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# Neonatal mono-colonization of germ-free mice with *Lactobacillus casei* enhances casein immunogenicity after oral sensitization to cow's milk

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# Neonatal mono-colonization of germ-free mice with *Lactobacillus casei* enhances casein immunogenicity after oral sensitization to cow's milk

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**Abbreviations: BLG**, β-lactoglobulin; **CAS**, whole casein fraction; **CMP**, cow's milk protein; **GF**, germ-free mice; **CV**, conventional mice; **LC**, *L. casei* mono-colonized mice;

**Keywords:** allergy, casein, germ-free mice, *Lactobacillus casei,* neonatal colonization

**Abstract**

**Scope:** Food allergy is an increasing global health problem and perinatal administration of probiotic bacteria is currently under investigation in order to prevent the development of allergic diseases. Here, we investigated the impact of neonatal mono-colonization of mice with *Lactobacillus casei* BL23 on an oral sensitization to cow's milk.

**Methods and results:** Mono-colonized (LC) mice were obtained by inoculating *L. casei* to germ-free (GF) parents. Nine-week-old GF, LC and conventional (CV) mice were orally sensitized to cow's milk with cholera toxin as adjuvant. Compared to GF and CV mice, LC mice developed higher casein-specific IgG responses. In contrast, no significant differences between GF and LC mice were observed for the humoral responses against whey proteins. Immunoblotting experiments performed on αS1-casein hydrolysates revealed the presence of small peptides immunoreactive with sera from LC mice but not from GF mice. After *in vitro* reactivation of splenocytes, secretion of IL-17 was higher in LC mice than in GF and CV mice.

**Conclusions:** Neonatal monocolonization by *L. casei* BL23 modulated the allergic sensitization toward food antigens. Furthermore, our data suggest that casein-specific humoral responses in LC mice were enhanced because of casein hydrolysis by *L. casei* into immunogenic peptides.

## 1. Introduction

Food allergy is a major public health problem and prevalence of IgE-mediated food allergy is increasing worldwide, especially in Western countries [1]. Food allergy results from the lack of induction of oral tolerance toward food antigens that has been associated with defective regulatory T cells (Treg) and with the development of inappropriate Th2-polarized immune responses resulting in the production of IgE antibodies specific of the allergens [2]. Genetic factors alone cannot explain the increase of the prevalence and several environmental changes have been proposed to underlie the development of allergic diseases [3]. The critical role of the gut microbiota in driving the development and the maturation of the host immune system has been revealed during the last decade [4, 5]. The hygiene hypothesis now suggests that a lower exposure of neonates to environmental microbes leads to an altered gut colonization by commensal microbiota, which could affect epithelial and immune system maturation and favor the development of inappropriate immune responses toward ordinarily harmless food antigens [6]. Recently, a lower intestinal microbial diversity has been be observed during the first months of life in children who developed allergic diseases afterwards in comparison with healthy children [7, 8]. Some studies also reported a lower colonization by lactobacilli species in those infants [9, 10]. Due to their properties to modulate host immune responses by reducing allergic sensitization through the stimulation of immunosuppressive Treg cells and/or by shifting the initial Th2 response toward a more balanced Th1/Th2/Th17 profile, probiotic strains of lactobacilli have been tested for pre and/or post-natal supplementation to prevent the development of allergic diseases [11]. Some beneficial effects have been observed for the prevention of atopic dermatitis, especially when supplementation was performed during pregnancy and after birth, thus underlying the importance of the perinatal period for probiotic intervention [12]. A mixture of three *Lactobacillus* strains has

also been recently shown to improve the integrity of intestinal barrier in adult germ-free (GF) mice and to diminish the severity of atopic dermatitis in a pilot study on children [13].

The role of the gut microbiota in the maturation process of the host immune system was particularly investigated in GF animals, whose gut-associated lymphoid tissue is underdeveloped [14, 15]. GF mice display defective regulatory T cell functions [16] and we previously showed that GF mice sensitized to cow's milk proteins (CMP) display a higher level of sensitization toward whey proteins, including the soluble β-lactoglobulin (BLG) and α-lactalbumin (ALA), than conventional (CV) mice [17-19]. In contrast, oral sensitization of GF mice toward insoluble casein (CAS) was less efficient than that to BLG and humoral responses toward CAS were not significantly different between GF and CV mice [19]. We also showed that delayed bacterial colonization of GF mice altered persistently the reactivity of the host immune system to oral sensitization [19]. Considering the importance of the early-life microbial exposure for the establishment of a well-balanced immune system [20], neonatal mono-colonization of GF mice could offer useful models to evaluate the immunomodulatory properties of bacterial strains of interest [21, 22].

The isolated plasmid-free *L. casei* BL23 strain has been widely used for genetic, biochemical and physiology studies [23]. This strain is easily transformable and anti-inflammatory properties has been previously demonstrated [24, 25]. Its genome has been recently sequenced [26]. We previously showed that the BL23 strain exhibited adjuvant properties that could inhibit the development of an allergic sensitization to BLG in adult BALB/c mice pretreated with a BLG-producing *L. casei* BL23 [27]. However, no significant effect could be observed when mice were pretreated with *L. casei* BL23 alone. In the present study, we

wanted to evaluate whether neonatal mono-colonization with wild-type *L. casei* BL23 would protect mice from an oral sensitization to cow's milk.

## 2. Materials and methods

### 2.1. Reagents, medium and bacterial strain

Unskimmed Ultra-High-Temperature-sterilized cow's milk (Candia, Paris, France) and cholera toxin (CT, Sigma-Aldrich, St Louis, MO, USA) were used for experimental sensitization. Whey proteins (BLG and ALA) and CAS and its four constituents (αS1-, αS2-, β and κ-caseins) were purified and characterized as previously described [28]. The *L. casei* BL23 strain (ATCC 393 cured of plasmid pLZ15 [23]) was cultured at 37°C in De Man-Rogosa-Sharpe (MRS) broth (Difco, BD, Le Pont de Claix, France).

### 2.2. Mice and house conditions

Conventional SOPF female BALB/cByJ (CV) mice were purchased from Charles River Laboratories (CRL, l'Arbresle, France). Germ-Free (GF) and *L. casei* mono-colonized (LC) mice were produced and bred in the animal facilities of Anaxem (Micalis institute, INRA Jouy-en-Josas, France). Mice were housed in Trexler-type isolators and received *ad libitum* autoclaved water and sterilized pelleted standard chow deprived of CMP (R03-40, SAFE, Augy, France). Neonatal colonization by *L. casei* was obtained by inoculating the strain to adult GF breeding pairs, before mating. The bacterial status of GF and LC mice was monitored as previously described [19, 29]. The level of gut colonization by *L. casei* was evaluated in 4-week-old mice at around $1.4 \times 10^9$ CFU/g feces and remained stable throughout the experiment (data not shown). Female offsprings were used for experimental sensitization. All experiments were performed in compliance with European regulations on care and protection of Laboratory Animals (EC Directive 86/609, French Law 2001-486, June 6, 2001), with permission 91–493 of French Veterinary Services.

