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RESEARCH ARTICLE

Induction of Probiotic Strain *Enterococcus faecium* EF1 on the Production of Cytokines, Superoxide Anion and Prostaglandin E_2 in a Macrophage Cell Line

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ABSTRACT

Immunological effects of probiotic strain *Enterococcus faecium* EF1 was evaluated through the production of cytokines, superoxide anion (O_2^-) and prostaglandin E_2 (PGE_2) in murine macrophage cell line RAW 264.7. In addition, the responses were contrasted with a pathogenic coliform, *Escherichia coli* strain K88. After 12-h co-incubation of RAW 264.7 with *E. faecium* EF1 or *E. coli* K88, the culture supernatants were collected for further analysis of cytokines (TNF-α, IFN-γ, IL-1β, IL-6, IL-12, IL-8 and IL-10) and inflammatory mediators including superoxide anion (O_2^-) and PGE_2. The results showed that, *E. faecium* EF1 induced an increased ($P<0.01$) release of TNF-α, IFN-γ, IL-6, IL-10 and O_2^- in RAW 264.7 cells. However, levels of cytokines induced by *E. faecium* EF1 were far lower ($P<0.01$) than those observed following co-culture with *E. coli* K88. In addition, when RAW 264.7 cells were first treated with *E. faecium* EF1 and then infected with *E. coli* K88, the production of IL-12 and O_2^- in response to *E. coli* K88 were significantly suppressed, indicating that *E. faecium* EF1 may limit the ability of macrophages to induce excessive inflammation even to potent inflammatory bacteria. Our current findings concluded that, *E. faecium* EF1 was capable of triggering a moderate innate inflammatory response on direct contact with murine macrophage cell line RAW264.7.

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INTRODUCTION

Macrophages are major immune cells in innate immune system and have ability to specifically respond to different antigens. They play an essential role in initiating an immune response by releasing an array of inflammatory cytokines and mediators, such as superoxide anion (O_2^-) and prostaglandin E_2 (PGE_2). Probiotics are "beneficial bacteria" usually containing lactic acid bacteria (LAB) including *Lactobacillus* spp., *Lactococcus* spp. or *Enterococcus* spp. strains (Tarasova *et al*., 2010). Immunological effects of LAB usually include the changes in secretion of immunoglobulins (Scharek *et al*., 2007), activation of macrophage activity and expression of cytokines, chemokines and other inflammatory factors. LAB isolated from kefir grain could up-regulate *in vitro* production of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-12 in RAW264.7 cells and murine peritoneal macrophages (Hong *et al*., 2009). Some LAB, such as *L. plantarum*, induced extracellular superoxide (O_2^-) from both, intestinal epithelial cells and underlying monocytes (Nissen *et al*., 2009). *E. faecium* is one of LAB with immune modulating properties. Previous studies have shown that oral administration of *E. faecium* L5 led to increase in expression of IL-10 and decrease in IL-8 expression in rats with dysbiosis (Tarasova *et al*., 2010) and examination of the pig small intestinal epithelial cells as well as macrophages cultured *in vitro* with *E. faecium* showed potent induction of pro-inflammatory cytokine IL-6 (Nissen *et al*., 2009). However, these effects have been proven as strain specific. Previously, we determined that oral administration of probiotic *E. faecium* EF1 could improve the balance of intestinal microflora, reduce the incidence of diarrhea (Huang *et al*., 2012) and modulate the immunological homeostasis in both, jejunal and ileal

mucosa of piglets during sucking period (Huang *et al*., 2012).

In this study, we used RAW264.7 cells, a widely used murine cell line with macrophage properties, as a model to evaluate the immunological effects of *E. faecium* EF1 on the production of cytokines (TNF-α, IFN-γ, IL-1β, IL-6, IL-12, IL-8, IL-10) and inflammatory mediators including $O_2^{\cdot-}$ and PGE_2; in addition, responses were compared against a pathogenic strain of *E. coli* K88.

