Journal of Neuroimmunology 268 (2014) 35–42

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Downmodulation of peripheral MOG-specific immunity by pVAXhsp65 treatment during EAE does not reach the CNS

Sofia Fernanda Gonçalves Zorzella-Pezavento [a], Fernanda Chiuso-Minicucci [a], Thais Graziela Donegá França [a], Larissa Lumi Watanabe Ishikawa [a], Larissa Camargo da Rosa [a], Priscila Maria Colavite [a], Camila Marques [b], Maura Rosane Valerio Ikoma [b], Célio Lopes Silva [c], Alexandrina Sartori [a,*]

[a] Department of Microbiology and Immunology, Biosciences Institute, Univ. Estadual Paulista (UNESP), Botucatu, São Paulo, Brazil
[b] Laboratório de Citometria de Fluxo - Fundação Dr. Amaral Carvalho, Jaú, São Paulo, Brazil
[c] Department of Biochemistry and Immunology, University of São Paulo (USP), Ribeirão Preto, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 15 October 2013
Received in revised form 11 December 2013
Accepted 30 December 2013

Keywords:
Experimental autoimmune encephalomyelitis
hsp65
DNA vaccine
CD4 + CD25 + Foxp3 + T cells

ABSTRACT

Most of the therapeutic strategies to control multiple sclerosis are directed to immune modulation and inflammation control. As heat shock proteins are able to induce immunoregulatory T cells, we investigated the therapeutic effect of a genetic vaccine containing the mycobacterial hsp65 gene on experimental autoimmune encephalomyelitis (EAE). Although pVAXhsp65 was immunogenic for mice with EAE and downmodulated specific cytokine induction by MOG, therapy was not able to decrease clinical severity nor to modify immunologic parameters in the CNS. These results indicate that hsp65, administered as a DNA vaccine, was not therapeutic for EAE.

1. Introduction

Multiple sclerosis (MS) is the most common inflammatory and demyelinating disease of the central nervous system (CNS). It is estimated that this disease affects up to two million people worldwide but its distribution throughout the different geographical regions is very uneven. MS is being particularly prevalent in temperate zones and, contrarily, much less common in subtropical and tropical areas. The interplay among factors as genetic background, environmental conditions and socio-economic network seems to define this contrasting incidence (Koch-Henriksen and Sorensen, 2011).

The generally accepted hypothesis is that the disease is triggered by the activation of autoaggressive T cells specific for myelin antigens, followed by an expanding neurodegenerative process (Zhang et al., 1994; Compston and Coles, 2008). These cells cross the blood–brain barrier (BBB), establish themselves at the CNS and mediate damage against the central neurons, their myelin sheaths and also to their axons (Fletcher et al., 2010). The interaction between different cells and mediators of the immune system and a variety of components of the CNS determines the pathogenesis of MS. Briefly, T self-reactive cells are somehow activated in the periphery and have the potential to differentiate into distinct effector T cell subsets as Th1 and Th17 (McFarland and Martin, 2007). These activated cells up-regulate integrins, cross the BBB, encounter the cognate self-antigens presented by resident or immigrant antigen presenting cells (APCs) in the CNS and release their signature cytokines. These cytokines, together with different cell types that are attracted to the inflamed region, cause direct or indirect local damage (Barnett et al., 2006). Tc, Th2, B cells, macrophages and an array of soluble mediators additionally contribute to local tissue destruction (Fletcher et al., 2010; Constantinescu et al., 2011). Resolution of the inflammatory process is often partial and is due to immunoregulatory mechanisms mediated by regulatory T or NK cells (McGeachy et al., 2005; Hao et al., 2010). The anti-inflammatory IL-10 cytokine has been largely associated with these immunoregulatory mechanisms (McGeachy et al., 2005).

Much of the knowledge related to MS immunopathogenesis is originated from experimental autoimmune encephalomyelitis (EAE). This is considered a very useful model to unravel the immunological interactions throughout the disease and also to test potential therapeutical or immunomodulatory drugs (Gold et al., 2006). Different from the human disease, EAE is deliberately induced by immunization with a myelin antigen associated with an adjuvant (Stromnes and Goverman, 2006; Seger et al., 2010; Zorzella-Pezavento et al., 2010). Most of the drugs that are in current use or that are already being submitted to clinical trials for MS treatment were validated through EAE studies. The main licensed drugs for MS therapy include type 1 interferons (Castro-Borrero et al., 2012), glatiramer acetate (Castro-Borrero et al.,

* Corresponding author at: Departamento de Microbiologia e Imunologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Botucatu, São Paulo, Distrito de Rubião Júnior s/n, Botucatu, São Paulo, 18618-970, Brazil. Tel.: +55 14 38800414.
E-mail address: sartori@ibb.unesp.br (A. Sartori).

0165-5728/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jneuroim.2013.12.015

2012), mitoxantrone (Hartung et al., 2002) and azathioprine (Casetta et al., 2007). Many other disease modifying therapies are under clinical trials as recently reviewed by Constantinescu et al. (2011).

Heat shock proteins (hsps) are a group of molecular chaperones that are categorized under distinct families according to their molecular weights. As the name suggests they can be up-regulated by stressing, including heat, conditions. However, they are constitutively and abundantly present in all living beings where they play many important roles. One of the most interesting aspects of these proteins is their high degree of conservation during evolution. Heat shock protein 60 (hsp60) is one of the most well studied members of the hsp family. Hsp60 from different bacterial species, also referred as hsp65, displays more than 95% sequence homology at both DNA and protein levels. An overall 55% homology is detected by comparing human and bacterial hsp60/65 (Grundtman et al., 2011). In addition, hsp60/65 was described as a dominant antigen during bacterial infections (Kaufmann et al., 1991) but also as a target self-antigen in pathological autoimmunity (van Eden et al., 2005).Therefore, the initially prevailing view was that this protein was involved in autoimmunity due to molecular mimicry with the human counterpart (Jones et al., 1993). Further knowledge revealed, however, that this molecule is endowed with strong immunoregulatory properties (van Eden et al., 2005; van Eden et al., 2007). As recently reviewed by Coelho and Faria (2012), the preferential direction of its effects will be determined by a diversity of factors as, for example, route of delivery, appropriate peptides and time point of its administration during the pathology. It has been shown by us and other groups that DNA vaccines containing the human or the mycobacterial hsp60/65 gene is able to confer protection against the development of diabetes and arthritis (Quintana et al., 2002; Quintana et al., 2003; Santos-Junior et al., 2005; Santos Júnior et al., 2007). Recently we also observed that this vaccine was able to decrease inflammation and modulate immune response in EAE induced in Lewis rats (Zorzella-Pezavento et al., 2010).

Considering this previous anti-inflammatory potential, we investigated the possible therapeutic effect of pVAXhsp65 on EAE development.

2. Materials and methods

2.1. Experimental design

Mice were initially submitted to EAE induction by immunization with MOG in Complete Freund's Adjuvant (CFA). A treatment with 3 or 4 pVAXhsp65 doses was started 5 days later. Efficacy of therapy with pVAXhsp65 on EAE was evaluated by clinical follow-up (weight variation and clinical score) and also by histopathological analysis of the CNS. The immunoregulatory effect of pVAXhsp65 was checked by specific induction of cytokines with MOG and rhsp65 and also by analyzing the presence of regulatory T cells (Treg) in the CNS and spleen. Non-immunized (named control) and pVAX (empty vector) injected animals were included as control groups.

