Influence of the route of administration on immunomodulatory properties of bovine beta-lactoglobulin-producing Lactobacillus casei

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1   **Abstract**

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3   Because of their intrinsic immunomodulatory properties, some lactic acid bacteria were

4   reported to modulate allergic immune responses in mice and humans. We recently developed

5   recombinant strains of *Lactobacillus casei* that produce β-Lactoglobulin (BLG), a major

6   cow's milk allergen. Here, we investigated immunomodulatory potency of intranasal and oral

7   administrations of recombinant *lactobacilli* on a subsequent sensitization of mice to BLG.

8   Intranasal administration of the BLG-producing *Lb. casei* stimulated serum BLG-specific

9   IgG2a and IgG1 responses, and fecal IgA response as well, but did not inhibit BLG-specific

10   IgE production. In contrast, oral administration led to a significant inhibition of BLG-specific

11   IgE production while IgG1 and IgG2a responses were not stimulated. After both oral and

12   intranasal administrations, production of IL-17 cytokine by BLG-reactivated splenocytes was

13   similarly enhanced, thus confirming the adjuvant effect of the *Lb. casei* strain. However, a

14   mixed Th1/Th2-cell response was evidenced in BLG-reactivated splenocytes from mice

15   intranasally pretreated, with enhanced secretions of Th1 cytokines (IFN-γ and IL-12) and Th2

16   cytokines (IL-4 and IL-5) whereas only production of Th1 cytokines, but not Th2-cytokines,

17   was enhanced in BLG-reactivated splenocytes from mice orally pretreated. Our results show

18   that the mode of administration of live bacteria may be critical for their immunomodulatory

19   effects.

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1   **Introduction**

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3   Cow's milk allergy (CMA) is the most common food allergy in early infancy [1].

4   Type I allergy generally corresponds to an inappropriate immune response characterized by a

5   disruption of the Th1/Th2 balance toward a Th2 profile with production of interleukin(IL)-4

6   and IL-5, and production of immunoglobulin (Ig)-E specific of allergens.. In milk allergic

7   children, ß-lactoglobulin (BLG), the most abundant whey protein, and casein induce the

8   highest specific IgE-response [2;3]. Although most patients outgrow CMA by 3 years of age,

9   IgE-mediated reactions to cow's milk proteins increase the risk for developing persistent

10 CMA and other atopies, such as asthma, atopic eczema, or egg allergy [4].

11   It has been recently suggested that a reduced microbial exposure in industrialized

12 countries could partially explain the increasing prevalence of allergic diseases [5;6]. On the

13 ground of the "hygiene hypothesis", two interpretations are currently proposed, based either

14 (i) on a lack of shifting of allergen-specific responses from the initial Th2 phenotype specific

15 of the neonatal immune response, toward the Th1 profile or (ii) on an impaired immune

16 suppression because of a reduced activity of T regulatory cells [5]. Comparisons of early

17 intestinal microbiota from allergic and healthy children have thus revealed a less prominent

18 colonization by lactobacilli or bifidobacteria in the allergic group [7;8]. As Th1 and Th2

19 responses inhibit each other's development [9], some strategies for preventing and modulating

20 allergic response consist in the mucosal delivery of food antigen by lactic acid bacteria (LAB)

21 in order to shift the allergen-specific Th2 response toward a more balanced Th1/Th2 profile,

22 [10]. In this regard, non-pathogenic, non-invasive and non-colonizing gram-positive LAB,

23 that exhibit intrinsic Th1-promoting effects or immunosuppressive properties against

24 inappropriate immune responses, provide attractive delivery systems.

1   Among the different LAB that are frequently used for the mucosal delivery of

2   therapeutic proteins, lactococci and lactobacilli display distinct properties in survival and

3   persistence in the digestive tract. *Lactobacillus casei* exhibit an optimal growth temperature

4   of 37°C and a high resistance to the gastric environment while *Lactococcus lactis* prefers a

5   growth temperature of 30°C and is more rapidly lysed in the digestive tract [11]. Viability of

6   the bacteria may be important since live bacterial vectors have been described to be more

7   effective than inactivated ones for the *in situ* delivery of therapeutic proteins to the intestinal

8   mucosa [12;13]. Moreover, lactobacilli, such as *Lb. plantarum*, have been described to be

9   more immunogenic than *Lc. lactis* [14;15]. In addition to physiological properties,

10   immunomodulatory capacities are also strain-specific. In a recent work, *Lc. lactis* MG1363

11   has been shown to exhibit a slightly pro-inflammatory profile, in an *in vitro* PBMC-based

12   assay, by inducing relatively high levels of the inflammatory cytokine IL-12 and low levels of

