**Lactobacillus rhamnosus** GG decreases TNF-α production in lipopolysaccharide-activated murine macrophages by a contact-independent mechanism

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**Summary**

Animal studies and human clinical trials have shown that *Lactobacillus* can prevent or ameliorate inflammation in chronic colitis. However, molecular mechanisms for this effect have not been clearly elucidated. We hypothesize that lactobacilli are capable of down-regulating pro-inflammatory cytokine responses induced by the enteric microbiota. We investigated whether lactobacilli diminish production of tumour necrosis factor alpha (TNF-α) by the murine macrophage line, RAW 264.7 gamma (NO–), and alter the TNF-α/interleukin-10 (IL-10) balance, *in vitro*. When media conditioned by *Lactobacillus rhamnosus* GG (LGG) are co-incubated with lipopolysaccharide (LPS) or lipoteichoic acid (LTA), TNF-α production is significantly inhibited compared to controls, whereas IL-10 synthesis is unaffected. Interestingly, LGG-conditioned media also decreases TNF-α production of *Helicobacter*-conditioned media-activated peri- neal macrophages. *Lactobacillus* species may be capable of producing soluble molecules that inhibit TNF-α production in activated macrophages. As overproduction of pro-inflammatory cytokines, especially TNF-α, is implicated in pathogenesis of chronic intestinal inflammation, enteric *Lactobacillus*-mediated inhibition of pro-inflammatory cytokine production and alteration of cytokine profiles may highlight an important immunomodulatory role for commensal bacteria in the gastrointestinal tract.

**Introduction**

Commensal species of the genus *Lactobacillus* represent the most commonly used probiotic bacteria in clinical studies. Probiotics are commensal microbes with positive health benefits beyond mere nutrition (Lilly and Stillwell, 1965). The ubiquitous presence of intestinal lactobacilli and their role as members of the autochthonous (indigenous) microbiota (Alvarez-Olmos and Oberhelman, 2001; Holzapfel et al., 2001; Reuter, 2001) have stimulated interest in their roles as gut-beneficial bacteria. By capsule endoscopy, Reuter (2001) describes the presence of multiple *Lactobacillus* species as indigenous intestinal bacteria residing in the gastrointestinal tracts of healthy children and adults. One study (Ahrne et al., 1998) showed that *Lactobacillus rhamnosus* was one of the three most commonly found intestinal lactobacilli found in the oral and rectal mucosa of healthy human individuals. Healthy rodents including mice are also commonly colonized by lactobacilli in the stomach and intestine (Tannock, 1997).

*Lactobacillus rhamnosus* GG (LGG) was isolated from the stool of a healthy individual in 1985 by S. Gorbach and B. Goldin (Gorbach, 2000) and subsequent studies showed beneficial effects in patients with colitis (Gorbach et al., 1987). LGG colonizes the gut of rodents (Banasaz et al., 2002) and humans (Alander et al., 1997). This strain has been shown to adhere to the colonic mucosa in human individuals (Alander et al., 1999) and can be recovered successfully from colonic mucosa and faeces. It survives for 1–3 days in most individuals and up to 7 days in 30% of subjects. In addition to its colonization ability, the presence of LGG affects mucosal immune responses. LGG stimulates mucosal IgA responses and enhances antigen uptake in Peyer’s patches (Gorbach, 2000).

As a potential probiotic agent, multiple studies have demonstrated the ability of LGG to colonize the intestinal tract and modulate mucosal epithelial and immune responses. LGG increased enterocyte proliferation and villous size in mono-associated gnotobiotic rats (Banasaz et al., 2002). LGG also modulates murine lymphocyte proliferation *ex vivo* following oral administration (Kirjavainen et al., 1999) and *L. paracasei* alters modulatory cytokine profiles of CD4+ T lymphocytes (von der Weid et al., 2001). In addition to adaptive immune responses, LGG has effects on innate immune...
responses. *Lactobacillus rhamnosus* GG activates nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription (STAT) signalling pathways in human macrophages (Miettinen et al., 2000) and *L. rhamnosus* stimulates interleukin-12 (IL-12) production by macrophages (Hessle et al., 1999). *Lactobacillus rhamnosus* GG also stimulates production of immunomodulatory cytokines such as IL-10 in children (Pessi et al., 2000) and may regulate pro-inflammatory responses in vivo. Effector cells of innate immunity, such as macrophages, dendritic cells and neutrophils, are the primary drivers for the majority of inflammatory responses (Janeway and Medzhitov, 2002). The thought that innate immunity dictates the course of both innate and adaptive responses to antigens as self or non-self emphasizes the role of the innate immunity in controlling inflammation.

