[Frontiers in Bioscience 12, 1300-1307, January 1, 2007]

Lactobacillus delbrueckii as a potential skin adjuvant for induction of type 1 immune responses

Laura Gomes Castanheira [1], Juciane Maria de Andrade Castro [1], Olindo de Assis Martins-Filho [2], Jacques Robert Nicoli [3], Leda Quercia Vieira [4] and Luis Carlos Crocco Afonso [1]

[1] Departamento de Ciências Biológicas, NUPEB, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Ouro Preto, MG, Brasil, [2] Centro de Pesquisas René Rachou, Fiocruz, Belo Horizonte, MG, Brasil, [3]Departamento de Microbiologia and [4] Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte MG, Brasil

TABLE OF CONTENTS

1. ABSTRACT

We evaluated the ability of *Lactobacillus delbrueckii* UFV H2b20, a probiotic candidate, to stimulate the production of inflammatory cytokines and to induce macrophage activation and Th1 differentiation in peripheral blood mononuclear cells (PBMC) from healthy volunteers. Our results show that PBMC stimulated with heat-killed *Lact. delbrueckii* produced elevated levels of IL-12, IFN-γ and TNF-α but no IL-10. IFN-γ production was IL-12 dependent with NK cells as the main source. Furthermore, PBMC infected with *Leishmania amazonensis* presented elevated microbicidal activity when co-incubated with *Lact. delbrueckii*. Finally, *Lact. delbrueckii* was capable of inducing *in vitro* differentiation of *L. amazonensis*-specific Th1 cells. These findings suggest that this probiotic may be used as an adjuvant in vaccination protocols.

2. INTRODUCTION

The immune response to several pathogens, such as *Leishmania sp., Trypanosoma cruzi, Toxoplasma gondii* and *Listeria monocytogenes* is dependent on the development of Th1 lymphocytes specific to the pathogen (1-4). It is assumed that vaccine development against such pathogens would require the development of a strong Th1 response that generally is not achieved by the simple inoculation of antigens from the pathogens. Hence, the study of adjuvants that would increase this response is greatly needed.

Several species of *Lactobacillus* have already been studied in their ability to induce cytokine production (5-8). However, data available in the literature shows that there is great variability amongst the several species/strains

of *Lactobacillus* regarding the profile of cytokines induced by these bacteria (9,10). *Lactobacillus delbrueckii* UFV H2b20 was isolated from feces of a newborn child and is currently being evaluated as a probiotic. Previous studies from our group showed that this strain increases macrophage activation in mice, accelerating the clearance of *Escherichia coli* from the blood of gnotobiotic mice (11). Furthermore, *Lact. delbrueckii* also induces the production of IFN-γ by spleen cells from normal mice (our unpublished results). In view of these findings, we decided to evaluate the ability of *Lact. delbrueckii* to induce *in vitro* production of pro-inflammatory cytokines by PBMC and also to test its use as a possible adjuvant capable of inducing the differentiation of naïve lymphocytes into Th1 cells. Our results show that *Lact. delbrueckii* not only induces the cytokines necessary for T cell differentiation (IL-12, TNF-α and IFN-γ), without increasing the production of anti-inflammatory cytokines (IL-10), but also that the amount of cytokines produced is sufficient to drive *in vitro* differentiation of T cells, suggesting that this probiotic candidate may be used as an adjuvant in vaccination protocols where the development of a Th1 response is necessary.

3. MATERIALS AND METHODS

3.1. Subjects

A total of 27 healthy volunteers were recruited for this study. Volunteers were from both sexes (17 male) ageing between 19 and 50 years (median 22). Volunteers were asked to answer a questionnaire and only participated in the study after signing an informed consent. This study has been approved by the ethical committee of Universidade Federal de Ouro Preto and is in agreement with the guidelines of the Brazilian Ministry of Health.

3.2. Microorganisms

Lactobacillus delbrueckii UFV H2b20, a strain of human origin, was isolated at the Federal University of Viçosa, Minas Gerais, Brazil. *Lact. delbrueckii* was maintained at -70°C in non-fat reconstituted dry milk containing 20% glycerol. The strain was grown in de Man, Rogosa and Sharp (MRS) broth (Merck) for 18 h at 37°C as previously described . Bacteria in the stationary growth phase were harvested by centrifugation (2000 *g*, 15 min, 5°C), washed three times with sterile PBS and subsequently adjusted to a final concentration of 10^{10} c.f.u./ ml in PBS. Bacteria were heat killed (121°C, 15 min).

Leishmania amazonensis (IFLA\BR\67\PH8) were cultured in Grace´s Medium supplemented with 20% fetal bovine serum (Nutricell, Campinas, SP, Brazil) and 50 mM garamicine. Metacyclic forms were isolated as described by Courret *et al*. (1999) and used to infect PBMC at a 3:1 parasite/cell ratio. *Leishmania* antigen was prepared by several cycles of freezing – thawing of stationary phase (five days of culture) promastigotes (12).