13   the anti-inflammatory cytokine IL-10. In contrast, the *Lactobacillus casei* BL23 strain has

14   been associated with an anti-inflammatory profile that has been correlated with protective

15   effects in a mouse model of acute colitis [16]. In a mouse model of experimental sensitization

16   to BLG, we previously showed that oral administrations of BLG-producing lactococci to mice

17   induced an immune response that partially prevented the development of a BLG-specific IgE

18   response [17;18]. This preventive effect was attributed to the Th1-adjuvant properties of *Lc.*

19   *lactis* to induce a specific Th1 response down-regulating a further Th2 one. Because strain-

20   specific properties can be used to promote different modulation of humoral or cellular

21   responses, we investigated whether anti-inflammatory properties of the BL23 strain could also

22   counter-regulate or suppress the development of an allergic-type sensitization.

23   For this purpose, BLG-producing BL23 *lactobacilli* have been generated and BLG-

24   production has been improved by fusing the BLG protein to a secretion signal peptide and

25   carrier proteins and by optimizing the induction protocol of BLG production [19]. We then

1   investigated the ability of the BLG-producing *Lb. casei* (LC$_{BLG}$) to modulate immune

2   response in a mouse model of allergic sensitization to BLG. As the route of administration

3   was described to affect the development of an immune response, the present study also aimed

4   to further evaluate the effect of administrations of recombinant *Lb. casei* provided through

5   oral and intranasal routes. We also compared the immunomodulatory properties of LC$_{BLG}$

6   with co-delivery of *Lb. casei* and purified BLG (LC+BLG) in order to determine the influence

7   of *in situ* production of BLG.

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1   **Materials and Methods**

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3   **Media and reagents.** *Lb. casei* was grown at 37°C in MRS broth (Difco, BD, Le Pont

4   de Claix, France). When required, erythromycin and chloramphenicol (5 µg/mL) were added.

5   For promoter induction, nisin (Sigma, St Louis, MO, USA) was added at a final concentration

6   of 25 ng/mL. BLG was purified from cow's milk as previously described [3]. Endotoxin

7   levels in BLG preparations were below 0.1 EU/mg (QCL-1000 kit, Lonza Walkersville, Inc.,

8   MD, USA).

9   **Animals.** Female BALB/c mice were purchased from CERJ (Le Genest Saint-Isle,

10   France), and were housed under normal husbandry conditions, with a diet deprived of milk

11   proteins. Seven weeks old mice were used. All experiments were performed accordingly to

12   European Community rules of animal care, and with authorization 91-244 of the French

13   Veterinary Services.

14   **Preparation of live bacterial inocula.** Overnight cultures of *Lb. casei* strains were

15   centrifuged (8,000 x g, 10 min, 20°C) and cell pellets were resuspended in fresh medium.

16   After 1h30 at 37°C, nisin was added (25 ng/mL). After 2h at 37°C, *Lb. casei* cultures were

17   centrifuged (8,000 x g, 10 min, 20°C). Cell pellets were washed once and resuspended in one

18   volume of saline buffer (NaCl 0.9%), giving a concentration around 5×10^{10} cells/mL.

19   Amount of recombinant BLG was quantified by enzyme-linked immunosorbent assay [20]

20   and was evaluated to be about 109 ± 34 µg/mL of bacterial inoculum ($\pm$ standard deviation, n

21   = 10). As described in a previous work, the produced BLG is mainly located intracellularly

22   [19].

23   **Mucosal pretreatments.** Groups of 7 mice received intragastrically (300 µL of

24   bacterial inoculum) or intranasally (10 µL of bacterial inoculum), the control strain (LC,

25   BL23(*int:nisRK*) transformed with the plasmid pVE3655), the recombinant *Lb. casei* (LC$_{BLG}$,

1   BL23(*int*:*nisRK*) transformed with the plasmid pSEC:LEISS-Nuc-BLG [19]), the control

2   strain mixed with purified BLG (166 µg/mL of bacterial inoculum, LC+BLG), or saline

3   buffer (Saline). Oral and intranasal pretreatments were administered twice, for five

4   consecutive days, on days 1 to 5 and on days 22 to 26. For intranasal delivery, a short and

5   light anesthesia of the mice was achieved with isoflurane (AErrane, Baxter S.A., Lessines,

6   Belgique) and preparations were delivered into the nares using a micropipette. Mice were then

7   sensitized by intraperitoneal (i.p.) injections of 5 µg of BLG emulsified with incomplete

8   Freund's adjuvant (IFA, Difco Laboratories, Detroit, MI, USA) on day 33. A group of 7 naive

9   mice were left untreated and unsensitized. Blood samples and fresh feces were collected on

10   day 30 to evaluate the immunogenicity of the *Lb. casei* preparations and on day 50 in order to

11   measure immunomodulation of the primary response after i.p. sensitization to BLG. Mice

12   received a second i.p. injection on day 54 and on day 72, spleens were collected for *in vitro*

13   reactivation with BLG.