In these experimental studies, we investigated the ability for LGG to specifically inhibit pro-inflammatory cytokine production by the innate immune system. With a murine macrophage model, we demonstrate that LGG specifically inhibits TNF-α production independent of apoptosis or cytotoxic effects. *Lactobacillus rhamnosus* GG secretes soluble factors including proteins that diminish TNF-α production by lipopolysaccharide (LPS)- or lipoteichoic acid (LTA)-activated macrophages independent of effects on other cytokines. Furthermore, the TNF-α-inhibitory effects of LGG antagonize stimulatory effects of *Helicobacter pylori* or *Helicobacter hepaticus* conditioned media.

**Results**

**LGG-mediated inhibition of TNF-α production by LPS-activated macrophages**

We developed an in vitro bioassay to look at the ability of *Lactobacillus* species to downregulate inflammatory responses in cultured macrophages (Fig. 1). Cells of the innate immune system utilize germ line-encoded pattern recognition receptors (PRRs) to recognize pathogen- or commensal-associated molecular patterns (P/CAMPs). One such P/CAMP is bacterial LPS, which serves as a ligand to the PRR, Toll-like receptor 4 (TLR4) (Poltorak et al., 1998; Lien et al., 2000). We used RAW 264.7 macrophages, a transformed peritoneal macrophage line from BALB/c mice, as reporter cells (Raschke et al., 1978). Both wild-type RAW 264.7 macrophages and a spontaneous mutant, RAW 264.7 gamma NO(−) were compared. The gamma NO(−) cell is a spontaneous mutant requiring both IFN-γ and LPS for production of nitric oxide and full activation (Lowenstein et al., 1993). Briefly, RAW 264.7 macrophages were cultured and exposed to LPS, and macrophage culture supernatants were collected at 30 min, 1, 3, 5, 7, 9, 12 and 24 h post activation. Maximal TNF-α secretion, after LPS activation, was reached at approximately 5 h, with no significant differences when compared to 24 h post activation, as measured by quantitative ELISA. Levels of TNF-α production were noted to be higher in wild-type macrophages versus the gamma NO(−) cells (levels per 50 000 cells: >2500 pg ml⁻¹ and 2000–2500 pg ml⁻¹ respectively). It must be noted that these levels may overestimate the levels of TNF-α homotrimers as quantitative ELISAs are designed to detect all forms of TNF-α including monomers and dimers.

Viable, intact ultraviolet light (UV)-killed, and sonicated *Lactobacillus* cells had different effects on LPS-mediated activation of macrophages in co-incubation experiments. Exposure of macrophages to either intact viable or UV-killed bacteria did not induce TNF-α secretion, whereas bacterial cell sonicates elicited high levels of TNF-α (data not shown). Both intact viable and UV-killed LGG cells failed to abrogate TNF-α production when macrophages were co-exposed to LPS.

![Fig. 1. Schematic of LGG-macrophage bioassay. Macrophages are stimulated with purified LPS from *E. coli*. Activation is characterized by morphologic changes, such as vacuolization and extrusion of cellular processes. Additionally, activation also results in secretion of pro-inflammatory cytokines, such as TNF-α. The presence of putative immunomodulins made by lactobacilli, may block LPS-mediated production of TNF-α.](image-url)
Bacterial cell-free conditioned media from *Escherichia coli* Nissle and different lactobacilli were tested for effects on pro-inflammatory cytokine output (Fig. 2). Immuno-modulatory effects were observed with cell-free conditioned media derived from LGG (LGG-cm) indicating the presence of an immunomodulin, or a soluble molecule modulating the immune response. In the presence of LGG-cm, TNF-α secretion is markedly reduced in LPS-activated macrophages when compared to macrophages exposed to LPS alone (P < 0.025). The ability of LGG to inhibit LPS-induced TNF-α production in macrophages depended on the relative concentrations of LPS and putative bacterial immunomodulins. As the concentration of LPS is increased, the ability of LGG-cm to modulate TNF-α response is diminished (data not shown). Conversely, maintaining the LPS concentration at 2 ng well⁻¹ and varying the amount of LGG-cm yielded similar results. In order to control for lactic acid production and reduced pH effects, acidified media was tested and did not affect TNF-α levels without the presence of LGG-cm. Conditioned media derived from other lactic acid bacteria did not inhibit TNF-α secretion and was inconsistent with general pH effects due to lactic acid production.