2.2. Animals

Female C57BL/6 mice (8–10 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 ethical guidelines adopted by the Brazilian College of Animal Experimentation. All experimental protocols were approved by the local Ethics Committee (Ethics Committee for Animal Experimentation, Medical School, Universidade Estadual Paulista).

2.3. Genetic vaccine construction and purification

The vaccine pVAXhsp65 was derived from the pVAX vector (Invitrogen, Carlsbad, CA, USA), previously digested with BamH I and Not I (Gibco BRL, Gaithersburg, MD, USA) by inserting a 3.3 kb fragment corresponding to the *M. leprae* hsp65 gene and the CMV intron A. The empty pVAX vector was used as a control. DH5α *E. coli* transformed with plasmid pVAX or the plasmid carrying the hsp65 gene (pVAXhsp65) were cultured in LB liquid medium (Gibco BRL, Gaithersburg, MD, USA) containing kanamycin (100 μg/ml). The plasmids were purified using the Concert High Purity Maxiprep System (Gibco BRL, Gaithersburg, MD, USA). Plasmid concentrations were determined by spectrophotometry at $\lambda = 260$ and 280 nm by using the Gene Quant II apparatus (Pharmacia Biotech, Buckinghamshire, UK).

2.4. Treatment with pVAXhsp65

C57BL/6 mice already immunized with MOG plus CFA were injected with 3 or 4 pVAXhsp65 doses (100 μg each) by intramuscular route (quadriceps muscle). The first dose was administered 5 days after EAE induction and subsequent doses were administered 5 days apart. Control groups received the same volume of saline or the same concentration of pVAX (empty vector).

2.5. EAE induction

MOG35–55 peptide (MEVGWYRSPFSRVVHLYRNGK) was synthetized by Proteimax, São Paulo, Brazil. EAE was induced as previously described (Zorzella-Pezavento et al., 2013). Briefly, mice were subcutaneously immunized with 150 μg of MOG35–55 peptide emulsified in complete Freund's adjuvant (CFA) containing 5 mg/ml of mycobacteria. Mice also received 2 doses, 0 and 48 h after immunization, of 200 ng of *Bordetella pertussis* toxin (Sigma Aldrich, St. Louis, MO, USA) intraperitoneally. Animals were daily inspected and disease intensity was graded as: 0 — no disease, 1 — limp tail, 2 — weak/partially paralyzed hind legs, 3 — completely paralyzed hind legs, 4 — complete hind and partial front leg paralysis, 5 — complete paralysis/death.

2.6. Isolation of mononuclear cells infiltrated in the CNS

Mice were anesthetized with ketamine/xylazine and perfused with 10 ml of saline solution. Brain and spinal cord sections (cervical, thoracic and lumbar) were excised, macerated and maintained in 4 ml of RPMI medium (Sigma Aldrich, St. Louis, MO, USA) supplemented with 2.5% collagenase D (Roche Diagnostics, Indianapolis, IN, USA) at 37 °C, 5% CO_2 incubator. Forty-five min later suspensions were washed in RPMI and centrifuged at 450×*g* for 15 min at 4 °C. Cells were then re-suspended in percoll (GE Healthcare, Uppsala, Sweden) 37% and gently laid over percoll 70% in tubes of 15 ml. The tubes were centrifuged at 950×*g* for 20 min with centrifuge breaks turned off. After centrifugation the ring containing mononuclear cells was collected, washed in RPMI and centrifuged at 450×*g* for 5 min. Cellular suspensions were then re-suspended in complete RPMI medium, counted and analyzed.

2.7. Spleen and CNS cell culture conditions

Mice with EAE treated with 3 or 4 pVAXhsp65 doses were euthanized 30 days after EAE induction. Spleen and CNS-isolated cells were collected and adjusted to 5×10^6 cells/ml and 2×10^5 cells/ml, respectively. Cells were cultured in complete RPMI medium (RPMI supplemented with 5% of fetal calf serum, 20 mM glutamine and 40 IU/ml of gentamicin). Spleen cells were stimulated with MOG (20 μg/ml), rhsp65 (10 μg/ml) or Concanavalin A - Sigma Aldrich (10 μg/ml). CNS-isolated cells were re-stimulated *in vitro* with MOG (50 μg/ml) or rhsp65 (10 μg/ml). Cytokine levels were evaluated 48 h later by enzyme-linked immunosorbent assay (ELISA) in culture supernatants using IFN-γ and IL-10 BD OptEIA Sets (Becton Dickinson, San Jose, CA, USA) and IL-6, IL-17 and TNF-α Duosets (R&D Systems, Minneapolis, MN, USA). The assays were performed according to the manufacturer's instructions.

2.8. Percentage of CD4 + CD25 + Foxp3 + T cells in spleen and CNS

Spleen cells were collected and the red blood cells were lysed with Hank's buffer containing NH_4Cl. Mononuclear cells infiltrated in the brain and spinal cord were isolated as described before and adjusted to 2.5×10^6 cells/100 μl. Spleen and CNS infiltrating cells were then incubated with 0.5 μg of fluorescein isothiocianate (FITC) anti-mouse CD4 (clone GK1.5) and 0.25 μg of allophycocyanin (APC) anti-mouse CD25 (clone PC61.5) for 20 min at room temperature. A staining for Foxp3 was then performed utilizing the phycoerythrin (PE) anti-mouse/rat Foxp3 Staining Set (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. After incubation, the cells were fixed in paraformaldehyde 1%. The cells were analyzed by flow cytometry using the FACSCalibur (Becton Dickinson, San Jose, CA, USA) and BD CellQuest Pro software (Becton Dickinson, San Jose, CA, USA).

2.9. Evaluation of inflammatory infiltrates in the CNS

A histological analysis was performed in the CNS at the 30th day after EAE induction. After euthanasia and blood withdrawal, brain and lumbar spinal cord samples were removed and fixed in 10% formaldehyde. Tissues were dehydrated in graded ethanol and embedded in a 100% paraffin block. Serial sections with 5 μm thickness were cut and stained with hematoxylin and eosin. Five photomicrographs were obtained by each animal with a Nikon microscope.

2.10. 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 non-normal 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. DNA vaccine containing the mycobacterial hsp65 gene is immunogenic for mice with EAE

Homologous prime-boost strategies employing 3 or 4 doses of pVAXhsp65 were similarly able to induce a specific immune response against hsp65 in mice with EAE. Spleen cells re-stimulated *in vitro* with rhsp65 produced high levels of IFN-γ (Fig. 1a) and IL-10 (Fig. 1e) in comparison to the EAE group. This increase was not observed in the group injected with pVAX (empty vector). The levels of TNF-α (Fig. 1b), IL-6 (Fig. 1c) and IL-17 (Fig. 1d) in these cultures were also elevated but they were similar to the ones found in the EAE group.