14   **Quantification of BLG-specific serum antibodies.** Blood samples were collected

15   from the retro-orbital venous plexus and sera were stored at -20°C until further assays. BLG-

16   specific IgG1, IgG2a and IgE were measured as previously described [18;21].

17   **Quantification of BLG-specific IgA in fecal extracts.** BLG-specific IgA secretion

18   was monitored in pooled fecal extracts from each group of mice. Fresh fecal pellets were

19   added to PBS containing 50 µg/mL bacitracin, 300 µg/mL benzamidin, 80 µg/mL leupeptin,

20   20 µg/mL chymostatin, 25 µg/mL pepstatin and 200 µM phenylmethylsulfonyl fluoride

21   (Sigma), and incubated on rotary shaker for 4h at 4°C. Suspensions were then centrifuged at

22   $15,000 \times g$ for 10 min at 4°C. The supernatant was collected and total protein concentration

23   was determined by the BCA protein assay (Pierce). Fecal samples (0.1 mg protein/mL),

24   prepared in EIA buffer (0.1 M phosphate buffer, 0.1% BSA, 0.15 M NaCl, 0.01% sodium

25   azide, 0.1% Tween) were incubated on plates coated with 5 µg/mL BLG. Specific IgA were

1   detected using a goat polyclonal serum anti-mouse IgA (Southern Biotechnology Associates,

2   Birmingham, AL, USA) labelled with acetylcholinesterase [22].

3   **Cytokine production.** Spleens were harvested and pooled in RPMI-10 (RPMI 1640

4   medium supplemented with 10% fetal calf serum, 2 mM L-Glutamine, 100 U penicillin, 100

5   µg/mL streptomycin). After lysis of red blood cells (180 mM NH_4Cl, 17 mM Na_2EDTA) and

6   several washes, splenocytes were resuspended in RPMI-10. Cells were incubated for 60 h at

7   37°C (5% CO_2) in 96-well culture plates (in quadruplicate at 10^6 cells/well) in the presence of

8   BLG (20 µg/mL) or ovalbumin (20 µg/mL, negative control). After incubation, culture plates

9   were centrifuged and supernatants were collected and stored at - 80°C until further assay.

10   Cytokine levels were analyzed using the Bio-plex multiple cytokine assay system

11   according to the manufacturer's recommendations (BioRad, Hercules, CA, USA). TGF-β was

12   assayed using CytoSets^{TM} kit (Biosource International Europe, Nivelles, Belgium). Results

13   are expressed in pg/mL after subtraction of baseline levels determined in ovalbumin-

14   stimulated cultures.

15   **Statistical analyses.** Data were analyzed using the non-parametric Mann-Whitney test

16   to compare the different treatments with the saline control. Statistical analyses were

17   performed with GraphPad Prism 5.01 software and a p< 0.05 was considered significant (*

18   P<0.05, ** P<0.01 and *** P<0.001).

19

1   **Results**

2

3   ***Immunogenicity of the recombinant Lb. casei.*** Intranasal administration of the mix

4   LC+BLG led to a significant production of serum BLG-specific IgG1 (64 ± 18 µg/mL,

5   p<0.001) and IgG2a (3.8 ± 1.8 µg/mL, p<0.001) while oral administration of LC+BLG did

6   not induce significant production of BLG-specific IgG1 and IgG2a. No BLG-specific IgG

7   were detected in sera from mice pretreated with the saline solution, with *Lb. casei* alone or

8   with LC$_{BLG}$. We did not detect any secretion of BLG-specific IgA in fecal samples from any

9   group of mice.

10

11   ***BLG-specific antibody responses in sera after sensitization to BLG***. Intranasal and

12   oral pretreatments with LC$_{BLG}$ resulted in two distinct modulations of the immune response

13   induced by i.p. sensitization to BLG (Fig. 1). After intranasal administration, production of

14   BLG-specific IgG2a and IgG1 were significantly stimulated compared to the saline control.

15   With a 17-fold mean increase, IgG2a production appeared to be preferentially enhanced since

16   IgG1 production underwent only a 5-fold mean increase. In sera from mice pretreated with the

17   mix LC+BLG, BLG-specific IgG2a mean concentration was 43-fold higher than that found in

18   the saline control while IgG1 response was only 4-fold higher than the saline control. IgE

19   production was significantly inhibited after intranasal administration of the mix LC+BLG but

20   not of LC$_{BLG}$. In contrast, oral pretreatments with LC$_{BLG}$ or with LC+BLG resulted in a

21   significant inhibition of IgE production compared to the saline control. An inhibition of the

22   IgG1 response was also observed in mice orally pretreated with the mix LC+BLG while no

23   significant difference could be observed for the BLG-specific IgG2a responses (Fig. 1).

