Immunology Letters 166 (2015) 6–12

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Divergent signaling pathways regulate IL-12 production induced by different species of Lactobacilli in human dendritic cells

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ARTICLE INFO

Article history:
Received 1 April 2015
Received in revised form 28 April 2015
Accepted 4 May 2015
Available online 11 May 2015

Keywords:
Lactobacillus reuteri
Dendritic cells
IL-12
MAP kinases
Probiotics

ABSTRACT

Recent studies have indicated that different strains of Lactobacilli differ in their ability to regulate IL-12 production by dendritic cells (DCs), as some strains are stronger inducer of IL-12 while other are not and can even inhibit IL-12 production stimulated by IL-12-inducer Lactobacilli.

In this report we demonstrate that *Lactobacillus reuteri* 5289, as previously described for other strains of *L. reuteri*, can inhibit DC production of IL-12 induced by *Lactobacilllus acidophilus* NCFM. Remarkably, *L. reuteri* 5289 was able to inhibit IL-12 production induced not only by Lactobacilli, as so far reported, but also by bacteria of different genera, including pathogens.

We investigated in human DCs the signal transduction pathways involved in the inhibition of IL-12 production induced by *L. reuteri* 5289, showing that this potential anti-inflammatory activity, which is also accompanied by an elevated IL-10 production, is associated to a prolonged phosphorilation of ERK1/2 MAP kinase pathway.

Improved understanding of the immune regulatory mechanisms exerted by Lactobacilli is crucial for a more precise employment of these commensal bacteria as probiotics in human immune-mediated pathologies, such as allergies or inflammatory bowel diseases.

1. Introduction

Intestinal balance requires that mucosal immune system is able to discriminate effectively between pathogenic and harmless inhabitants of the gut. The cross-talk between the mucosa-associated immune system and microbiota is critical in mucosal tissue homeostasis as well as in protection against infectious and inflammatory diseases occurring at mucosal sites [1]. In the gut there are two main subsets of DCs, conventional myeloid DC (mDCs) [2] and plasmacytoid DCs [3–5]; both subsets are involved in the regulation of the intestinal immune system. Dendritic cells (DCs) reside in an immature stage in peripheral tissues and are scattered throughout the gut mucosa. Sub-epithelial DCs can sample intestinal antigens by protruding their dendrites across epithelial tight junctions into the gut lumen or through direct interaction with bacteria that have gained access via M cells [6]. They use pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), to sense various pathogen-associated molecular patterns (PAMPs) and thus distinguish the different members of the microbiota [7–9]. Upon exposition of DCs to antigenic stimulus, they undergo a maturation process, in which the expression of co-stimulatory molecules and induction of cytokines take place. Thus, mature DCs migrate to secondary lymphoid organs through afferent lymph [10–12]; here DC activate the acquired immune response and dictate whether a Th1, Th2, Th17 or T-regulatory response is induced [13]. Many studies have revealed that intestinal DC can either be tolerogenic or immunogenic, depending on their type and state of activation. The discriminative factors in this respect are production of cytokines; mainly IL-12, IL-10, IL-23, IL-6, IL-1β, TNF-α, TGF-β as well as the expression of various maturation markers, CD40, CD54, CD80, CD83, CD86 and MHC complex [14,15]. When IL-12 production is induced in DCs, polarization is driven towards

Abbreviations: *L.*, *Lactobacillus*; DC, dendritic cell; iDCs, immature dendritic cells; mDCs, myeloid dendritic cells; LAB, lactic acid bacteria; Gram$^+$, Gram positive; Gram$^-$, Gram negative.

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http://dx.doi.org/10.1016/j.imlet.2015.05.001
0165-2478/

Th1 cells, while expression of IL-1β, IL-6, TGFβ and IL-23 leads to Th17 cells, and induction of IL-10 generates T-regulatory cells [16]. Conceptually, probiotics have been defined as live microorganisms, that, when administered in adequate amounts confer a health benefit on the host [17]. Several Lactobacilli and bifidobacteria are recognized as probiotics due to their health promoting effects, such as exclusion and inhibition of intestinal pathogens, increase of the gut barrier integrity, and modulation of the host immune system at local and systemic levels [18]. Clinical applications of lactic acid bacteria (LAB) include prevention and treatment of allergy in particular atopic dermatitis in children and inflammatory bowel diseases as well as prevention of viral infections [19]. Interestingly, recent studies have demonstrated that different strains of LAB posses the ability to finely regulate myeloid DCs maturation, polarizing the subsequent T cell activity toward Th1, Th2, or even Treg responses [15,20]. Moreover, also NK-DC crosstalk has been demonstrated to play a pivotal role in the immunomodulation by commensal bacteria [13,21]. Indeed, interactions between NK cells and DC are likely to occur in the gut-associate lymphoid tissue, where NK reside among intraepithelial lymphocytes [22].