As the modulatory activity of LGG-cm seemed to be concentration-dependent, we examined whether the ability to inhibit LPS-mediated TNF-α production (with 2 ng LPS/well) by the putative immunomodulin was bacterial-density dependent. LGG-cm collected at 4, 8 and 24 h post inoculation were compared. These three time points represented early log, mid-log and late logarithmic/early plateau phases of LGG growth, respectively, based on absorbance spectrophotometry. The immunomodulatory activity was most potent in LGG-cm harvested at 24 h, whereas conditioned media of bacteria in log phase had only partial immunomodulatory activity. Re-challenge experiments were performed to determine the longevity of the TNF-α inhibitory activity. Macrophages were stimulated using LGG-cm with LPS or LPS alone. At the end of 5 h post activation, cell culture media was removed and replenished with fresh media. After 24 h, both LGG with LPS or LPS-treated cells were re-challenged with LPS alone. TNF-α was detectable in both groups, showing that the putative immunomodulin blocks TNF-α in a reversible manner (Fig. 3).

Macrophages and other immune cells recognizing P/CAMPs via PRRs are thought to require soluble cofactors in serum, such as soluble CD14 (sCD14) and LPS-binding proteins (LBP) (Muta and Takeshige, 2001). Bioassays were performed in serum-free media and TNF-α was measured in LPS-exposed cells. In our *in vitro* system, LPS-induced TNF-α production by macrophages was independent of serum-soluble cofactors, although there was a slight, but insignificant, difference in the production of TNF-α in serum-deprived cells compared to serum-supplemented macrophages. Importantly, LGG immunomodulatory activity was retained in the absence of serum (Fig. 4).

**LGG-mediated inhibition of TNF-α production by LTA-activated macrophages**

Other pathogen or commensal associated molecular pattern (P/CAMP) biomolecules, such as Gram-positive bacterial lipoteichoic acid (LTA), have been shown to activate macrophages via PRRs (Schwandner et al., 1999; Takeuchi et al., 1999). To explore the Toll-like receptor (TLR2)-mediated pathway, LGG-conditioned media was added to LTA-activated macrophages. Indeed, LGG-cm inhibited TNF-α secretion by macrophages induced by LTA from...
Staphylococcus aureus, Enterococcus faecalis or Bacillus subtilis. In this assay, LTA was able to induce TNF-α levels that were comparable to that of LPS. It is worth mentioning that whereas concentrations of LTA used in the bioassays were more than 10 times that of LPS (25 ng per 50,000 cells and 2 ng per 50,000 cells respectively), the same amount of LGG-cm inhibited TNF-α secretion for both LTA- and LPS-activated macrophages (see Experimental procedures). However, when macrophages were exposed to both LPS and LTA, the TNF-α inhibitory activity of LGG is partially reduced (Fig. 5). These results suggest that dual stimulation of TLR2 and TLR4-mediated pathways partially overcomes the block in TNF-α production.

Evaluation of cytokine profiles and bacterial-bacterial antagonism

To further understand the implications of TNF-α inhibition by LGG on the cytokine network of the innate immune response, we evaluated cytokine profiles of LPS-stimulated macrophages in the presence or absence of LGG-cm. Bioassays were performed and cytokines quantified by the Luminex LabMAP 100™ System (Martins et al, 2002). Interleukin-1β (IL-1β), IL-10, IL-12 and TNF-α, but not granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-6 (IL-6) and interferon-gamma (IFN-γ), were detected in culture supernatants of LPS-stimulated macrophages. Levels of IL-1β and IL-10 in LGG-treated-LPS-stimulated macrophages were comparable to quantities produced by LPS-stimulated cells. An 85% reduction was observed in TNF-α levels in LGG-treated LPS-stimulated cells (125 pg ml⁻¹) compared to LPS alone (850 pg ml⁻¹), similar to ELISA data. Interestingly, the levels of IL-10 were unaffected whether macrophages were exposed to LPS alone or co-incubated with LGG-cm. LGG-treated macrophages had diminished TNF-α/IL-10 ratios compared to LPS alone (Fig. 6) indicating a net immunomodulatory effect.

