Inoculation with *Mycobacterium plhei* inhibits allergic inflammation in a rabbit model of ovalbumin (ova) sensitization*

La inoculación con Mycobacterium phlei inhibe la inflamación alérgica en un modelo de conejos sensibilizados con ovoalbúmina (OVA)

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Resumen

La prevalencia de enfermedades atópicas y otros desórdenes autoinmunes tipo Th2 se ha incrementado en los países occidentales. Una teoría lo explica mediante la "hipótesis higiénica", la cual plantea que una disminución en la exposición a microbios del medio ambiente conduce a afecciones atópicas. El objetivo del estudio es determinar el efecto de la inoculación con *Mycobacterium phlei* sobre la respuesta alérgica en conejos sensibilizados en estadios tempranos post nacimiento en mucosa rectal. Treinta y dos conejos fueron divididos en 4 grupos: G1: Control normal; G2: Sensibilizado subcutáneo (SC) con ovoalbúmina (OVA); G3: Sensibilizado SC con OVA y desafiado por vía rectal con el mismo antígeno (OVA); G4: Sensibilizados SC con OVA y desafiados vía rectal con OVA previamente inoculados con *M. phlei* los días 1, 30 y 60 luego del nacimiento. Fueron utilizados los siguientes anticuerpos monoclonales: ratón anti-conejo CD4; ratón anti-conejo CD8 y ratón anti-conejo CD25 (receptor de Il-2). Las células marcadas se visualizaron mediante biotina-estreptavidina y AEC como cromógeno. **Eosinófilos:** G1: 1,2 (0,6), G2: 3,1 (0,2), G3: 13,1 (0,8), G4: 1,33 (0,4); G4 vs G3 $p<0,05$. **Mastocitos:** G1: 8,7 (1,3), G2: 17,6 (2,5), G3: 6,0 (1,8), G4: 7,2 (1,7); G4 vs G2 $p<0,001$. **CD4:** G1: 8,35 (0,7), G2: 13,4 (0,9), G3: 8,26 (0,6), G4 17,2 (1,29); G4 vs G1 and G3 $p<0,05$. **CD8:** G1: 6,28 (0,5), G2: 8,25 (0,8), G3: 10,77 (0,5), G4: 11,29 (1,1); **CD25:** G1: 13,2 (1,2), G2: 15,1 (1,9), G3: 20,0 (1,4), G4: 14,41 (1,9); G4 vs G3 $p<0,05$. En base a la evidencia presentada en este trabajo, concluimos que la inoculación temprana con *M. phlei* podría inhibir mediante modulación inmune la respuesta alérgica en conejos sensibilizados y desafiados con OVA.

Palabras clave

Conejo, inflamación alérgica, mucosa rectal, Mycobacterium phlei, ovalbúmina, sensibilización.

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Abstract

The prevalence of atopic disease and other TH2 autoimmune disorders have increased in Western countries. One theory explains that fact by the hygiene hypothesis, which suggests that a decrease exposure to microbes in the environment leads to atopic disorders. The aim of the present study is to determine the effect of *Mycobacterium phlei* inoculation on allergic response from sensitized rabbits at early stages after birth in rectal mucosa. Thirty two rabbits were divided in 4 groups: G1: Normal control; G2: Ovalbumin (OVA) sensitized subcutaneously (SC); G3: OVA sensitized SC and OVA rectal challenged and G4: OVA sensitized SC and OVA rectal challenged previously inoculated with *M. phlei* at days 1; 30 and 60 after birth. The following monoclonal antibodies were used: anti-rabbit CD4 for CD4+ T cells identification; anti-rabbit CD8 and anti-rabbit CD25 (IL-2 receptor marker). Botin-streptavidin system was used for detection of marked cells and AEC as chromogen. **Eosinophils:** G1: 1,2 (0,6), G2: 3,1 (0,2), G3: 13,1 (0,8), G4: 1,33 (0,4); G4 vs G3 p<0,05. **Mast cells:** G1: 8,7 (1,3), G2: 17,6 (2,5), G3: 6,0 (1,8), G4: 7,2 (1,7); G4 vs G2 p<0,001. **CD4:** G1: 8,35 (0,7), G2: 13,4 (0,9), G3: 8,26 (0,6), G4 17,2 (1,29); G4 vs G1 and G3 p<0,05. **CD8:** G1: 6,28 (0,5), G2: 8,25 (0,8), G3: 10,77 (0,5), G4: 11,29 (1,1); **CD25:** G1: 13,2 (1,2), G2: 15,1 (1,9), G3: 20,0 (1,4), G4: 14,41 (1,9); G4 vs G3 p<0,05. Based on the evidence presented in this work, we conclude that early inoculation with M. phlei may inhibit by immune modulation allergic response in ovoalbumin sensitized and challenged rabbits.