**2.3. Cellularity of Peyer's patches**

Peyer's patches were excised from small intestines from 8-week-old naïve GF, MX and CV mice (n=6/group). Peyer's patches were then squeezed in buffer D-PBS, glucose 0.1% and filtered through 70 µm cell strainer (BD Falcon, NJ). Total cell count was determined with Guava ViaCount assay, according to the manufacturer's recommendation (EMD Millipore Corporation, Hayward, CA).

**2.4. Histology of intestinal sections**

Small intestines of naïve 8-week-old GF, LC and CV mice (n=3/group) were embedded in OCT compound (Miles Scientific, Naperville, IL) and frozen with isopentane in liquid nitrogen. The 5µm-thick sections were fixed in 95% ethanol at -20 °C for 5 min. Endogenous peroxidase was blocked with 3% $H_2O_2$ for 10 min and nonspecific binding was blocked with Avidin/Biotin blocking kit (Invitrogen, Camarillo, CA). Slides were then incubated with biotinylated anti-mouse CD4 mAb (clone GK1.5, 20 µg/mL, eBioscience, San Diego, USA) in PBS 1% BSA. After 45 min of incubation with streptavidin-horseradish peroxidase (Pharmingen™ BD, BD Biosciences, San Diego, CA), slides were developed with VECTOR® NovaRED™ (Vector, Burlingame, CA) as substrate. Finally, the sections were counterstained with hematoxylin (Mayer haemalum, RAL diagnostics, Martillac, France). Slides were scanned with Nanozomer Digital Pathology virtual viewer and analyzed using NDP viewer software (Hamamatsu, Japan).

**2.5. Oral sensitization to CMP**

Nine-week-old GF, LC and CV mice (n=10/group) were orally sensitized with 200µL of cow's milk mixed with CT (10µg/mouse) on day 1, 8, 15, 22, and 29 (Fig. E1). Blood samples were

collected on day 26 and 36 [19]. Spleen and mesenteric lymph nodes (MLN) cells were collected after sacrifice on day 42 for *in vitro* reactivation and flow cytometry analysis. Of note, administration of saline solution or CT alone to GF and CV mice has been previously tested and no signal specific to CMP has ever been detected [19].

**2.6. Humoral immune responses**

Appropriate dilutions of sera (1:50 for IgE, 1:200 for IgG2a, and 1:10.000 for IgG1) in EIA buffer (0.1M phosphate buffer, 0.1% BSA, 0.15M NaCl) were incubated overnight on passively CMP-coated on 96-well microtiter plates (Nunc Immunoplate Maxisorp) at 5µg/mL. Allergen-specific IgE, IgG1 and IgG2a levels were determined as previously described [19, 30]. Briefly, after washing (0.01 M phosphate buffer pH 7.4, 0.05% Tween 20), IgG1, IgG2a or IgE binding was revealed by incubation with goat anti-mouse IgG1 and IgG2a polyclonal affinity-purified antibodies (Southern Biotechnology Associates, Birmingham, AL, USA) or with a rat anti-mouse IgE monoclonal antibody (clone LOME-3, Serotec, Oxford, England) labelled with acetylcholinesterase [31]. Results are reported as absorbance units (AU) at 414 nm.

**2.7. Cellular immune responses**

At the end of the experiment, within each group of mice, all mesenteric lymph nodes (MLN) were pooled (1 pool/group) and spleens were pooled by pairs (5 pools/group). Spleens were homogenized in RPMI-10 (RPMI 1640 medium supplemented with 10% fetal calf serum, 2mM L-glutamine, 100U penicillin, 100mg/mL streptomycin). After lysis of red blood cells in 180mM $NH_4Cl$, 17mM $Na_2EDTA$ buffer, splenocytes were resuspended in RPMI-10. Cells ($2.10^6$ cells/well) were incubated for 60h at 37°C (5% $CO_2$) in 96-well culture plates in the presence of BLG (20 µg/mL), CAS (20 µg/mL) or Dulbecco's Phosphate-Buffered Saline (D-PBS, Life-Technologies, UK) as negative control. Cytokine levels (IL-4, IL-10, IFN-γ and IL-17) were

assayed in culture supernatant by using BioPlex technology with Milliplex map kit (Millipore, Billerica, USA) according to the manufacturer's recommendations. Results are represented as cytokine secretions (pg/mL) in supernatants of reactivated splenocytes after subtraction of cytokines assayed in supernatants of D-PBS-reactivated splenocytes, thus corresponding to specific production.

**2.8. Flow cytometry**

Spleen cell suspensions were analyzed for regulatory T cells. After blocking of non-specific binding sites by addition of Fc block (Mouse BD Fc Block TM, clone 2.4G2, BD Pharmingen), spleen cell suspensions were analyzed by FSC/SSC gating and extracellular staining with anti-CD4 antibody (FITC Rat anti-Mouse CD4, clone GK1.5, BD Pharmingen) and intra-cellular staining with anti-Fox P3 antibody (anti-Fox P3, clone 3G3, kit MACS Miltenyi Biotec, Germany), according to the supplier's recommendations. The acquisition was carried out using the flow cytometry system Guava EasyCyte Plus (Millipore) using the Guava cytosoft$^{TM}$ software (Data Acquisition and Analysis software version 5.3).

**2.9. αS1-casein hydrolysis**

Hydrolysis of purified αS1-casein was performed using bovine plasmin (5U/mL, Roche Diagnostics) for 0 to 18h at pH 8 and at 37°C, using a ratio of 0.004U/mg of purified allergen and with chymosin (from calf-stomach, 25U/mg solid, Sigma-Aldrich) for 0 to 21h at pH 6.2 and at 30°C, using a ratio of 0.8U/mg of purified allergen [32]. Hydrolysis were stopped by adding protease inhibitor cocktail (Sigma-Aldrich) and samples were immediately frozen at -20°C.

**2.10. Immunoblot analyses**

SDS-PAGE and immunoblot analyses were performed in reducing conditions using apparatus and reagents from Invitrogen (Life Technologies, Carlsbad, CA, USA). Casein hydrolysates (2µg/well) and molecular-weight markers (Novex® Sharp prestained protein standard) were loaded on NuPage Novex® 12% Bis-Tris gels. Electrophoresis was performed in MES buffer. After transfer to PVDF membrane (Hybond-P, GeHealthcare Life Sciences), immunoblot analyses were performed with mice sera as primary antibody (1:10,000) and HRP-conjugated goat anti-mouse IgG as secondary antibody (1:20,000 ThermoScientific, Waltham, MA USA). Membranes were incubated with ECL Prime Western blotting detection reagent (Ge Healthcare Life Sciences) for 5 min and then revealed using photographic film (CLXposure, ThermoScientific) and X-ray developer reagents from Kodak (Carestream, Rochester, NY USA).