3.2. MOG-specific immunity is downmodulated by treatment with pVAXhsp65

Treatment with pVAXhsp65 significantly reduced the production of IFN-γ (Fig. 2a), IL-6 (Fig. 2b) and IL-17 (Fig. 2c) by spleen cells re-stimulated *in vitro* with MOG. IL-10 levels (Fig. 2d) in these cultures were slightly lower than the levels found in the EAE control group; however these differences were not significant. A very similar profile of cytokine production was observed when the cultures were stimulated with ConA (Fig. 3). In both cases, the control EAE group injected with the empty vector (pVAX) showed no immunomodulation.

3.3. Clinical disease severity is not improved by pVAXhsp65 treatment

The EAE control group, comprising C57BL/6 mice immunized with MOG but not treated, developed the first EAE signs 12 days after disease induction. The maximal degree of paralysis, indicating an acute phase, occurred around day 16, when the average clinical score reached 2.0. From this time on the animals showed a decrease in the clinical score, partially recovering from paralysis. However the recovery was not complete and the disease stabilized around day 22

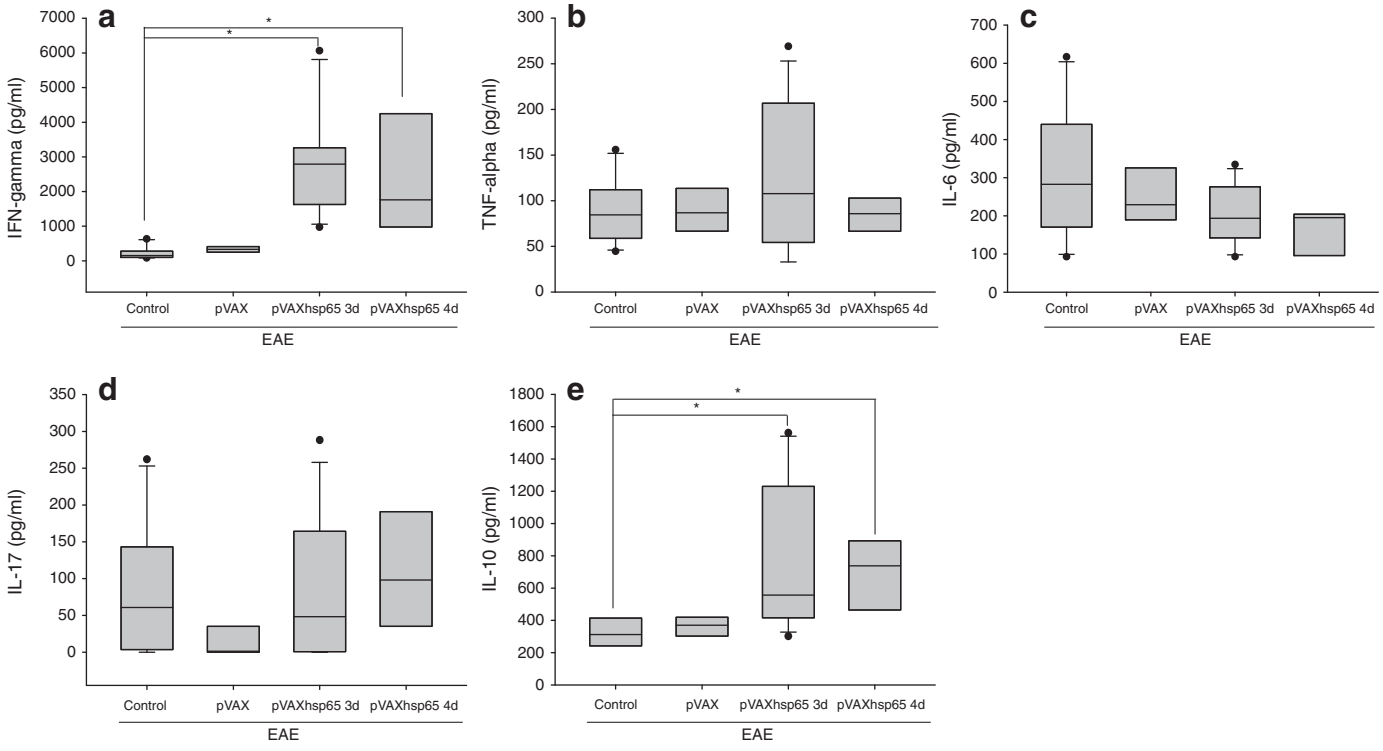

Fig. 1. Immunogenicity of pVAXhsp65 in mice with EAE. C57BL/6 mice were submitted to EAE induction and then immunized with pVAXhsp65. Cytokine production was tested 30 days after EAE induction. IFN-γ (a), TNF-α (b), IL-6 (c), IL-17 (d) and IL-10 (e) production was assayed in spleen cell cultures re-stimulated *in vitro* with rhsp65. Data were presented as median (25–75% ranges) of 6 mice and representative of two independent experiments. *represents the difference between immunized and control group. $p < 0.05$.

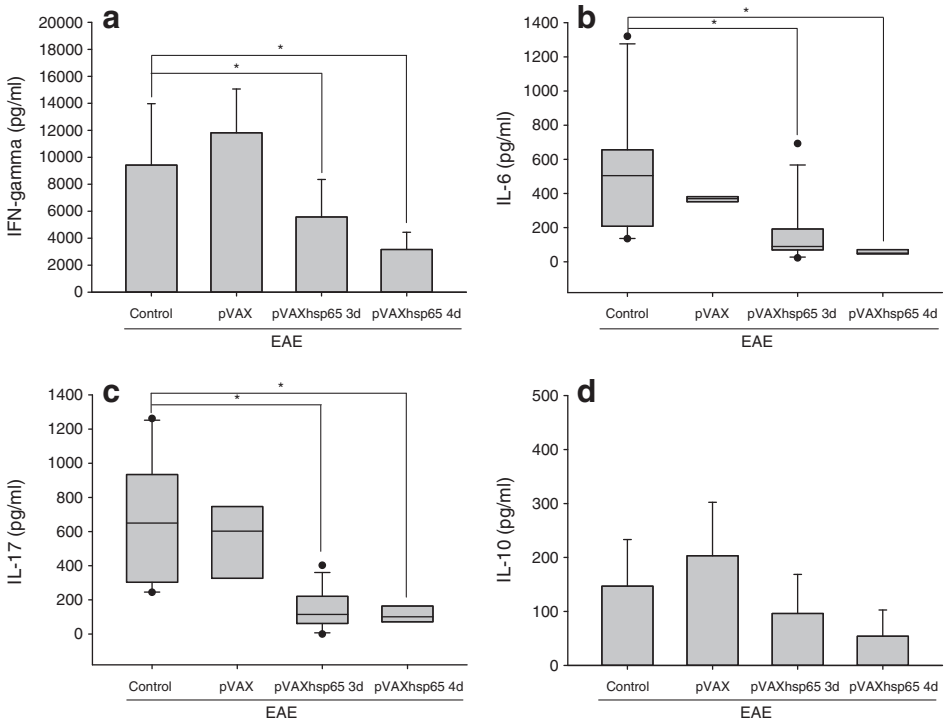

Fig. 2. Effect of therapeutical vaccination with pVAXhsp65 on MOG-specific immune response. C57BL/6 mice were submitted to EAE induction and then injected with 3 or 4 pVAXhsp65 doses. Cytokine production was tested 30 days after EAE induction. IFN-γ (a), IL-6 (b), IL-17 (c) and IL-10 (d) production was assayed in spleen cell cultures re-stimulated *in vitro* with MOG. Data were presented as mean ± SE or median (25–75% ranges) of 6 mice and representative of two independent experiments. * represents the difference between immunized and control group. $p < 0.05$.

with an average score of 0.5. Treatment with 3 or 4 pVAXhsp65 doses did not influence onset, duration or disease severity (Fig. 4a). Variation in body weight showed an expected course characterized by a significant weight drop during the acute phase. Complete weight recovery was observed when the animals achieved the chronic disease phase (Fig. 4b).