24

1   ***BLG-specific IgA responses in fecal extract.*** Only intranasal administration of LC_{BLG},

2   but not of LC+BLG, stimulated the secretion of fecal BLG-specific IgA, as compared to the

3   saline control (Fig. 2). The secretion of BLG-specific IgA was also detected in fecal extracts

4   from mice orally pretreated with LC_{BLG} but the increase was weaker than that observed after

5   the intranasal pretreatment.

6

7   ***Cytokine responses after BLG reactivation.*** Secretion of IL-17 by BLG-reactivated

8   splenocytes from mice pretreated with LC_{BLG} or with LC+BLG was 8-fold enhanced as

9   compared with the saline control or the LC control (Fig. 3). The enhancement of IL-17

10   secretion was not affected by the route of administration used for the pretreatments. In

11   contrast, the profile of Th1/Th2 cytokines secreted by BLG-reactivated splenocytes appeared

12   to be affected by the mode of administration of the *Lb. casei* preparations. The BLG-

13   reactivation of splenocytes from mice intranasally pretreated with LC_{BLG} or LC+BLG

14   stimulated the secretion of Th1 cytokines, IFN-γ and IL-12, concomitantly with Th2

15   cytokines, IL-4, IL-5, and IL-10, as compared with the saline control. After oral

16   administration of LC_{BLG} or LC+BLG, BLG-reactivation of splenocytes led to enhanced

17   secretions of Th1 cytokines IFN-γ and IL-12, but without increase of IL-5 or IL-10

18   productions. A 2-fold increase of IL-4 production was still detectable in the group orally

19   pretreated with LC_{BLG} but this enhancement remained weaker than the 4-fold increase

20   observed after intranasal pretreatment. We did not detect any significant release of TGF-β.

21

1   **Discussion**

2

3   The mucosal milieu influences the way the immune system processes an antigen and

4   thereby affects the pattern of antibody and T-cell responses [23]. For example, previous work

5   showed that intranasal immunization of mice with recombinant lactococci strains producing

6   HPV-16 E7 antigen was more effective than intragastric immunization to induce an antigen-

7   specific mucosal and systemic immune response [14]. In addition to structural and functional

8   characteristics of the host mucosal tissues, the physiological state of the LAB *in situ* may also

9   influence their immunomodulatory properties. Indeed, while LAB are immediately in contact

10   with the mucosal nasopharyngeal tissues after intranasal administration, the bulk of bacteria

11   reaches the ileum only one hour after intragastric administration, which is sufficient for *Lb.*

12   *casei* to initiate its physiological adaptation to the harsh intestinal environment [24]. This may

13   be of critical importance since growth phase of *Lactobacillus* strains has been described to

14   affect an antigen-specific antibody response [25]. Taken together, these considerations

15   prompted us to compare the modulation of allergic-type responses after oral or intranasal

16   administrations of a BLG-producing *Lb. casei*.

17   We first evaluated whether administration of *Lb. casei* preparations was able to initiate

18   a BLG-specific immune response. In contrast to oral administration, intranasal application of

19   *Lb. casei* plus soluble BLG (LC+BLG) induced a detectable BLG-specific IgG response. The

20   absence of a significant systemic response after oral administration of LC+BLG was probably

21   due to an extensive digestion of the soluble BLG during the intestinal transit. Moreover,

22   mucosal administration of LC_{BLG} failed to induce significant antibody response. Soluble

23   BLG, mixed with lactobacilli, thus appeared to be more efficient to induce a systemic

24   response than the recombinant BLG that mostly remained entrapped inside the recombinant

25   LC_{BLG} [19]. Compared to the mucosal BLG-specific immune responses induced after delivery

1 of recombinant BLG-producing *Lc. lactis* [18], administration of LC$_{BLG}$ did not induce any

2 detectable BLG-specific response. In contrast to the pro-inflammatory profile of *Lc. lactis*, the

3 intrinsic anti-inflammatory properties of the BL23 strain may thus prevent the development of

4 a systemic or mucosal immune response [16]. In this regard, an anti-inflammatory profile may

5 be detrimental for immunization through mucosal applications but could still be advantageous

6 for inhibiting the development of excessive immune responses.

