The Role of M. leprae Hsp65 Protein and Peptides in the Pathogenesis of Uveitis

Alessandra Gonçalves Commodaro,1 Eliana Blini Marengo,2 Jean Pierre S. Peron,3 Wesley Brandao,3 Christina Arslanian,3 Robson Lopes Melo,4 Estevam J. Baldon,5 Rubens Belfort Jr.,1 Osvaldo Augusto Sant’Anna,5 and Luiz Vicente Rizzo2

1 Department of Ophthalmology, São Paulo Hospital, Federal University of São Paulo, R. Botucatu 820, 04023-062 São Paulo, SP, Brazil
2 Hospital Israelita Albert Einstein, Avenue Albert Einstein 627-701, 2 Subsolo Bloco A, 05651-901 São Paulo, SP, Brazil
3 Department of Immunology, University of São Paulo, R. Prof. Dr. Lineu Prestes 1730, 05508-900 São Paulo, SP, Brazil
4 Center for Applied Toxicology (CAT/CEPID), Butantan Institute, Avenue Vital Brazil 1500, 05503-900 São Paulo, SP, Brazil
5 Immunochemistry Laboratory, Butantan Institute, Avenue Vital Brazil 1500, 05503-900 São Paulo, SP, Brazil

Correspondence should be addressed to Osvaldo Augusto Sant’Anna, gbrazil@usp.br

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1. Introduction

Experimental autoimmune uveitis (EAU) is an organ-specific, T-cell-mediated disease that targets the posterior pole of the eye [1] and is characterized by granuloma formation in the neural retina, destruction of photoreceptor cells, and blindness [1, 2].

The Hsp60 family comprises molecules that are immunodominant in several infectious processes [3]. The Mycobacterium leprae Hsp65 is part of this family-denominated chaperonins and one of the major immuno-reactive proteins in mycobacteria [3]. It is noteworthy that this protein shares approximately 50% amino acid identity with its mammalian homologue, suggesting their participation in the autoimmune diseases by molecular mimicry mechanism between self and exogenous Hsp molecules [4, 5]. The role of the Hsp65 has been intensively investigated in the pathogenesis of arthritis and diabetes [6, 7], murine systemic lupus erythematosus [8], ocular manifestations of Behcet’s disease, and acute anterior uveitis [9, 10]. High anti-Hsp65 antibodies titers were correlated to the retinopathy in type 1 diabetes patients [11] and with the development and progression of atherosclerosis lesions [12, 13].

Moreover, in animals with acute EAE, lesions were accentuated by increased expression of Hsp60, primarily by infiltrating cells. In chronic EAE, Hsp60 was found predominantly on CNS components, particularly oligodendrocytes...
and astrocytes, showing the participation of Hsp in CNS [14]. In addition, the 180–188 mycobacterial Hsp65 epitope, which is cross-reactive with a self-antigen in joint cartilage, is able to initiate and induce resistance to subsequent attempts to produce the adjuvant arthritis [6, 15].

Our group previously exposed that the passive administration of wild-type *M. leprae* Hsp65 interfered with endogenous equilibrium by enhancing the entropy of the immunobiological system, as expressed by the early death of the (NZB×NZW)F1; experimental lupus mice [8] and aggravation of the ocular disease in experimental autoimmune Uveitis [16]. Following these studies, the biological effects and the primary sequence of *M. leprae* Hsp65 Leader pep and K409A pep synthetic peptides, which cover residues 352–371, were shown [17].

In this context, in the present study, we evaluated the effects of the K409A mutant Hsp65 protein and the Leader pep and K409A pep peptides administration in the pathogenesis of EAU. We showed that K409A and Leader pep were able to aggravate the disease. On the other hand, mice injected with K409A pep did not develop the disease when compared to controls, K409A, and Leader pep.

2. Methods

2.1. Animals. Six- to eight-week-old B10.RIII mice were obtained from the animal facilities at the University of São Paulo, Brazil. All animals were housed under specific pathogen-free conditions and handled under ethical conditions. The Animal Care Committee of the Institute of Biomedical Sciences at the University of São Paulo approved all the procedures used in this study.

2.2. Expression of the Recombinant K409A Mutant M. leprae Hsp65 in Escherichia coli and Purification. Expression and purification of the recombinant K409A mutant *M. leprae* Hsp65 was done as described in [8].

