intriguingly, IL-10 levels in these cultures were significantly downregulated in DNA injected mice. When these splenic cells were restimulated with rhs65, the expected high IFN-\gamma and IL-10 levels previously observed in pVAXhsp65 immunized mice were not observed. As IL-10 has been described as
one of the most effective anti-inflammatory cytokines in EAE and MS [44], we reasoned that IL-10 producer cells, specific for MOG\(_{35-55}\) or hsp65, had migrated to the CNS and partially controlled inflammation. This possibility was tested by stimulating mononuclear cells eluted from CNS with MOG\(_{35-55}\) and rhsp65. Mice previously immunized with pVAXhsp65 produced significantly higher levels of IFN-\(\gamma\) and IL-10 in response to rhsp65, but not MOG\(_{35-55}\), after in vitro restimulation. As nonstimulated cell cultures did not produce cytokines (data not shown), we hypothesized that these were hsp65 specific cells. Vaccination also did not alter the frequency of Foxp3+ Treg cells in the periphery or in the CNS, suggesting that the classical CD4+CD25+Foxp3+ Treg cells are not responsible for IL-10 production. It is possible therefore that other cell types that do not express Foxp3 are the source of this anti-inflammatory cytokine [45].

As the protocol that we used to isolate CNS cells was based on centrifugation over discontinuous percoll gradients, a variety of cells could be present in these CNS cell cultures as neuronal cells, astrocytes, oligodendrocytes, microglial cells, and infiltrating leukocytes, as described by Pino and Cardona, 2011 [46]. Then, distinct specific or even
Figure 4: Effect of previous vaccination with pVAXhsp65 on peripheral immune response. C57BL/6 mice were immunized with 4 pVAXhsp65 doses and then submitted to EAE induction. Cytokine production was assessed 30 days after EAE induction. IFN-γ (a), IL-6 (b), IL-17 (c), and IL-10 (d) production were assayed in spleen cell cultures restimulated in vitro with MOG35–55 or rhsp65. The percentage of CD4+CD25+Foxp3+ T cells was evaluated in the total number of spleen cells 30 days after EAE induction (e). Data were presented by mean ± SE of 6 mice and are representative of two independent experiments. * represents the difference between DNA injected groups and control group with EAE. p < 0.05.
Figure 5: Effect of previous vaccination with pVAXhsp65 at the CNS. C57BL/6 mice were immunized with 4 pVAXhsp65 doses and then submitted to EAE induction. Cytokine production was assessed 30 days after EAE induction. IFN-γ (a), TNF-α (b), IL-6 (c), and IL-10 (d) production were assayed in CNS infiltrating cells cultures restimulated in vitro with MOG$_{35-55}$ or rhsp65. The percentage of CD4$^+$CD25$^+$Foxp3$^+$ T cells was evaluated in the total number of mononuclear cells from CNS 30 days after EAE (e). Data were presented by mean ± SE of 5 mice. * represents the difference between immunized and control group with EAE. $p < 0.05$. 
nonspecific cells could be the source of this regulatory cytokine. Various cell types as macrophages, dendritic cells, Tr1, and B regulatory cells [47, 48] are described as being able to produce IL-10 and to contribute to EAE and MS recovery. Many reports have also highlighted the role of hsp60/65 in activation of B cells [49], T cells [50], Treg cells [51], and maturation of dendritic cells [52]. Specially concerning hsp65 formulated as a DNA vaccine, Fontoura et al., 2015 [53], described that DNAhsp65 immunization of C57BL/6 mice induced a subtype of IL-10 producing B cell able to reduce the production of proinflammatory cytokine mRNAs in the spleen. Hsp65 was also able to attenuate the development of airway hyperresponsiveness and inflammation in BALB/c mice through modulation of dendritic cell function [54]. These findings endorse the possibility that hsp65 responsive cells able to produce IL-10 are present in the CNS and could mediate the protective effect observed in this work.

The vector also triggered a protective effect even though it was discrete comparing to the one elicited by the vaccine. This finding suggests that the immunoregulatory ability of pVAXhsp65 partially depends upon the plasmid vector itself. In this regard, CpG motifs in the plasmid vector could trigger an anti-inflammatory immune response. This possibility is supported by some literature reports. Quintana et al., 2000 [55], showed that injection of empty plasmid DNA or CpG oligonucleotides inhibited diabetes in NOD mice due to a shift to Th2 profile. More recently, it has been demonstrated that CpG-DNA sequences were capable of inducing a Th2 response in human endothelial cells through the inhibition of proinflammatory cytokines and enhanced IL-10 expression [56].