**2.11. Statistical Analyses**

Data on humoral responses were analyzed using nonparametric tests (Kruskal-Wallis test with Dunn's multiple comparison test or Mann-Whitney test). Data on cytokine secretions from splenocytes were analyzed using one-way ANOVA with Bonferroni multiple comparaison test. Statistical analyses were performed with GraphPad Prism 5.01 software and $p < 0.05$ was considered significant (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

## 3. Results

### 3.1. Impact of neonatal mono-colonization with *L. casei* on gut immune system

In order to assess the impact of neonatal gut colonization by *L. casei* BL23 on 8 week-old mice, just before performing experimental sensitization, we assessed the cellularity of Peyer's patches and the expansion of CD4+ lymphocyte subpopulation in the lamina propria. As previously reported [33], the absence of gut microbiota in GF mice was characterized by hypoplastic Peyer's patches and neonatal monocolonization with the BL23 did not increase the cellularity of the Peyer's patches (Fig. 1). Moreover, qualitative examination of ileal sections stained with anti-CD4 antibodies also indicated a very low frequency of CD4+ cells in the lamina propria of GF mice compared to CV mice (Fig. 2), as previously reported [33]. The presence of CD4+ cells in LC mice was as low as that observed in GF mice and confirmed that colonization with *L. casei* BL23 alone could not induce a proper maturation of the gut immune system.

### 3.2. Impact of neonatal mono-colonization with *L. casei* on oral sensitization to CMP

- Humoral responses against whey proteins

Anti-BLG IgE responses were higher in GF and LC mice than in CV mice at day 26 but differences were no more statistically significant at day 36 (Fig. 3). As previously observed [19], the level of IgG1 responses against BLG in GF mice was significantly higher than that in CV mice (Fig. 4). This higher level of BLG-specific IgG1 antibodies was also observed in LC mice, with no significant differences between GF and LC mice. The differences were not limited to Th2 antibody responses since productions of anti-BLG IgG2a were comparable between GF and LC

mice and remained significantly higher than those measured in CV mice (Fig. 4). In addition, the levels of specific IgG1 and IgG2a responses toward ALA, another soluble whey protein, were similar between GF and LC mice and were higher than those observed in CV mice (Fig. 4).

- Humoral responses against caseins

In contrast to whey proteins, no significant differences were observed between GF and CV mice for the CAS-specific IgG1 and IgG2a responses (Fig 4) and IgE responses (Fig. 3). However, LC mice still exhibited higher anti-CAS IgG1 and IgG2a responses than CV mice ($p<0.01$, Kruskal-Wallis test and Dunn's multiple comparison test). Of note, when considering only mice with immature gut immune system, *i.e.* GF and LC mice, the IgG1 and IgG2a responses against CAS were also significantly different (Fig. 2, $p<0.05$ Mann-Whitney test). The humoral responses was then further analyzed using the four constituents of CAS, the αS1-, αS2-, β- and κ-caseins. The IgG1 and IgG2a responses against αS1-, αS2- and κ-caseins were also significantly higher in LC mice than in CV mice ($p<0.01$, Kruskal-Wallis test and Dunn's multiple comparison test, Fig. 4). As previously reported, β-casein was poorly immunogenic in our experimental model [19]. Although no significant IgG2a response was observed against β-casein in any group of mice (data not shown), a β-casein specific IgG1 response was detected and was significantly higher in LC mice than in CV mice (Fig E2). When comparing GF and LC mice, the IgG1 and IgG2a responses against αS1- and κ-caseins were also significantly different (Fig. 5, $p<0.05$ Mann-Whitney test).

- Cellular responses

Frequency of splenic CD4+Foxp3+ cells was significantly higher in sensitized CV mice than in GF mice and was not affected by neonatal gut colonization by *L. casei* BL23 (Fig. 6). After

reactivation of splenocytes by BLG or CAS, no significant difference between the different groups was observed for IL-4 and IL-10 secretions (Fig. 7A). Specific secretion of IFN-γ was detected only in splenocyte supernatants from CV mice. In contrast, BLG and CAS-reactivated splenocytes from LC mice secreted higher amount of IL-17 than CV and GF mice. This higher level of IL-17 secretion was similarly observed after reactivation of pooled MLN cells (Fig. 7B).

**3.3 IgG immunoreactivity of αS1-casein hydrolysates**

After having checked that *L. casei* BL23 exhibited a proteolytic activity against different CAS constituents (Fig. E3 and [34]), we hypothesized that *in vivo* proteolytic degradation of CAS by *L. casei* could lead to the production of immunogenic peptides and then explain the enhanced immune responses of LC mice against CAS and, in particular, against αS1-casein. We then further investigated the IgG responses of sensitized mice toward αS1-casein hydrolysates.

*In vitro* digestion of purified αS1-casein was performed in order to generate high amount of CAS peptides. Two different enzymes, chymosin and plasmin, were used to get different profiles of breakdown products and digestion kinetics were optimized in order to get a large panel of breakdown peptides (Fig. 8A). Immunoreactivity of different hydrolysates of αS1-casein was then evaluated by immunoblot analysis with pooled sera from GF, LC and CV mice (Fig. 7B) and with individual sera from GF and LC mice (Fig. E4). Although high MW breakdown products (> 15 kDa) were recognized by sera from all groups of mice, immunoreactive peptides at around 5-6 kDa from plasmin breakdown products could be detected with sera from LC mice and, to a much lesser extent, with sera from CV mice but not with sera from GF mice (Fig 8B). Small immunoreactive peptides were also detected among αS1-casein breakdown products generated by chymosin digestion with a LC individual serum (Fig. E4). We also tested

whether *in vitro* digestion of αS1-casein by *L. casei* could produce a specific pattern of immunoreactive peptides. The hydrolytic activity of *L. casei* on αS1-casein was confirmed as indicated by decreasing amount of intact αS1-casein and the appearance of immunoreactive breakdown products between 15 kDa and 20 kDa (Fig. E3). However, complementary smaller peptides were not detected, maybe because of their rapid degradation by *L. casei*.

**4. Discussion**

Gnotobiotic mice models provide powerful tools to study the interaction of the gut microbiota with the host immune system. However, in these models, the period of life in which the bacteria of interest are associated to GF mice is of crucial importance as it can significantly affect the host immune responses. Sudo *et al* (1997) showed that oral tolerance could be induced in GF mice neonatally colonized with *Bifidobacterium infantis* but not in GF mice colonized at older age [35]. It is increasingly apparent that early-life microbial exposure provides a narrow "window of opportunity" for the proper maturation of the gut immune system [20]. In this regard, we wanted to evaluate the impact of a neonatal mono-colonization of GF mice by the strain *L. casei* BL23 on a subsequent allergic sensitization.

For this purpose, mother-to-offspring mono-colonization of GF mice was performed. First, it appeared that the presence of *L. casei* alone could not induce the proper maturation of the gut immune system as observed in CV mice since GF and LC mice displayed hypoplastic Peyer's patches and an absence of CD4+ cell expansion in the lamina propria.

