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# Intragastric administration of *Lactobacillus casei* BL23 induces regulatory FoxP3+RORyt+ T cells subset in mice

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## RESEARCH ARTICLE

### Abstract

Many studies have highlighted the immunomodulatory properties of the probiotic strain *Lactobacillus casei* BL23. Recently, we demonstrated the ability of this strain to modulate the Th2-oriented immune response in a mouse model of cow's milk allergy based on the induction of a Th17-biased immune response. The probiotic function of *L. casei* has been also linked to gut-microbiota modifications which could be potentially involved in the immune regulation; however, its precise mechanism of action remains poorly understood. In this regard, recent studies suggest that gut microbiota induces a specific subset of CD4+FoxP3+ Treg cells that also express RORyt+, the specific transcription factor of Th17 cells. This new type of regulatory T cells, called type 3 Treg, displays suppressive function during intestinal inflammation, participating in inflammation control. We thus explored the ability of *L. casei* BL23 to specifically induce type 3 Treg cells, both *in vitro* and *in vivo*. Our results showed that intragastric administration of *L. casei* BL23 to mice induces local and systemic FoxP3+ RORyt+ type 3 Treg cells that could then participate in the beneficial effects of *L. casei* BL23 in different intestinal-related disorders.

**Keywords:** *Lactobacillus casei*, type 3 regulatory T cells, FoxP3, RORyt, immunomodulation

## 1. Introduction

Lactic acid bacteria (LAB) have been used for centuries for preserve and produce fermented foods. The most representative LAB species belong to *Lactobacillus* genus. Some LAB strains naturally colonise human mucosal surfaces such as gastrointestinal tract and vagina and are then considered part of the human microbiota (Walter, 2008). Several studies have demonstrated the immunomodulatory properties of different strains of lactobacilli (Foligne *et al.*, 2007; Kechaou *et al.*, 2013; Santos Rocha *et al.*, 2014). For instance, *Lactobacillus casei* BL23 represents a potential candidate strain for the treatment of intestinal-related disorders such as intestinal inflammation and food allergies (Del Carmen *et al.*, 2011; Hazebrouck *et al.*, 2009).

Accordingly, we previously demonstrated that intragastric administration of recombinant *L. casei* BL23 expressing a major cow's milk allergen partially corrects the Th2-

biased immune response in a mouse model of cow's milk allergy (Hazebrouck *et al.*, 2006, 2009). Additionally, oral treatment with the BL23 strain protects mice against 1,2-dimethylhydrazine (DMH)-induced colorectal cancer (Lenoir *et al.*, 2016). Interestingly, in both models, the immunomodulation was related to the induction of a fine-tuned Th17-oriented immune response, although the cellular mechanisms underlying lactobacillus-mediated protection are currently unclear (Lenoir *et al.*, 2016).

The plasticity of the CD4+ T cells is a well-documented fact. Upon environmental stimuli, CD4+ T cells differentiate into diverse subsets that express lineage-specific transcription factors, such as T-bet, GATA-3, RORyt and Foxp3 for Th1, Th2, Th17 and T regulatory cells (Treg), respectively (Weaver *et al.*, 2007). This capacity may hold the key to managing immune response properly; nevertheless, the decision of T cells to balance into either Th or Treg cells is strongly driven by cytokine signals from environment (Yang *et al.*, 2008; Zhou *et al.*, 2008). In an elegant study,

Ayyoub and coworkers found that a significant proportion of Tregs also express high levels of ROR $\gamma$ t (Ayyoub *et al.*, 2009). Lately, the ability of Treg double-positive FoxP3+ROR $\gamma$ t+ to suppress effector T cells was reported in a mouse model of inflammation (Tartar *et al.*, 2010). In this context, Ohnmacht and coworkers have recently shown that gut microbiota, could induce a specific subset of regulatory CD4+FoxP3+ T cells that also express the Th17 transcription factor, ROR $\gamma$ t (Ohnmacht *et al.*, 2015). These FoxP3+ROR $\gamma$ t+ double positive cells, designated as type 3 Treg cells, were shown to be involved in the regulation of type 2 immune responses and allergic reactions (Ohnmacht *et al.*, 2015). Lochner and coworkers also described the enhanced suppressive abilities of ROR $\gamma$ t+ Treg cells during intestinal inflammation (Lochner *et al.*, 2015). Considering the immunomodulatory properties of *L. casei* BL23, these findings encouraged us to investigate the ability of *L. casei* BL23 to induce this specific subset of Treg cells.