7 Recently, Daniel *et al.* reported that intranasal vaccination with a *Lb. plantarum*

8 NCIMB8826 producing the birch pollen Bet v1 allergen led to a shift towards a non-allergic

9 Th1 response with reduced specific IgE and enhanced IgG2a, IgG1 and IgA levels [15]. In the

10 present work, intranasal pretreatment with the BLG-producing *Lb. casei* also resulted in an

11 increase of the BLG-specific IgG2a, IgG1 and fecal IgA but did not significantly affect the

12 IgE production. A significant inhibition of IgE production was nevertheless observed after

13 intranasal pretreatment with the mix LC+BLG. Considering that intranasal administration of

14 LC+BLG, but not LC$_{BLG}$, could initiate, by itself, an early production of BLG-specific IgG,

15 we suggest that the early IgG response was partially protective, maybe by providing

16 "blocking" activities interfering with the allergen presentation to T cells, as previously

17 suggested [15;26;27].

18 In contrast to the intranasal pretreatment, oral administration of LC$_{BLG}$ or LC+BLG

19 resulted in a significant inhibition of IgE production without enhancing the BLG-specific

20 IgG1 or IgG2a responses. Oral administration of LC+BLG led even to a significant inhibition

21 of BLG-specific IgG1. Immunomodulation of the allergic sensitization thus appeared to be

22 significantly affected by the route of administration of the *Lb. casei* preparations. This was

23 confirmed by the profile of cytokines secreted by BLG-reactivated splenocytes, since only

24 intranasal administration of LC$_{BLG}$ or LC+BLG led to enhanced secretion of the Th2-

25 cytokines IL-5 and IL-10. These differences may thus result from the activation of distinct

1   dendritic and T cell populations at the mucosal site of delivery. This was previously described

2   for induction of mucosal tolerance to ovalbumin [23]. Pulmonary dendritic cells isolated after

3   nasal administration of ovalbumin (OVA) induce Tr1-like regulatory cells while mesenteric

4   lymph node dendritic cells isolated after oral administration of OVA, induce Th3-like

5   regulatory cells [28]. The distinct modulations of allergic sensitization after intranasal and

6   oral applications could thus depend on *Lb. casei* intrinsic ability to drive locally, or not, the

7   development of IL-10-secreting regulatory T cells, as already observed for other species of

8   lactobacilli [29]. This effect on the development of regulatory T cells could explain why the

9   *Lb. casei* BL23 strain exhibit beneficial effects in experimental mouse models of Th1 and

10   Th2-biased immune diseases. Indeed, oral administrations of the BL23 strain also provided a

11   significant protection in two different models of acute TNBS- and moderate DSS-colitis

12   [16;30]. In a similar way, the *Lb. plantarum* NCIMB8826 induced too a significant protection

13   toward acute colitis, although moderate compared to the BL23 strain. In correlation with its

14   pro-inflammatory profile, *L. lactis* MG1363 failed to prevent significantly TNBS-induced

15   colitis [16].

16   Stimulation of IFN-γ and IL-12 productions by BLG-reactivated splenocytes, after

17   both intranasal and oral pretreatments, confirmed that inhibition of the IgE response was

18   likely due to the induction of a moderate counter-regulatory Th1 immune response and not to

19   the establishment of a specific oral tolerance toward BLG. We previously showed that, when

20   purified BLG was orally administered with *L. lactis*, oral tolerance was abrogated because of

21   the bacterial adjuvanticity [17]. In a recent study, OVA-specific tolerance was induced by

22   means of oral administrations of an OVA-producing *L. lactis* strain [31]. However, induction

23   of oral tolerance was favored by the fact that the transgenic mice used in this work did not

24   respond to the Th1 adjuvant effect of *L. lactis* [31]. In our study, the adjuvant properties of

25   *Lb. casei* have also prevented the establishment of a specific tolerance to BLG. The BLG-

1   reactivation of splenocytes from mice pretreated with LC$_{BLG}$ or LC+BLG led also to the

2   secretion of high levels of IL-17. This cytokine is important in host defense against

3   extracellular bacteria and against fungi [32] and some microbial lipopeptides were described

4   to induce the production of IL-17 in Th cells [33]. Even if *Lb. casei* BL23 is not pathogenic,

5   this strain is not a commensal bacterium of laboratory mice and thereby remains a foreign

6   microorganism for the gut immune system. Administration of large amount of lactobacilli

7   seems then to activate immune mechanisms leading to the stimulation of a Th17-oriented

8   response that could also prevent the induction of tolerance [34].

