Immunobiology 220 (2015) 701–710

Contents lists available at ScienceDirect

Immunobiology

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

Modulatory activity of *Lactobacillus rhamnosus* OLL2838 in a mouse model of intestinal immunopathology

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ARTICLE INFO

Article history:
Received 6 August 2014
Received in revised form 9 January 2015
Accepted 10 January 2015
Available online 19 January 2015

Keywords:
Enteropathy
Gluten
Immunomodulation
Lactobacilli
Transgenic mice

ABSTRACT

Gut microbiota and probiotic strains play an important role in oral tolerance by modulating regulatory and effector cell components of the immune system. We have previously described the ability of Lactobacilli to influence both the innate and adaptive immunity to wheat gluten, a food antigen, in mouse. In this study, we further explored the immunomodulatory mechanisms elicited in this model by testing three specific probiotic strains, namely *L. rhamnosus* OLL2838, *B. infantis* ATCC15697 and *S. thermophilus* Sfi39. *In vitro* analysis showed the all tested strains induced maturation of bone marrow derived dendritic cells (DCs). However, only *L. rhamnosus* induced appreciable levels of IL-10 and nitric oxide productions, whereas *S. thermophilus* essentially elicited IL-12 and TNF-α. The anti-inflammatory ability of OLL2838 was then tested *in vivo* by adopting mice that develop a gluten-specific enteropathy. This model is characterized by villus blunting, crypt hyperplasia, high levels of intestinal IFN-γ, increased cell apoptosis in lamina propria, and reduced intestinal total glutathione (GSHtot) and glutathione S-transferase (GST) activity. We found that, following administration of OLL2838, GSHtot and GST activity were enhanced, whereas caspase-3 activity was reduced. On the contrary, this probiotic strain failed in recovering the normal histology and further increased intestinal IFN-γ. Confocal microscopy revealed the inability of the probiotic strain to appropriately interact with enterocytes of the small intestine and with Peyer's patches in treated mice. In conclusion, these data highlighted the potential of *L. rhamnosus* OLL2838 to recover specific toxicity parameters induced by gluten in enteropathic mice through mechanisms that involve induction of low levels of reactive oxygen species (ROS).

Introduction

Diseases caused by a dysfunctional interaction between food and the body are as numerous as they are broad, ranging from gastrointestinal to respiratory. The system at the cornerstone of this interaction is the immune system. It is noteworthy that one of the most relevant areas of research currently being pursued is the concomitant effects of diet on the gut microbiota and on the immune system (Kau et al., 2011). In particular, it has been suggested that a healthy microbiota exists when there is a balance between symbionts, commensal organisms, and pathobionts. Alterations in this balance can lead to dysbiosis, which has been implicated in inflammatory, autoimmune, and allergic diseases (Rescigno, 2014). Importantly, gut microbiota appears to be a master regulator of immune equilibrium, an activity primarily conducted by Lactobacilli and Bifidobacteria.

The ability of the microbiota to influence APC responses in the gut was initially supported by studies demonstrating that bone marrow-derived dendritic cells (DCs) incubated with *Lactobacillus*

Abbreviations: APC, antigen presenting cell; CFSE, carboxyfluorescein succinimidyl ester; DCs, dendtitic cells; GSHtot, glutathione; GST, glutathione S-transferase; MLN, mesenteric lymph node; MLR, mixed leukocyte reaction; PP, Peyer's patches; ROS, reactive oxygen species.

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http://dx.doi.org/10.1016/j.imbio.2015.01.004
0171-2985/

species have enhanced ability to promote Treg cell differentiation *in vitro* and to prevent intestinal inflammation after adoptive transfer *in vivo* (Foligne et al., 2007a). This effect is dependent on Toll-like receptor (TLR) 2 and myeloid differentiation primary response gene 88 (MyD88) expression by DCs.

The current knowledge on the mechanisms by which the intestinal microbiota modulates both regulatory and effector components of the immune system underlines the importance of enterocyte and phagocyte interplay. In particular, factors released by intestinal epithelial cells (Iliev et al., 2009) influence the activity of $CD103^+$ DCs, critically involved in driving differentiation of intestinal Treg cells. Besides $CD103^+$ DCs, two populations of $CD11c^+$ macrophage/DC cells have been characterized in mouse, based on the expression of CX3CR1, a chemokine receptor. $CD11c^+$ $CX3CR1^{high}$ $F4/80^+$ macrophages produce IL-10 required to activate Treg (Rivollier et al., 2012). These cells are sessile, a characteristic conferred by gut microbiota, as they can migrate out of the intestine after antibiotic treatment (Diehl et al., 2013). By contrast, $CD11c^+$ $CX3CR1^{int}$ cells develop under intestinal inflammation, are able of migrating to the draining lymph node and activate inflammatory Th1 T cells (Rivollier et al., 2012). $CD11c^+$ $CX3CR1^{high}$ cells along with Peyer's patch DCs, continuously sample the intestinal lumen, elaborating tolerogenic signals. The precise mechanisms by which the intestinal microbiota can influence the development and function of APCs largely remain to be elucidated. More recently, microbial productions of butyrate and niacin have been associated to the induction of anti-inflammatory properties in phagocytes (Singh et al., 2014). In line with these data, we recently demonstrated the ability of metabolites secreted by *L. gasseri* to influence the crosstalk between enterocyte and DCs that may contribute to the beneficial effects of these bacteria in gut homeostasis (Luongo et al., 2013).