3.3. PBMC isolation

Peripheral blood mononuclear cells from healthy volunteers were isolated from blood samples over a Ficoll-Hypaque gradient (13) and diluted to 3 x 10^6 cells/ml in RPMI 1640 supplemented with 10% of fetal bovine serum, 50 mM garamicine, 2 mM L-glutamine, 50 μM mercaptoethanol and 25 mM HEPES.

3.4. Leishmanicidal Activity

For the evaluation of leishmanicidal activity, PBMC were added in duplicate to round cover slips placed in the bottom of flat-bottom 24-well tissue culture plates. Cells were infected with metacyclic promastigotes of *L. amazonensis* in a 3:1 parasite/cell ratio in the presence or absence of *Lact. delbrueckii* for 72h at 37°C, 5% CO_2. A total of 200 macrophages/monocytes were analyzed and the percentages of infected cells, as well as the number of parasites per cell, were evaluated by optical microscopy of the cover slips stained by a modified Romanowsky method (Panótico Rápido, Laborclin).

3.5. Depletion of NK cells

$CD56^+$ NK cells were isolated from PBMC by magnetic cell sorting (MACs) using a negative selection kit according to manufacturers instructions (NK cell isolation kit - Miltenyi Biotec). The degree of cell depletion was confirmed by flow-cytometry analysis.

3.6. Cytokine production

For the evaluation of cytokine production by total PBMC and PBMC depleted of NK cells triggered by *Lact. delbrueckii* stimulus, cells were stimulated with heat-killed bacteria at a 10:1 and 50:1 bacteria/cell ratio at 37°C, 5% CO_2, in the presence or absence of the anti-IL-12 monoclonal antibody (C8.6.2) (100 μg/ ml – final concentration). Culture supernatants were collected after 24 h for cytokine evaluation. IFN-γ and IL-12 were measured in supernatants from PBMC by sandwich ELISA as described by elsewhere (14). TNF-α detection was carried out by the same procedure, using mouse anti TNF-α monoclonal antibodies (B1549.2 and biotinylated B1547.1) and for IL-10, 9D7 followed by biotinylated 12G8 monoclonal antibodies. Recombinant cytokines (R&D Systems, Minneapolis, USA) were used as standards.

3.7. Induction of *Leishmania*-specific Th1 lymphocytes

PBMC (3 x 10^6 cells/ml) from volunteers that had not been in contact with *Leishmania* parasites or antigens were stimulated with *L. amazonensis* antigen (50 μg /ml) in the presence or absence of heat killed *Lact. delbrueckii* (50 bacteria per PBMC) and incubated for 14 d at 37°C, 5% CO_2. After 14 days, cultures were submitted to centrifugation over a Ficoll-Hypaque gradient in order to recover live cells. These were re-stimulated with *L. amazonensis* antigen alone in the presence of autologous feeder cells previously treated with mitomycin C (50 μg/ml) for 1 h. Culture supernatants were harvested after seven days (21st d) for IFN-γ evaluation and phenotypic characterization of the stimulated cells by flow cytometry was performed.

3.8. Flow cytometry analysis

PBMC (2 x 10^5 cells) were washed in sterile PBS and stained with anti-CD3-FITC, CD4-PE and CD8-PE (BD Biosciences, USA) and CD56-PE (Dako A/S, Glostrup, Denmark). Cytometric analysis was performed on FacScan (BD Biosciences) and data analyzed using Win MDI 2.8 software (http://facs.scripps.edu/software.html).