9   We also compared administration of LC$_{BLG}$ and LC+BLG in order to determine

10   whether *in situ* expression of BLG could affect the BLG-specific immune response. After

11   LC$_{BLG}$ or LC+BLG administration, BLG-reactivation of splenocytes led to similar levels of

12   cytokines secretion, especially for IL-17. Adjuvanticity of *Lb. casei* thus appears to be

13   effective even if BLG is not produced by *Lb. casei*. Moreover, partial inhibition of IgE

14   production was observed after both intranasal and oral administration of LC+BLG while only

15   oral administration of LC$_{BLG}$ could prevent the IgE response. This raises the question whether

16   use of recombinant BLG-producing *Lb. casei* is really advantageous. On the other hand,

17   development of recombinant LAB can be useful by avoiding the need for large scale

18   purification of allergen. It may also improve antigen uptake at the intestinal mucosa by

19   preventing its digestive degradation. It is noteworthy that the presence of BLG-specific IgA in

20   feces was detected only in mice pretreated with LC$_{BLG}$ and not with LC+BLG. Entrapment of

21   BLG into recombinant *Lb. casei* membranes may thus stimulate IgA production through the

22   simultaneous presentation to the mucosal immune system of BLG and *Lb. casei*-associated

23   molecular patterns that could activate the TLR-mediated signaling pathways [35]. This

24   phenomenon should be further investigated since the protective capacity of secretory IgA has

25   been reported as alleviating factors for the severity of some allergic symptoms [2;36].

1   The present work shows that oral and intranasal administrations of a recombinant *Lb.*

2   *casei* BL23 strain induce modulations of immune responses through distinct mechanisms.

3   Intranasal administration appeared to be particularly effective to stimulate the systemic IgG1

4   and IgG2a responses, thus confirming the advantage of intranasal over intragastric route of

5   immunization to induce an antigen-specific humoral response [14]. On the other hand, an

6   efficient suppression of BLG-specific IgE production was observed after oral pretreatments

7   without enhancing the systemic antibody responses or the Th2-oriented cellular response.

8   Oral administration of recombinant *Lb. casei* BL23 could thus offer more attractive

9   perspectives to inhibit the development of food allergic responses. This needs to be further

10   investigated in therapeutic settings.

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1   *Figure 1.* BLG-specific IgG1, IgG2a, and IgE responses in mice intranasally (A) or orally (B)

2   pretreated with *Lb. casei* preparations. Mice (n=7 per group) were administered saline

3   solution (Saline), control *Lb. casei* (LC), control *Lb. casei* plus soluble BLG (LC+BLG) or

4   BLG-producing *Lb. casei* (LC$_{BLG}$) and then were i.p. sensitized to BLG (see Materials and

5   Methods). BLG-specific IgG1, IgG2a and IgE concentrations were determined by quantitative

6   immunoassays on day 50. No BLG-specific antibodies were detected in sera from naive mice

7   bled on the same day (data not shown). Means are indicated. Significantly different from

8   saline pretreated group (* $P < 0.05$, ** $P<0.001$ and *** $P < 0.001$).

9   *Figure 2.* BLG-specific IgA responses in fecal extracts after mucosal applications of *Lb. casei*

10 preparations. Mice (n=7 per group) were intranasally (*i.n.*) or orally (*oral*) administered saline

11 solution (Saline, white bars), control *Lb. casei* (LC, horizontal hatched bars), control *Lb. casei*

12 plus soluble BLG (LC+BLG, grey bars) or BLG-producing *Lb. casei* (LC$_{BLG}$, black bars) and

13 then were sensitized to BLG (see Materials and Methods). BLG-specific IgA levels on day 50

14 were reported as absorbance units (AU) at 414 nm. Naive mice (vertical hatched bars) were

15 left unpretreated and unsensitized.

16 *Figure 3.* Cytokines secretions by BLG-reactivated splenocytes from mice pretreated with *Lb.*

17 *casei* preparations. Mice (n=7 per group) were intranasally (*i.n.*) or orally (*oral*) administered

18 saline solution (Saline, white bars), control *Lb. casei* (LC, horizontal hatched bars), control

19 *Lb. casei* plus soluble BLG (LC+BLG, grey bars) or BLG-producing *Lb. casei* (LC$_{BLG}$, black

20 bars) and then were i.p. sensitized to BLG (see Materials and Methods). Naive mice (vertical

21 hatched bars) were left unpretreated and unsensitized. Cells were incubated for 60 h at 37°C

22 (5% CO_2). Results are represented as cytokines secretions in supernatants of BLG-reactivated

23 splenocytes after substraction of cytokines assayed in supernatants of ovalbumin-reactivated