Moreover, neonatal mono-colonization of mice by *L. casei* alone did not correct the exaggerated reactivity of the host immune system toward an oral sensitization as observed in GF mice. Indeed, GF and LC mice exhibited a level of sensitization toward CMP significantly higher than that of CV mice. We even observed an enhanced IgG responses toward caseins in LC mice. In addition, increased secretions of IL-17 were measured after *in vitro* CMP-reactivation of splenocytes and MLN cells from LC mice. Accordingly, such enhanced IL-17 secretions has been also observed in adult CV mice partially protected from BLG-sensitization because of preventive oral administrations of BLG-producing *L. casei* BL23 [27]. In that previous work, the inhibition of an allergic sensitization was due to the stimulation of counter-

regulating Th1/Th17 responses and not due to the induction of suppressive regulatory responses. Considering the approach of neonatal mother-to-offspring mono-colonization, a similar absence of immune modulation was also observed with the wild-type probiotic *Lactobacillus plantarum* NCIMB8826 on a systemic sensitization toward a major birch pollen allergen [21]. It thus showed that mono-colonization with bacteria displaying strong Th17 or Th1 adjuvant properties [21, 27], even neonatally associated, are not necessarily sufficient to prevent the development of an allergic sensitization. In contrast, neonatal colonization of mice with a strain of *Bifidobacterium longum* displaying only suppressive regulatory responses, significantly inhibited the development of both Th1 and Th2 responses after systemic sensitization [22]. Altogether, these results suggest that, in models of neonatal colonization of GF mice, only probiotic strains displaying strong immunosuppressive properties can prevent the development of an allergic sensitization while stimulation of pro-Th1 and/or -Th17 responses was ineffective. However, it remains to determine whether such induced hyporesponsiveness of the host immune system, that is beneficial for the prevention of an allergic sensitization, could not be detrimental for the development of protectives humoral responses, expected during vaccination or pathogen infections.

One striking result was the humoral responses against CAS that were enhanced in LC mice. Both IgG1 and IgG2a specific responses were increased thus showing that the presence of *L. casei* in LC mice did not favor the development of Th1 responses over Th2 responses. We previously observed that sensitization of GF mice to CAS tended to be less efficient than sensitization to whey proteins [19]. Roth-Walter *et al* also showed that soluble BLG and ALA, but not insoluble CAS, are readily transcytosed through enterocytes while uptake of CAS micelles occurs mainly through Peyer's patches [36, 37]. We then hypothesized that sensitization of GF mice to insoluble CAS was less efficient than that to soluble BLG, because

of an impaired uptake of CAS in Peyer's patches that are hypoplastic in GF mice compared to CV mice [33]. We thus wondered whether proteolytic capacities of *L. casei* could be involved in the increased IgG responses to CAS. Indeed, lactic acid bacteria are known for their ability to degrade CMP and the production of bioactive peptides [38, 39]. Therefore, hydrolysis of CAS by *L. casei* into soluble peptides that could be transcytosed through enterocytes, could increase immune responses to CAS. The paracellular transport of soluble peptides across the intestinal barrier could be also considered since the intestinal barrier integrity in GF or mono-colonized mice can be impaired [13, 36, 40]. We then assessed the recognition of αS1-casein hydrolysates by sera from CMP-sensitized mice. Interestingly, small peptides around 5 kDa, obtained by digestion of αS1-casein with chymosin or plasmin, were shown to be immunoreactive with sera from LC mice but not with sera from GF mice. Thus, IgG responses against αS1-casein in LC mice recognized a higher number of epitopes, probably located on peptides generated *in vivo* by the proteolytic activity of *L. casei*. In this regard, we confirmed the ability of *L. casei* BL23 to hydrolyze αS1-casein *in vitro* with the production of immunoreactive fragments between 15 to 20 kDa. To a lesser extent, the small plasmin- and chymosin-derived peptides were also recognized by sera from CV mice. This is in agreement with the uptake of CAS through mature Peyer's patches of CV mice and an efficient processing by antigen-presenting cells. Whether degradation of CAS by the mouse gut microbiota, in which lactobacilli represent a dominant genus [41], could also enhance the specific humoral responses against CAS remains to be determined. Nevertheless, immunoreactivity of these peptides in CV mice was much lower than in LC mice, according to their lower level of sensitization to CMP. These results are, to our knowledge, the first experimental evidence in a gnotobiotic murine model that *L. casei* can enhance *in vivo* the immunogenic response to

CAS. They also further illustrate the complex interactions between the host, its gut microbiota and its diet that can modulate the immunogenicity and allergenicity of food proteins.

In conclusion, neonatal mono-colonization of BALB/c mice with *L. casei* BL23 affected the humoral responses to caseins but not to whey proteins in response to an oral sensitization. This effect was probably due to the ability of *L. casei* to hydrolyze *in vivo* insoluble CAS into soluble immunogenic peptides. However, considering that the BL23 strain was first selected for its adjuvant properties, we cannot exclude other immunomodulatory mechanisms as suggested by the enhanced secretion of IL-17 by reactivated spleen and MLN cells from LC mice. The impact of *L. casei* on the integrity of the intestinal barrier should be also investigated in order to evaluate the paracellular transport of soluble peptides. On the other hand, the impact of neonatal association with *L. casei* alone was rather narrow, as indicated by the absence of significant maturation of some host lymphoid structures as observed in GF mice. This is also in agreement with the requirement of a highly diversified gut microbiota for the establishment of a proper intestinal homeostasis.

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Author contributions: MAM and SM performed the experiments, HB provided purified allergens and supervised digestion experiments. SR supervised production and breeding of GF and LC mice. KAP and SH supervised the study. MAM, KAP and SH wrote the manuscript. All authors read and approved the final manuscript.

The authors have declared no conflict of interest.

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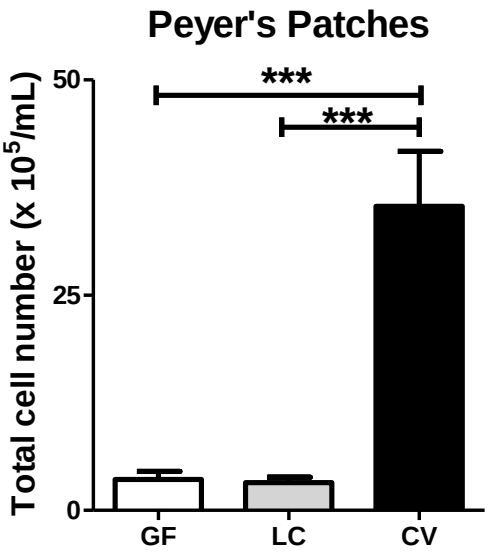


**Fig. 1: Cellularity of Peyer's patches.** Peyer's patches were excised from small intestines from 8-week-old naïve GF, LC and CV mice (n=6/group) and total cell count was determined with Guava ViaCount assay. Results represent mean ± SEM of two different experiments (one-way ANOVA with Bonferroni multiple comparison test).