As Gram-negative bacterial-derived products stimulate naive macrophages, we wanted to establish whether LGG prevented TNF-α production induced by E. coli or pathogenic helicobacters. In our assay, conditioned media of Gram-negative bacteria such as E. coli, H. pylori or H. hepaticus, were capable of inducing TNF-α secretion by macrophages. However, neither H. pylori- or H. hepaticus-derived P/CAMPs present in conditioned media were as potent as E. coli-derived P/CAMPs in stimulating TNF-α secretion in macrophages. Intra-genus comparison of
macrophage activation shows that *H. pylori*-conditioned media elicits about 900 pg ml⁻¹ TNF-α while *H. hepaticus* produces approximately half of *H. pylori*-induced levels (Fig. 7). In the presence of LGG-cm, TNF-α induction is significantly inhibited indicating antagonism of LGG-derived immunomodulins vs. *Helicobacter*-derived immunostimulatory factors (*P* < 0.01). It is interesting to note that induction by *E. coli* is not affected by the addition of LGG-cm. *Lactobacillus rhamnosus* GG may inhibit TNF-α only when LPS (or an immunostimulatory P/CAMP) of a given nature or particular threshold concentration is present (Fig. 7).

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immunomodulin has a protein or peptide component that inhibits TNF-α production in macrophages.

**Discussion**

In summary, these results indicate that *L. rhamnosus* GG specifically inhibits TNF-α production and reduces TNF-α/IL-10 ratios in a murine macrophage model. The net effect is immunomodulatory in nature. Other *Lactobacillus* species did not have such a modulatory effect, demonstrating that specific immune effects may be species- or strain-specific. This effect is serum- and contact-independent, requiring the presence of soluble LGG immunomodulins for complete modulatory activity. Other NF-κB-dependent cytokines such as interleukin-12 (IL-12) are not inhibited and IL-10 production is unaffected. Thus, this modulatory effect appears to be specific for TNF-α and may be NF-κB-independent. Intestinal lactobacilli produce soluble protein factors that presumably bind to cell surface receptors and somehow inhibit synthesis or secretion of TNF-α independent of pro-apoptotic effects or cell necrosis.

Most lactobacilli ferment carbohydrates into lactic acid when cultured under reduced oxygen tension. We observed that acidified MRS medium did not inhibit LPS-mediated TNF-α production and that acidified MRS medium did not induce TNF-α production in naïve macrophages. If lactic acid and reduced pH diminished TNF-α production, *L. rhamnosus* GG-mediated inhibition of TNF-α production would be shared with media conditioned by other lactic acid bacteria. As we have found that TNF-α production was inhibited by less than 10% of *Lactobacillus* strains tested, it seems highly unlikely that lactic acid or other acid metabolites inhibit TNF-α production. Instead, lactic acidosis increases TNF-α production in rat peritoneal macrophages (Jensen et al., 1990).

The results obtained with other NF-κB-regulated cytokines such as IL-6 and IL-12 may appear to be inconsistent with other findings. The literature commonly includes studies with LPS concentrations that are considerably higher (100 ng ml⁻¹–10 μg ml⁻¹). In fact, Pennanen et al. (1995) documented the lack of detectable expression of murine IL-6 by RAW 264.7 cells exposed to 100 ng ml⁻¹ LPS for 6 h. These results were consistent with our data even when LPS concentrations were adjusted for macrophage cell numbers. Our macrophage bioassay included lower LPS concentrations (0.5 ng ml⁻¹) that may preclude detection of IL-6 by quantitative ELISA or Luminex LabMAP assays in short-term incubation experiments (5 h). The lower limit of the quantitative ELISA (BioSource) for murine IL-6 was 3 pg ml⁻¹, whereas the lower limit of detection of the Luminex LabMAP (multiplex) assay for murine IL-6 was 10 pg ml⁻¹. Interleukin-12 was not inhibited by LGG-cm and demonstrated an inverse relationship with respect to TNF-α production. This result was not unexpected as TNF-α and IL-12 negatively co-regulate one another (Ma, 2001). The authors speculate that, since the bioassay assesses the short-term (5 h) response to LPS stimulation, macrophages primarily secrete TNF-α initially. When TNF-α is inhibited by LGG-cm, reduced levels of TNF-α may stimulate IL-12 expression.

TNF-α represents a potent pro-inflammatory cytokine produced by activated macrophages, which stimulates Th1 immune responses. TNF-α production in LPS-activated macrophages is dependent on NF-κB activation. NF-κB is considered to be a key transcriptional regulator of pro-inflammatory genes important in host innate immune responses. Inhibition of TNF-α production may be secondary to interference with NF-κB activation, blocking transcription of TNF-α. With respect to LGG and murine macrophages, this pathway does not appear to be affected because other NF-κB-regulated genes such as IL-12 are not diminished. Preliminary data (not shown) based on real-time quantitative RT-PCR experiments indicate that TNF-α mRNA levels are unaffected in LPS- or LTA-activated macrophages exposed to LGG-cm. Instead, it appears that TNF-α inhibition is post-transcriptional in nature. At this time, it is unclear whether LGG-cm affects mRNA processing, translation, post-translational processing, or protein trafficking and secretion.

