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Note

Disruption of Lipid Rafts Enhances the Effect of Lactobacilli on the Production of Tumor Necrosis Factor-Alpha in Mononuclear Blood Cells

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We investigated, to determine whether disruption of lipid rafts would influence the effect of three selected strains of *Lactobacillus* on TNF-alpha production by peripheral blood mononuclear cells. Two strains increased TNF-alpha production; and the third one reduced cytokine levels. Disruption of rafts changed the immunomodulatory effect of the three strains. This is the first report on a potential role of rafts in lactobacilli-cell interaction.

Key words: lactobacilli; lipid rafts; tumor necrosis factor-alpha

Probiotic lactic acid bacteria (LAB) such as lactobacilli have numerous beneficial effects on human health, including modulation of the immune system.[1,2] During the last few years, there has been an increasing interest in probiotic microorganisms as immunity-enhancing agents for new therapeutic regimens.[3,4] *In vitro* and *in vivo* studies with LAB strains have been performed, and have found that the capacity to induce anti- and pro-inflammatory cytokine production varies between bacterial species and even between strains.[5,6] To date, the mechanisms of this effect remain unclear.

Lipid rafts are specialized plasma membrane microdomains of eukaryotic cells enriched in cholesterol, sphingolipids, and GPI-proteins that serve as major assembly and sorting platforms in signal transduction complexes.[7] In addition to this role in cell signaling, many pathogen bacteria, such as *Escherichia coli* and *Pseudomonas aeruginosa*, interact specifically with target cells *via* these microdomains.[8,9] Indeed, lipid raft integrity is required at different steps of bacterial infection.[10] While the field of pathogen-lipid raft interactions is rapidly advancing and giving rise to new possibilities for the treatment of disease,[11–13] the potential role of these microdomains in the interaction between host cell and non-pathogenic bacteria, *e.g.*, probiotic LAB, has not yet been investigated.

In this study, we investigated to determine whether disruption of lipid rafts would influence the effect of three selected strains belonging to the genus *Lactobacillus* on tumor necrosis factor alpha (TNF-α) production by peripheral blood mononuclear cells (PBMCs).

Three strains stored in the Centro de Referencia para Lactobacilos (CERELA) Culture Collection (Tucumán, Argentina) were selected for this study: *Lactobacillus acidophilus* strain CRL1014, isolated from human infant feces, and *Lactobacillus reuteri* strain CRL1098, a lactic acid bacterium of sourdough origin, were used because of their probiotic properties;[14,15] *Lactobacillus rhamnosus* strain CRL1036 (derived from type strain ATCC 7469) was selected because *Lactobacillus rhamnosus* strains have been reported to induce variable immune responses and might be useful in the prevention and treatment of some diseases.[16,17] The bacteria were grown in MRS broth[18] at 37 °C up to an OD at 560 nm of about 0.50. Then they were collected by centrifugation and resuspended in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) for co-culture assays. The number of viable microorganisms (colony-forming units/ml) was determined by the agar plate method. Heat-killed lactobacilli were obtained by heating the bacteria in suspension at 80 °C for 30 min. Then viability was tested on MRS agar by the absence of growth after incubation at 37 °C for 72 h. Human peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained by density centrifugation through Ficcol-Paque.[19] The cells were then washed and resuspended in RPMI 1640 with 10% fetal bovine serum for further assays. Cell viability was determined by the trypan blue exclusion method. To disrupt lipid rafts, PBMCs were treated with 10 mM methyl-β-cyclodextrin (MβCD) (Sigma, Steinheim, Germany) in RPMI 1640 medium

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for 15 min at 37 °C. Visualization of the cellular cholesterol content was detected by filipin staining.[20] Different concentrations of LAB strains (0, 1.0×10^6, 1.0 and 2.0×10^7 cfu/ml) were co-incubated with 1.0×10^6 PBMCs in RPMI 1640 medium for various periods of time (0.5, 1, 2, 4, and 6 h) in 5% CO_2 at 37 °C. At the indicated times, the cells and bacteria were removed by centrifugation, and the supernatant was stored at −20 °C for further determination of TNF-α. Simultaneously, PBMCs were incubated under the same conditions in the absence of bacteria, and the TNF-α detected in supernatant was considered the basal value. Control and MβCD-treated cells (1.0×10^6 cells/ml) were incubated with live or heat-killed lactobacilli at optimal lactobacilli strain:cell ratio and incubation time previously determined for each strain. The TNF-α in the co-culture supernatants was measured by chemiluminescent immunometric assay (IMMULITE). Experimental data were expressed as mean ± SD. The significance of differences in mean values was determined by analysis of variance (ANOVA) and Tukey's test. Values of $P < 0.05$ were considered statistically significant.