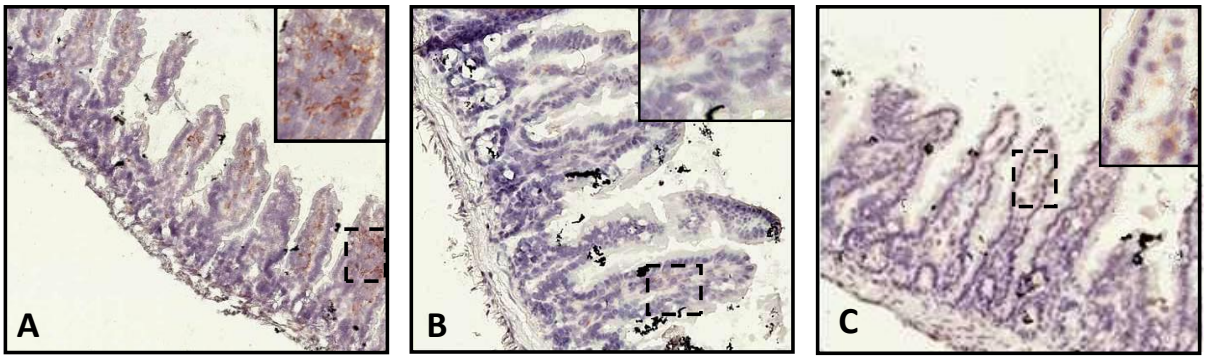


**Fig. 2:** Histological examination of small intestine from CV (A), LC (B) and GF (C) BALB/c mice. Slides have been incubated with anti-CD4 mAb. Inset show higher magnification. Representative mice are shown (n=3/group).

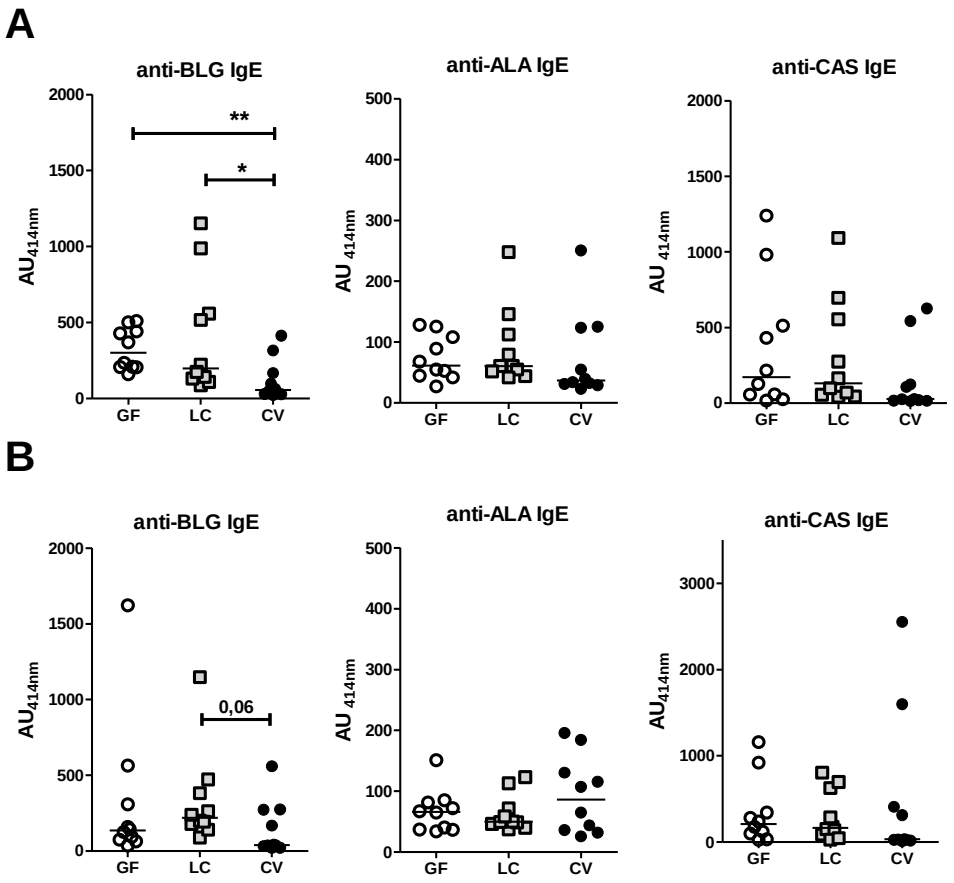


**Fig. 3:** IgE responses at day 26 (A) and 36 (B) against BLG, ALA and CAS. Nine-week-old GF, LC and CV mice received five oral administrations of cow's milk with cholera toxin as adjuvant on day 1, 8, 15, 22, and 29 and blood samples were collected on day 26 and 36. The dilutions used 1:50. Assays were performed as duplicate on sera from 10 mice/group. Results are reported as absorbance units (AU) at 414 nm and median is shown (Kruskal-Wallis test and Dunn's multiple comparison test).