2.3. Peptide Synthesis. Leader pep (ENSDSDYDREKQLQERLAKLA) of *M. leprae* Hsp65 and K409A pep (ENSDSDYDREKQLQERLAKLA) of the mutated form K409A, both covering residues 352–371, were synthesized using the Fmoc (N-(9-fluorenyl)methoxycarbonyl) procedure [18] in a Shimadzu PSSM8 peptide synthesizer (Shimadzu, Tokyo, Japan). The Fmoc-amino acids were purchased from Novabiochem (Nottingham, UK). The synthetic peptides were purified by preparative reversed-phase chromatography (reversed-phase HPLC), and the purity and identity of the peptides were confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on Ettan MALDI-TOF/Pro instrument (Amersham Biosciences, Buckinghamshire, UK) and by analytical reversed-phase high profile liquid chromatography (HPLC) (Shimadzu Inc., Tokyo, Japan).

2.4. Induction of EAU and In Vivo Treatment with Hsp65 Molecules. Mice \( n = 4–6 \text{group} \) were immunized subcutaneously (s.c.) at the base of the tail with 40 μg of interphotoreceptor retinoid binding (IRBP) 161–180 peptide emulsified in 0.2 mL of complete Freund’s adjuvant (CFA) (v/v). At the same time, mice were injected intraperitoneally (i.p.) with 0.5 μg of Bordetella pertussis toxin (PTX) in 0.1 mL as an additional adjuvant and followed by single-dose inoculation of 2.5 μg of K409A, Leader pep, or K409A pep i.p.

2.5. Histopathology EAU. Eyes were collected and prepared for histopathological evaluation at the end of each experiment (day 21 after immunization). The eyes were immersed for 1 h in phosphate-buffered glutaraldehyde 4%, transferred into phosphate-buffered formaldehyde 10% for 24 h, and replaced with ethanol 70% until processing. Fixed and dehydrated tissue was embedded in paraffin wax, and 4–6 μm sections were cut through the papillary-optic nerve plane. Sections were stained by hematoxylin and eosin. Presence or absence of disease was evaluated in a double-blinded fashion by examining six sections cut at different levels for each eye. Severity of EAU was scored on a scale of 0 (no disease) to 4 (maximum disease) in half-point increments, according to a semiquantitative system described previously [1], according to lesion type, size, and number. In brief, the minimal criterion to score an eye as positive by histopathology was inflammatory cell infiltration of the ciliary body, choroids, or retina (EAU grade 0.5). Progressively higher grades were assigned for the presence of discrete lesions in the tissue such as vasculitis, granuloma formation, retinal folding and/or detachment, and photoreceptor damage [1].

2.6. Determination of Cytokine Production. Spleen cells harvested 21 days after immunization were cultured in 24-well plates (10⁶ cells/well) and stimulated with 30 μg/mL IRBP. Supernatants were collected for cytokine analysis after 72 h and stored at −80°C until assayed. The level of IL-10 was assessed by ELISA using kit from BD/ Pharmingen (La Jolla, CA), and the level of IL-17 was assessed by ELISA using kit from eBioscience (San Diego, CA). All kits were used according to the manufacturer’s instructions.

2.7. Intracellular Cytokine Staining and FACS Analysis. For detection of intracellular expression of IFN-γ, cells from draining lymph nodes and spleens collected at day 21 after immunization were labeled with anti-CD4 monoclonal antibody, fixed and permeabilized, stained intracellularly, and analyzed by flow cytometry evaluating the CD4 and IFN-γ expression. Cells were left unstimulated or stimulated overnight with IRBP (30 μg/mL) in the presence of either Golgiplug or Golgistop at the recommended concentrations (BD Pharmingen). All samples were acquired on a FACSCalibur (BD Biosciences), and data were analyzed with FlowJo software (TreeStar).

2.8. Statistical Analysis. Data are expressed as mean ± SD. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California, USA). Parametric Student’s t-test was employed, and P values <0.05 were considered significant.
3. Results

3.1. K409A, Leader Pep, and K409A Pep in the Disease Development in Mice. The animals were immunized and injected with K409A, Leader pep, or K409A pep. We observed that K409A and Leader pep amplified the development of the disease compared with K409A pep and control group (Figure 1). On the other hand, animals injected with K409A pep did not develop the disease (Figure 1). The histopathologic examination shows vasculitis and presence of inflammatory cells in the vitreous in mice with EAU on day 21 (Figures 2(a) and 2(b), controls). In animals injected with K409A (Figures 2(c) and 2(d)) and Leader pep (Figures 2(e) and 2(f)), it is possible to observe the presence of inflammatory cells in the vitreous and retinal folds. On the other hand, in animals injected with K409A pep we can observe a normal organization of the retina (Figures 2(g) and 2(h)).