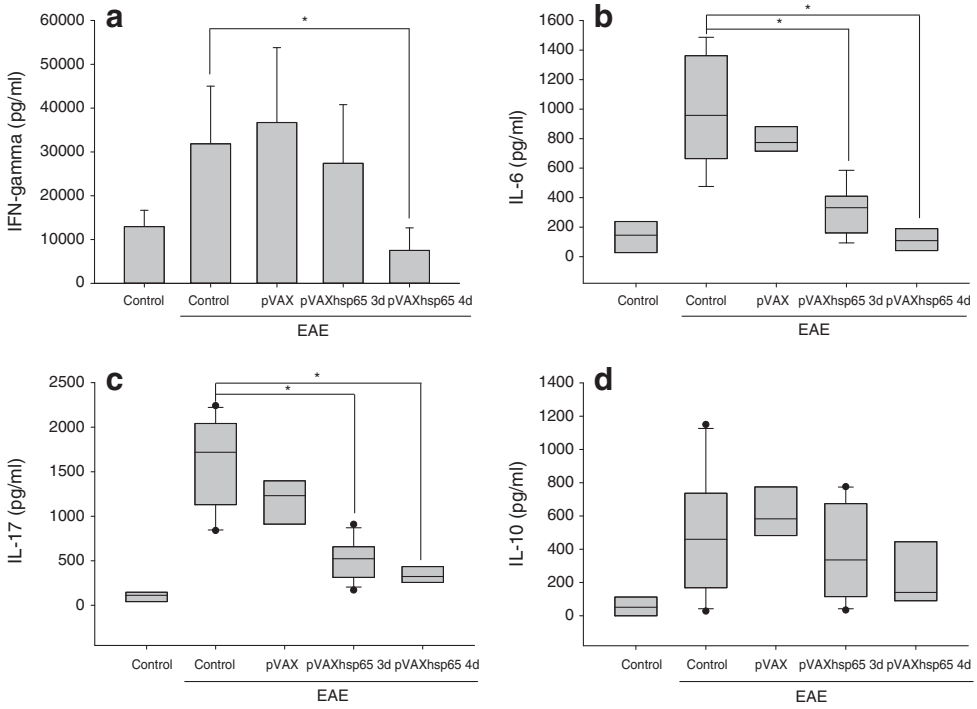

Fig. 3. Effect of therapeutical vaccination with pVAXhsp65 on polyclonal immune response. C57BL/6 mice were submitted to EAE induction and then injected with 3 or 4 pVAXhsp65 doses. Cytokine production was tested 30 days after EAE induction. IFN-γ (a), IL-6 (b), IL-17 (c) and IL-10 (d) production was assayed in spleen cell cultures re-stimulated *in vitro* with ConA. Data were presented as mean ± SE or median (25–75% ranges) of 6 mice and representative of two independent experiments. * represents the difference between immunized and control group. $p < 0.05$.

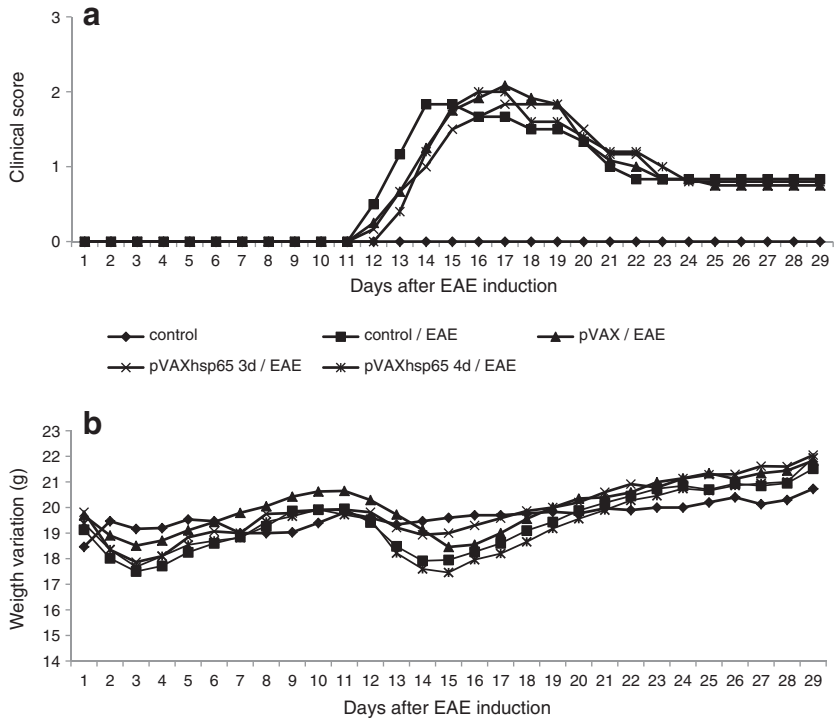

Fig. 4. Effect of treatment with pVAXhsp65 on EAE development. C57BL/6 mice were submitted to EAE induction and then injected with 3 or 4 pVAXhsp65 doses. Clinical score (a) and weight variation (b) were daily evaluated. Data were presented by mean ± SE of 6 mice and representative of three independent experiments.

3.4. Cytokine production by cells from the CNS

Cells isolated from the CNS obtained from mice with EAE produced high levels of TNF-α (Fig. 5b), IL-6 (Fig. 5c) and IL-10 (Fig. 5d), but much lower levels of IFN-γ (Fig. 5a), in response to *in vitro* stimulation with hsp65. Treatment with pVAXhsp65 or its control, the empty vector, did not alter this pattern. These cytokines were also induced by MOG stimulation but again no differences were determined by previous immunization (Fig. 5a, b, c and d).