MATERIALS AND METHODS

Bacterial strains and growth conditions: Probiotic *E. faecium* EF1 used for experiment was isolated and identified by the Institute of Feed Science, Zhejiang University. Strains were maintained at −80 °C in 10% (v/v) glycerol with de Man, Rogosa, and Sharpe (MRS) broth (Oxoid, England). Then *E. faecium* EF1 was cultured in MRS broth at 37 °C under anaerobic conditions to log-phase growth (16 h) without shaking. Pathogenic *E. coli* strain K88 was provided by China Veterinary Culture Collection (Beijing, China). The *E. coli* bacteria were cultured in Luria-Bertani (LB) (Oxoid, England) medium at 37 °C for 8 h before use. All bacterial cells were harvested by centrifugation (10 min at 6000 rpm), washed twice with sterile PBS buffer (pH 7.4) and re-suspended in the same buffer to a final concentration of 2×10^8 cfu/ml.

Growth and maintenance of cell cultures: The murine macrophage cell line RAW 264.7 (ATCC TIB71), obtained from Shanghai Institutes for Biological Sciences (Shanghai, China), was maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, USA), supplemented with 10% fetal calf serum (Gibco), penicillin (100 U/ml, Sigma-Aldrich, St. Louis, USA) and streptomycin (100 µg/ml, Sigma). Cell lines were routinely grown in 25 cm^2 cell culture flasks (Corning, USA) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air (Nissen *et al*., 2009). After overnight culture, viable cells were enumerated using trypan blue dye (0.5% Trypan-blue, Amersco, Solon, USA) in PBS (0.1 mM, pH 7.4) exclusion, and 2 ml aliquots containing 1×10^6 viable cells were seeded into each well of a 24-well tissue culture plates and allowed to adhere for 48 h at 37 °C with a 5% CO_2 to 95% air mixture in an incubator (Thermo Electron Corporation, USA). Spent culture medium was removed and the cell surfaces were washed with D-Hank's (Gibco), and then 0.5 ml fresh DMEM complete medium without antibiotics was added to each well of a 24-well plates immediately (Cross *et al*., 2004).

Co-culture of cells with bacterial strains: RAW 264.7 cells with different bacterial treatments were designated as group I, II, III, and IV, respectively. Group I served as control by adding 0.5 ml sterile PBS (pH 7.4) into wells of 24-well plates containing RAW 264.7 cells. Activation of cells was made by adding 0.5 ml of suspensions of *E. faecium* EF1 (group II) and *E. coli* K88 (group III) at 2×10^8 CFU/ml to 24-well plates. In group IV, RAW 264.7 cells were incubated for 1 h with *E. faecium* EF1. Then cells were extensively washed and incubated with *E. coli* K88 for an additional 11 h of incubation. Each group had six replicated wells. After 12 h of incubation, cell culture supernatants were collected from the wells and clarified by centrifugation and stored at −80°C for analysis of the cytokines, superoxide anion and prostaglandin E_2.

Measurement of cytokine levels: Supernatants were determined for the concentrations of tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-12 (IL-12), interleukin-10 (IL-10), interleukin-8 (IL-8) and prostaglandin E_2 (PGE_2) using the murine Enzyme-Linked Immunosorbent Assay Kit (ELISA Kit; R&D Systems, Inc.) according to the manufacturer's instructions. Superoxide anion ($O_2^{\cdot-}$) was determined using the detection kit provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Statistical analysis: Data were analyzed using the one-way analysis of variance procedure of SPSS 16.0 for Windows. Differences between treatments were detected with Fisher's least significant difference (LSD) test.

RESULTS

Comparative induction of cytokines through *E. faecium* EF1 and *E. coli* K88 in RAW 264.7 macrophages: *E. coli* K88 (group III) was found as a strong inducer of all of the tested cytokines, while *E. faecium* EF1 (group II) could only induce the release of TNF-α, IFN-γ and IL-10 when compared to the control (group I) ($P<0.01$) as shown in Figure 1. Moreover, the cytokine responses induced by *E. faecium* EF1 were at much lower levels than by *E. coli* K88. Findings showed that *E. faecium* EF1 and *E. coli* K88 induced a similar up-regulation of IL-6. However, *E. faecium* EF1 had no detectable effect on IL-8, IL-1β and IL-12 secretion, and manifested a reduced inflammatory response.