3.2. Decrease of T CD4+ IFN-γ+ Cell Population in Lymph Nodes and Spleens of Mice Injected with K409A Pep. It is known that autoreactive Th1 cells mediate EAU, and thus its induction correlates with the production of IFN-γ by T cells. In this work, we observe a significant increase in the frequency of CD4+IFN-γ+ T cells in lymph nodes (Figure 3(a)) of animals injected with Leader pep when compared to those of K409A, K409A pep, and control groups. Also, there is a decrease of CD4+IFN-γ+ T cells in lymph nodes and spleen of the K409A pep mice group compared to the other experimental groups (Figures 3(a) and 3(b)). These results corroborate with our score results where we showed that mice injected with K409A pep presented lower scores of the disease when compared to mice injected with K409A, Leader pep, and controls.

3.3. Analysis of IL-10 and IL-17 Levels in Spleen. The immunosuppressive cytokine IL-10 regulates EAU susceptibility and may be a factor in genetic resistance to EAU [19]. In this work, we showed an increase in IL-10 levels by mice injected with K409A pep when compared to other groups. This result corroborates the EAU score presented by K409A pep mice, in which the animals did not develop the disease.
4. Discussion

Recently, we showed that EAU scores are increased in IRBP-immunized animals and inoculated with recombinant wildtype M. leprae Hsp65. Interestingly, this was associated with a higher expansion of CD4+IFN-γ+ (Th1) and CD4+IL-17+ T cells (Th17), and with higher levels of IFN-γ [16]. Moreover, Marengo and collaborators still demonstrated that WT Hsp65 inoculation accelerates death in mice which spontaneously develop the Systemic Lupus Erythematosus (SLE) [8].

In the present work, we showed higher EAU scores in animals injected with K409A and Leader pep. However, animals injected with K409A pep did not develop the disease. Histopathological analysis showed that animals injected with K409A pep did not show lesions of retina when compared to controls, otherwise K409A and Leader pep presented retinal folds, vasculitis, and inflammatory infiltrating cells in the vitreous.

To evaluate the effect of K409A, Leader pep, and K409A pep in the periphery, it was evaluated the CD4+ T cells producing IFN-γ in spleen and lymph nodes of mice induced to EAU. In this model, the increase in CD4+ IFN-γ + T-cell population in lymph nodes and spleens was associated with the increased scores observed in the K409A, Leader pep, and control groups. Friedland and collaborators demonstrated that the secretion of proinflammatory cytokines from monocytes activated by mycobacterial 65 kDa Hsp may be important in the host immune response and in the development of antigen-specific T-cell-mediated immunity [20].

Th17 cells were recently described as crucial for the development of EAU [21–25]; however, it was not observed differences in IL-17 levels by splenocytes at day 21 after-immunization between the experimental groups. Thus, we
may assume that, at this phase, the Th17 cells are in the eye. On the other hand, different from that we observed in the present work, it was previously shown an expansion of CD4+IL-17+ T cells in mice injected with rHsp65 [16].

It is known that the immunosuppressive cytokine IL-10 regulates EAU susceptibility and may be a factor in genetic resistance to EAU [19]; the results showed that spleen cells from animals injected with K409A pep produced higher levels of IL-10. The increase of IL-10 levels can be associated with the absence of the disease, as IL-10 is a potent suppressive cytokine. Moreover, as we did not evaluate Tregs, it is also possible that higher levels of IL-10 observed in Leader pep mice group may be derived from this population such as Tr1 cells or IL-10 secreting Foxp3+ cells.

In 2006, our group showed that rGal-1 administration was able to decrease the disease in mice with EAU, and this fact was associated with increased levels of IL-10 and decreased levels of IFN-γ [26]. A study with experimental arthritis showed that antibodies against the Hsp molecule suppress inflammation by inhibiting the proinflammatory effect of the Hsp on the innate immune system. The increase in IL-10 secretion in the inflammatory site can skew the local cytokine profile from an inflammatory to an anti-inflammatory response and thus explain the mechanism of protection against inflammation by these antibodies [27].

The opposite effects of the K409A protein and their correspondent peptide which covers the 352–371 residues, the K409A pep, in EAU score were unexpected. In (NZB×NZW)F1 mice, which present the H-2d, e alleles, the inoculation of the Leader pep and K409A pep shows resembled effects to their respectively proteins in the survival time, showing a amplified effect [17]. Here, in the EAU susceptible B10.RIII mice, the H-2d allele can explain at least in part their divergent effect. We can speculate that an important participation of the genetic background and immunobiological factors occurs. EAU is a organ-specific disease mediated by T-cells commitment, and SLE is a systemic disease characterized mainly by humoral self-response. Furthermore, differences at the H-2 loci in B10.RIII and (NZB×NZW)F1 mice suggest different or selective antigen-binding core and presentation to the immune system. Also, we can consider that the short length of the K409A pep facilitates its antigen processing of these cells, being more effective than the K409A protein. Some theoretical experiments based on molecules interaction by structural modeling studies can clarify the MHC molecules peptide binding to better comprehend the in vivo findings observed to the mutant forms.