24 splenocytes, thus corresponding to specific production.
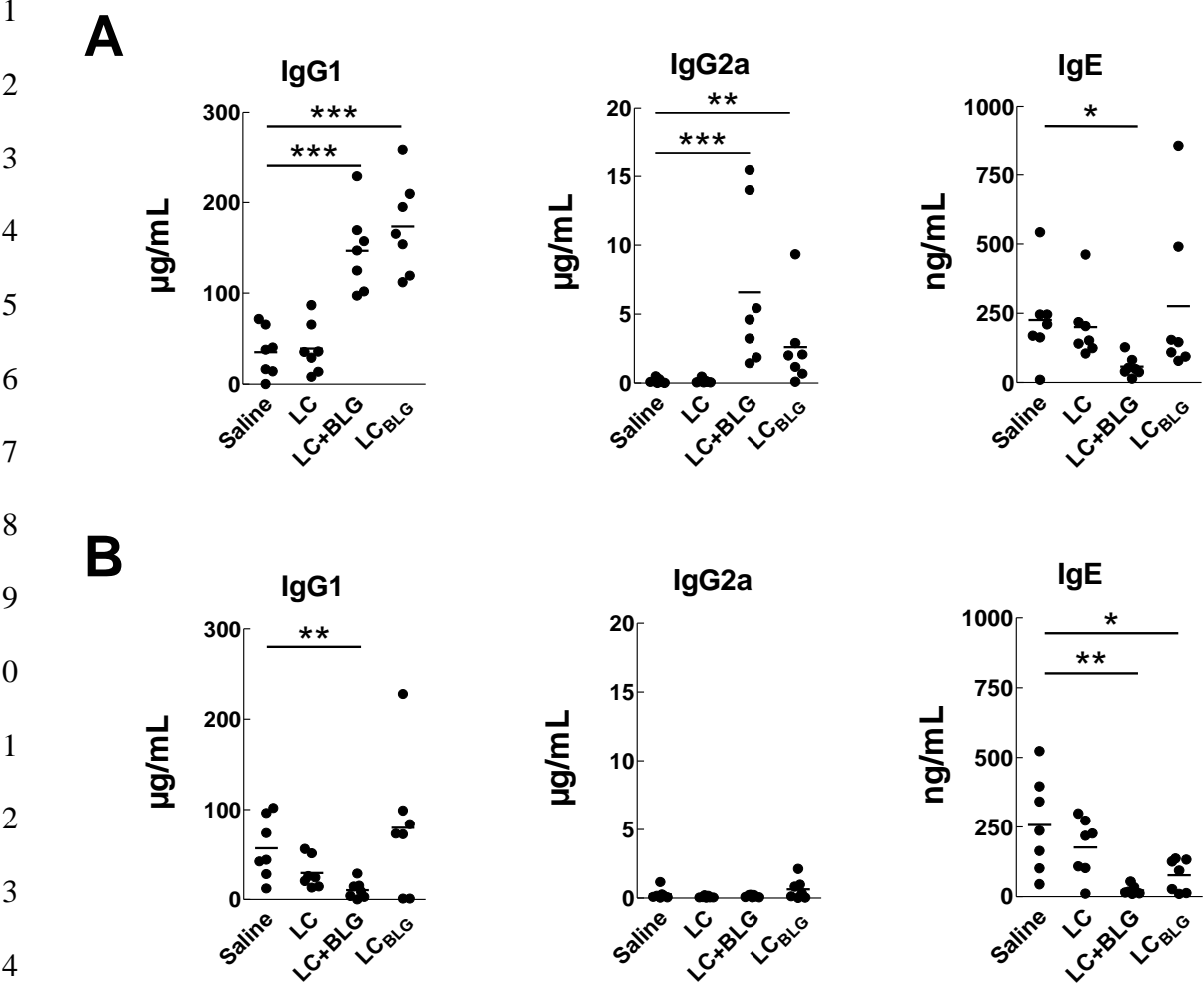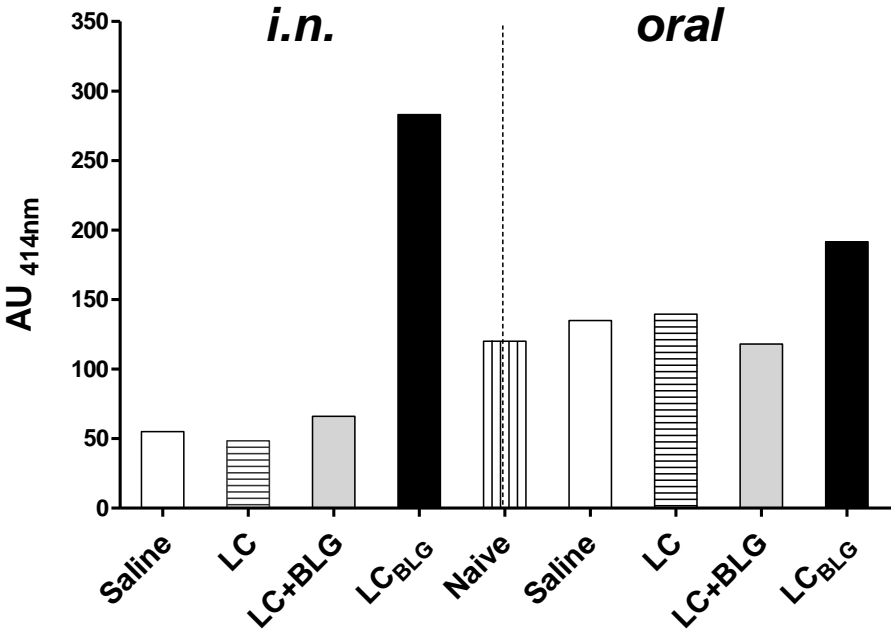