The capacities of *L. acidophilus* CRL1014, *L. rhamnosus* CRL1036, and *L. reuteri* CRL1098 to modulate TNF-α production was investigated in co-cultures with PBMCs. Each strain was co-incubated with 1.0×10^6 PBMCs at various bacteria:cell ratios and incubation times and TNF-α was quantified in culture supernatant by chemiluminescent immunometric assay (Fig. 1). Cytokine secretion was modified by three strains in different ways, depending on the microorganism concentration and the incubation time. *L. acidophilus* induced secretion of TNF-α, at ratios of 10:1 and 20:1 (bacteria:cell), reaching a maximum of 2.3-fold higher than the basal value (149 pg/ml) produced by PBMCs alone, at a ratio of 20:1 (bacteria:cell) at 4 h incubation (Fig. 1). Co-culture with *L. rhamnosus* showed a slight but significant stimulation of cytokine production, and the effect was similar at the ratios of 10:1 and 20:1 (bacteria:cell). The highest increase (1.3-fold higher than the basal level) was reached at 0.5 h, and was maintained up to 6 h, incubation. In contrast, in co-culture of *L. reuteri* with PBMCs, TNF-α secretion was reduced by 33% with respect to the basal level, at a ratio of 20:1 (bacteria:cell) at 4 h, incubation. The three strains tested in co-culture with the same amount of bacteria and cells, did not induce changes in TNF-α production (data not shown). Our results confirm the heterogeneous capacity of *Lactobacillus* strains to modulate cytokine production, reported previously in murine macrophages.[6] Accordingly, individual variations in the bacteria concentration can, in addition to the presence of different strains, generate different outcomes after interaction with cells and thus determine whether stimulatory or inhibitory response occurs, as observed in this study. This might be due to different cellular receptor expression in response to exposure to gram-positive bacteria.[21]

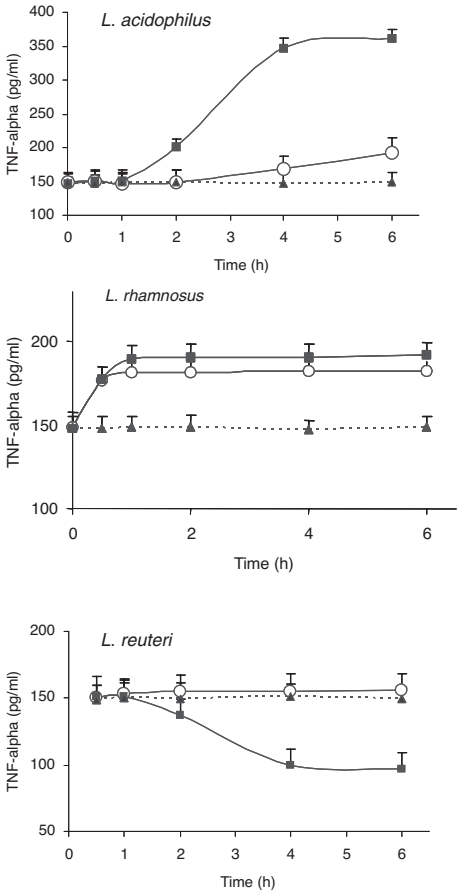

Fig. 1. Modulation of TNF-α Production by LAB.

PBMCs (1×10^6) were co-cultured with *L. acidophilus* CRL 1014, *L. rhamnosus* CRL 1036, and *L. reuteri* CRL 1098 at different bacteria:cell ratios, and incubation times. TNF-α was detected in the co-culture supernatant by chemiluminescent immunometric assay. Each value represents the mean ± SD obtained from three different experiments. Bacteria:cell ratios = 0:1 (▲), 10:1 (○), and 20:1 (■).

To disrupt the lipid rafts, PBMCs were depleted of cholesterol with 10 mM MβCD. The cells were then stained with filipin, a fluorescent polyene that binds to cholesterol.[20] Untreated cells were used as the control. Images showed that treatment with MβCD caused a strong reduction in filipin fluorescence intensity as compared to the control cells. Cells viability was not affected since MβCD-treated cells excluded trypan blue at about 97%. The effect of lipid raft disruption on the modulation of TNF-α induced by the three *Lactobacillus* strains was investigated using co-cultures with MβCD-treated PBMCs, at the optimal incubation time and bacteria:cell ratio as determined above. First, we tested whether treatment to disrupt lipid rafts would affect cytokine secretion. There were no differences in TNF-α production by lipid rafts-disrupted cells with respect to control cells (Fig. 2), indicating that MβCD treatment did not modify cytokine secretion. When 1.0×10^6 MβCD-treated PBMCs were co-cultured with *L. acidophilus* (20:1 bacteria:cell ratio, 4 h, incubation), the stimulatory effect on TNF-α production was significant