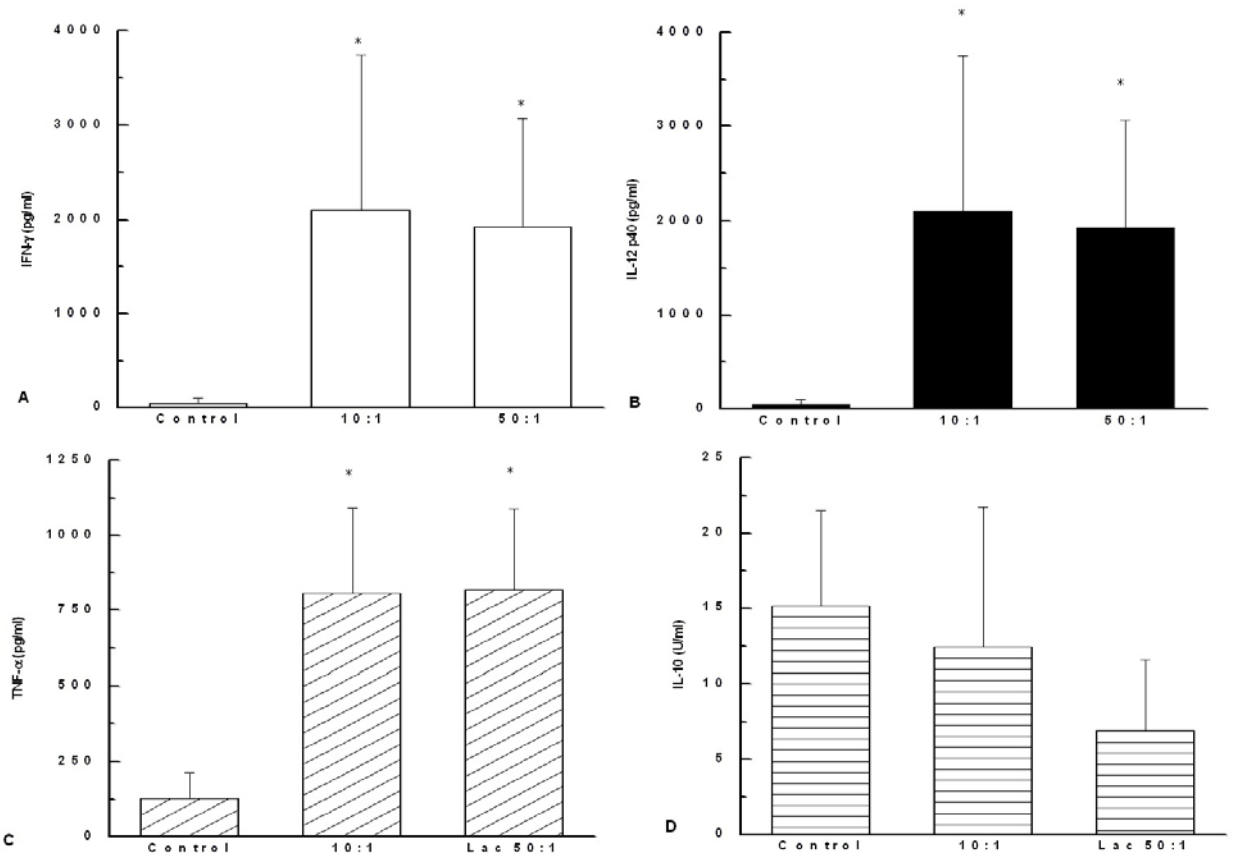

Figure 1. Cytokine production by PBMC from healthy volunteers stimulated with *Lact. delbrueckii* UFV H2b20. PBMC were stimulated for 24 h with heat-killed *Lact. delbrueckii* at the indicated bacteria:cell ratios. Cytokine concentration was determined by ELISA. (A) IFN-γ; (B) IL-12p40; (C) TNF-α; (D) IL-10. Bars represent the mean + SD of cytokine concentration from seven volunteers, except for IL-12 (n = 5). * indicates $P < 0.05$ as compared to control (unstimulated) cultures.

3.9. Statistical analysis

Data were analyzed by paired two tail Student's *t* test. $p \leq 0.05$ was considered significant.

4. RESULTS

We have previously observed that heat-killed *Lact. delbrueckii* is able to induce dose-dependent cytokine production by PBMC from normal volunteers 24 h after stimulation (data not shown). In the present study, we expanded these results to a larger number of volunteers. The results shown in Figure 1 demonstrate that, when used at a bacteria:cell ratio of 10:1 or 50:1, *Lact. delbrueckii* is able to significantly induce IFN-γ, IL-12 and TNF-α production ($P < 0.05$). We also observed that, at these doses, no IL-10 production, in addition to basal levels, was stimulated by the bacteria, indicating a clear type 1 cytokine profile.

IL-12 is a major inducer of IFN-γ production (15). However, while T cells can produce IFN-γ independently of the action of IL-12, production of IFN-γ by NK cells is deeply dependent on the presence of IL-12 (16). Thus, in order to investigate the involvement of NK cells in the production of IFN-γ in our experiments, we decided to inhibit IL-12 activity in our *Lact. delbrueckii*-stimulated cultures. As shown in Figure 2, the addition of an antibody against IL-12 severely decreased the ability of PBMC to produce IFN-γ upon stimulation with *Lact. delbrueckii*, suggesting a possible role of NK cells in the IFN-γ production in this system. To further characterize the involvement of NK cells in the IFN-γ production, we depleted these cells from the PBMC pool prior to the incubation with *Lact. delbrueckii*. We observed that depletion of NK cells (usually greater than 99%) practically abolished IFN-γ production by PBMC (Figure 3) implicating these cells as the major source of this cytokine in our experiments.

In order to verify whether the amount of IFN-γ being produced by *Lact. delbrueckii*-stimulated cells had biological significance, we decided to evaluate the ability of this cytokine to induce macrophage activation. *L. amazonensis* is an intracellular parasite that inhabits macrophages of its mammalian hosts. Elimination of *Leishmania sp.* from infected macrophages is dependent on the presence of IFN-γ produced both by NK and T lymphocytes (17-19). Thus, we incubated PBMC with *L. amazonensis* promastigotes in the presence or absence of *Lact. delbrueckii* and evaluated the development of this