3.5. Quantification of Foxp3 + T cells

The frequency of CD4 + CD25 + Foxp3 + T cells was evaluated in the spleen and also in the mononuclear cells isolated from the CNS. A

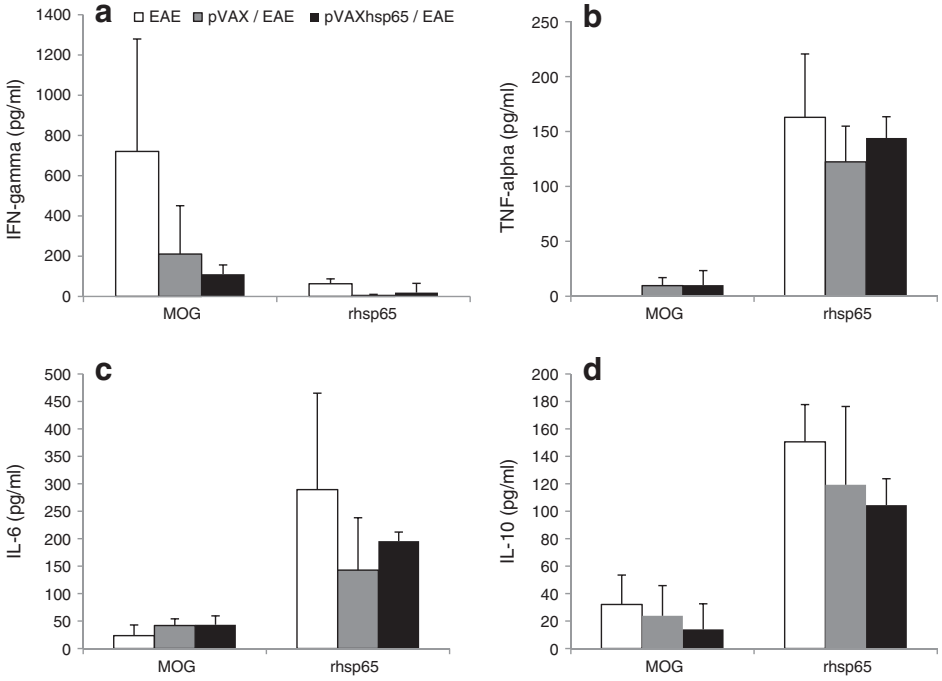

Fig. 5. Cytokine production by cells from the CNS. C57BL/6 mice were immunized with 4 pVAXhsp65 doses and then submitted to EAE induction. Cytokine production was tested 30 days after EAE induction. IFN-γ (a), TNF-α (b), IL-6 (c) and IL-10 (d) production was assayed in CNS infiltrating cell cultures re-stimulated *in vitro* with MOG or rhsp65. Data were presented by mean ± SE of 5 mice.

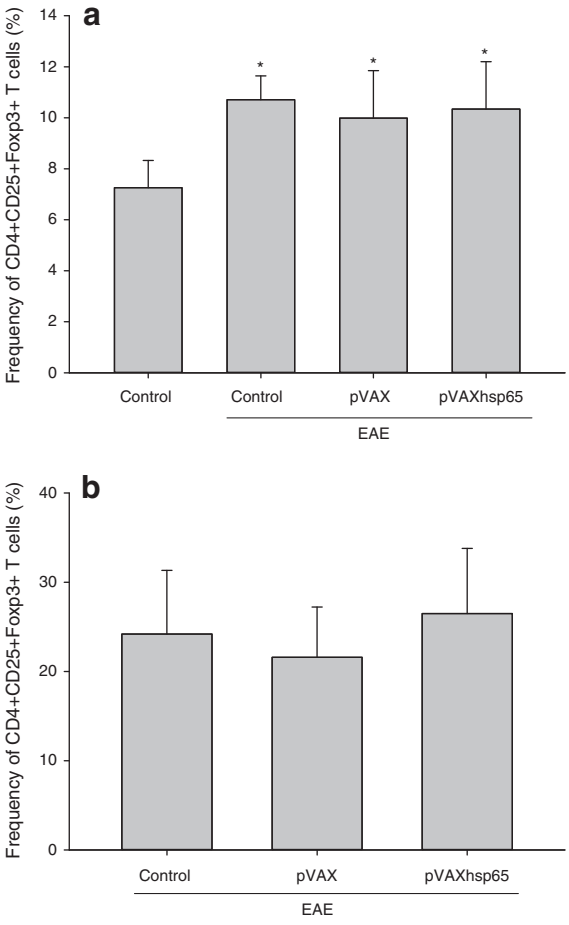

Fig. 6. Effect of treatment with pVAXhsp65 on the percentage of regulatory T cells in spleen and CNS. Frequency of CD4 + CD25 + Foxp3+ T cells in the spleen (a) and CNS (b). This evaluation was determined 30 days after EAE induction by flow cytometric analysis. Data were presented by mean ± SE of 4–6 mice. * represents the difference between immunized and control group. $p < 0.05$.

significant increase was observed in the spleen (Fig. 6a) of the EAE groups in comparison to the control group (not submitted to EAE induction). Therefore, previous immunization with the vaccine or vector injection did not modify Treg frequency. Contrasting with the small increment of Treg cells in the lymphoid peripheral organ, a very high proportion, around 25%, of the CNS infiltrating mononuclear cells was Foxp3 + T cells (Fig. 6b). Treatment with the vector or the DNA vaccine did not change the Foxp3 + T cells frequency in this target organ.

3.6. Inflammation is not affected by treatment with pVAXhsp65

Typical lesions, characterized by an intense perivascular inflammatory infiltrate were observed in the brain (Fig. 7a, b and c) and lumbar spinal cord (Fig. 7d, e and f). A visual inspection indicated that treated and non-treated animals presented comparable degrees of inflammation.

4. Discussion

In this investigation we evaluated if the treatment with a DNA vaccine containing the hsp65 gene from *Mycobacterium leprae* could improve the clinical condition of mice with experimental encephalomyelitis. This approach was based on the widely accepted concept that heat shock proteins are potent immunomodulatory molecules (Keijzer et al., 2012). Hsps are ubiquitous self-antigens that are highly expressed in inflamed tissues. The prokaryotic counterparts, that are immunodominant antigens, display large areas of sequence homology with the human hsps. Even though this homology can trigger an autoimmune disease (Rajaiah and Moudgil, 2009) it can also induce T cell regulation of chronic inflammation (Hauet-Broere et al., 2006).

We initially examined the characteristics of the specific immune response induced by immunization with 3 or 4 pVAXhsp65 doses in mice whose encephalomyelitis was already induced. These pVAXhsp65 immunized animals exhibited a clear cellular immune response. Spleen cells from immunized animals produced high levels of IFN-γ and IL-10; these levels were similar to the ones found in normal (without EAE) immunized mice (not shown). The levels of TNF-α, IL-6 and IL-17 were, however, similar to the ones produced by the EAE control group. Interestingly, the control EAE group also presented a recall response to *in vitro*