## 2. Experimental procedures

### Preparation of live bacterial inoculum

*L. casei* BL23 (Acedo-Félix *et al.*, 2003) was grown at 37 °C on De Man, Rogosa and Sharpe (MRS; Difco, BD, Le Pont de Claix, France) agar plates. A single colony was selected and grown in 5ml of MRS broth at 37 °C for 18 h in closed tubes, without agitation, as previously described (Hazebrouck *et al.*, 2007). A 100  $\mu$ l aliquot was then transferred into 10 ml of fresh MRS broth and incubated at 37 °C until an optical density at 600 nm of 1 was reached. Bacterial cultures were then centrifuged and washed three times with phosphate buffered saline (PBS). For *in vitro* assays, bacterial concentration was adjusted at  $1 \times 10^7$  cfu/ml in RPMI medium supplemented with 10% foetal calf serum (FCS) and 2 mM L-glutamine. To perform *in vivo* experiments, bacteria were resuspended in saline buffer (NaCl 0.9%) at 1 $\times$  the volume of the pellet sediment collected, which resulted in a concentration of  $\sim 5 \times 10^{10}$  cells/ml, as described (Hazebrouck *et al.*, 2009).

### Splenocytes isolation and *in vitro* co-incubation with *Lactobacillus casei*

Spleens were recovered from six naïve BALB/cJ mice (five-week-old; Centre Elevage René Janvier, Le Genest-Saint-Isle, France) as previously described (Lenoir *et al.*, 2016). Briefly, splenocytes were recovered by mechanical disruption using gentleMACS<sup>®</sup> Dissociator (Miltenyi Biotec GmbH, Germany). After red blood cell lysis (180 mM NH<sub>4</sub>Cl, 17 mM Na<sub>2</sub>EDTA), spleen cells were recovered and pooled in RPMI medium supplemented with 10% FCS and 2 mM L-glutamine. The splenocytes were then co-incubated with *L. casei* BL23 for 1 h (at 37 °C and 10% of CO<sub>2</sub>), using a ratio of 10 bacteria per splenic cell. After co-incubation, splenocytes were centrifuged and washed twice with PBS,

in order to remove bacteria from the culture. Recovered spleen cells were resuspended in supplemented RPMI containing antibiotics (50  $\mu$ g/ml of gentamicin, 100 units/ml of penicillin and 100  $\mu$ g/ml of streptomycin), and cell culture was pursued for 4 days. The co-incubation assays were performed at least in triplicate. A mock control was prepared in a similar fashion without bacteria (untreated splenocytes). On day 4, spleen cells were centrifuged and collected for cell phenotype analyses by flow cytometry.