In group IV, RAW 264.7 cells were pre-treated with *E. faecium* EF1 for 1 h, and incubated with *E. coli* K88 for an additional 11 h. We found that, the secretion of TNF-α, IFN-γ, IL-8, IL-10, IL-1β by macrophages increased remarkably ($P<0.01$), while the secretion of IL-12 was noticeably suppressed ($P<0.01$).

Comparative induction of superoxide anion ($O_2^{\cdot-}$) and prostaglandin E_2 (PGE_2) through *E. faecium* EF1 and *E. coli* K88 in RAW 264.7 macrophages: We observed higher levels of $O_2^{\cdot-}$ in RAW 264.7 cells incubated with *E. faecium* EF1 (group II) and *E. coli* K88 (group III) (Figure 2). However, *E. faecium* EF1 was less potent in inducing $O_2^{\cdot-}$ release when compared to K88 (group III). Besides, the levels of $O_2^{\cdot-}$ in group IV were lower than those induced by *E. coli* K88 only, suggesting that *E. faecium* EF1 may down-regulate the ability of RAW 264.7 cells to generate oxidative burst in response to microorganisms.

E. faecium EF1 had no influence upon PGE_2 production in RAW 264.7 cells. In group III, PGE_2 was secreted at a level significantly ($P<0.01$) higher than control and *E. faecium* EF1-treated group. In addition, when cells were pre-treated with EF1, the PGE_2 levels in response to *E. coli* K88 were much higher ($P<0.01$) than in RAW 264.7 cells pre-treated only with medium (DMEM).

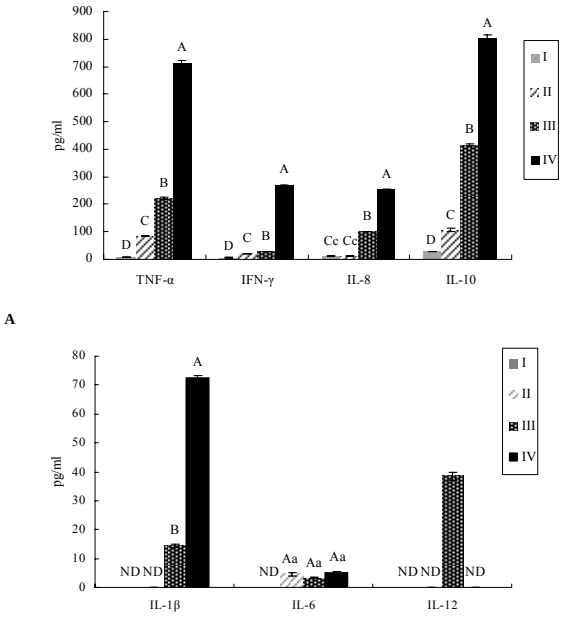

Fig. 1: Comparative cytokine production in four groups (I,II,III &IV) of bacteria-stimulated macrophage cell line (RAW 264.7) induced under *in vitro* condition. (a) TNF-α, IFN-γ, IL-8 and IL-10 and (b) IL-1β, IL-6 and IL-12. Values were expressed as mean±SD, n = 6. The error bars indicate standard deviations. Different capital letters indicate significant difference between groups for each cytokine (P<0.01); Different small letters indicate significant difference between groups for each cytokine (P<0.05); ND: Non-detectable.

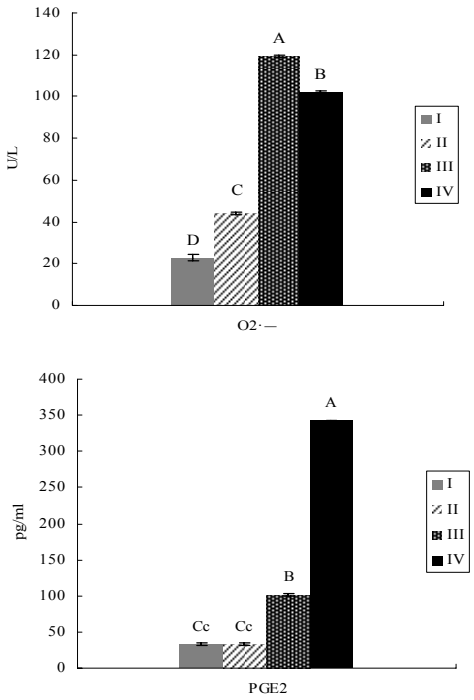

Fig. 2: Comparative superoxide anion (O_2^-) and prostaglandin E_2 (PGE_2) production in different groups of bacteria-stimulated macrophage cell line (RAW 264.7) induced under *in vitro* condition. (a) superoxide anion (O_2^-) and (b) prostaglandin E_2 (PGE_2). Values were expressed as mean±SD, n = 6. The error bars indicate standard deviations. Different capital letters indicate significant difference (P<0.01) between groups for O_2^- and PGE_2, respectively; Different small letters indicate significant difference (P<0.05) between groups for O_2^- and PGE_2, respectively.