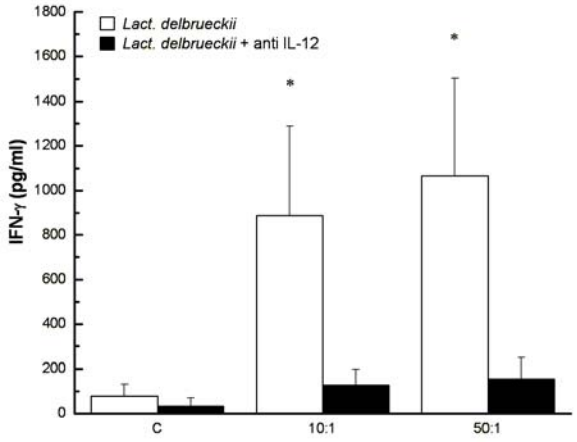

Figure 2. IFN-γ production induced by *Lact. delbrueckii* is IL-12 dependent. PBMC were stimulated for 24 h with heat-killed *Lact. delbrueckii* at the indicated bacteria:cell ratio in the presence (solid bars) or absence (open bars) of an IL-12-neutralizing monoclonal antibody. IFN-γ concentration was determined by ELISA. Bars represent the mean + SD of cytokine concentration from four volunteers. * indicates $P < 0.05$ as compared to control cultures.

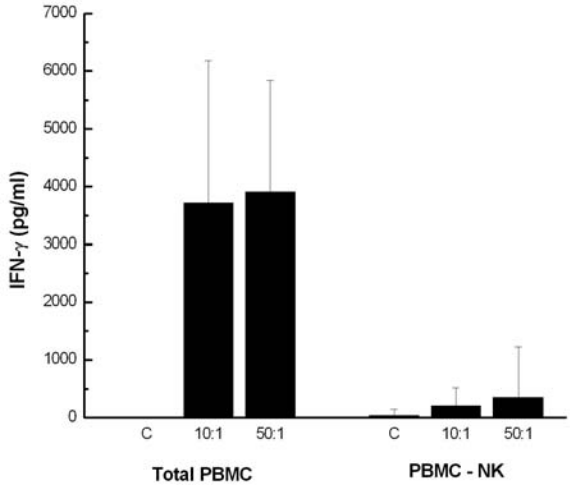

Figure 3. NK cells are the main source of IFN-γ production in *Lact. delbrueckii* stimulated cultures. Total or NK cell depleted-PBMC were stimulated with *Lact. delbrueckii* at the indicated bacteria:cell ratio for 24 h. IFN-γ concentration was determined by ELISA. Bars represent the mean + SD of cytokine concentration from seven volunteers. * indicates $P < 0.05$ as compared to total PBMC

parasite in the cells. As shown in Figure 4, the presence of *Lact. delbrueckii* in the cultures reduced the number of infected macrophages as well as the number of parasites per cell. The macrophage activation was associated with increased IFN-γ, TNF-α and IL-12 production as shown in Figure 5. Furthermore, our results showed no significant differences ($P > 0.05$) in the production of IL-10 (a macrophage deactivating cytokine) by infected cells, regardless of the presence of heat-killed bacteria even though a tendency to a decreased IL-10 production was observed in the presence of the bacteria.

Since the main goal of this study was to investigate the possibility of the use of *Lact. delbrueckii* as an adjuvant for Th1 induction, we decided to verify if the cytokine production induced by the bacteria would be sufficient to drive the differentiation to naïve T cells into *L. amazonensis*-specific Th1 cells. To this purpose, we incubated PBMC from normal volunteers with *L. amazonensis* antigen in the presence or absence of *Lact. delbrueckii* and compared the ability of these cells to induce the production of IFN-γ upon re-stimulation with the parasite antigen alone. As shown in Figure 6A, cells derived in the presence of *Lact. delbrueckii* were able to produce significantly more IFN-γ upon re-stimulation with *L. amazonensis* antigen, suggesting the differentiation of these cells into Th1 cells. Since, as mentioned above, IFN-γ can be produced by several cell types, we characterized the phenotype of the cells present in the culture after the re-stimulation with the parasite antigen. We observed an increased percentage of $CD4^+$ cells in cultures primed with *L. amazonensis* antigen in the presence of *Lact. delbrueckii* when compared to those primed with the parasite alone (Figure 6B). On the other hand, no change in the percentage of $CD8^+$ or NK cells was detected. This result suggests that the source of the increased *Leishmania*-specific IFN-γ production could be associated to $CD4^+$ cells.

5. DISCUSSION

In this study we present data indicating that *Lact. delbrueckii* UFV H2b20, a probiotic candidate, is able to induce the production of several cytokines by stimulated human PBMC. The ability of lactobacilli to induce cytokine production has been demonstrated in several occasions both in mice and humans. However, the kind of cytokine produced as well as its concentration is dependent on the species/strain of *Lactobacillus* used. For example, while *Lact. paracasei, Lact. rhamnosus* and *Lact. plantarum* were able to induce IL-12 production by human PBMC, only *Lact. rhamnosus* induced IL-10 production . In addition, *Lact. rhamnosus* has been shown to induce IFN-γ production while *Lact. bulgaricus* failed to do so (20). These studies indicate that the cytokine profile induced by each species/strain of *Lactobacillus* may show a distinct immunomodulatory potential, which will determine its therapeutic use. In the case of *Lact. delbrueckii*, we were able to show that this microorganism induces the production of cytokines that are involved with the stimulation of inflammatory responses (Figs. 1A-C). Furthermore, we also showed that the production of IL-10, an anti-inflammatory cytokine, was not stimulated in our cultures (Figure 1D).