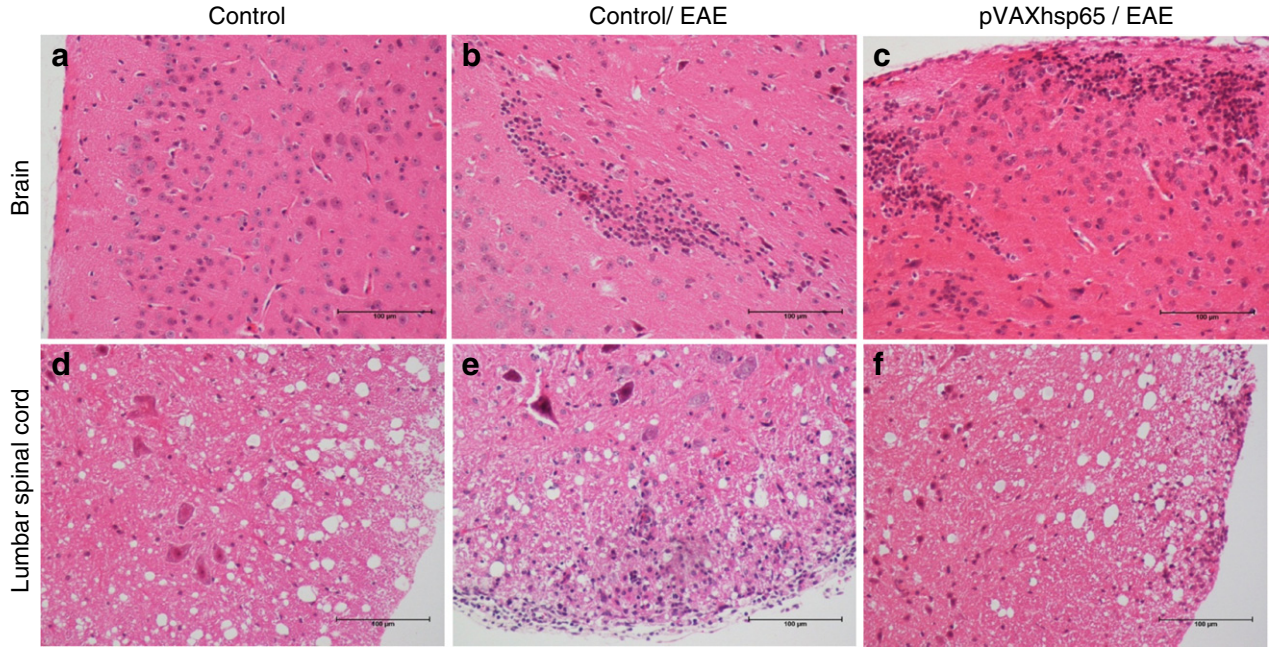

Fig. 7. Effect of treatment with pVAXhsp65 on CNS inflammation. C57BL/6 mice were submitted to EAE induction and then injected with 4 pVAXhsp65 doses. Brain (figures a, b, c) and lumbar spinal cord (d, e, f) inflammatory infiltrates of control mice without EAE (a, d), control mice with EAE (b, e) or mice treated with 3 pVAXhsp65 doses (c, f) were evaluated 30 days after EAE induction. Panel is representative of 6 animals/group.

stimulation with rhsp65. These animals produced small amounts of IFN-γ and IL-10 and also elevated levels of IL-6 and IL-17. A possible explanation for these is the presence of hsp65 in the CFA that is used in disease induction (Wong et al., 2003).

The regulatory ability of hsp65 was clearly observed in this investigation. The treatment with both therapeutic protocols (3 and 4 DNA vaccine doses) downmodulated the production of encephalitogenic cytokines by spleen cells stimulated with MOG. The production of IFN-γ, TNF-α, IL-6 and IL-17 was significantly smaller in these cultures, in comparison to cultures from EAE non-treated group. Generally, the reduction detected in the group treated with 4 doses was more accentuated than the one observed in animals treated with 3 doses. This immunomodulatory effect was also detectable when spleen cells were stimulated with the polyclonal activator ConA, suggesting the activation of an extensive mechanism of immunoregulation. The treatment with the empty vector did not trigger this effect, indicating that is was related to the hsp65 gene. This pVAXhsp65 downmodulatory effect over some pro-inflammatory cytokines was already observed by our group in experimental autoimmunity models (Santos-Junior et al., 2005; Zorzella-Pezavento et al., 2010).

Unexpectedly, this accentuated immunomodulatory activity was not associated with any improvement in the clinical status of the animals. Treated mice showed body weight loss and clinical scores very similar to non-treated animals. pVAXhsp65 treatment was also not able to quantitatively modify the CNS inflammatory process. These findings also contrasted with the previous experience of our and other groups. The therapeutic anti-inflammatory potential of this kind of construction was already well characterized in various experimental models as arthritis (Santos-Junior et al., 2005), tuberculosis (Silva et al., 2005), hypersensitivity associated with esquistosomiasis (Frantz et al., 2011) and, more recently, also in a model of allergy (Fonseca et al., 2012). In relation to MS and its corresponding experimental model there are no reports designed to investigate the benefit of this molecule as a therapeutical measure. As we already demonstrated that a prophylactic immunization with pVAXhsp65 was able to significantly decrease brain inflammation in a rat EAE model (Zorzella-Pezavento et al., 2010), we could think that pVAXhsp65 is more potent to avoid naïve cell-priming than to control already activated T cells. There is yet no direct experimental proof that this is the reason of this unsuccessful therapy. This possibility is, however, reinforced by the findings of Korn et al. (2007). These authors elegantly demonstrated that Foxp3 + cells isolated from the CNS were effective in suppressing naïve MOG-specific T cells but failed to control CNS-derived encephalitogenic T effector cells that secreted IL-6 and TNF-α.

It is also important to consider that EAE in Lewis rats and in C57BL/6 mice present very distinct profiles of progression. In Lewis rat EAE is characterized by a monophasic pathology with little demyelination and edema in the CNS that is followed by total recovery after an acute episode. Otherwise, EAE in C57BL/6 mice is a chronic-progressive disease associated with demyelination and axonal damage (Gold et al., 2006). In this scenario, we could think that immunomodulatory cells and/or mediators elicited by pVAXhsp65 probably found a refractory environment in the CNS of C57BL/6 sick mice. The work of Korn et al. (2007) supports this possibility.

The immunoregulatory ability of hsp65 has been attributed to different mechanisms as tolerance induction, deviation of Th1 to Th2 polarization, suppression of Th17, apoptosis of pathogenic T cells and expansion/activation of regulatory T cells as Tr1 and Th3 (Quintana et al., 2002; Ulmansky et al., 2002; Satpute et al., 2007; Moudgil and Durai, 2008; Rajaiah and Moudgil, 2009; Coelho and Faria, 2012). Interestingly, we observed that immunization with pVAXhsp65 not only significantly downmodulated IL-17 specifically induced by MOG but also decreased the production of other encephalytogenic cytokines as IFN-γ and IL-6. These three cytokines have been described as highly encephalitogenic (Fletcher et al., 2010; Jadidi-Niaragh and Mirshafiey, 2011). It is therefore tempting to speculate that downmodulation of the specific autoimmune response is occurring but is not reaching the CNS. This possibility is, at least partially, supported by our data obtained from analysis of the CNS immunity. In this case, the previous vaccination did not modify the MOG specific cytokine induction. Alternatively, we could think that this type of construction (genetic vaccine) and the employed route of administration are not indicated to induce tolerance and immunoregulation. In line with this possibility, it is widely described that genetic vaccines administered by intramuscular route induce mainly Th1 and Tc effector cells (Silva et al., 2009). This was clearly exemplified by investigations that demonstrated the induction of protection against experimental tuberculosis by this vaccine (Bonato et al., 2004; de Paula et al., 2007). However, our own experience indicated that this genetic construction was able to protect mice against experimental arthritis and diabetes (Santos-Junior et al., 2005; Santos Júnior et al., 2007; Santos Junior et al., 2009). As the ability of this vaccine to protect against EAE in both mice and rat (Zorzella-Pezavento et al., 2010) is clearly more discrete, we postulate that the characteristics of experimental encephalomyelitis could make it more resistant to immunomodulation by pVAXhsp65. Factors as hsp60 expression at the CNS, permeability of the BBB and type of regulatory T cell able to infiltrate in this target tissue must be evaluated to better understand the meaning of this result.