A similar anti-inflammatory effect of this genetic vaccine was previously described by our research group in arthritis [28] and diabetes [30, 31, 57] experimental models. The potential of pVAXhsp65 as a prophylactic vaccine against diabetes was also established by us in both homologous and heterologous prime-boost strategies. NOD mice were clinically protected by immunization with pVAXhsp65. The vector also determined immunomodulation but its protective effect against insulinitis was very discrete. Interestingly, protection coincided with the influx of CD25+ cells and increased staining for IL-10 in the islets [30]. We also demonstrated that the combination of pVAXhsp65 with BCG, in a heterologous prime-boost protocol, was highly effective to prevent diabetes in NOD mice [57]. In addition, we observed that this vaccine decreased lumbar inflammation and downmodulated peripheral IL-10 production in an EAE rat model [12] similarly to the findings showed in the present investigation. Again, the empty plasmid prompted a similar but less pronounced effect. This protective effect of hsp65 in distinct inflammatory conditions is highly supported by findings of many other authors [58–61].

Concerning EAE development, the immunoregulatory effect of hsp65 has also been demonstrated by employing distinct formulations containing this heat shock protein. For example, oral administration of a recombinant Lactococcus lactis strain that produces hsp65 prevented the development of EAE in C57BL/6 mice. This protection was confirmed by the reduced inflammatory cell infiltrate and absence of injury signs in the spinal cord [62]. Also recently, Billetra et al., 2012 [63], found that intranasal treatment of EAE with the peptide RatP2 derived from hsp60 determined a significant clinical improvement which was superior to therapy with glatiramer acetate.

5. Conclusion

Previous vaccination with pVAXhsp65 was able to reduce EAE clinical manifestations and also triggered higher IL-10 production at the CNS. These findings reinforce the potential of hsp65 to be explored as an adjuvant therapy in this and other autoimmune pathologies.

Competing Interests

The authors declare no conflict of interests.

Acknowledgments

The study was supported by São Paulo Research Foundation (FAPESP) Grant no. 2011/07528-5 and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) Grant no. 301770/2009-3.

References

[1] J. M. Fletcher, S. J. Lalor, C. M. Sweeney, N. Tübridy, and K. H. G. Mills, “T cells in multiple sclerosis and experimental autoimmune encephalomyelitis,” Clinical and Experimental Immunology, vol. 162, no. 1, pp. 1–11, 2010.
[2] C. A. Dendrou, L. Fugger, and M. A. Friese, “Immunopathology of multiple sclerosis,” Nature Reviews Immunology, vol. 15, no. 9, pp. 545–558, 2015.
[3] L. Steinman, “Assessment of animal models for MS and demyelinating disease in the design of rational therapy,” Neuron, vol. 24, no. 3, pp. 511–514, 1999.
[4] G. C. Furtado, M. C. G. Marcondes, J.-A. Latkowski, J. Tsai, A. Wensky, and J. J. Lafaille, “Swift entry of myelin-specific T lymphocytes into the central nervous system in spontaneous autoimmune encephalomyelitis,” Journal of Immunology, vol. 181, no. 7, pp. 4648–4655, 2008.
[5] R. A. O’Connor, C. T. Prendergast, C. A. Sabatos et al., “Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis,” Journal of Immunology, vol. 181, no. 6, pp. 3750–3754, 2008.
[6] W. I. McDonald and T. A. Sears, “The effects of experimental demyelination on conduction in the central nervous system,” Brain, vol. 93, no. 3, pp. 583–598, 1970.
[7] M. Sanford, K. A. Lyseng-Williamson, E. G. Celius et al., “Subcutaneous recombinant interferon-β-1a (Rebif®): a review of its use in the treatment of relapsing multiple sclerosis,” Drugs, vol. 71, no. 14, pp. 1865–1891, 2011.
[8] K. P. Johnson, “Glatiramer acetate for treatment of relapsing-remitting multiple sclerosis,” Expert Review of Neurotherapeutics, vol. 12, no. 4, pp. 371–384, 2012.
[9] E. Waubant, “Overview of treatment options in multiple sclerosis,” Journal of Clinical Psychiatry, vol. 73, no. 6, 2012.
[10] I. M. Stromnes and J. M. Goverman, “Passive induction of experimental allergic encephalomyelitis,” *Nature Protocols*, vol. 1, no. 4, pp. 1952–1960, 2006.