In our study, we measured only the levels of the p40 subunit of IL-12, which would not guarantee the presence of significant levels of active IL-12. However, we also demonstrate that, by using a IL-12 neutralizing

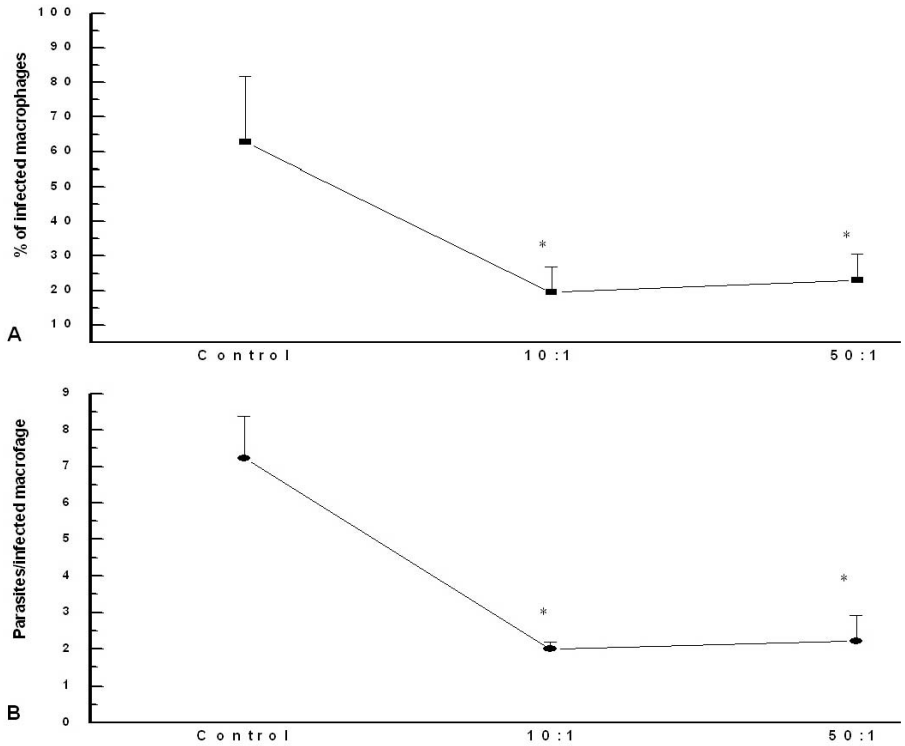

Figure 4. *Lactobacillus delbrueckii* induces macrophage leishmanicidal activity. PBMC were infected with *L. amazonensis* metacyclic promastigotes at a ratio of 3 parasites per cell in the presence or absence of *Lact. delbrueckii* at the indicated bacteria:cell ratio for 72 h. (A) Percentage of infected macrophages. (B) Number of parasites per infected macrophage. Symbols represent the mean + SD from 4 volunteers. * indicates $P < 0.05$ as compared to cultures without *Lact. delbrueckii*.