Key words

Allergic inflammation, Mycobacterium phlei, ovalbumin, rabbit, rectal mucosa, sensitization.

Introduction

In the last decades, the prevalence of atopic disease and other TH2 autoimmune disorders have increased in Western countries[1, 8, 35]. One theory explains that fact by the hygiene hypothesis, which suggests an altered exposure to microbes in the environment, due to improved sanitation and personal hygiene, smaller family sizes, shorter duration of breastfeeding, immunizations and lack of serious childhood infections results in alteration of immunoregulation[26, 32, 34]. This hypothesis is based on an inbalance in T-helper (Th) type response and Th cell-regulatory mechanisms due to the lack of microbial stimulation[16, 25, 31].

In fact, some authors describes an increased rate of allergy due to reduced exposure to environmental mycobacteria or mycobacterial products[14, 36]. In mice BCG vaccination leads to Th1 profile in airway allergic responses (reduce levels of immunoglobulin E, eosinophils, interleukin-4, and interleukin-5) to ovalbumin sensitization[10]. Inoculation of *M. vaccae* or BCG reduced asthmatic responses in mice[15, 38]. A similar pattern is observed in rats with BCG (a suppression of atopic symptoms with a reduced TH2 responses, IgE and Il-4)[19, 20, 21].

In a mouse model intranasal application of BCG reduced eosinophil numbers and TH2 response to ovalbumin[10, 24]. In some animal models it is already demonstrated that mycobacteria can restore immune homeostasis[6]. Mycobacteria are Gram-positive aerobic bacteria belonging to the family Mycobacteriaceae and are one of several mycolic-acid-containing genera within the order Actinomycetales[17, 29]. *M. phlei*, a commensal Mycobacterium non pathogenic for rabbit is widely distributes in the nature and has been used as adjuvant for different kinds of vaccines[4].

We have previously developed a rabbit model of allergic inflammation in rectal mucosa after ovalbumin challenge in sensitized animals[33]. Rabbit *(Oryctolagus cuniculus)* is one of the animal species frequently employed as an experimental model in human and veterinary research[11, 23].

The aim of the present study is to determine the effect of *M. phlei* inoculation on allergic response from sensitized rabbits at early stages after birth.

Materials and methods

Thirty two adult New Zealand rabbits were divided in 4 groups:

1. Group 1: (n=8) Normal control.

2. Group 2: (n=8) Ovalbumin (OVA) sensitized subcutaneously (SC).

3. Group 3: (n=8) OVA sensitized SC and OVA rectal challenged.

4. Group 4: (n=8) OVA sensitized SC and OVA rectal challenged previously inoculated with *M. phlei* at days 1, 30 and 60 after birth. *M. plhei* inoculation was developed by subcutaneous administration of 3x107 bacilli per rabbit (final volume 0,1ml).

As we described in previous works, rabbits from group 2, 3 and 4 were twice subcutaneously sensitized with 70 µg OVA (Grade V Sigma) in 30 mg ALUM/ml (aluminium hydroxide)[2, 3].

An interval of 15 days among sensitizations was developed. After 15 days post sensitization, rabbits were 24 hours fasten and G3 and G4 animals were rectal challenged with 50 mg OVA in 5 ml of Phosphate Buffered Saline (PBS).

Animals were sacrificed with sulfuric ether 4 hours after challenged, according to considerations of Ethical Cometee of Rosario School of Medicine and samples from rectum were obtained from all groups. Material for immunohistochemistry were snap frozen in liquid nitrogen with OCT protector and stored at –20 °C and cut at 8 micrometers.

Samples for histology were paraffin embedded and cut at 6 micrometers.