Commensal bacteria are known to produce immunoregulatory factors that may enhance infection in the host by modulating immune responses (Wilson et al., 1998). Such immunomodulins may have important roles in maintaining intestinal health and quenching systemic inflammatory responses. *Lactobacillus paracasei* induces populations of regulatory CD4+ T cells which produce high levels of...
the modulatory cytokines, IL-10 and transforming growth factor β (TGF-β) (von der Weid et al., 2001). Lactobacilli modulate cytokine production in bone marrow-derived dendritic cells with a net effect of altering overall cytokine profiles in a species-dependent manner (Christensen et al., 2002). Non-virulent Salmonella strains regulate NF-κB-dependent induction of pro-inflammatory cytokine production by preventing ubiquitination of the NF-κB inhibitory subunit, IκBα (Neish et al., 2000).

Prokaryotes have developed mechanisms for inhibiting pro-inflammatory cytokine responses and facilitating long-term colonization and microbial-host co-existence. Lactobacilli may exert different effects on both mucosal and systemic cytokine levels in rodent models (Ha et al., 1999; Tejada-Simon et al., 1999) and highlight the importance of examining quantitative differences in cytokine synthesis. These seemingly disparate results emphasize the importance of distinguishing experimental studies with lysates versus intact cells or conditioned media. Additionally, different species or strains of any genus may have distinct biologic effects. The biologic unit of importance for pathogenesis and commensalism is ultimately the clone. In support of the strain differences, studies have demonstrated the strain-dependence of immunopotentiating effects of Lactobacillus delbrueckii (Nagafuchi et al., 1999).

Pathogenic bacteria produce proteins that diminish TNF-α expression in host immune cells by different mechanisms and presumably facilitate systemic spread and proliferation. For example, Brucella suis produces a major outer membrane protein, Omp25, that inhibits TNF-α production by human macrophages during infection (Jubier-Maurin et al., 2001). Anthrax lethal factor produced by Bacillus anthracis cleaves two mitogen-activated protein kinases (MAPKKs) in macrophages, causing a substantial reduction in the production of nitrogen oxide (NO) and TNF-α in response to lipopolysaccharide or IFN-γ (Pellizzari et al., 1999). The intestinal pathogen Yersinia enterocolitica expresses a protein YopP that interferes with TNF-α production in murine monocyte-macrophages by interfering with the NF-κB and MAPK pathways (Boland and Cornelis, 1998).

Probiotic Lactobacillus species have been effective in several animal models and clinical trials. Administration of L. reuteri to IL-10 deficient mice resulted in amelioration of colitis in treated animals and apparent shifts in the nature of the intestinal microbiota (Madsen et al., 1999; Madsen et al., 2000). In the acidic acid-induced rat colitis model, L. reuteri and L. rhamnosus GG yielded beneficial effects and diminished mucosal inflammation (Holma et al., 2001). Different species of Lactobacillus have been included in modern probiotic formulations for the treatment of antibiotic-associated colitis, viral gastroenteritis and inflammatory bowel disease in human patients. Oral ingestion of Lactobacillus rhamnosus GG has reduced recurrence risk in antibiotic-associated colitis (Bennett et al., 1996). Administration of Lactobacillus reuteri has reduced the length of disease and ameliorated symptoms due to rotaviral gastroenteritis (Shornikova et al., 1997). Finally, the administration of a mixture of Lactobacillus and Bifidobacterium sp. (VSL#3) in ulcerative colitis patients following colectomy has reduced recurrence of flare-ups in chronic pouchitis (Gionchetti et al., 2000).

Probiotic organisms including members of the genus Lactobacillus offer intriguing possibilities as anti-inflammatory biotherapeutic agents. Increased interest in probiotics for the treatment of inflammatory and infectious diseases of the gastrointestinal tract has generated enthusiasm for new therapeutic regimens, but the optimal bacterial strains for these purposes require further investigation. A more complete understanding of the molecular mechanisms of immunomodulation will facilitate the development of next-generation probiotics and will enhance our understanding of host-microbial interactions. Co-evolution of host and commensal organisms serve as a valuable context for framing the scientific questions as we proceed. Clearly commensal bacteria including lactobacilli interact intimately with the host mucosa beyond simple adherence. The production of surface-bound and secreted factors trigger particular eukaryotic signalling pathways and ultimately affect the production of specific host proteins. Such molecular interactions will shed insights and uncover new mechanisms into the regulation of mucosal inflammation and host immune responses.