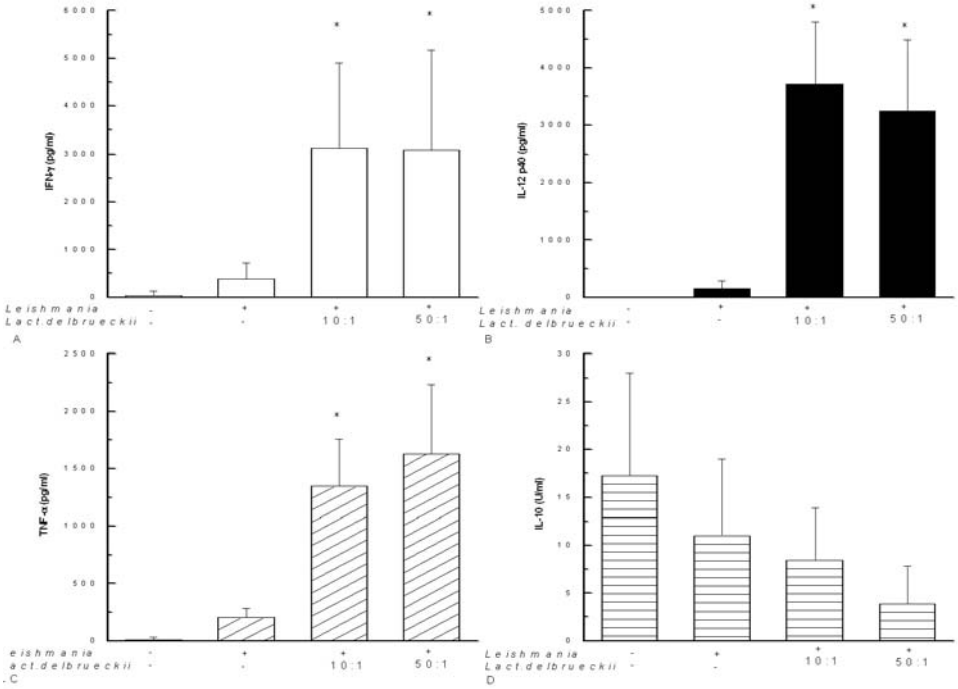

Figure 5. Cytokine production by PBMC infected with *L. amazonensis* in the presence of *Lact. delbrueckii*. PBMC were infected with *L. amazonensis* metacyclic promastigotes at a ratio of 3 parasites per cell in the presence or absence of *Lact. delbrueckii* at the indicated bacteria:cell ratio. Supernatants were harvested after 72 h for cytokine measurement by ELISA. (A) IFN-γ; (B) IL-12p40; (C) TNF-α; (D) IL-10. Bars represent the mean value + 1 SD from 5 volunteers. * indicates $P < 0.05$ as compared to cultures without *Lact. delbrueckii*.

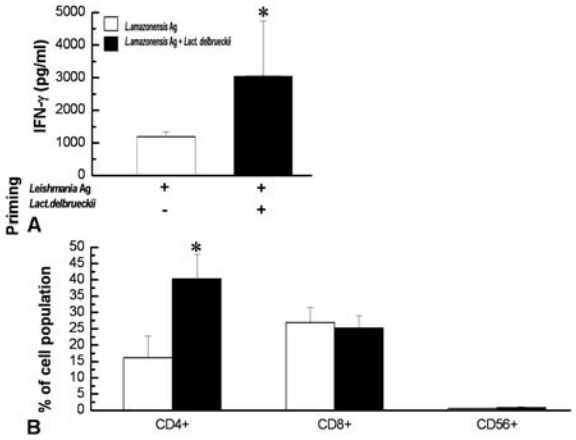

Figure 6. *Lactobacillus delbrueckii* drives the differentiation of naïve T cells into *L. amazonensis*-specific Th1 lymphocytes. PBMC were primed with *L. amazonensis* antigen (50 μg/ ml) in the presence or absence of *Lact. delbrueckii* (50:1). At 14 d after priming cells were re-stimulated with *L. amazonensis* antigen alone in the presence of autologous feeder cells. Seven days after re-stimulation cultures were evaluated for A. IFN-γ production and B. Phenotypic characterization of stimulated cells. Bars represent the mean value + 1 SD from six volunteers for cytokine measurement and three for phenotypic characterization. * indicates $P < 0.05$ as compared to cells primed without *Lact. delbrueckii*.

monoclonal antibody, a great reduction in the production of IFN-γ was observed, indicating that significant amounts of active IL-12 p70 was induced (Figure 2). Similarly, the biological activity of the IFN-γ produced early by NK cells (Figure 3) in our experiments was confirmed by the decrease in parasitism observed in macrophages infected with *L. amazonensis* promastigotes (Figs. 4 and 5). Furthermore, our results show that both the IL-12 and IFN-γ produced were able to induce the *in vitro* differentiation of naïve T cells into Th1 cells specific for *L. amazonensis* (Figure 6). We also confirmed (Figure 3) the observation made previously that treatment of PBMC with *Lactobacillus* is able to induce the activation of NK cells (21). This result is important in the context of induction of type 1 responses against intracellular parasites, since we have previously demonstrated that depletion of NK cells in mice interfere with the development of IFN-γ-producing T $CD4^+$ cells induced by the administration of IL-12 in association with *L. major* antigens (22).

We did not characterize the molecules involved in the induction of cytokine production in our system. However, several possible candidates can be appointed as responsible for this activity, i.e. lipoteichoic acid, peptidoglycan and unmethylated CpG-containing DNA motifs. These molecules, present in Gram-positive bacteria, such as lactobacilli, have been shown to stimulate cytokine production through the activation of Toll-Like receptors (23-30). Current studies are investigating this issue.

A large number of clinical applications for probiotic bacteria have been demonstrated over the years (31). Amongst the probiotic microorganisms, the use of lactobacilli has been suggested in several occasions and many studies demonstrate the feasibility of their use as live vectors in oral vaccination (32). However, oral vaccination usually does not induce the development of inflammatory responses necessary for the control of intracellular pathogens, such as *Leishmania* and *Toxoplasma.* In these cases, cutaneous immunization is more prone to induce the milieu necessary for Th1 development. A recent study has shown that Lactobacilli have the ability induce the differentiation of Th1 type cells (33). Our study further expands this observation by showing that lactobacilli may be used as an adjuvant to drive the differentiation of T cells specific to a different antigen other than the bacterium itself. The utilization of non-pathogenic bacteria, or their products, in vaccination protocols has obvious advantages and deserves further investigation.