The following monoclonal antibodies were used:

1. Mouse anti-rabbit CD4 (KEN-4, BALB/c IgG 2a, AbD SEROTEC, UK)[22].

2. Mouse anti-rabbit CD8 (12.C7, BALB/c IgG1, AbD SEROTEC, UK)[7].

3. Mouse anti-rabbit CD25 ((Kei-α1BALB/c IgG2b, AbD SEROTEC, UK)[22].

Botin-streptavidin system (Sero tec Ltd. Oxford, UK) was used for detection of marked cells and AEC as chromogen. Frozen samples were fixed in cold acetone for 10 minutes and then incubate for 60 minutes at 37 °C with monoclonal antibodies. Cromotope II technique was employed to detection of eosinophils and Alcian blue pH < 1 was used for mast-cells in rectum.

Positive cells were analyzed in 200 high power fields in each group and expressed as cell mean per microscopical field by morphometric analysis[9]. Results were statistical analyzed.

Specific anti-OVA IgE titres were evaluated by passive cutaneous anaphylaxis PCA[5].

The results were expressed as mean. The comparison between variables in different experimental groups was made by analysis of variance by ranks of Kruskal-Wallis or Mann-Whitney U test, using the statistical program Medcalc 3.6.

Samples were considered different when they had a significance level <0.05.

Results

Specific anti-OVA-IgE levels were evaluated by positive passive cutaneous anaphylaxis test (PCA) possitive at 160 fold dilutions in sensitized groups (G2, G3, G4). Histology of samples showed patchy mucosal edema, lymphangiectasia and eosinophil infiltration in the experimental OVA sensitized and challenged groups (G3 and G4). Eosinophils were spread on the mucosa

underlying the epithelium. There were no visualized histopathological modifications in the control (G1) and the sensitized and unchallenged group (G2). Different cell population quantification were summarized in table 1.

Table 1. Cell populations in rectal mucosa from normal rabbits, ova sensitized rabbits and sensitized and locally challenged rabbits. Positive cells per high power field arithmetic mean and SE.

Markers	*G1: Control N = 200*	*G2: sensitized n = 200*	*G3: sensitized and challenged n = 200*	*G4: sensitized and challenged, treated with M. phlei n = 200*
CD 4	8,35 ± 0,7	13,4 ± 0,9	8,26 ± 0,6	17,26 ± 1,2*
CD 8	6,28 ± 0,5	8,25 ± 0,8	10,77 ± 0,5	11,29 ± 1,1
CD 25	13,2 ± 1,2	15,1 ± 1,9	20 ± 1,4	14,41 ± 1,9*
Mast cells	8,7 ± 1,3	17,6 ± 2,5	6,0 ± 1,8	7,2 ± 1,7**
Eosinophils	1,2 ± 0,6	3,1 ± 0,2	13,1 ± 0,8	1,33 ± 0,4**

* Different from G2 and G3 p<0,05. ** Different from G2 (p<0,001). * Different from G3 (p<0,05). ** Different from G3 (p<0,05)

CD4, CD8 and CD25 positive cells from different groups were visualized in figures 1, 2, 3 and 4.

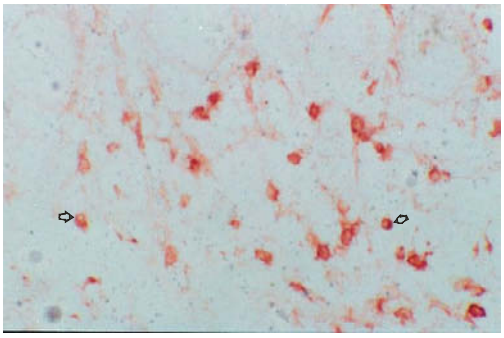

Figure 1. CD4 positive cells in the rectal mucosa. Sensitized and challenged rabbits. Observe the nucleus lack of staining. x 400. (Group 2).

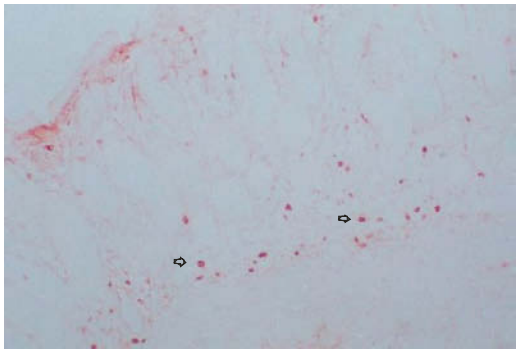

Figure 2. CD 4 positive cells adjacent to the bottom of crypts from G3, sensitized and challenged rabbitss. x 100.