Figure 5 is based on the linear equation \( y = a + i \): this theoretical model presumes that in anautoimmune process, it must be considered the entropy production \( i \) during the disease progression where the slope \( a \) is the intensity and degree of the process aggravation; \( a \), although variable to each individual, is a constancy for everyone, increasing at all illness episode \( i \). It was formulated the principle that the immunological history of an individual is unique and irreversible, being cumulative in an autoimmune process, staying progressively far from the physiological equilibrium [8]; thus, as for other autoimmunities syndromes, the uveitis is not reversible in the sense that the original immune state before the beginning of the disease cannot be reacquired. The general physiological conditions could be disrupted by similar homologous proteins, such as the M. leprae Hsp that, along the life time, proceeds performing a pathological role. At each new episode, the entropy production is higher, and the disease progression could only be controlled or delayed.

In conclusion, K409A and Leader pep were able to aggravate EAU development when administrated at the same time with the immunization. Moreover, mice injected with Leader pep presented high scores of the disease associated with increase percentage of CD4+IFN-γ+ T cells. However, K409A pep was able to inhibit the development of EAU, and this fact can be associated with an increase observed in IL-10 levels by spleen cells and with a decrease in the percentage of CD4+IFN-γ+ T cells. The results of this study would contribute significantly to understanding of the role of the Hsp molecules in the pathogenesis of ocular autoimmune diseases. They also support the concept that immune responses to Hsp have potential importance in exacerbating and perpetuating organ-restricted autoimmune diseases.

**Conflict of Interests**

All the authors declare that they have no conflict of interests.

**Authors’ Contribution**

A. G. Commodaro, E. B. Marengo, O. A. Sant’Anna and L. V. Rizzo contributed equally to this work.

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References

[1] R. R. Caspi, F. G. Roberge, C. C. Chan et al., “A new model of autoimmune disease. Experimental autoimmune uveoretinitis induced in mice with two different retinal antigens,” Journal of Immunology, vol. 140, no. 5, pp. 1490–1495, 1988.

[2] L. V. Rizzo, P. Silver, B. Wiggert et al., “Establishment and characterization of a murine CD4+ T cell line and clone that induce experimental autoimmune uveoretinitis in B10.A mice,” Journal of Immunology, vol. 156, no. 4, pp. 1654–1660, 1996.

[3] J. E. R. Thole, P. Hindersson, J. De Bruyn et al., “Antigenic relatedness of a strongly immunogenic 65 kDa mycobacterial protein antigen with a similarly sized ubiquitous bacterial common antigen,” Microbial Pathogenesis, vol. 4, no. 1, pp. 71–83, 1988.

[4] A. K. Dudani and R. S. Gupta, “Immunological characterization of a human homolog of the 65-kilodalton mycobacterial antigen,” Infection and Immunology, vol. 57, no. 9, pp. 2786–2793, 1989.

[5] Q. Xiao, K. Mandal, G. Schett et al., “Association of serum soluble heat shock protein 60 with carotid atherosclerosis: clinical significance determined in a follow-up study,” Stroke, vol. 36, no. 12, pp. 2571–2576, 2005.

[6] W. Van Eden, J. E. R. Thole, R. Van der Zee et al., “Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis,” Gynecologic Oncology, vol. 29, no. 1, pp. 171–173, 1988.

[7] I. R. Cohen, “Autoimmunity to chaperonins in the pathogenesis of arthritis and diabetes,” Annual Review of Immunology, vol. 9, pp. 567–589, 1991.

[8] E. B. Marengo, L. V. de Moraes, M. Faria et al., “Administration of M. leprae Hsp65 interferes with the murine lupus progression,” PLoS ONE, vol. 3, no. 8, Article ID e3025, 2008.

[9] H. Direskeneli, A. Hasan, T. Shinnick et al., “Recognition of B-cell epitopes of the 65 kDa HSP in Behçet’s Disease,” Scandinavian Journal of Immunology, vol. 43, no. 4, pp. 464–471, 1996.

[10] M. Huhtinen, M. Puolakkainen, K. Laasila, M. Sarvas, A. Karma, and M. Leirisalo-Repo, “Chlamydial antibodies in patients with previous acute anterior uveitis,” Investigative Ophthalmology and Visual Science, vol. 42, no. 8, pp. 1816–1819, 2001.