6. ACKNOWLEDGEMENTS

This work was supported by funds from FAPEMIG (CBB 2818/97), CNPq (521118/98-0) and CAPES. The authors want to thank Dr. Elizabeth Neumann (Centro Universitário Belo Horizonte, Belo Horizonte, MG, Brazil) for technical and scientific assistance and Dr. Célia Alencar de Morais (Universidade Federal de Viçosa, Viçosa, MG, Brazil) for kindly providing Lact. delbrueckii UFV H2b20. L.C.C Afonso, O.A. Martins-Filho, L.Q.Vieira and J.R. Nicoli are research fellows from CNPq.

7. REFERENCES

1. Scott, P.: The role of TH1 and TH2 cells in experimental cutaneous leishmaniasis. *Exp Parasitol* 68, 369-372 (1989)

2. Brener, Z. and Gazzinelli, R. T.: Immunological control of *Trypanosoma cruzi* infection and pathogenesis of Chagas' disease. *Int Arch Allergy Immunol* 114, 103-110 (1997)

3. Denkers, E. Y. and Gazzinelli, R. T.: Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. *Clin Microbiol Rev* 11, 569-588 (1998)

4. Tripp, C. S., Wolf, S. F., and Unanue, E. R.: Interleukin 12 and tumor necrosis factor-α are costimulators of interferon-γ production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is an physiologic antagonist. *Proc Natl Acad Sci USA* 90, 3725-3729 (1993)

5. Christensen, H. R., Frokiaer, H., and Pestka, J. J.: Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. *J Immunol* 168, 171-178 (2002)

6. Cross, M. L., Ganner, A., Teilab, D., and Fray, L. M.: Patterns of cytokine induction by gram-positive and gram-

negative probiotic bacteria. *FEMS Immunol Med Microbiol* 42, 173-180 (2004)

7. Haller, D., Serrant, P., Granato, D., Schiffrin, E. J., and Blum, S.: Activation of human NK cells by staphylococci and lactobacilli requires cell contact-dependent costimulation by autologous monocytes. *Clin Diagn Lab Immunol* 9, 649-657 (2002)

8. Morita, H., He, F., Fuse, T., Ouwehand, A. C., Hashimoto, H., Hosoda, M., Mizumachi, K., and Kurisaki, J.: Cytokine production by the murine macrophage cell line J774.1 after exposure to lactobacilli. *Biosci Biotechnol Biochem* 66, 1963-1966 (2002)

9. Hessle, C., Hanson, L. A., and Wold, A. E.: Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. *Clin Exp Immunol* 116, 276-282 (1999)

10. Mienttinen, M., Matikainen, S., Vuopio-Varkila, J., Pirhonen, J., Varkila, K., Kurimoto, M., and Julkunen, I.: Lactobacilli and Streptococci induce Interleukin-12 (IL-12), IL-18, and Gamma Interferon production in human peripheral blood mononuclear cells. *Infect Immun* 66, 6058-6062 (1998)

11. Neumann, E., Oliveira, M. A. P., Cabral, C. M., Moura, L. N., Nicoli, J. R., Vieira, E. C., Cara, D. C., Podoprigora, G. I., and Vieira, L. Q.: Monoassociation with *Lactobacillus acidophilus* H2b20 stimulates the mononuclear phagocyte system of germfree mice. *Braz J Med Biol Res* 31, 1565-1573 (1998)

12. Afonso, L. C. C. and Scott, P.: Immune responses associated with susceptibility of C57BL/10 mice to *Leishmania amazonensis*. *Infect Immun* 61, 2952-2959 (1993)

13. Kanof, M. E., Smith, P. D., and Zola, H.: Isolation of whole mononuclear cells from peripheral blood and cord blood. In:Current Protocols in Immunology.Eds:Coligan JE, Kruisbeek AM, Marguiles DH, Shevach EM, and Strober W, *John Wiley & Sons,* New York 7.1.0-7.1.7 (1996)

14. Toledo, V., Mayrink, W., Gollob, K., Oliveira, M., Costa, Cd, Genaro, O., Pinto, J., and Afonso, L.: Immunochemotherapy in American cutaneous leishmaniasis: immunological aspects before and after treatment. *Mem Inst Oswaldo Cruz* 96, 89-98 (2001)

15. D'Andrea, A., Rengaraju, M., Valiante, N. M., Chehimi, J., Kubin, M., Aste, M., Chan, S. H., Kobayashi, M., Young, D., Nickbarg, E., Wolf, S. F., and Trinchieri, G.: Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *J Exp Med* 176, 1387-1398 (1992)

16. Gazzinelli, R. T., Hieny, S., Wynn, S., Wolf, S., and Sher, A.: IL-12 is required for the T- lymphocyte independent induction of interferon-γ by an intracellular parasite and induces resistance in T-deficient hosts. *Proc Natl Acad Sci USA* 90, 6115-6119 (1993)

17. Belosevic, M., Finbloom, D. S., van der Meide, P. H., Slayter, M. V., and Nacy, C. A.: Administration of monoclonal anti IFN-γ antibodies in vivo abrogates natural resistance of C3H/HeN mice to infection with *Leishmania major*. *J Immunol* 143, 266-274 (1989)