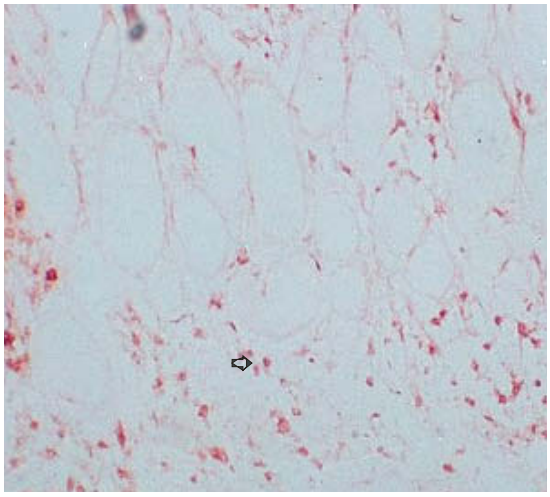

Figure 3. CD8 cells from group 4. X 100.

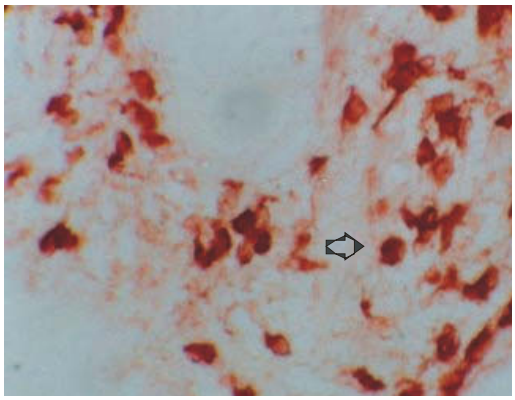

Figure 4. CD25 positive cells in the rectal mucosa from sensitized and challenged rabbits. x 400. (Group 3).

Discussion

Ovalbumin is a soluble antigen which, when subcutaneously administered, induces sensitization that elicits specific IgE antibodies[37]. Challenge with OVA in sensitized individuals produces an anaphylactic allergic response in the digestive tract. In previous works we found modifications in number of enteroendocrine cells, mast cells and eosinophils in rectal mucosa from OVA-sensitized and challenged rabbit[3, 11, 37]. These changes associated to high levels of specific anti-OVA IgE, indicated an immediate hypersensivity reaction.

In the present work we observed eosinophil infiltration, vascular congestion and lymphangiectasia

in group 2 and 3 (sensitized and challenged). Important strengths of our study are the objective histopathological findings of allergic inflammation in rectal mucosa in sensitized animals.

Such signs become apparent 4 hours after OVA challenge. In reference to *M. Phlei* inoculated rabbits, our study demonstrated an inhibition of allergic inflammation in rectal mucosa vs group 2 and 3.

As seen in results referred to reduced eosinophil infiltration and mast cell degranulation in rabbits inoculated with *M. phlei* we conclude that early contact with mycobacteria inhibits in some way allergic infiltration and inflammation. These results are similar to that found by different authors at airways in mice[15, 38, 39]. We demonstrated a statistic significant increase in number of CD4+ cellular populations in animals from G2 and G4 as compared to control. Sensitization and oral challenge induce certain immune cells populations recruitment and migration phenomena with a decrease in naive lymphocytes.

Conversely the immune activation of T cells is evidenced by an enhance of CD25 (interleukin-2 receptor) membrane receptor in sensitized and challenged rabbits (G3), these increase is strongly inhibited in *M. Phlei* inoculated animals (G4). These data is similar to that found by Saavedra *et al.*[30], showing evidence that mycobacterial cell-surface glycolipid are able to reduce antigen-induced proliferation of human CD4+ and CD8+ T-cell subsets associated with a decrease of cells expressing the T-cell surface activation markers CD25 and CD69.

Different mechanisms could explain these findings. Exposition to mycobacteria at early stages of life may downregulate allergic response through the induction of T regulatory cells and production of anti-inflammatory cytokines[12, 13]. Some authors have demonstrated that simultaneous mycobacteria inoculation and allergen sensitization may stimulate a Th1 profile able to suppress Th2 activity significantly[18].

In our model stimulation of T cells with OVA by subcutaneous sensitization only induced Th2-dominant responses, whereas concomitant stimulation with both OVA and *M. plhei* could caused induction of antigen-specific Th1 responses, with secretion of IFN-g, mediated by IL-12 as described by C. Obihara[27, 28].