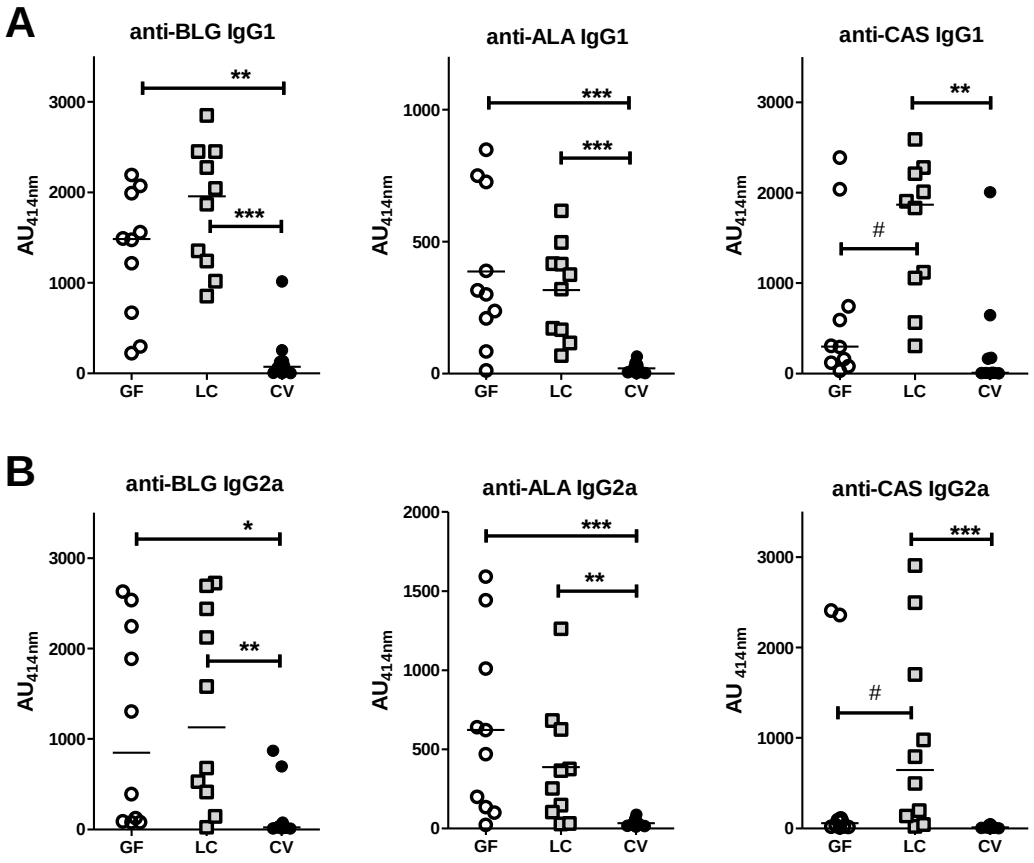


**Fig. 4:** IgG1 (A) and IgG2a (B) responses to BLG, ALA and CAS in CM-sensitized mice. Nine-week-old GF, LC and CV mice received five oral administrations of cow's milk with cholera toxin as adjuvant on days 1, 8, 15, 22, and 29 and blood samples were collected on day 36. The dilutions used are 1:10000 for specific IgG1 assay and 1:200 for specific IgG2a assay. Assays were performed in duplicate on individual sera from 10 mice/group. Results are reported as absorbance units (AU) at 414 nm and median is shown (*, Kruskal-Wallis test and Dunn's multiple comparison test and #, Mann-Whitney test between GF and LC mice).

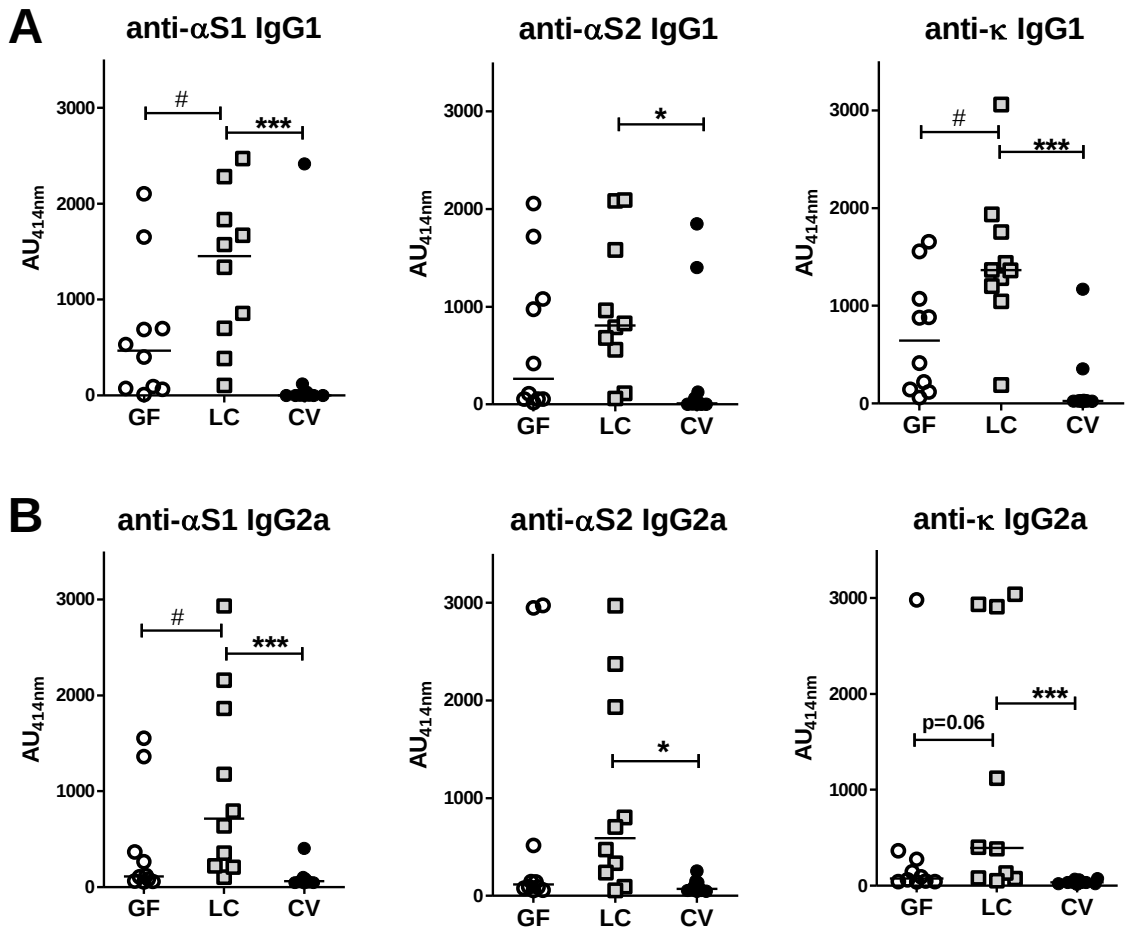


**Fig. 5:** IgG1 (A) and IgG2a (B) responses toward αS1-, αS2- and κ-caseins in CM-sensitized mice. Nine-week-old GF, LC and CV mice received five oral administrations of cow's milk with cholera toxin as adjuvant on day 1, 8, 15, 22, and 29. The dilutions used are 1:10000 for specific IgG1 assay and 1:200 for specific IgG2a assay. Assays were performed in duplicate on individual sera from 10 mice/group. Results are reported as absorbance units (AU) at 414 nm and median is shown (*, Kruskal-Wallis test and Dunn's multiple comparison test and #, Mann-Whitney test between GF and LC mice).

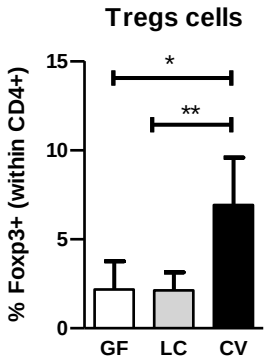


**Fig. 6:** Percentage of Foxp3+ cells within CD4+ cells in splenocytes, expressed as mean ± SEM, after oral sensitization to cow's milk of GF (white bar), LC (gray bar) and CV (black bar) mice.