18. Scott, P.: IFN-γ modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. *J Immunol* 147, 3149-3155 (1991)

19. Wang, Z. E., Reiner, S. L., Zheng, S., Dalton, D. K., and Locksley, R. M.: CD4+ effector cells default to the Th2 pathway in interferon-γ deficient mice infected with *Leishmania major*. *J Exp Med* 179, 1367-1371 (1994)

20. Pohjavuori, E., Viljanen, M., Korpela, R., Kuitunen, M., Tiittanen, M., Vaarala, O., and Savilahti, E.: *Lactobacillus* GG effect in increasing IFN-γ production in infants with cow's milk allergy. *J Allergy Clin Immunol* 114, 131-136 (2004)

21. Haller, D., Blum, S., Bode, C., Hammes, W. P., and Schiffrin, E. J.: Activation of human peripheral blood mononuclear cells by nonpathogenic bacteria in vitro: evidence of NK cells as primary targets. *Infect Immun* 68, 752-759 (2000)

22. Afonso, L. C. C., Scharton, T. M., Vieira, L. Q., Wysocka, M., Trinchieri, G., and Scott, P.: The adjuvant effect of Interleukin-12 in a vaccine against *Leishmania major*. *Science* 263, 235-237 (1994)

23. Optiz, B., Schröder, N. W. J., Spreitzer, I., Michelsen, K. S., Kirschning, C. J., Hallatschek, W., Zähringer, U., Hartung, T., Göbel, U. B., and Schumann, R. R.: Toll-like receptor-2 mediates *Treponema* glycolipid and lipoteichoic acid-induced NF-κB translocation. *J Biol Chem* 276, 22041-22047 (2001)

24. Schwandner, R., Dziarski, R., Wescher, H., Rothe, M., and Kirschning, C. J.: Peptidoglycan and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. *J Biol Chem* 274, 17046-17049 (1999)

25. Yoshimura, A., Lien, E., Ingalls, R. R., Tuomanen, E., Dziarski, R., and Golenbock, D. T.: Recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J Immunol* 163, 1-5 (1999)

26. Schröder, N. W. J., Morath, S., Alexander, C., Hamann, L., Hartung, T., Zähringer, U., Göbel, U. B., Weber, J. R., and Schumann, R. R.: Lipoteichoic acid of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor-2, lipopolysaccharide-binding

protein, and CD14, whereas TLR-4 and MD-2 are not involved. *J Biol Chem* 278, 15587-15594 (2003)

27. Matsuguchi, T., Takagi, A., Matsuzaki, T., Nagaoka, M., Ishikawa, K., Yokokura, T., and Yoshikai, Y.: Lipoteichoic acids from *Lactobacillus* strains eleicit strong tumor necrosis factor alpha-inducing activities in macrophages through Toll-like receptor 2. *Clin Diag Lab Immunol* 10, 259-266 (2003)

28. Bauer, S., Kirschning, C. J., Häcker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., and Lipford, G. B.: Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc Natl Ac Sci* 98, 9237-9242 (2001)

29. Takeshita, F., Leifer, C. A., Gursel, I., Ishii, K. J., Takeshita, S., Gursel, M., and Klinman, D. M.: Role of Toll-like receptor 9 in CpG DNA-induced activation of human cells. *J Immunol* 167, 3555-3558 (2001)

30. Matsuguchi, T., Takagi, A., Matsuzaki, T., Nagaoka, M., Ishikawa, K., Yokokura, T., and Yoshikai, Y.: Lipoteichoic acids from *Lactobacillus* strains elicit strong tumor necrosis factor alpha-inducing activities in macrophages through Toll-like receptor 2. *Clin Diagn Lab Immunol* 10, 259-266 (2003)

31. Mombeli, B. and Gismondo, M. R.: The use of probiotics in medical practice. *Int J Antimicrob Agents* 16, 531-536 (2000)

32. Maassen, C. B., Laman, J. D., Bak-Glashouwer, M. J., Tielen, F. J., Holten-Neelen, J. C., Hoogteijling, L., Antonissen, C., Leer, R. J., Pouwels, P. H., Boersma, W. J., and Shaw, D. M.: Instruments for oral disease-intervention strategies: recombinant *Lactobacillus casei* expressing tetanus toxin fragment C for vaccination or myelin proteins for oral tolerance induction in multiple sclerosis. *Vaccine* 17, 2117-2128 (1999)

33. Mohamadzadeh, M., Olson, S., Kalina, W. V., Ruthel, G., Demmin, G. L., Warfield, K. L., Bavari, S., and Klaenhammer, T. R.: Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. *Proc Natl Acad Sci U S A* 102, 2880-2885 (2005)

Key Words: Lactobacillus, Probiotics, Adjuvant, Leishmania

Send correspondence to: Dr Luís Carlos Crocco Afonso, Laboratório de Imunoparasitologia, Departamento de Ciências Biológicas / NUPEB, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, 35400-000 Ouro Preto MG, Brazil, Tel: 55-31-3559-1701, Fax: 55-31-3559-1680, E-mail: afonso@nupeb.ufop.br