DISCUSSION

Macrophages, specifically respond to different microbial stimuli, and release various cytokines and mediators which contribute to evoking and regression of inflammation. Thereby, in the present work, an *in vitro* approach was applied to study the immunological effects of *E. faecium* EF1 on production of cytokines (TNF-α, IFN-γ, IL-8, IL-10, IL-1β, IL-6 and IL-12) and inflammatory mediators (O_2^- and PGE_2) in a macrophage cell line RAW 264.7.

Comparative induction of cytokines through *E. faecium* EF1 and *E. coli* K88 in RAW 264.7 macrophages: Our results demonstrated that, *E. faecium* EF1 could induce an increased release of TNF-α and IFN-γ while leaving the release of IL-8 unchanged. TNF-α and IFN-γ are known as key pro-inflammatory cytokines considered to be important initiators of the inflammatory response in macrophages. Cross *et al*. (2004) demonstrated that *L. casei shirota* could readily induce the production of TNF-α by macrophage cell line J774A.1. Another study (Zoumpopoulou *et al*., 2008) showed that, *Lactobacillus plantarum* and *Streptococcus macedonicus* effectively induced the secretion of TNF-α and IFN-γ by human peripheral blood mononuclear cells. The induction of TNF-α and IFN-γ represents a direct illustration of immune stimulating properties of *E. faecium* EF1. Moreover, the stimulation of the immune system may improve the alertness of defensive system, thus preventing a subsequent strong inflammatory response.

The IL-8, a chemokine produced by macrophages and other cell types (Long *et al*., 2011), acts as a chemotactic factor that attracts and directs neutrophils to sites of inflammation (Brat *et al*., 2005). In our study, *E. faecium* EF1 induced a very low amount of IL-8 from macrophages. This correlates with the results of Cross *et al*. (2004), who previously reported that there was no significant change in levels of IL-8 secretion, following co-culture of lactobacilli with murine phagocytic cell lines.

In our case, while the pro-inflammatory cytokines were induced to a high level, it was accompanied by similarly strong induction of anti-inflammatory cytokine IL-10. The IL-10 is a potent down-regulator of the immune system as well as an activator of humoral immune responses by stimulating B cells (Groux and Cottrez, 2003). The up-regulation of IL-10 suggests *E. faecium* EF1 may play a homeostatic role by attenuating excessive inflammatory immune responses, confirming previous observations of inflammation suppressive function of *E. faecium* (Scharek *et al*., 2009). Conversely, it also suggests that this bacterium may exhibit adjuvant properties by activating humoral immune responses.

The IL-1β is a pro-inflammatory cytokine produced during an acute phase immune response to infection and inflammation (Hofstetter *et al*., 2007). It has been previously reported that *Lactobacillus reuteri* was capable of inducing noticeably higher levels of IL-1β from dendritic cells and mononuclear cells (Mohamadzadeh *et al*., 2005). Another pro-inflammatory cytokine is IL-12, which functions to induce a Th1-dominant immune response and is used as a potent adjuvant to enhance

cellular immunity (Afonso *et al*., 1994). IL-12 was previously demonstrated to be highly induced in macrophage cell lines stimulated with *L. casei shirota* (Cross *et al*., 2004), *Streptococcus thermophilus* and *Leuconostoc* strains (Kekkonen *et al*., 2008). However, it is well known that the effect of probiotic bacteria is strain dependent. Hong *et al*. (2009) failed to show that IL-12 was induced following co-incubation of *Lactobacillus kefiranofaciens* with RAW 264.7 cells. Our current findings showed that *E. faecium* EF1 had no detectable effect on IL-1β and IL-12 secretion, probably displaying a reduced inflammatory potential. It was possible that the secretion of IL-1β and IL-12 by macrophages occurred earlier than other cytokines. Furthermore, the half-life of cytokines is very short and the concentrations may be below detection limits (Hong *et al*., 2009).