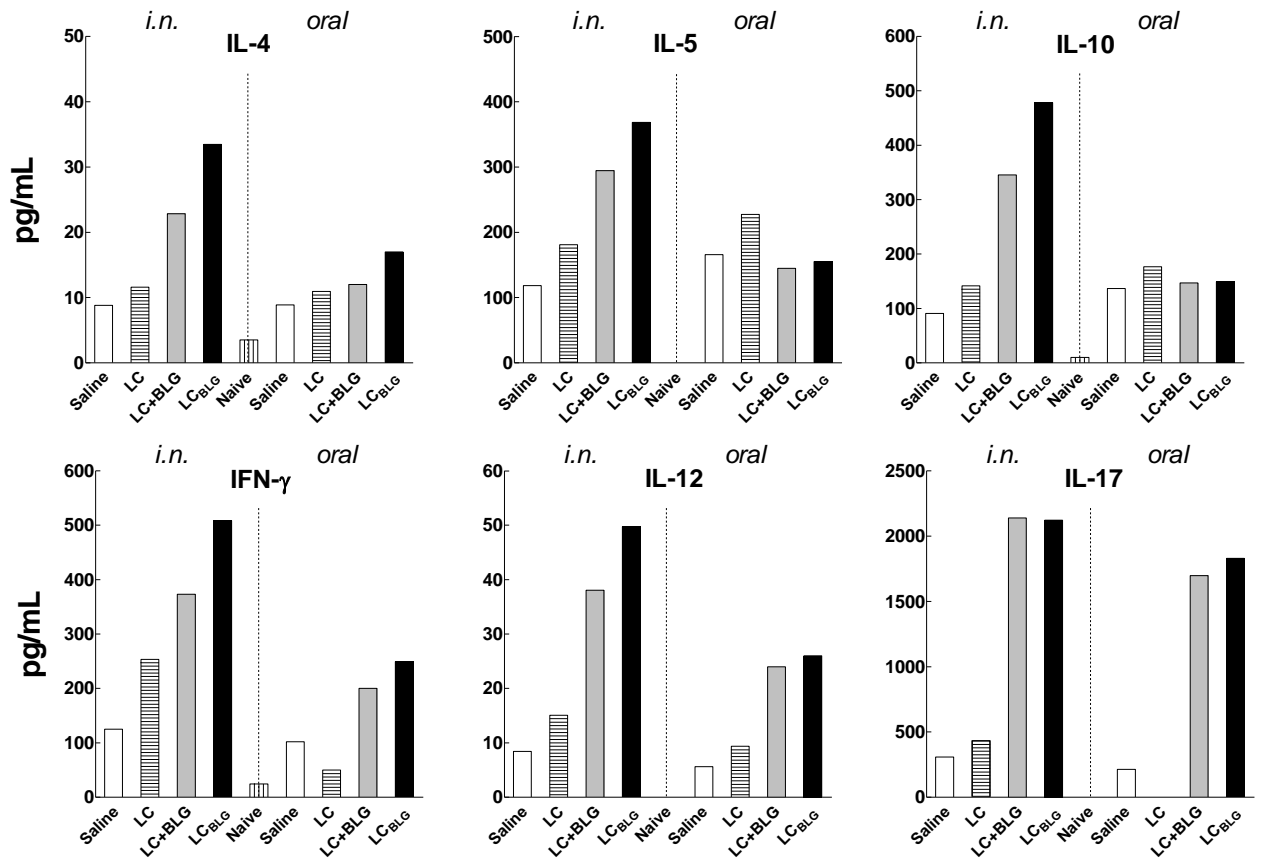
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16   *Figure 1.*

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