Based on the evidence presented in this work, we conclude that *M. phlei* (or *M. phlei* products) might be able to protect against the development of allergic inflammation and atopic disease. Although the exact mechanisms involved in the protective effect are not yet known, immune mechanisms involved in the defense against *Mycobacteria* and immune modulation could be reasons for observed effects on this animal model.

References

1. Barber DL, Mayer-Barber KD, Antonelli LR, Wilson MS, White S, Caspar P, Hieny S, Sereti I, Sher A. 2010. Th1 driven immune reconstitution disease in Mycobacterium avium infected mice. Blood. 23; 44: 256-64.

2. Bassan N, Vinuesa M, Pérez F, Roma S, Bernardi S, Lagrutta M. 1998. Mastocitos Azul Alciano positivos en la mucosa del ciego de conejos normales y esplenectomizados, sensibilizados y desafiados con ovoalbúmina. Analecta Veterinaria. 18: 15-20.

3. Bassan N, Vinuesa M, Roma S, Pérez F. 2002. Biological model for detection of food antigens. Arch Latinoam Nutr. 52: 249-256.

4. Bera AK, Bhattacharya D, Pan D, Manna B, Bandyopadhyay S, Das SK. 2010. Effect of heat killed Mycobacterium phlei on body weight gain and management of caecal coccidiosis in broiler chickens. Res Vet Sci. 27; 89 (2): 196-9.

5. Brandtzaeg P. 2002. Current understanding of gastrointestinal immunoregulation and its relation to food allergy. Ann. N. Y. Acad Sci 964:13 -45.

6. Briken V, Porcelli SA, Besra GS, Kremer L. 2004. Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response. Mol Microbiol. 53(2): 391-403.

7. Civin C.I., Gore S. 1993. Antigenic analysis of hematopoiesis: a review. J Hematother. 2(2): 137-144.

8. Downs S., Marks G., Sporik R., Belosouva E.G., Car N.G., Peat J.K. 2001. Continued increase in the prevalence of asthma and atopy. Arch Dis Child. 84: 20–23.

9. Ensari A, Marsh M, Loft D, Morgan S, Moriarty K. 1993. Morphometric analysis of intestinal mucosa. V-Quantitative histologic and immunocytochemical studies of rectal mucosae in gluten-sensitivity. Gut. 34:1225-1229.

10. Erb K, Holloway J, Sobeck A, Moll H, Le Gros G. 1998. Infection of mice with *Mycobacterium bovis* - Bacillus Calmette-Guerin (BCG) suppresses allergen-induced airway eosinophilia. J Exp Med. 187: 561–569.

11. Fekete S. 1987. Recent finding and future perspectives of rabbit's digestive physiology. Cuni Sciences. 4: 1-9.

12. Gruber C, Meinlschmidt G, Bergmann R. 2002. Is early BCG vaccination associated with less atopic disease? An epidemiological study in German preschool children with different ethnic backgrounds. Pediatr Allergy Immunol. 13: 177–181.

13. Guyot-Revol V, Innes JA, Hackforth S, Hinks T, Lalvani A. 2006. Regulatory T cells are expanded in blood and in disease sites in patients with tuberculosis. Am J Respir Crit Care Med. 173: 803–810.

14. Han ER, Choi IS, Eom SH, Kim HJ. 2010. Preventive effects of mycobacteria and their culture supernatants against asthma development in BALB/c mice. Allergy Asthma Immunol Res. 2(1):34-40.

15. Hopfenspirger MT, Agrawal DK. 2002. Airway hyperresponsiveness, late allergic response, and eosinophilia are reversed with mycobacterial antigens in ovalbumin-presensitized mice. J Immunol. 168(5): 2516-2522.

16. Hopkin JM. Atopy, asthma and the mycobacteria. 2000. Thorax. 55: 443–445.

17. Howard ST, Byrd TF. 2000. The rapidly growing mycobacteria: saprophytes and parasites. Microbes Infect. 2(15): 1845-1853.

18. Janssen R, Kruisselbrink A, Hoogteijling L. 2001. Analysis of recombinant mycobacteria as T helper type 1 vaccines in an allergy challenge model. Immunol. 102: 441–449.