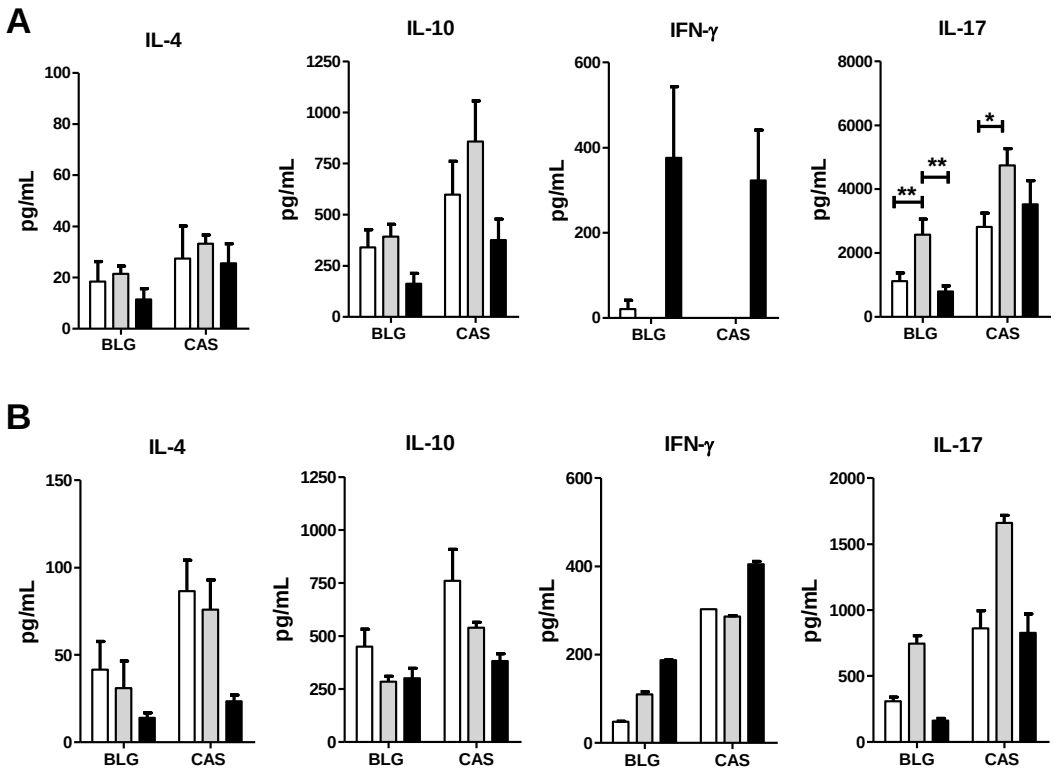


**Fig. 7:** Cytokine secretions by BLG- or CAS-reactivated splenocytes (A) and MLN cells (B). Results are represented as cytokine secretions (pg/mL) in supernatants of reactivated splenocytes after subtraction of cytokines assayed in supernatants of D-PBS-reactivated splenocytes, thus corresponding to specific production. Results are expressed as mean ± SEM. Data were analyzed using one-way ANOVA with Bonferroni multiple comparison test (*$p < 0.05$, **$p < 0.01$). No statistical analysis was performed on data obtained with pooled MLN cells of 10 mice/group (duplicates are shown).

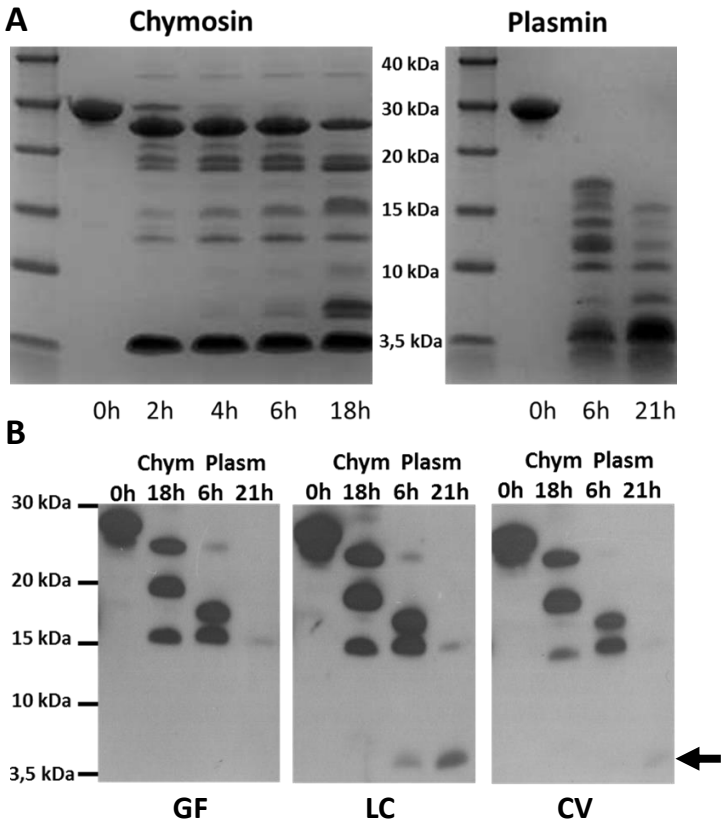


**Fig. 8: A,** Digestion of purified αS1-casein by chymosin or plasmin. Samples were collected at various time points and were analyzed by SDS-PAGE under reducing conditions. **B**, Immunoblot using pooled sera from each group of GF, LC or CV mice, on αs1-casein hydrolysates obtained after digestion with chymosin (18h) or plasmin (6h and 21h). Immunoreactive peptides at 3-5kDa are indicated by an arrow.

# Supplementary Materials and methods

### *In vitro* digestion of αS1-casein by *L. casei* BL23

The protocol of digestion was adapted from those described by Hebert *et al*, 2008 and Munoz-Provencio *et al*, 2012. Cells grown overnight in MRS broth at 37°C were pelleted by centrifugation (6,000 x g, 10 min at RT) and washed twice with phosphate-buffered saline (PBS, pH7). Cells were then suspended in 100 mM MES (morpholineethanesulfonic acid) buffer (pH7) supplemented with glucose (10 g/L) and $CaCl_2$ (5 mM) and adjusted to an $OD_{600}$ of 3 or 10. A solution of purified αS1-casein was also prepared in MES buffer (100 mM, pH7) at a concentration of 2 mg/mL. Cell suspensions were then mixed with the αS1-casein solution at a 1:1 volume ratio. After incubation at 37°C for 24h, cells were pelleted by centrifugation and supernatants (aliquots of 5µL) were analyzed by SDS-PAGE and immunoblot.

## Supplementary figures

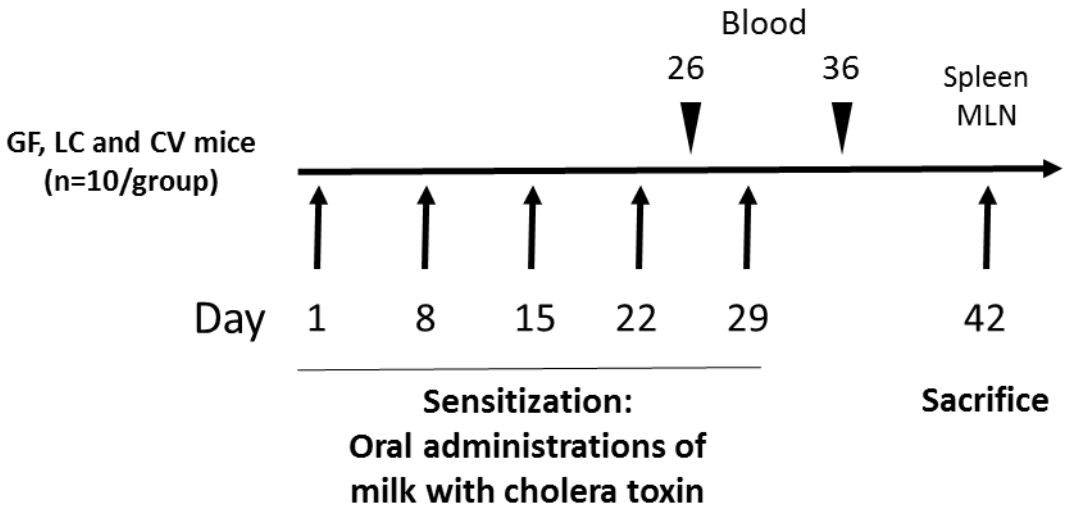


**Fig. E1: Oral sensitization of GF, LC and CV mice to cow's milk.** Experimental setting: Nine-week-old GF, LC and CV mice (n=10/group) were orally sensitized with 200µL of cow's milk mixed with CT (10µg/mouse) on day 1, 8, 15, 22, and 29. Blood samples were collected on day 26 and 36. Spleen and mesenteric lymph nodes (MLN) cells were collected after sacrifice on day 42 for *in vitro* reactivation and flow cytometry analysis.