Experimental procedures

Bacteriologic methods

Lactobacillus spp. (L. acidophilus ATCC 4796, L. animalis ATCC 35046, L. rhamnosus GG ATCC 53103, L. johnsonii ATCC 33200, L. murinus ATCC 35020, L. plantarum ATCC 14917, L. plantarum ATCC 49445, L. reuteri ATCC 53808, L. reuteri ATCC 55148, L. salivarius ATCC 11471) and E. coli Nissle (obtained from V. Fussing, Statens Serum Institut, Copenhagen, Denmark) were grown in Man, Rogosa, Sharpe (MRS) and Luria-Bertani (LB) media (Difco, Sparks, MD) respectively. Overnight cultures of lactobacilli were diluted to an OD₅₅₀ of 1.0 (representing approximately 10⁷ cells ml⁻¹) and further diluted 1:10 and grown for an additional 4, 8 and 24 h. Helicobacter pylori Sydney and Helicobacter hepaticus 381 were cultured for 48 h in Brucella broth (Difco) supplemented with 10% fetal bovine serum (FBS). Cultures were diluted 1:10 and grown for another 24 and 48 h. Bacterial cell-free conditioned media was collected by centrifugation at 8500 rcf for 10 min at 4°C. Conditioned media was separated from cell pellet and filtered through a 0.22 μm pore filter unit (Millipore, Bedford, MA). Intact UV-killed bacteria were prepared by washing lactobacilli in PBS and re-suspending cells to an OD₅₅₀ of 1. Bacterial cells were exposed to 2400 μJoules of UVₚₐₘ, light in a Stratalinker® UV Crosslinker (Stratagene, La Jolla, CA) and plated out on MRS agar to assess viability. Intactness of UV-killed cells was assessed by Gram-stain morphology.
Manipulation of conditioned media

Lactobacillus-conditioned media was treated with degradable enzymes and temperature shifts to determine the nature of immunomodulatory molecules possibly secreted by these microorganisms. Conditioned media was subjected to the following: three cycles of freezing and thawing, 15 min heating at 95°C, 15 min DNase I (Ambion, Austin, TX) treatment at T∞, or 20 min digestion at 37°C with Proteinase K or Protease E (Sigma, St Louis, MO), followed by a 10 min heat inactivation at 95°C. MRS broth was acidified with hydrochloric acid to a pH comparable to that of lactobacilli conditioned media (pH 4.0) and used as controls.

Cell cultures and bioassays

Mouse monocyte/macrophage cell lines, RAW 264.7 (ATCC TIB-71) and RAW 264.7 gamma NO (−) (ATCC CRL-2278), were used as reporter cells for studying inflammatory response pathways. RAW 264.7 cells were grown either in Dulbecco's modified Eagle's medium (for wild-type macrophages) or RPMI Medium 1640 (for gamma NO (−) cells) (Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 2% antibiotic (5000 units ml⁻¹ penicillin and 5 mg ml⁻¹ streptomycin, Sigma) at 5% CO₂, 37°C until 80–90% confluent. Approximately 5 x 10⁴ cells were seeded into 96-well cell culture clusters and allowed to adhere for 2 h prior to LPS activation and addition of conditioned media. Naïve RAW 264.7 cells were exposed to cell-free conditioned media, purified lipopolysaccharide (LPS) from E. coli (Sigma, St Louis, MO), followed by a 10 min heat inactivation at 95°C. MRS broth was acidified with hydrochloric acid to a pH comparable to that of lactobacilli conditioned media (pH 4.0) and used as controls.

Cytokine measurements

Production of TNF-α in macrophage cell culture supernatants was measured using a mouse TNF-α specific sandwich enzyme immunoassay (ELISA) (Biosource, Camarillo, CA). To study the cytokine milieu of activated macrophage culture in the presence of putative immunoregulators, mouse-specific cytokine antibody-bead kits for Luminex LabMAP 100™ Systems (Biosource) were used to detect and quantify IL-1β, IL-6, IL-10, IL-12 (p70 and p40 specific), TNF-α, IFN-γ and GM-CSF in culture supernatants in a Luminex 100 instrument (Luminex, Austin, TX).

Statistical analyses

All experiments were performed at least three times (each time in triplicate) and analysed using Independent Samples T-test (SPSS for WINDOWS version 11.0.1, SPSS, Chicago, IL) at a significance level of P < 0.05. Error bars in figures represent standard deviation (SD).