### *In vivo* experiments

All animal experiments were performed according to European Community rules of animal care and with the authorisation N° 91-368 368 of the French Veterinary Services. Experiments were covered by agreement No. 2009-DSV-074 from the Veterinary Inspection Department of Essonne (France). Three-week-old female BALB/cJ (CERJ) were housed under pathogen-free conditions in the animal facility of the Institute of Biology and Technology (iBiTeCS, CEA-Saclay, France). The immune response triggered by the intragastric administration of bacteria was evaluated as previously described (Hazebrouck *et al.*, 2009). Mice were intragastrically administered with *L. casei* BL23 ( $\sim 1.5 \times 10^8$  cfu/mouse/administration; n=8) or PBS as a control (n=8) for five consecutive days. Three days after the last administration, animals were sacrificed by cervical dislocation. Cells were recovered from the spleen, mesenteric lymph nodes (MLN) and Peyer's patches (PP) by mechanical disruption as previously reported (Adel-Patient *et al.*, 2011). The isolation of lamina propria (LP) cells was performed as described (Bernink *et al.*, 2013), with some modifications. Briefly, after spleen and MLN collection, the small intestine was removed and fat cleared. The intestinal content was removed by PBS flushing, PP were recovered and the small intestine was then opened and cut into small pieces. In order to eliminate epithelial cells, intestinal sections were incubated three times in a solution of Hank's balanced salt solution (without Ca<sup>2+</sup> and Mg<sup>2+</sup>) containing 2mM of EDTA and 10 mM of HEPES (15 min, 37 °C and 250 rpm). The remaining tissues were then digested with a solution of RPMI containing 10 mM of HEPES, 25  $\mu$ g/ml collagenase (Libérase, Roche, Sigma-Aldrich) and 10 U/ml of DNase I (Roche, Sigma-Aldrich) (45 min, 37 °C and 250 rpm). LP cells were recovered from digested tissue by mechanical disruption using the gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells from spleen, MLN and PP were then phenotyped by flow cytometry. Each mouse was treated individually.

### Phenotyping of T cells populations

All cells were recovered in a solution of PBS containing 2% FCS and 1 mM EDTA, and afterwards filtered through a 70-100  $\mu$ m cell strainer. Fc receptors were blocked using anti-CD16/CD32 (clone 2.4G2, BD Pharmingen, Franklin

Lakes, NJ, USA). T cell populations were analysed using the following antibodies: anti-CD4-FITC (clone GK1.5, BD Pharmingen), anti-CD4-PE-CY7 (clone GK1.5, eBioscience, San Diego, CA, USA), anti-CD4-BV785 (clone RM4-5, Biolegend, San Diego, CA, USA), anti-FoxP3-PE (Miltenyi Biotech) and anti-RORyt-FITC (clone 46419, Imgenex, San Diego, CA, USA), anti-IL-17A-PerCP-Cy5.5 (clone eBio17B7, eBioscience). Approximately 70,000 cells were acquired using a NovoCyte flow cytometer (ACEA Bioscience, Inc., San Diego, CA, USA), and in some experiments, a Guava EasyCyte Plus cytometer (GuavaTechnologies, Hayward, CA, USA) was used. Analyses were performed using NovoExpress™ software (Version 1.0.0, ACEA Biosciences, Inc.). Samples were first inspected in all light scatter patterns and fluorescence channels to confirm quality, and abnormal populations were excluded. Unlabelled processed samples were used for reporting the percentage of positive cells (Johansson *et al.*, 2014). Lymphocytes were typed as follows: Treg were defined as CD4+FoxP3+; Th17 as CD4+RORyt+ and type 3 Treg as FoxP3+RORyt+ within CD4+ population (Ohnmacht *et al.*, 2015). Frequency of interleukin (IL)-17 producing cells within the CD4+ cells was also measured (CD4+IL17+ cells).