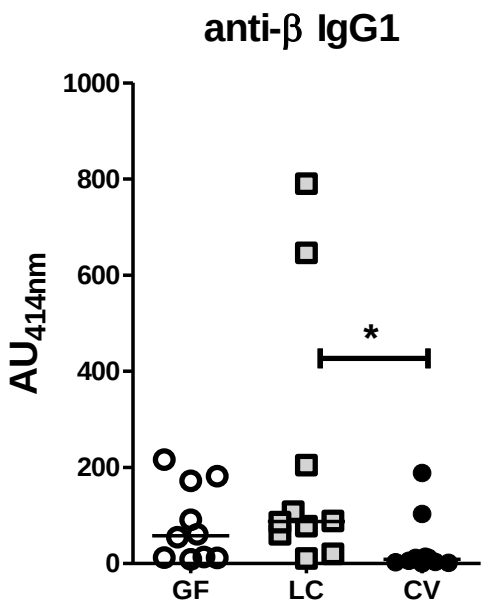


**Fig E2**: IgG1 response toward β-casein. Nine-week-old GF, LC and CV mice received five oral administrations of cow's milk with cholera toxin as adjuvant on day 1, 8, 15, 22, and 29. The dilutions used are 1:10000. Assays were performed in duplicate on individual sera from 10 mice/group. Results are reported as absorbance units (AU) at 414 nm and median is indicated (Kruskal-Wallis test and Dunn's multiple comparison test).

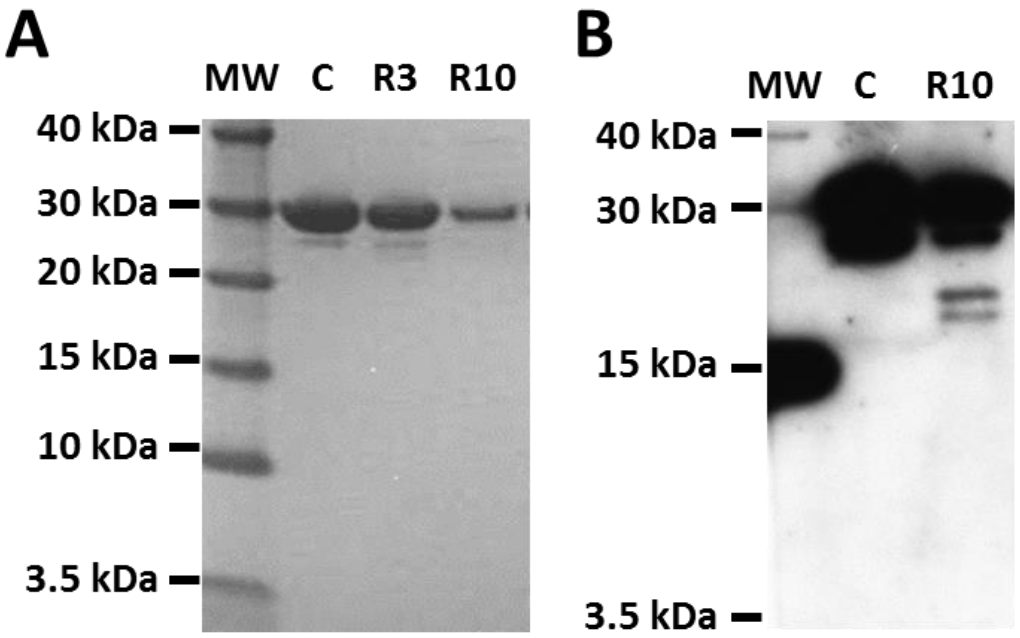


**Fig. E3:** Digestion of αS1-casein by *L. casei* BL23 determined by SDS-PAGE (A) and immunoblot analysis (B). Digestion of αS1-casein was performed for 24h at 37°C by mixing a solution of αS1-casein (2 mg/mL) with a suspension of bacterial cells adjusted at an $OD_{600}$ of 3 (R3) or 10 (R10). C is a sample of αS1-casein incubated without bacterial cells. Immunoblot analysis was performed with the serum from an LC mouse also used in Fig. E4.

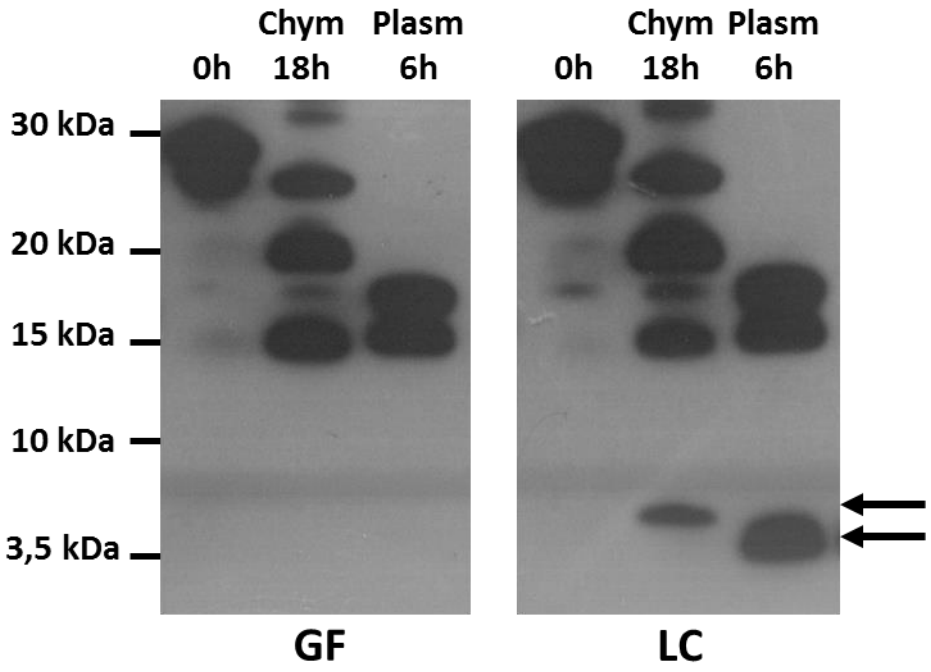


**Fig. E4:** Immunoblot using sera from individual GF and LC mice, on αS1-casein hydrolysates obtained after digestion with chymosin (18h) or plasmin (6h). Immunoreactive peptides at 3-5kDa among chymosin- or plasmin-derived fragments are indicated by arrows.