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References

Ahrne, S., Nobaek, S., Jeppsson, B., Adlerberth, I., Wold, A.E., and Molin, G. (1998) The normal Lactobacillus flora of healthy human rectal and oral mucosa. J Appl Microbiol 85: 88–94.
Alander, M., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T., and von Wright, A. (1997) Recovery of Lactobacillus rhamnosus GG from human colonic biopsies. Lett Appl Microbiol 24: 361–364.
Alander, M., Satokari, R., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T., and von Wright, A. (1999) Persistence of colonization of human colonic mucosa by a probiotic strain, Lactobacillus rhamnosus GG, after oral consumption. Appl Environ Microbiol 65: 351–354.
Alvarez-Olmos, M.I., and Oberhelman, R.A. (2001) Probiotic agents and infectious diseases: a modern perspective on a traditional therapy. Clin Infect Dis 32: 1567–1576.
Banasaz, M., Norin, E., Holma, R., and Midtvedt, T. (2002) Increased enterocyte production in gnotobiotic rats mono-associated with Lactobacillus rhamnosus GG. Appl Environ Microbiol 68: 3031–3034.
Bennett, R.G., Gorbach, S.L., Goldin, B.R., Chang, T.W., Laughon, B.E., Greenough, W.B., III, and Bartlett, J.G. (1996) Treatment of relapsing Clostridium difficile diarrhea with Lactobacillus GG. Nutrition Today Supplement 31: 355–38S.
Boland, A., and Cornelis, G.R. (1998) Role of YopP in suppression of tumor necrosis factor alpha release by macrophages during Yersinia infection. Infect Immun 66: 1878–1884.
Christensen, H.R., Frokiaer, H., and Pestka, J.J. (2002) Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J Immunol 166: 171–178.
Gionchetti, P., Rizzello, F., Venturi, A., Brigidi, P., Matteuzzi, D., Bazzocchi, G., et al. (2000) Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. Gastroenterology 119: 305–309.
Gorbach, S.L. (2000) Probiotics and gastrointestinal health. Am J Gastroenterol 95: S2–S4.
Gorbach, S.L., Chang, T.W., and Goldin, B. (1987) Successful treatment of relapsing Clostridium difficile colitis with Lactobacillus GG. Lancet 2: 1519.
Ha, C.L., Lee, J.H., Zhou, H.R., Ustunol, Z., and Pestka, J.J. (1999) Effects of yogurt ingestion on mucosal and systemic cytokine gene expression in the mouse. J Food Prot 62: 181–188.
Hessle, C., Hanson, L.A., and Wold, A.E. (1999) Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. Clin Exp Immunol 116: 276–282.
Holma, R., Salmenpera, P., Lohi, J., Vapaatalo, H., and Korpeila, R. (2001) Effects of Lactobacillus rhamnosus GG and Lactobacillus reuteri R2LC on acetic acid-induced colitis in rats. Scand J Gastroenterol 36: 630–635.

Holzapfel, W.H., Haberer, P., Geisen, R., Bjorkroth, J., and Schillinger, U. (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. Am J Clin Nutr 73: 3655–373S.

Janeway, C.A., Jr, and Medzhitov, R. (2002) Innate immune recognition. Annu Rev Immunol 20: 197–216.

Jensen, J.C., Buress, C., and Norton, J.A. (1990) Lactic acidosis increases tumor necrosis factor secretion and transcription in vitro. J Surg Res 40: 350–353.

Jubier-Maurin, V., Boigegrain, R.A., Cloeckaert, A., Gross, A., Alvarez-Martinez, M.T., Terraza, A., et al. (2001) Major outer membrane protein Omp25 of Brucella suis is involved in inhibition of tumor necrosis factor alpha production during infection of human macrophages. Infect Immun 69: 4823–4830.

Kirjavainen, P.V.E., Nezami, H.S., Salminen, S.J., Ahokas, J.T., and Wright, P.F. (1999) Effects of orally administered viable Lactobacillus rhamnosus GG and Propionibacterium freudenreichii subsp. shermanii JS on mouse lymphocyte proliferation. Clin Diagn Laboratory Immunol 6: 799–802.

Lien, E., Means, T.K., Heine, H., Yoshimura, A., Kasumoto, S., Fukase, K., et al. (2000) Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. J Clin Invest 105: 497–504.

Lilly, D.M., and Stillwell, R.H. (1965) Probiotics: growth promoting factors produced by microorganisms. Science 147: 747–748.

Lowenstein, C.J., Alley, E.W., Raval, P., Snowman, A.M., Snyder, S.H., Russell, S.W., and Murphy, W.J. (1993) Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. Proc Natl Acad Sci USA 80: 9730–9734.