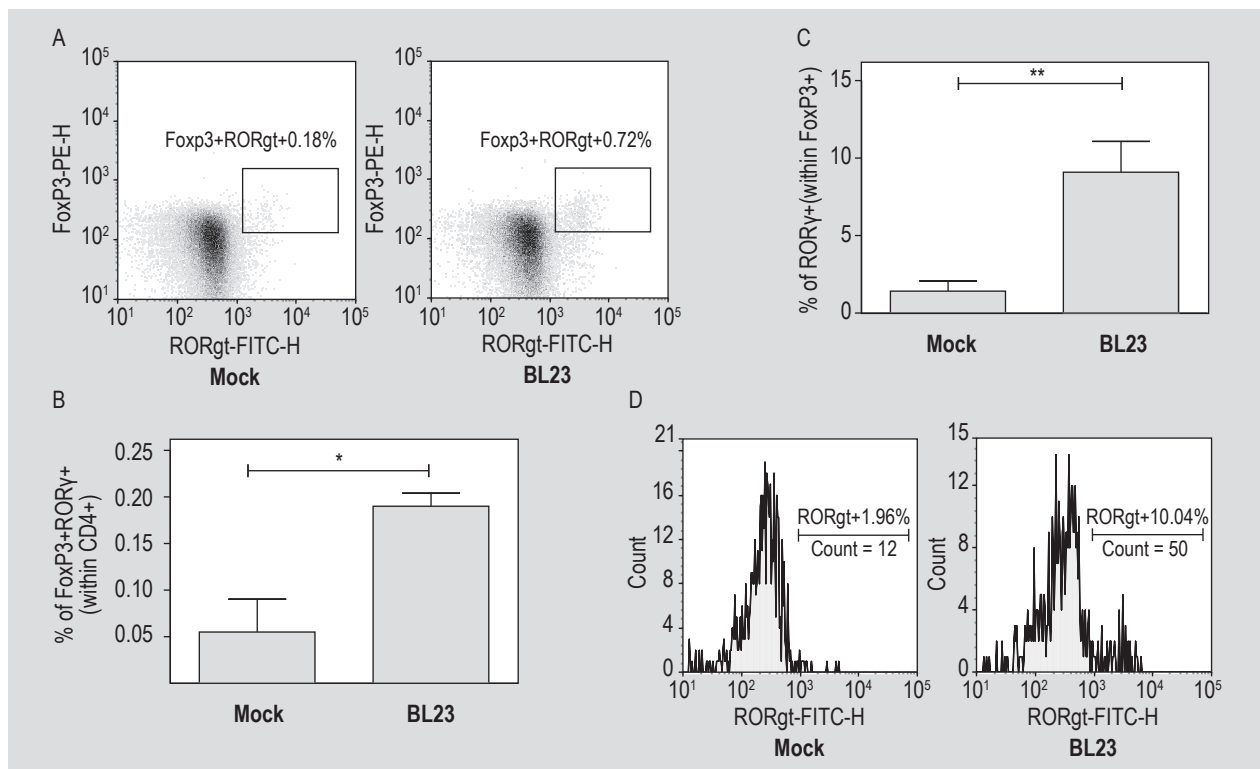
### Statistical analysis

Data analysis was performed using the GraphPad Prism V.5.01 (GraphPad, La Jolla, CA, USA). Experiments were analysed using an unpaired *t* test. The two-tailed unpaired Mann-Whitney test was used to evaluate differences between two groups. In all experiments, a value of  $P < 0.05$  was considered significant (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ ).

## 3. Results

### Co-incubation of splenocytes with *Lactobacillus casei* BL23 enhanced frequency of FoxP3+RORyt+ double positive T cells

The induction of FoxP3+RORyt+ double positive T cells by *L. casei* BL23 was first evaluated by an *in vitro* approach using splenocytes from naïve mice. The frequency of FoxP3+RORyt+ cells was initially assessed within the whole population of singlet live cells. As shown in Figure 1A, the interaction of splenocytes with *L. casei* BL23 led to a significant increase of FoxP3+RORyt+ double positive cells ( $P < 0.05$ ). We then analysed the frequency of FoxP3+RORyt+ cells within CD4+ cells. Although, the frequency of this Treg