19. Ke X, Huang J, Chen Q, Hong S, Zhu D. 2010. Protective effects of combined *Mycobacterium bovis* BCG and interleukin-12 vaccination on airway inflammation in a murine model of allergic asthma. Clin. Invest. Med. 1; 33 (3): 196-202.

20. Koh YI, Choi IS, Kim WY, Lee HC, Lee J. 2001. Effects of BCG infection on Schultz-Dale reaction, allergen-specific IgE levels, and Th2 immune response in sensitized rats. Korean J Intern Med. 16(3): 180-186.

21. Koh YI, Choi IS, Kim WY. 2001. BCG infection in allergen-presensitized rats suppresses Th2 immune response and prevents the development of allergic asthmatic reaction. J Clin Immunol. 21(1): 51-59.

22. Kotani M. 1993. Generation and characterization of monoclonal antibodies against rabbit CD4, CD5 and CD11a antigens. J Imm Meths. 157: 241-252.

23. Mage RG. 1998. Immunology of lagomorphs. En: Handbook of Vertebrate Immunology, pp. 223-260. Eds. Pastoret P, Griebel P, Bazin H, Govaerts A. San Diego: Academic Press.

24. Major T, Wohlleben G, Reibetanz B, Erb KJ. 2002. Application of heat killed *Mycobacterium bovis*-BCG into the lung inhibits the development of allergen-induced Th2 responses. Vaccine, 20: 1532-1540.

25. Mills KH. 2004. Regulatory T cells: friend or foe in immunity to infection? Nat. Rev Immunol. 4: 841–855.

26. Obihara C, Bardin P. 2008. Hygiene hypothesis, allergy and BCG: a dirty mix? Clin Exp Allergy. 8(38): 388–392.

27. Obihara CC, Beyers N, Gie RP, Potter PC, Marais BJ, Lombard C, Enarson D, Kimpen JLL. 2005. Inverse association between *Mycobacterium tuberculosis* infection and atopic rhinitis in children. Allergy. 60: 1121–1125.

28. Obihara CC, Kimpen JLL, Gie RP. 2006. Mycobacterium tuberculosis may protect against allergy in a tuberculosis endemic area. Clin Exp Allergy. 36: 70–76.

29. Pitulle C., Dorsch M., Kazda J., Wolters J., Stackebrandt E. 1992. Phylogeny of rapidly growing members of the genus Mycobacterium. Int J Syst Bacteriol. 42: 337–343.

30. Saavedra R, Segura E, Tenorio EP, López-Marín LM. 2006. Mycobacterial trehalose-containing glycolipid with immunomodulatory activity on human CD4+ and CD8+ T-cells. Microbes Infect. 8(2): 533-540.

31. Sano K, Haneda K, Tamura G, Shirato K. 1999. Ovalbumin (OVA) and Mycobacterium tuberculosis bacilli cooperatively polarize anti-OVA T-helper (Th) cells towards a Th1-dominant phenotype and ameliorate murine tracheal eosinophilia. Am J Respir Cell Mol Biol. 20: 1260–1267.

32. Smit JJ, Folkerts G, Nijkamp FP. 2004. Mycobacteria, genes and the hygiene hypothesis. Curr. Opin. Allergy Clin Immunol. 4: 57–62.

33. Sreekumar E, Das SK. 2001. Mycobacterium phlei as an oral immunomodulator with Newcastle disease vaccine. Indian J Exp Biol. 39(10): 989-992.

34. Strachan D. Hay fever, hygiene and household size. 1989. BMJ. 299: 1259–1260.

35. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. 1998. Lancet. 351: 1225–1232.

36. Trajkovic V, Natarajan K., Sharma P. 2004. Immunomodulatory action of mycobacterial secretory proteins. Microbes Infect. 6(5): 513-519.

37. Vinuesa M, Tanaka Y, Hakugawa J, Jae B, Katayama I. 1997. In-situ expression of interleukin-4, 5 and 6 in Peyer's Patch from Ovalbumin (OVA)-sensitized BALB/c mice after oral challenge. Int. Allergol. 46: 243-247.

38. Wang C, Rook G. 1998. Inhibition of an established allergic response to ovalbumin in BALB/c mice by killed Mycobacterium vaccae. Immunology. 93: 307–313.

39. Zuany-Amorim C, Sawicka E, Manlius C. 2002. Suppression of airway eosinophilia by killed Mycobacterium vaccae-induced allergen-specific regulatory T-cells. Nat Med 8: 625–629.