Ma, X. (2001) TNF-alpha and IL-12: a balancing act in macrophage functioning. Microbes Infect 3: 121–129.

Madsen, K.L., Doyle, J.S., Jewell, L.D., Tavernini, M.M., and Fedorak, R.N. (1999) Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice. Gastroenterology 116: 1107–1114.

Madsen, K.L., Doyle, J.S., Tavernini, M.M., Jewell, L.D., Rentie, R.P., and Fedorak, R.N. (2000) Antibiotic therapy attenuates colitis in interleukin 10 gene-deficient mice. Gastroenterology 118: 1094–1105.

Martins, T.B., Pasi, B.M., Pickering, J.W., Jaskowski, T.D., Litwin, C.M., and Hill, H.R. (2002) Determination of cytokine responses using a multiplexed fluorescent microsphere immunoassay. Am J Clin Pathol 116: 346–353.

Miettinen, M., Lehtonen, A., Jukkunen, I., and Matikainen, S. (2000) Lactobacilli and Streptococci activate NF-kappa B and STAT signaling pathways in human macrophages. J Immunol 164: 3733–3740.

Muta, T., and Takeshige, K. (2001) Essential roles of CD14 and lipopolysaccharide-binding protein for activation of toll-like receptor (TLR) 2 as well as TLR4 Reconstitution of. Eur J Biochem 268: 4580–4589.

Nagafuchi, S., Takahashi, T., Yajima, T., Kuwata, T., Hirayama, K., and Itoh, K. (1999) Strain dependency of the immunopotentiating activity of Lactobacillus delbrueckii subsp bulgaricus. Biosci Biotechnol Biochem 63: 474–479.

Neish, A.S., Gewirtz, A.T., Zeng, H., Young, A.N., Hobert, M.E., Karmali, V., et al. (2000) Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. Science 289: 1560–1563.

Pellizzari, R., Guidi-Rontani, C., Vitale, G., Mock, M., and Montecucco, C. (1999) Anthrax lethal factor cleaves MKK3 in macrophages and inhibits the LPS/IFN gamma-induced release of NO and TNFalpha. FEBS Lett 462: 199–204.

Pennanen, N., Lapinjoki, S., Palander, A., Urtti, A., and Monkkonen, J. (1995) Macrophage-like RAW 264 cell line and time-resolved fluoroimmunoassay (TRFIA) as tools in screening drug effects on cytokine secretion. Int J Immunopharmacol 17: 475–480.

Pessi, T., Sutas, Y., Hurme, M., and Isolauri, E. (2000) Interleukin-10 generation in atopic children following oral Lactobacillus rhamnosus GG. Clin Exp Allergy 30: 1804–1808.

Pollorak, A., He, X., Smirnova, I., Liu, M.Y., Du Huffel, C.V.X., Birdwell, D., et al. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282: 2085–2088.

Raschke, W.C., Baird, S., Ralph, P., and Nakoinz, I. (1978) Functional macrophage cell lines transformed by Abelson leukemia virus. Cell 15: 261–267.

Reuter, G. (2001) The Lactobacillus and Bifidobacterium microflora of the human intestine: composition and succession. Curr Issues Intest Microbiol 2: 43–53.

Schwandner, R., Dziarski, R., Wescce, H., Rotte, M., and Kirschning, C.J. (1999) Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J Biol Chem 274: 17406–17409.

Shornikova, A.V., Casas, I.A., Mykkanen, H., Salo, E., and Vesikari, T. (1997) Bacteriotherapy with Lactobacillus reuteri in rotavirus gastroenteritis. Pediatr Infect Dis J 16: 1103–1107.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., et al. (1999) Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. Immunity 11: 443–451.

Tannock, G.W. (1997) Normal microbiota of the gastrointestinal tract of rodents. In Gastrointestinal Microbiology. Mackie, R.I., White, B.A., and Isaacson, R.E. (eds). New York: Chapman & Hall, pp. 187–215.

Tejada-Simon, M.V., Ustunol, Z., and Pestka, J.J. (1999) Effects of lactic acid bacteria ingestion of basal cytokine mRNA and immunoglobulin levels in the mouse. J Food Prot 62: 287–291.

von der Weid, T., Buliard, C., and Schiffrin, E.J. (2001) Induction by a lactic acid bacterium of a population of CD4 (+) T cells with low proliferative capacity that produce transforming growth factor beta and interleukin-10. Clin Diagn Laboratory Immunol 8: 695–701.

Wilson, M., Seymour, R., and Henderson, B. (1998) Bacterial perturbation of cytokine networks. Infect Immun 66: 2401–2409.