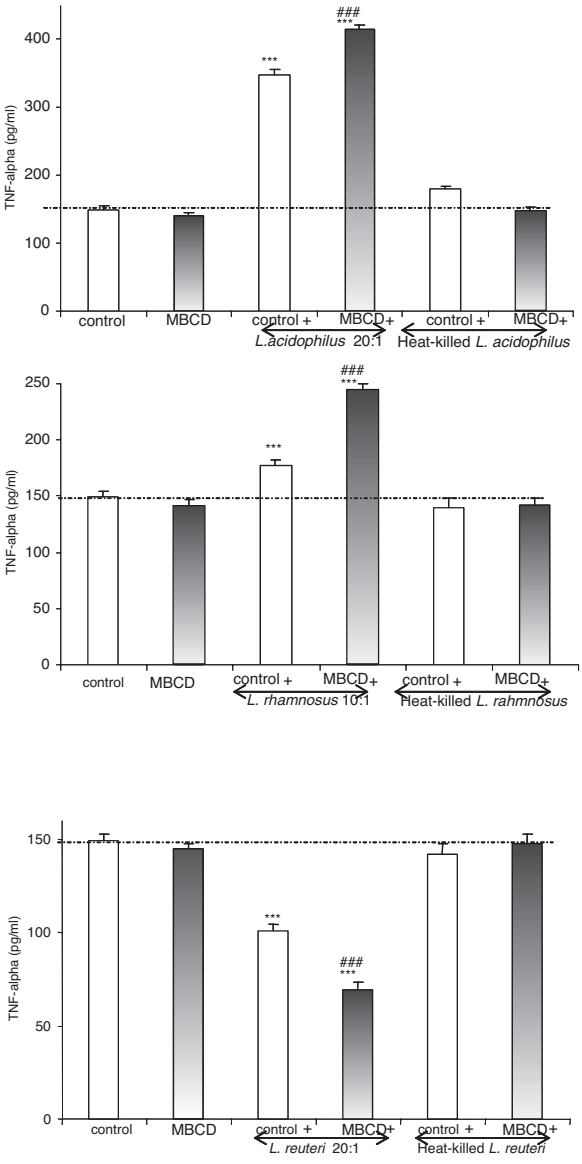

Fig. 2. Disruption of Lipid Rafts Changed TNF-α Production Induced by LAB.

PBMCs were co-cultured with live and heat-killed LAB at different bacteria:cell ratios. TNF-α production was detected in the co-culture supernatant. Control and MβCD bars represent TNF-α secreted by intact- and disrupted-lipid raft cells respectively, incubated in absence of bacteria. Statistical significance as compared with control by ANOVA test ($^{***}P < 0.001$), and as compared with MBCD ($^{\#\#\#}P < 0.02$).

higher in the MβCD-treated PBMCs than in the control cells (3.5-fold higher) and than in co-cultures of control cells with bacteria (1.3-fold higher) (Fig. 2). Similarly, co-culturing MβCD-treated PBMCs with *L. rhamnosus* (10:1 bacteria:cell ratio, 0.5 h, incubation) produced a slight but significant increase in TNF-α production with respect to the control (1.6-fold higher). The inhibitory effect of *L. reuteri* on TNF-α production was also increased in co-cultures with MβCD-treated PBMCs as compared to the control cells (53% inhibition) and to the co-culture of control cells with bacteria (32% inhibition). Heat-killed LAB strains failed to change TNF-α secretion by either control or MβCD-treated PBMCs (Fig. 2). This kind of response might have been the result of changes in LAB immunoestimulatory surface structures, due to heat treatment, which might have diminished their capacity to interact with the cells.[22] Pathways proposed for cytokine production induced by LAB is the direct interaction with immunocompetent cells,[23] but no information is available concerning the molecular mechanisms involved in this cell-microbial interaction, particularly the potential role of lipid rafts.

In this study, we found that live cells of lactobacilli modulate secretion of TNF-α and that this effect was enhanced by disruption of lipid raft. Previous reports have shown that key molecules for immune response, such as CD14, are constitutively or inducibly accumulated in lipid rafts, and that the entire bacterial-host cell interaction is based on the recruitment of other multiple signaling molecules within clusters of lipid rafts.[24] Thus, different combinational associations of receptors within activation clusters determine different responses to a variety of bacterial stimuli.[25] Disruption of lipid rafts integrity can lead to re-localization of the molecules involved in bacteria-cell interactions,[13] resulting in a modified cellular response like that observed in this study.

This is the first report that suggests a potential role of lipid rafts in the LAB-immune cell interaction. Defining the role of lipid rafts, might help in getting a better understanding of host-LAB interaction mechanisms, and hopefully facilitate the development of new probiotics more effective in the treatment of infectious and inflammatory diseases.

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