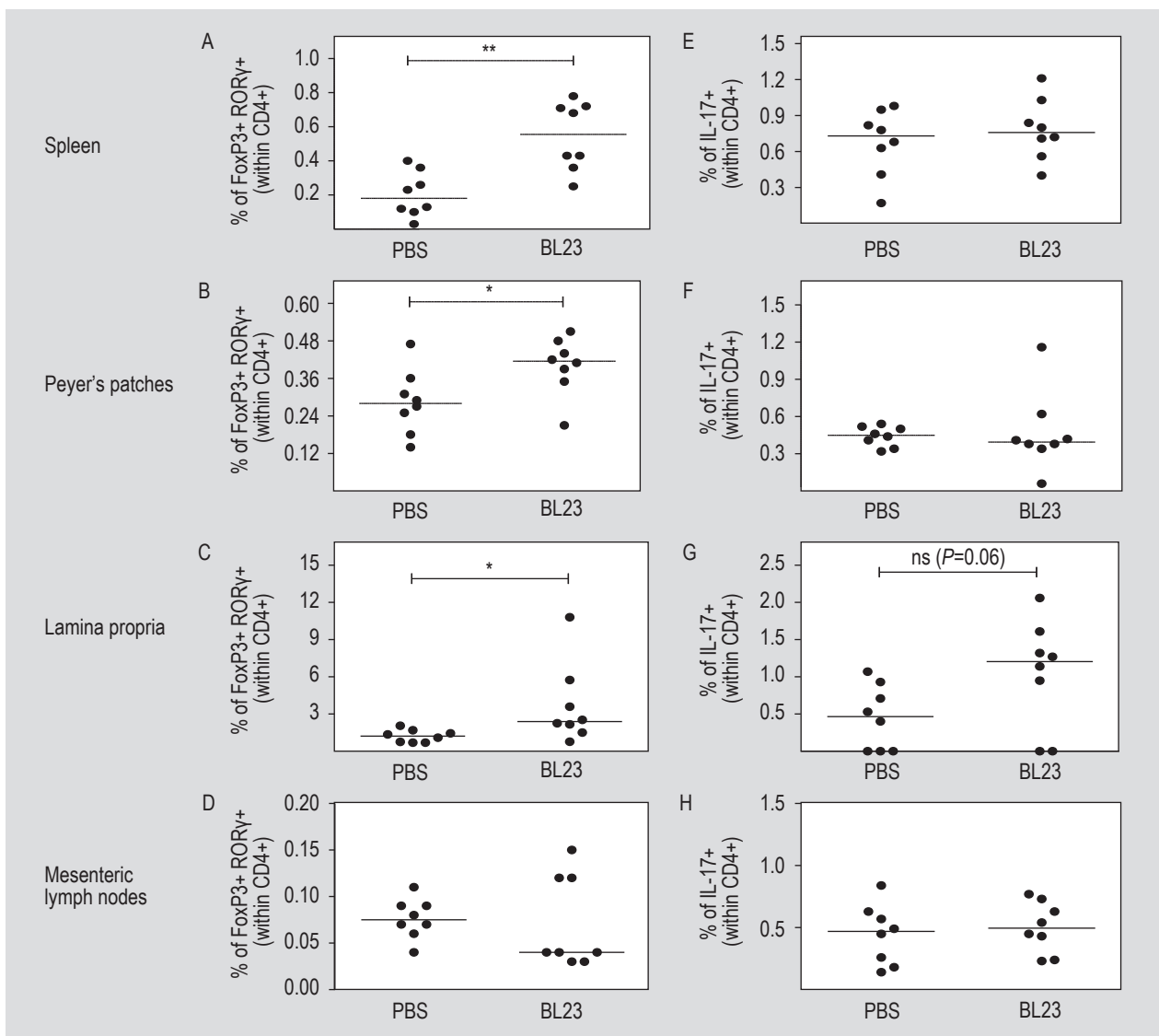
**Figure 1.** Analysis of regulatory T cells induced by *Lactobacillus casei* BL23 *in vitro*. Gating strategies are shown in Supplementary Figure S1. (A) Representative dot plot showing the frequency of FoxP3+RORyt+ double-positive cells within whole single-gated cells. (B) Type 3 Treg cells were defined as percent of double-positive FoxP3+ RORyt+ cells within CD4+ population. (C) Expression of RORyt+ within the CD4+FoxP3+-gated cells. (D) Histogram showing the frequency of RORyt+ within FoxP3+ cells. Results (mean  $\pm$  standard deviation) are representative of at least two independent experiments performed in triplicate. Unpaired *t* test was used to compare data, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

subset was very low within CD4+ cells population (mean  $0.1\% \pm 0.01$  standard deviation), we demonstrated a 3-fold increase of this population after interaction with *L. casei* ( $P < 0.05$ , Figure 1B). To confirm this observation we also analysed the expression of RORyt+ within the FoxP3+ cells (Figure 1C and 1D). Actually, *L. casei* BL23 enhanced the expression of RORyt+ within the FoxP3+ cells ( $P < 0.01$ ).