[11] J. Seger, S. F. G. Zorzella-Pezavento, A. C. Pelizon, D. R. Martins, A. Domingues, and A. Sartori, “Decreased production of TNF-alpha by lymph node cells indicates experimental autoimmune encephalomyelitis remission in Lewis rats,” *Memorias do Instituto Oswaldo Cruz*, vol. 105, no. 3, pp. 263–268, 2010.

[12] S. F. G. Zorzella-Pezavento, F. Chiuso-Minicucci, T. G. D. França et al., “Immunization with pVAXhsp65 decreases inflammation and modulates immune response in experimental encephalomyelitis,” *NeuroImmunoModulation*, vol. 17, no. 5, pp. 287–297, 2010.

[13] F. Petermann and T. Korn, “Cytokines and effector T cell subsets causing autoimmune CNS disease,” *FEBS Letters*, vol. 585, no. 23, pp. 3747–3757, 2011.

[14] J. D. Fontenot, M. A. Gavin, and A. Y. Rudensky, “Foxp3 programs the development and function of CD4+CD25+ regulatory T cells,” *Nature Immunology*, vol. 4, no. 4, pp. 330–336, 2003.

[15] M. G. Roncarolo, S. Gregori, M. Battaglia, R. Bacchetta, K. Sakaguchi, N. Sakaguchi, M. Asano, M. Itoh, and M. Toda, “Programs the development and function of CD4+CD25+ regulatory T cells,” *Nature Immunology*, vol. 155, no. 3, pp. 1151–1164, 1995.

[16] X. Yuan and T. R. Malek, “Cellular and molecular determinants for the development of natural and induced regulatory T cells,” *Human Immunology*, vol. 73, no. 8, pp. 773–782, 2012.

[17] M. G. Roncarolo, G. Strippoli, G. Battaglia, L. Bacchetta, K. Fleischhauer, and M. K. Levings, “Interleukin-10-secreting type 1 regulatory T cells in rodents and humans,” *Immunological Reviews*, vol. 212, pp. 28–50, 2006.

[18] H. L. Weiner, “Induction and mechanism of action of transforming growth factor-β-secreting Th3 regulatory cells,” *Immunological Reviews*, vol. 182, pp. 207–214, 2001.

[19] I. Cortese, J. Ohayon, K. Fenton et al., “Cutaneous adverse events in multiple sclerosis patients treated with daclizumab,” *Neurology*, vol. 86, no. 9, pp. 847–855, 2016.

[20] W. Castro-Borrero, D. Graves, T. C. Frohman et al., “Current and emerging therapies in multiple sclerosis: a systematic review,” *Therapeutic Advances in Neurological Disorders*, vol. 5, no. 4, pp. 205–220, 2012.

[21] H. Hegen, M. Auer, and F. Deisenhammer, “Pharmacokinetic considerations in the treatment of multiple sclerosis with interferon-β,” *Expert Opinion on Drug Metabolism and Toxicology*, vol. 11, no. 12, pp. 1803–1819, 2015.

[22] D. M. Harrison and P. A. Calabresi, “Promising treatments of tomorrow for multiple sclerosis,” *Annals of Indian Academy of Neurology*, vol. 12, no. 4, pp. 283–290, 2009.

[23] A. H. Cross and R. T. Naismith, “Established and novel disease-modifying treatments in multiple sclerosis,” *Journal of Internal Medicine*, vol. 275, no. 4, pp. 350–363, 2014.

[24] W. Van Eden, G. Wick, S. Alban, and I. Cohen, “Stress, heat shock proteins, and autoimmunity: how immune responses to heat shock proteins are to be used for the control of chronic inflammatory diseases,” *Annals of the New York Academy of Sciences*, vol. 1113, pp. 217–237, 2007.

[25] C. I. Silva, V. L. D. Bonato, R. R. Dos Santos-Júnior, C. R. Zárate-Bladés, and A. Sartori, “Recent advances in DNA vaccines for autoimmune diseases,” *Expert Review of Vaccines*, vol. 8, no. 2, pp. 239–252, 2009.

[26] C. Keijzer, L. Wieten, M. van Herwijnen, R. van der Zee, W. van Eden, and F. Broere, “Heat shock proteins are therapeutic targets in autoimmune diseases and other chronic inflammatory conditions,” *Expert Opinion on Therapeutic Targets*, vol. 16, no. 9, pp. 849–857, 2012.