The role of IL-6 is pivotal as signaling molecule leading either to pro- or anti-inflammatory response (Wang and Hasselgren, 2002). We showed that, after 12-h co-incubation of RAW 264.7 with *E. faecium* EF1, the levels of IL-6 were significantly higher than that in control group, which agrees with the results of previous reports of strong IL-6 up-regulation following co-culture of *E. faecium* with pig small intestinal epithelial cells and pig blood monocytes (Nissen *et al*., 2009). Hong *et al*. (2009) demonstrated that *Lactobacillus kefiranofaciens* had strong potential to induce production of IL-6 in RAW 264.7 cells.

E. faecium EF1 was indeed recognized by macrophages as evidenced by the increase in release of cytokines. However, levels of cytokines induced by *E. faecium* EF1 were far lower than those observed following co-culture of RAW 264.7 cells with *E. coli* K88. Our results indicated that, *E. faecium* EF1 might be less inflammatory and could initiate a more moderate inflammatory response than *E. coli* K88. Moreover, *E. faecium* EF1 affected the ability of RAW 264.7 cells to respond to strong immunogens *E. coli* K88. Pre-culture of RAW 264.7 cells with *E. faecium* EF1 was capable of markedly up-regulating production of TNF-α, IFN-γ, IL-8, IL-10 and IL-1β but decreasing IL-12 secretion following co-culture with *E. coli* K88. The down-regulation of IL-12 indicated that activated macrophages may become hyper-responsive to *E. coli* K88 on activation, thus suggesting that *E. faecium* EF1 may act on macrophages limiting their ability to induce inflammation even to potent inflammatory bacteria.

Comparative induction of superoxide anion (O_2^-) and prostaglandin E_2 (PGE_2) through *E. faecium* EF1 and *E. coli* K88 in RAW 264.7 macrophages: Prostaglandin E_2 (PGE_2), a pro-inflammatory mediator synthesized in substantial amounts at inflammatory sites (Giuliano and Warner, 2002), plays crucial role in regulation of various processes of acute and chronic inflammation. *Lactobacillus bulgaricus* increased PGE_2 production by Caco-2 cells (Takamura1 *et al*., 2011), while the probiotic mixture VSL#3 failed to induce PGE_2 secretion in intestinal epithelial cells (Otte *et al*., 2009). Current results demonstrated that *E. faecium* EF1 induced secretion of PGE_2 at lower levels than *E. coli* K88, consistent with low concentrations of IL-1β observed in RAW 264.7 cells. These results agree with the research of Franczak *et al*. (2010), who stated that the synthesis and secretion of PGE_2 might be stimulated by IL-1β.

Upon phagocytosis, macrophages can produce a burst of superoxide anion (O_2^-) and other reactive oxygen species to attack the unwelcome invading bacteria (Johnston *et al*., 1978). However, its uncontrolled release can also cause harm to the body. Thus, a balanced release of O_2^- is essential for intestinal homeostasis. Vesterlund *et al*. (2007) reported that probiotic lactobacilli induced low respiratory burst in peripheral blood mononucleocytes. Another study (Balcázar *et al*., 2007) reported that, superoxide anion production was significantly enhanced in kidney phagocytes from rainbow trout fed with *Lactobacillus sakei*. Our current findings demonstrated that *E. faecium* EF1 induced lower levels of superoxide anion (O_2^-) in macrophages when compared to *E. coli* K88. Moreover, when RAW 264.7 cells were pre-treated with *E. faecium* EF1, the superoxide anion (O_2^-) levels in response to *E. coli* K88 were much lower than cells treated only with *E. coli* K88. The results of present study indicated that, *E. faecium* EF1 might possess the ability to avoid excessive immune stimulation of respiratory burst in activated macrophages.

In summary, we demonstrated that *E. faecium* EF1 was capable of triggering a moderate innate inflammatory response upon direct contact with murine macrophage cell line RAW264.7 when compared to *E. coli* K88, but the mechanism needs to be determined more fully.

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