Dysbiosis appears to play a role also in food-mediated diseases like celiac disease (CD), a wheat gluten intolerance on an immune basis (Sanz et al., 2011). Therefore, the objective to ameliorate this inflammatory condition by restoring a normal microbiota in CD patients is worth pursuing. Our *in vivo* studies reported the ability of Lactobacilli to exert modulatory effects both on the innate and adaptive immunity in mice with gluten sensitivity (D'Arienzo et al., 2008, 2009). Very recently, we reproduced a gluten-specific enteropathy in a transgenic mouse model, characterized by activation of innate immunity (Mazzarella et al., 2014). Herein, we adopted this model to evaluate the anti-inflammatory properties of a specific *L. rhamnosus* strain, encoded OLL2838 (Miyauchi et al., 2009). The results of this study indicated that OLL2838 was effective in reducing the cytotoxic status but not the immune-mediated mucosal damage in enteropathic mice.

Materials and methods

Mice

Transgenic mice, which express the human HLA-DQ8 molecule, associated to gluten intolerance, in the absence of endogenous mouse class II (Cheng et al., 1996) were maintained in pathogen-free conditions at our animal facility (accreditation no. DM.161/99). Animals were reared on a gluten-free diet and used at the age of 6–12 weeks. These studies were approved by the national institutional review committee.

Bacterial strains and culture conditions

Lactobacillus rhamnosus (from human intestine) was deposited as strain OLL2838 in the Patent Microorganisms Depositary, National Institute of Technology and Evaluation, Japan. *Streptococcus thermophilus* Sfi39 (from a dairy product) and *Bifidobacterium longum* subsp. *infantis* ATCC15697 (from human intestine) were generous gifts from Prof. Ricca (University "Federico II", Naples) and Dr. Guglielmetti (DiSTAM, University of Milan), respectively. Based on their characteristics, the three strains are considered probiotic strains (Table 1) (Miyauchi et al., 2009; Matsumoto et al., 2002; Khalil, 2009). Working cultures were grown in de Man Rogosa Sharpe (MRS) broth (*L. rhamnosus*), M17 + 0.5% lactose (*S. thermophilus*) or MRS + 0.05% cysteine (*B. infantis*) for 24 h at 37 °C under micro-aerobic conditions. All media were from Difco (Difco Ltd., Detroit, MI, USA). The cell concentration of individual strains was evaluated by measuring the optical density at 600 nm and converting this value to the corresponding CFU ml^{-1} value. Fluorescent-labeled *L. rhamnosus* was prepared as described in Hiramatsu et al. (2011). Briefly, carboxyfluorescein succinimidyl ester (CFSE) dissolved in PBS to a final concentration of 50 μg/ml was added to the bacteria suspension (1×10^9 CFU/ml), and the mixture was allowed to react at 37 °C for 1 h in the dark. After extensive washing steps the bacterial cell suspension was used for oral administration.

Preparation of bone marrow derived DCs

DCs were generated according to a published method (Lutz et al., 1999). In brief, bone marrow cells from the femurs of 6–12 weeks old DQ8 mice were flushed and cell aliquots (2×10^6) were diluted in 10 ml of RPMI supplemented with 10% FCS and 20 ng/ml GM-CSF (culture medium) before to be seeded in 100-mm petri dishes (Falcon, Heidelberg, Germany). On day 3, 10 ml of culture medium were added and on day 7, 10 ml of the culture medium were replaced by freshly prepared medium. On day 8, non-adherent DCs were harvested by gently pipetting. Cell aliquots (1×10^6/ml) were then placed in 24-well plates and incubated in the presence of LPS and/or bacterial strains.

In vivo *treatments*

DQ8 transgenic mice were intragastrically administered 500 μg of gliadin on days 0, 3, 5 and 7. *L. rhamnosus* (1×10^{10} CFU/dose, four doses) was administered 4 days before or co-administered on the same day by os. A sub-enteropathic dose of indo (1.5 mg/100 ml; Sigma–Aldrich, Milan, Italy) was given in the drinking water and changed every 3–4 days. Mice were sacrificed on day 10. Mesenteric lymph nodes (MLNs) were isolated for assessment of IFN-γ levels. Small intestine fragments were collected for biochemical and immuno-morphometric measurements. In some experiments CFSE-labeled bacterial cells were administered orally at a dose of 1×10^9 CFU/mouse. Mice were then sacrificed over a time course (0, 2, 4, 6, and 24 h) after administration to remove Peyer's patches (PP) and small intestinal fragments for confocal microscopy.

Morphometry and confocal microscopy

Tissue fragments were placed on black filter paper and covered with normal saline, and their mucosal surfaces were immediately examined under a stereomicroscope. The tissue was then embedded in OCT compound (Tissue Tek, Miles Laboratories, Elkhart, IN, USA), snap-frozen in liquid nitrogen, and prepared for histological evaluation or fluorescence confocal microscopy. Cryostat sections (5 μm) were air-dried at room temperature and fixed in acetone. Sections were finally stained with Mayer's hematoxylin and mounted. The villus height and crypt depth were measured with an ocular micrometer. Twenty to 30 individual measurements were made on each slide, and average crypt depths and villus heights were calculated. For confocal microscopy, cryostat sections were repeatedly washed at room temperature in Tris buffered