[27] Y. Zhong, H. Tang, X. Wang et al., “Intranasal immunization with heat shock protein 60 induces CD4+CD25+GARP+ and type 1 regulatory T cells and inhibits early atherosclerosis,” *Clinical and Experimental Immunology*, vol. 183, no. 3, pp. 452–468, 2016.

[28] R. R. Santos-Júnior, A. Sartori, M. De Franco et al., “Immuno-modulation and protection induced by DNA-hsp65 vaccination in an animal model of arthritis,” *Human Gene Therapy*, vol. 16, no. 11, pp. 1338–1345, 2005.

[29] S. R. Satpute, R. Rajaiya, S. K. Polumuri, and K. D. Moudgil, “Tolerization with Hsp65 induces protection against adjuvant-induced arthritis by modulating the antigen-directed interferon-γ, interleukin-17, and antibody responses,” *Arthritis and Rheumatism*, vol. 60, no. 1, pp. 103–113, 2009.

[30] R. Rodrigues Dos Santos Jr., A. Sartori, V. L. Deperon Bonato et al., “Immune modulation induced by tuberculosis DNA vaccine protects non-obese diabetic mice from diabetes progression,” *Clinical and Experimental Immunology*, vol. 149, no. 3, pp. 570–578, 2007.

[31] R. R. Santos, A. Sartori, D. S. Lima et al., “DNA vaccine containing the mycobacterial hsp65 gene prevented insulin in MLD-STZ diabetes,” *Journal of Immune Based Therapies and Vaccines*, vol. 7, article no. 4, 2009.

[32] A.-H. Zhu, L. Jin, J.-J. Liu, M.-Y. Liu, A.-J. Lv, and Y.-L. Zheng, “Intranasal vaccination with mycobacterial 65-kD heat-shock protein can prevent insulitis and diabetes in non-obese diabetic mice,” *Chinese Journal of Cellular and Molecular Immunology*, vol. 27, no. 11, pp. 1165–1168, 2011.

[33] C. Grundtman, B. Jakic, M. Buszko et al., “Mycobacterial heat shock protein 65 (mbHSP65)-induced atherosclerosis: preventive oral tolerization and definition of atheroprotective and atherogenic mbHSP65 peptides,” *Atherosclerosis*, vol. 242, no. 1, pp. 303–310, 2015.

[34] S. F. G. Zorzella-Pezavento, F. Chiuso-Minicucci, T. G. D. França et al., “Downmodulation of peripheral MOG-specific immunity by pVAXhsp65 treatment during EAE does not reach the CNS,” *Journal of Neuroimmunology*, vol. 268, no. 1–2, pp. 35–42, 2014.

[35] A. R. O. Watson and W. T. Lee, “Differences in signaling molecule organization between naive and memory CD4+ T lymphocytes,” *Journal of Immunology*, vol. 173, no. 1, pp. 33–41, 2004.

[36] S. A. dos Santos, C. R. Zárate-Bladés, F. C. de Sá Galetti et al., “A subunit vaccine based on biodegradable microspheres carrying rHsp65 protein and KLK protects BALB/c mice against tuberculosis infection,” *Human Vaccines*, vol. 6, no. 12, pp. 1047–1053, 2010.

[37] S. F. G. Zorzella-Pezavento, F. Chiuso-Minicucci, T. G. D. França et al., “Persistent inflammation in the CNS during chronic EAE despite local absence of IL-17 production,” *Mediators of Inflammation*, vol. 2013, Article ID 519627, 10 pages, 2013.

[38] L. A. N. Mimura, F. Chiuso-Minicucci, T. F. C. Fraga-Silva et al., “Association of myelin peptide with vitamin D prevents EAE despite local absence of IL-17 production,” *Memorias do Instituto Oswaldo Cruz*, vol. 105, no. 3, pp. 263–268, 2010.

[39] I. A. Soellner, J. Rabe, V. Mauri, J. Kaufmann, K. Addicks, and S. Kuerten, “Differential aspects of immune cell infiltration and neurodegeneration in acute and relapse experimental autoimmune encephalomyelitis,” *Journal of Neuroimmunology*, vol. 275, no. 4, pp. 350–363, 2014.
autoimmune encephalomyelitis,” *Clinical Immunology*, vol. 149, pp. 519–529, 2013.