However, despite great improvements in understanding interactions between probiotics and immune cells it remains unknown how they exert their immune-regulatory effects. To date, no clear picture of these properties on a genus, specie or strain level is available. Understanding the mechanisms beyond the immune homeostasis ensured by the gut microflora is of key importance to the elucidation of disease etiology and refinement of probiotic treatments for specific diseases. This study was specifically undertaken with the objective of assessing the immunomodulatory efficacy of *Lactobacillus reuteri* 5289 in human monocyte derived dendritic cells. DCs were exposed to various bacterial stimuli and then the effects of the simultaneous presence of IL-12 inducing bacteria such as *Lactobacillus acidophilus*, *Staphylococcus aureus*, *Pseudomonas aerugionosa* and an IL-12 weaker producer, i.e. *L. reuteri* 5289, were investigated by analysing the expression of pro- and anti-inflammatory cytokines, co-stimulatory molecules and signal transduction. We found that *L. reuteri* 5289, as previously observed with other strains of *L. reuteri*, is enabled with the capability of inducing IL-10 release by DCs and of inhibiting IL-12 production by DC upon stimulation by IL-12-inducer bacteria; remarkably, we observed that the inhibition of IL-12 production induced by *L. reuteri* was associated with prolonged ERK1/2 MAP kinase phosphorylation.

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. acidophilus NCFM and *L. reuteri* ATCC PTA 5289 were supplied by Pr. Lisbeth Nielsen (Center for Biological Sequence Analysis, Technical University of Lyngby, Denmark) while *Escherichia coli* 10536, *Pseudomonas aeruginosa* 25853 and *S. aureus* 43300 were provided by Dr. Angela Filocamo (Laboratory of Microbiology, University of Messina, Italy). The cultures were harvested at the stationary growth phase by centrifugation (5000 × *g*, 5 min) and washed twice in PBS. All bacteria were killed by 20 min exposure to UV light and stored at −80 °C. Killing was verified by plating the UV-exposed bacteria on nutritive agar plates (Merck, Darmstadt, Germany).

2.2. Generation of monocyte derived dendritic cells

Human PBMCs were obtained from heparinised blood of healthy volunteers using Ficoll method of separation based on density gradient, then dendritic cells were derived as previously described [23]. Briefly, adherence-isolated monocytes were cultured for 6 days in RPMI-1640 medium (Bio-Whittaker, Cambrex Company, Verriers, Belgium) containing 10% (v/v) heat-inactivated fetal calf serum (Sigma-Aldrich), penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (4 mM), and supplemented with 10^3 U/ml IFN-α and 25 ng/ml GM-CSF (Schering-Plough, Kenilworth, N.J.) as differentiation factor. After 3 days of incubation fresh medium containing IFN-α and granulocyte-macrophage colony-stimulating factor was added. After 6 days, the CD14 cells were differentiated into non-adherent immature DCs. Ninety to 95% of the cells expressed the DC markers.

2.3. Stimulation of dendritic cells with bacteria

Immature DCs were resuspended at the density of 5×10^5 cells/ml in fresh RPMI-1640 medium and seeded in 48-well tissue culture plates at a final volume of 500 μl/well. Pro-inflammatory bacteria (*L. acidophilus* NCFM, *E. coli* 10536, *P. aeruginosa* 25853 and *S. aureus* 43300) were added (at ratios 1/100 DC/bacteria) alone or in combination with *L. reuteri* 5289. The cell cultures were incubated for 48 h at 37 °C in 5% CO^2. LPS (*E. coli*; Sigma-Aldrich, St Louis, Mo) was used as positive control at a final concentration of 0.1 μg/ml.

2.4. Immunostaining and flow cytometry

DCs exposed to various treatments are harvested and washed twice in phosphate buffer solution (PBS). The following antibodies were used for staining: PE-conjugated anti-mouse CD80, FIITC-conjugated anti-mouse CD83 (BD Bioscience, San Jose, CA). Nonspecific binding is evaluated by matched isotype controls. DCs were analysed using a BD FACS array flow cytometer based on counting 10,000 cells. The level of expression is measured as the geometric mean of fluorescence (MFI).

2.5. Cytokine quantification in culture supernatants

After exposure of immature DCs to various conditions for 48 h, culture supernatants were harvested and stored at a temperature of −30 °C until use. The production of IL10 and IL12 (p70) was quantified using FlowCytomix multiple analyte detection system (E-Bioscience, San Diego, CA, USA) following manufacturer instructions.

2.6. MAPK phosporilation

The expression of p38 and ERK1/2 MAP kinase phosphorylation was estimated by flow cytometry using directed antibodies (BD Bioscience, San Jose, CA). Immature DCs were exposed to bacteria for different time points, then cells were fixed in 2% formaldehyde for 10 min at 37 °C. After washing, permeabilization of cell membrane was done with freshly prepared 90% cold methanol for 30 min on ice. Cells were washed twice in incubation buffer (PBS + BSA at 0.5%), labelled with antibodies anti-p38 and anti-ERK1/2 for 45mn, and washed afterwards in incubation buffer. The signal transduction cascade has been evaluated using a flow cytometer technology (FACSCanto II; Becton Dickinson, Moutanin View, CA).

2.7. Statistical analysis

Data are expressed as means ± SEM of three independent experiments. Student's matched pair *t*-test was used to compare means and P values lower than 0.05 were considered statistically significant.