**Intragastric administration of *Lactobacillus casei* BL23 positively modulates type-3 Treg cells**

We then explored the *in vivo* effect of intragastric administration of *L. casei* BL23 on the equilibrium of Treg/Th17 cells. Mice (n=8/group) were treated for

five consecutive days with *L. casei* BL23 or PBS. The distribution of CD4+ cells, as well as that of FoxP3+ or RORyt+ cells within the CD4+ population was not affected by *L. casei* administration in any of the lymphoid organ studied (Supplementary Figure S3). Conversely, within the CD4+ population, a statistically significant increase in FoxP3+RORyt+ double positive cells was observed in the spleen ( $P < 0.01$ ), PP ( $P < 0.05$ ), and LP ( $P < 0.01$ ) of mice treated with *L. casei* BL23 compared with the control group (Figure 2A-C). Furthermore, a concomitant trend to the reduction of this cell subset was observed in MLN in five out eight mice) (Figure 2D). Conversely, the production of IL-17 by CD4+ T cells was not significantly affected after the intragastric administration of *L. casei* BL23 (Figure 2E-



**Figure 2. Effect of intragastric administration of *Lactobacillus casei* BL23 on the Th17 and T regulatory FoxP3+ RORyt+ cell populations cells from splenocytes, Peyer's patches, lamina propria and mesenteric lymph nodes. (A-D) Percentage of double-positive FoxP3+ RORyt+ cells within CD4+ population and (E-H) percentage of interleukin (IL)-17+ cells within CD4+ T cells. Each data point represents one individual mouse and lines represent the median values. The Mann-Whitney test was used for statistical analyses. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .**

H), although a tendency ( $P=0.06$ ) to increase was observed in the LP. This thus suggests that classical Th17 cells were not induced by *L. casei* administration.

#### 4. Discussion

Different studies have reported the beneficial properties of some bacteria, thus considered as probiotic. However, even if some underlying mechanisms have been evoked (for a review, see Van Baarlen *et al.*, 2013), little is known about the immune mechanisms engaged by 'good' bacteria to positively affect the host immune system. In this context, Ohnmacht and colleagues have elucidated a potential mechanism induced by the gut microbiota and regulating type 2 immune response. This mechanism involves the induction of a new Treg subset designated as type 3 Treg (Ohnmacht *et al.*, 2015). Alongside suppressive and anti-inflammatory features, the particularity of this novel T subset is the concomitant expression of FoxP3, the transcription factor associated to Treg cells, and RORγt+, the transcription factor associated to Th17 cells (Lochner *et al.*, 2015). Accordingly, our results showed evidence that *L. casei* BL23 increases the frequency of type 3 Treg both *in vitro* and *in vivo*. The induction of this Treg population could explain, at least in part, the immunomodulatory capacity already demonstrated for *L. casei* BL23. Further studies are underway to confirm this hypothesis, notably their role in the protective effects of *L. casei* BL23 on different pathologies, such as intestinal inflammation or food allergies.

#### Supplementary material

**Supplementary material** can be found online at <https://doi.org/10.3920/BM2016.0174>.

**Figure S1.** Gating strategy used to assess the frequency of type 3 RORγt+ Treg induced after *in vitro* stimulation of splenocytes with *Lactobacillus casei*.

**Figure S2.** Gating strategy used to assess cellular population in mice orally administrated with *Lactobacillus casei*.

**Figure S3.** Influence of oral administration of *Lactobacillus casei* BL23 on cellular responses (T cells, regulatory T cells and Th17 cells).

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#### Conflict of interest

The authors declare that they have no conflict of interest.

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