The evaluation of Foxp3 + T cells in the spleen indicated that the EAE development itself was already associated with a significant increase in the percentage of these cells. The therapeutic vaccination with pVAXhsp65, however, did not increase the percentage of this type of regulatory cell in the spleen. As the downmodulation of encephalitogenic cytokines is occurring in the absence of an increase in Foxp3 + cells in spleen, it is possible that other T cell regulatory subsets are being activated. The presence of hsp65-reactive T cells presenting a regulatory potential has been described mainly in experimental arthritis (Tanaka et al., 1999; Prakken et al., 2001; Kamphuis et al., 2005). Some studies have also suggested that hsps are antigens seen by natural regulatory T cells (Nishikawa et al., 2005). However, in our work, in mice immunized with pVAXhsp65 there was no increase of nTreg cells. The contribution of hsp65-specific T cells for both, pathogenesis and immunoregulation during EAE and MS is not well established. Only a few reports were published in this area suggesting that hsp60 is expressed in brain cells (Selmaj et al., 1992) and that hsp60 specific clones are present in the brain (Quintana et al., 2012). Altogether these results indicate that hsp65 administered as a genetic vaccine is able to downmodulate immunity against MOG but this effect is not associated with a protective activity against EAE development.

Financial support

The study was supported by São Paulo Research Foundation (FAPESP) grant # 2011/07528-5 and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

Barnett, M.H., Henderson, A.P., Prineas, J.W., 2006. The macrophage in MS: just a scavenger after all? Pathology and pathogenesis of the acute MS lesion. Mult. Scler. 12, 121–132.

Bonato, V.L., Gonçalves, E.D., Soares, E.G., Santos Júnior, R.R., Sartori, A., Coelho-Castelo, A.A., Silva, C.L., 2004. Immune regulatory effect of pHSP65 DNA therapy in pulmonary tuberculosis: activation of CD8 + cells, interferon-gamma recovery and reduction of lung injury. Immunology 113, 130–138.

Casetta, I., Iuliano, G., Filippini, G., 2007. Azathioprine for multiple sclerosis. Cochrane Database Syst. Rev. 17 CD003982.

Castro-Borrero, W., Graves, D., Frohman, T.C., Flores, A.B., Hardeman, P., Logan, D., Orchard, M., Greenberg, B., Frohman, E.M., 2012. Current and emerging therapies in multiple sclerosis: a systematic review. Ther. Adv. Neurol. Disord. 5, 205–220.

Coelho, V., Faria, A.M., 2012. HSP60: issues and insights on its therapeutic use as an immunoregulatory agent. Front. Immunol. 2, 97.

Compston, A., Coles, A., 2008. Multiple sclerosis. Lancet 372, 1502–1517.

Constantinescu, C.S., Farooqi, N., O'Brien, K., Gran, B., 2011. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). Br. J. Pharmacol. 164, 1079–1106.

de Paula, L., Silva, C.L., Carlos, D., Matias-Peres, C., Sorgi, C.A., Soares, E.G., Souza, P.R., Bladés, C.R., Galleti, F.C., Bonato, V.L., Gonçalves, E.D., Silva, E.V., Faccioli, L.H., 2007.