[40] T. F. C. Fraga-Silva, L. A. N. Mimura, S. F. G. Zorzella-Pezavento et al., “Tolerogenic vaccination with MOG/VitD overcomes aggravating effect of C. albicans in experimental encephalomyelitis,” *CNS Neuroscience & Therapeutics*, vol. 22, no. 10, pp. 807–816, 2016.

[41] J. P. S. Peron, K. Yang, M.-L. Chen et al., “Oral tolerance reduces Th17 cells as well as the overall inflammation in the central nervous system of EAE mice,” *Journal of Neuroimmunology*, vol. 227, no. 1-2, pp. 10–17, 2010.

[42] M. Li, Y. Li, X. Liu, X. Gao, and Y. Wang, “IL-33 blockade suppresses the development of experimental autoimmune encephalomyelitis in C57BL/6 mice,” *Journal of Neuroimmunology*, vol. 247, no. 1-2, pp. 25–31, 2012.

[43] F. Chiuso-Minicucci, L. L. W. Ishikawa, L. A. N. Mimura et al., “Treatment with vitamin D/MOG association suppresses experimental autoimmune encephalomyelitis,” *PLoS ONE*, vol. 10, no. 5, Article ID e0125836, 2015.

[44] F. Jadidi-Niaragh and A. Mirshafiey, “Regulatory T-cell as P.A.PinoandA.E.Cardona,” *Isolation of brain and spinal cord T. F. C. Fraga-Silva, L. A. N. Mimura, S. F. G. Zorzella-J. M. Rodgers and S. D. Miller, “Cytokine control of inflammation by modulating the function of dendritic cells,” *Journal of Immunology Research*, vol. 11, no. 2, pp. 1789–1795, 2011.

[45] F. J. Quintana, A. Rotem, P. Carmi, and I. R. Cohen, “Vaccination with empty plasmid DNA or CpG oligonucleotide inhibits diabetes in nonobese diabetic mice: modulation of spontaneous 60-kDa heat shock protein autoimmunity,” *The Journal of Immunology*, vol. 165, no. 11, pp. 6148–6155, 2000.

[46] N. Fitzner, L. Zahner, C. Habich, and V. Kolb-Bachofen, “Stimulatory type A CpG-DNA induces a Th2-like response in human endothelial cells,” *International Immunopharmacology*, vol. 11, no. 11, pp. 430–437, 2013.

[47] F. Chiuso-Minicucci, L. C. Da Rosa, F. Chiuso-Minicucci, S. F. G. Zorzella-Pezavento et al., “Bacille Calmette-Guérin/DNAhsp65 prime-boost is protective against diabetes in non-obese diabetic mice but not in the streptozotocin model of type 1 diabetes,” *Clinical and Experimental Immunology*, vol. 173, no. 3, pp. 430–437, 2013.

[48] D. Harats, N. Yacov, B. Gilburd, Y. Shoefeld, and J. George, “Oral tolerance with heat shock protein 65 attenuates Mycobacterium tuberculosis-induced and high-fat-diet-driven atherosclerotic lesions,” *Journal of the American College of Cardiology*, vol. 40, no. 7, pp. 1333–1338, 2002.

[49] J. Liang, Z. Aihua, W. Yu, L. Yong, and L. Jingjing, “HSP65 serves as an immunogenic carrier for a diabetogenic peptide P277 inducing anti-inflammatory immune response in NOD mice by nasal administration,” *Vaccine*, vol. 28, no. 19, pp. 3312–3317, 2010.

[50] C. Rodriguez-Narciso, M. Pérez-Tapia, R. M. Rangel-Cano et al., “Expression of Mycobacterium leprae HSP65 in tobacco and its effectiveness as an oral treatment in adjuvant-induced arthritis,” *Transgenic Research*, vol. 20, no. 2, pp. 221–229, 2011.

[51] R. M. Rezende, R. P. Oliveira, S. R. Medeiros et al., “Hsp65-producing *Lactococcus lactis* prevents experimental autoimmune encephalomyelitis in mice by inducing CD4+LAP+ regulatory T cells,” *Journal of Autoimmunity*, vol. 40, no. 1, pp. 45–57, 2013.

[52] R. Billetta, N. Ghahramani, O. Lider et al., “Epitope-specific immune tolerization ameliorates experimental autoimmune encephalomyelitis,” *Clinical Immunology*, vol. 145, no. 2, pp. 94–101, 2012.