[11] R. Weltgasser, M. Lechleitner, T. Koch et al., “Antibodies to heat-shock protein 65 and neopterin levels in patients with type 1 diabetes mellitus,” Experimental and Clinical Endocrinology and Diabetes, vol. 111, no. 3, pp. 127–131, 2003.

[12] Q. Xu, J. Willeit, M. Marosi et al., “Association of serum antibodies to heat-shock protein 65 with carotid atherosclerosis,” Lancet, vol. 341, no. 8840, pp. 255–259, 1993.

[13] J. George, D. Harats, B. Gilburd et al., “Immunolocalization of β2-glycoprotein I (apolipoprotein H) to human atherosclerotic plaques: potential implications for lesion progression,” Circulation, vol. 99, no. 17, pp. 2227–2230, 1999.

[14] Y. L. Gao, C. F. Brosnan, and C. S. Raine, “Experimental autoimmune encephalomyelitis: qualitative and semiquantitative differences in heat shock protein 60 expression in the central nervous system,” Journal of Immunology, vol. 154, no. 7, pp. 3548–3556, 1995.

[15] S. M. Anderton, R. Van der Zee, A. Noordzij, and W. Van Eden, “Differential mycobacterial 65-kDa heat shock protein T cell epitope recognition after adjuvant arthritis-inducing or protective immunization protocols,” Journal of Immunology, vol. 152, no. 7, pp. 3656–3664, 1994.

[16] E. B. Marengo, A. G. Commodaro, J. P. S. Peron et al., “Administration of Mycobacterium leprae RHis65 aggravates experimental autoimmune uveitis in mice,” PLoS ONE, vol. 4, no. 11, Article ID e7912, 2009.

[17] E. B. Marengo, L. de Moraes, and R. L. Melo, “A Mycobacterium leprae Hsp65 mutant as a candidate for mitigating lupus aggravation in mice,” PLoS One, vol. 6, no. 9, Article ID e24093, 2011.

[18] E. Atherton and R. Sheppard, Solid Phase Peptide Synthesis—A Practical Approach, IRL Press, Oxford, UK, 1989.

[19] R. K. Agarwal, R. Horai, A. M. Viley et al., “Abrogation of anti-retinal autoimmunity in IL-10 transgenic mice due To reduced T cell priming and inhibition of disease effector mechanisms,” Journal of Immunology, vol. 180, no. 8, pp. 5423–5429, 2008.

[20] J. S. Friedland, R. Stoughton, D. G. Remick, and G. E. Griffin, “Mycobacterial 65-kD heat shock protein induces release of proinflammatory cytokines from human monocyctic cells,” Clinical and Experimental Immunology, vol. 91, no. 1, pp. 58–62, 1993.

[21] Y. Ke, K. Liu, G. Q. Huang et al., “Anti-inflammatory role of IL-17 in experimental autoimmune uveitis,” Journal of Immunology, vol. 182, no. 5, pp. 3183–3190, 2009.

[22] A. Amadi-Obi, C. R. Yu, X. Liu et al., “TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1,” Nature Medicine, vol. 13, no. 6, pp. 711–718, 2007.

[23] R. Rajaiah, M. Puttabyatappa, S. K. Polumuri, and K. D. Moudgil, “Interleukin-27 and interferon-γ are involved in regulation of autoimmune arthritis,” Journal of Biological Chemistry, vol. 286, no. 4, pp. 2817–2825, 2011.

[24] W. Ishida, K. Fukuda, T. Higuchi, M. Kajisako, S. Sakamoto, and A. Fukushima, “Dynamic changes of microRNAs in the eye during the development of experimental autoimmune uveoretinitis,” Investigative Ophthalmology and Visual Science, vol. 52, no. 1, pp. 611–617, 2011.

[25] A. G. Commodaro, J. P. S. Peron, C. T. Lopes et al., “Evaluation of experimental autoimmune uveitis in mice treated with FTY720,” Investigative Ophthalmology and Visual Science, vol. 51, no. 5, pp. 2568–2574, 2010.

[26] M. A. Toscano, A. G. Comodaro, I. M. Ilarriguret et al., “Galectin-1 suppresses autoimmune retinal disease by promoting concomitant Th2- and T regulatory-mediated anti-inflammatory responses,” Journal of Immunology, vol. 176, no. 10, pp. 6323–6332, 2006.

[27] R. Ulmansky, C. J. Cohen, F. Szafer et al., “Resistance to adjuvant arthritis is due to protective antibodies against heat shock protein surface epitopes and the induction of IL-10 secretion,” Journal of Immunology, vol. 168, no. 12, pp. 6463–6469, 2002.