Comparison of different delivery systems of DNA vaccination for the induction of protection against tuberculosis in mice and guinea pigs. Genet. Vaccines Ther. 24 (5), 2.
Fletcher, J.M., Lalor, S.J., Sweeney, C.M., Tubridy, N., Mills, K.H., 2010. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. Clin. Exp. Immunol. 162, 1–11.
Fonseca, D.M., Wowk, P.F., Paula, M.O., Campos, L.W., Gembre, A.F., Turato, W.M., Ramos, S.G., Dias-Baruffi, M., Barboza, R., Gomes, E., Silva, C.L., Russo, M., Bonato, V.L., 2012. Recombinant DNA immunotherapy ameliorate established airway allergy in a IL-10 dependent pathway. Clin. Exp. Allergy 42, 131–143.
Frantz, F.G., Ito, T., Cavassani, K.A., Hogaboam, C.M., Lopes Silva, C., Kunkel, S.L., Faccioli, L.H., 2011. Therapeutic DNA vaccine reduces schistosoma mansoni-induced tissue damage through cytokine balance and decreased migration of myofibroblasts. Am. J. Pathol. 179, 223–229.
Gold, R., Linington, C., Lassmann, H., 2006. Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. Brain 129, 1953–1971.
Grundtman, C., Kreutmayer, S.B., Almanzar, G., Wick, M.C., Wick, G., 2011. Heat shock protein 60 and immune inflammatory responses in atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 31, 960–968.
Hao, J., Liu, R., Piao, W., Zhou, Q., Vollmer, T.L., Campagnolo, D.I., Xiang, R., La Cava, A., Van Kaer, L., Shi, F.D., 2010. Central nervous system (CNS)-resident natural killer cells suppress Th17 responses and CNS autoimmune pathology. J. Exp. Med. 207, 1907–1921.
Hartung, H.P., Gonsette, R., König, N., Kwiecinski, H., Guseo, A., Morrissey, S.P., Krapf, H., Zwingers, T., 2002. Mitoxantrone in Multiple Sclerosis Study Group (MIMS): mitoxantrone in progressive multiple sclerosis: a placebo-controlled, double-blind, randomised, multicentre trial. Lancet 360, 2018–2025.
Hauet-Broere, F., Wieten, L., Guichelaar, T., Berlo, S., van der Zee, R., Van Eden, W., 2006. Heat shock proteins induce T cell regulation of chronic inflammation. Ann. Rheum. Dis. 65, iii65–iii68.
Jadidi-Niaragh, F., Mirshafiey, A., 2011. Th17 cell, the new player of neuroinflammatory process in multiple sclerosis. Scand. J. Immunol. 74, 1–13.
Jones, D.B., Coulson, A.F., Duff, G.W., 1993. Sequence homologies between hsp60 and autoantigens. Immunol. Today 14, 115–118.
Kamphuis, S., Kuis, W., de Jager, W., Teklenburg, G., Massa, M., Gordon, G., Boerhof, M., Rijkers, G.T., Uiterwaal, C.S., Otten, H.G., Sette, A., Albani, S., Prakken, B.J., 2005. Tolerogenic immune responses to novel T-cell epitopes from heat-shock protein 60 in juvenile idiopathic arthritis. Lancet 366, 50–56.
Kaufmann, S.H., Schoel, B., van Embden, J.D., Koga, T., Wand-Württenberger, A., Munk, M.E., Steinhoff, U., 1991. Heat-shock protein 60: implications for pathogenesis of and protection against bacterial infections. Immunol. Rev. 121, 67–90.
Keijzer, C., Wieten, L., van Herwijnen, M., van der Zee, R., Van Eden, W., Broere, F., 2012. Heat shock proteins are therapeutic targets in autoimmune diseases and other chronic inflammatory conditions. Expert Opin. Ther. Targets 16, 849–857.
Koch-Henriksen, N., Sorensen, P.S., 2011. Why does the north–south gradient of incidence of multiple sclerosis seem to have disappeared on the northern hemisphere? J. Neurol. Sci. 311, 58–63.
Korn, T., Reddy, J., Gao, W., Bettelli, E., Awasthi, A., Petersen, T.R., Bäckström, B.T., Sobel, R.A., Wucherpfennig, K.W., Strom, T.B., Oukka, M., Kuchroo, V.K., 2007. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. Nat. Med. 13, 423–431.
McFarland, H.F., Martin, R., 2007. Multiple sclerosis: a complicated picture of autoimmunity. Nat. Immunol. 8, 913–919.
McGeachy, M.J., Stephens, L.A., Anderton, S.M., 2005. Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4 + CD25 + regulatory cells within the central nervous system. J. Immunol. 175, 3025–3032.
Moudgil, K.D., Durai, M., 2008. Regulation of autoimmune arthritis by self-heat-shock proteins. Trends Immunol. 29, 412–418.
Nishikawa, H., Kato, T., Tawara, I., Saito, K., Ikeda, H., Kuribayashi, K., Allen, P.M., Schreiber, R.D., Sakaguchi, S., Old, L.J., Shiku, H., 2005. Definition of target antigens for naturally occurring CD4(+) CD25(+) regulatory T cells. J. Exp. Med. 201, 681–686.
Prakken, B.J., Wendling, U., van der Zee, R., Rutten, V.P., Kuis, W., van Eden, W., 2001. Induction of IL-10 and inhibition of experimental arthritis are specific features of microbial heat shock proteins that are absent for other evolutionarily conserved immunodominant proteins. J. Immunol. 167, 4147–4153.
Quintana, F.J., Carmi, P., Mor, F., Cohen, I.R., 2002. Inhibition of adjuvant arthritis by a DNA vaccine encoding human heat shock protein 60. J. Immunol. 169, 3422–3428.
Quintana, F.J., Carmi, P., Mor, F., Cohen, I.R., 2003. DNA fragments of the human 60-kDa heat shock protein (HSP60) vaccinate against adjuvant arthritis: identification of a regulatory HSP60 peptide. J. Immunol. 171, 3533–3541.
Quintana, F.J., Farez, M.F., Izquierdo, G., Lucas, M., Cohen, I.R., Weiner, H.L., 2012. Antigen microarrays identify CNS-produced autoantibodies in RRMS. Neurology 78, 532–539.
Rajaiah, R., Moudgil, K.D., 2009. Heat-shock proteins can promote as well as regulate autoimmunity. Autoimmun. Rev. 8, 388–393.
Santos Júnior, R.R., Sartori, A., Bonato, V.L., Coelho Castelo, A.A., Vilella, C.A., Zollner, R.L., Silva, C.L., 2007. Immune modulation induced by tuberculosis DNA vaccine protects non-obese diabetic mice from diabetes progression. Clin. Exp. Immunol. 149, 570–578.
Santos Junior, R.R., Sartori, A., Lima, D.S., Souza, P.R., Coelho-Castelo, A.A., Bonato, V.L., Silva, C.L., 2009. DNA vaccine containing the mycobacterial hsp65 gene prevented insulitis in MLD-STZ diabetes. J. Immune Based Ther. Vaccines 7, 4.
Santos-Junior, R.R., Sartori, A., De Franco, M., Filho, O.G., Coelho-Castelo, A.A., Bonato, V.L., Cabrera, W.H., Ibañez, O.M., Silva, C.L., 2005. Immunomodulation and protection induced by DNA-hsp65 vaccination in an animal model of arthritis. Hum. Gene Ther. 16, 1338–1345.
Satpute, S.R., Soukhareva, N., Scott, D.W., Moudgil, K.D., 2007. Mycobacterial Hsp65-IgG-expressing tolerogenic B cells confer protection against adjuvant-induced arthritis in Lewis rats. Arthritis Rheum. 56, 1490–1496.
Seger, J., Zorzella-Pezavento, S.F.G., Pelizon, A.C., Martins, D.R., Domingues, A., Sartori, A., 2010. Decreased production of TNF-alpha by lymph node cells indicates experimental autoimmune encephalomyelitis remission in Lewis rats. Mem. Inst. Oswaldo Cruz 105, 263–268.
Selmaj, K., Brosnan, C.F., Raine, C.S., 1992. Expression of heat shock protein-65 by oligodendrocytes in vivo and in vitro: implications for multiple sclerosis. Neurology 42, 795–800.
Silva, C.L., Bonato, V.L., Coelho-Castelo, A.A., De Souza, A.O., Santos, S.A., Lima, K.M., Faccioli, L.H., Rodrigues, J.M., 2005. Immunotherapy with plasmid DNA encoding mycobacterial hsp65 in association with chemotherapy is a more rapid and efficient form of treatment for tuberculosis in mice. Gene Ther. 12, 281–287.
Silva, C.L., Bonato, V.L., dos Santos-Júnior, R.R., Zárate-Bladés, C.R., Sartori, A., 2009. Recent advances in DNA vaccines for autoimmune diseases. Expert Rev. Vaccines 8, 239–252.
Stromnes, I.M., Goverman, J.M., 2006. Passive induction of experimental allergic encephalomyelitis. Nat. Protoc. 1, 1952–1960.
Tanaka, S., Kimura, Y., Mitani, A., Yamamoto, G., Nishimura, H., Spallek, R., Singh, M., Noguchi, T., Yoshikai, Y., 1999. Activation of T cells recognizing an epitope of heat-shock protein 70 can protect against rat adjuvant arthritis. J. Immunol. 163, 5560–5565.
Ulmansky, R., Cohen, C.J., Szafer, F., Moallem, E., Fridlender, Z.G., Kashi, Y., Naparstek, Y., 2002. Resistance to adjuvant arthritis is due to protective antibodies against heat shock protein surface epitopes and the induction of IL-10 secretion. J. Immunol. 168, 6463–6469.
van Eden, W., Hauet-Broere, F., Berlo, S., Paul, L., van der Zee, R., de Kleer, I., Prakken, B., Taams, L., 2005. Stress proteins as inducers and targets of regulatory T cells in arthritis. Int. Rev. Immunol. 24, 181–197.
van Eden, W., Wick, G., Albani, S., Cohen, I., 2007. Stress, heat shock proteins, and autoimmunity: how immune responses to heat shock proteins are to be used for the control of chronic inflammatory diseases. Ann. N. Y. Acad. Sci. 1113, 217–237.
Wong, D.A., Yip, P.C., Tse, D.L., Tung, V.W., Cheung, D.T., Kam, K.M., 2003. Routine use of a simple low-cost genotypic assay for the identification of mycobacteria in a high throughput laboratory. Diagn. Microbiol. Infect. Dis. 47, 421–426.
Zhang, J., Markovic-Plese, S., Lacet, B., Raus, J., Weiner, H.L., Hafler, D.A., 1994. Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. J. Exp. Med. 179, 973–984.
Zorzella-Pezavento, S.F.G., Chiuso-Minicucci, F., França, T.G.D., Ishikawa, L.L.W., Martins, D.R., Silva, C.L., Sartori, A., 2010. Immunization with pVAXhsp65 decreases inflammation and modulates immune response in experimental encephalomyelitis. Neuroimmunomodulation 17, 287–297.
Zorzella-Pezavento, S.F.G., Chiuso-Minicucci, F., França, T.G.D., Ishikawa, L.L.W., da Rosa, L.C., Marques, C., Ikoma, M.R., Sartori, A., 2013. Persistent inflammation in the CNS during chronic EAE despite local absence of IL-17 production. Mediat. Inflamm. 2013, 519627.