

RESEARCH ARTICLE

Delayed bacterial colonization of the gut alters the host immune response to oral sensitization against cow's milk proteins

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Scope: Cow's milk allergy is the most prevalent food allergy in infants whose immune system development is critically stimulated during postnatal gut colonization by commensal bacteria. Allergenic potential of cow's milk β -lactoglobulin (BLG) and caseins (CAS) was investigated in germ-free (GF) BALB/c mice and in GF mice conventionalized (CVd) at 6 weeks of age.

Methods and results: Oral sensitization to cow's milk in the presence of cholera toxin led to higher BLG-specific IgE, IgG1, and IgG2a responses in GF mice than in conventional (CV) mice. No significant difference was observed for CAS-specific IgE responses although IgG1 responses to α S1- and κ -caseins were higher in GF mice than in CV mice. CVd mice, orally inoculated with fecal preparations from CV mice, also displayed biased antibody responses compared to CV mice. Secretion of Th2 cytokines by BLG- and CAS-reactivated splenocytes of CVd mice was similar to that of GF mice whereas cytokine production by reactivated cells from mesenteric lymph nodes of CVd mice was equivalent to that of CV mice.

Conclusion: Oral sensitization to BLG and CAS was differentially affected by the absence of gut microbiota and delayed bacterial colonization altered persistently the host immune response to oral sensitization against food antigens.

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1 Introduction

Prevalence of IgE-mediated food allergy is currently increasing in Western countries and affects up to 5% of young children [1]. Allergy to cow's milk, generally the first food introduced into the infant's diet, is the most common food allergy in the first year of life, affecting 1 to 3% of infants [1, 2]. Although cow's milk allergy usually resolves by the age of 4 years, it increases the risk to develop allergic asthma, atopic

eczema, or egg allergy [3]. Cow's milk allergy is characterized by the development of an IgE response toward several cow's milk proteins (CMP). The major allergens of cow's milk are β -lactoglobulin (BLG) and α -lactalbumin from the whey fraction and α S1-, α S2-, β -, and κ -caseins from the whole casein (CAS) fraction [4].

The hygiene hypothesis suggests that the increasing prevalence of allergic and inflammatory diseases in Western countries is related to excessive cleanliness and diminished exposure to microbes [5, 6]. Bacterial gut colonization, which is a major stimulus for the development and the homeostasis of the gut immune system, is thereby altered [7]. Delayed bacterial colonization of the gut has been reported in Western infants and a reduced diversity in the composition of early gut microbiota has been observed in allergic children in comparison with healthy children [8–10].

The critical role of the gut microbiota has also been evidenced in germ-free (GF) mice, which display immature mucosal and systemic lymphoid tissues [7, 11]. GF mice have been shown to develop Th2-biased immune responses

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Abbreviations: BLG, β -lactoglobulin; CAS, whole casein fraction; CMP, cow's milk protein; CT, cholera toxin; CV, conventional; CVd, conventionalized; GF, germ-free; MLN, mesenteric lymph nodes; TTGE, temporal temperature gradient gel electrophoresis; UHT, ultra high temperature

[12–14] and to display defective immunosuppressive functions [14–16]. In a previous work, the propensity of GF mice to develop higher antibody responses than conventional (CV) mice was used to evaluate the intrinsic allergenic and immunogenic potential of BLG and CAS by performing intraperitoneal administrations without adjuvant [17]. However, the absence of IgE response against CAS suggested that this mode of administration was inadequate for studying the allergenic potential of CAS. In this regard, oral route appears to be the most relevant route for administering food antigens. However, it generally leads to the induction of oral tolerance. Therefore, the use of an adjuvant is required to abrogate oral tolerance induction. In this regard, cholera toxin (CT) has been successfully used to orally sensitize CV mice to CMP [18, 19] and to sensitize GF mice to whey proteins or ovalbumin [13, 20].

In the present work, the allergenic and immunogenic properties of BLG, CAS, and α -lactalbumin were evaluated in GF and CV mice. In order to address the effect of the food matrix while preserving the sterility of GF mice, we used ultra high temperature (UHT) sterilized milk to perform oral sensitization. It is also noteworthy that baked milk proteins have been associated with severe and persistent milk allergy in children [21]. In addition, we investigated the impact of delayed gut colonization by commensal bacteria on the host immune reactivity to oral sensitization against CMP.

2 Materials and methods

2.1 Media and reagents

UHT-sterilized unskimmed cow's milk (Candia, Paris, France) and cholera toxin (CT, Sigma-Aldrich, St. Louis, MO, USA) were used for oral sensitization of mice. Brain Heart Infusion broth (BHI, Difco, BD, Le Pont de Claix, France) was used for the preparation of fresh fecal suspensions. CMP were isolated, purified, and characterized, as previously described [22].

2.2 Animals and housing conditions

Conventional SOPF female BALB/cByJ (Cv) mice were purchased from Charles River Laboratories (CRL, l'Arbresle, France) and used after having checked the absence of milk protein in their diet. GF BALB/cByJ mice were bred in the GF animal facilities of Anaxem (INRA, Jouy en Josas, France). GF mice were housed in sterile trexler-type isolators (Getinge-La Calhène, Vendôme, France). Autoclaved tap water and sterilized pelleted standard chow deprived of cow's milk protein (R03–40, SAFE, Augy, France) were given ad libitum. The GF status was monitored by aerobic and anaerobic culture of feces and by microscopic examination of fecal preparations.

All experiments were performed with permission 91–493 of French Veterinary Services.

2.3 Experimental protocols

In the first set of experiments, 7-wk-old GF and CV mice (GF Milk and CV Milk, $n = 8/\text{group}$) were orally sensitized on day 1, 8, 15, 22, and 29 with 200 μL of cow's milk mixed with CT (10 $\mu\text{g}/\text{mouse}$). Blood samples from the retro-orbital venous sinus and fecal samples were collected on day 20, 27, and 34. Control GF and CV mice (GF Ctrl and CV Ctrl; $n = 8/\text{group}$) received saline buffer and were bled on the same days. The administration of CT alone has been previously tested on CV and GF mice ([18] and preliminary results) and no signal specific to CMP has ever been detected. On day 41, spleens were collected for *in vitro* reactivation.

In a second set of experiments, 6-wk-old GF mice were orally inoculated with a 1:100 dilution of fecal homogenate freshly prepared from CV mice. Mice received a second administration of fresh fecal suspension 1 week later. These conventionalized (CVd) mice and CV mice were housed in the same isolator for 4 weeks in order to assure optimal gut colonization by bacteria. On the day preceding the experimental sensitization, pooled feces from CVd and CV mice were collected and stored at -80°C for further characterization by PCR-temporal temperature gradient gel electrophoresis (TTGE). Oral sensitization was then performed as described in the first experiment. Blood samples from the retro-orbital venous sinus and fecal samples were collected on day 26 and 36. On day 41, spleens and mesenteric lymph nodes (MLN) were collected for *in vitro* reactivation.

2.4 DNA isolation, 16S rRNA gene amplification, and TTGE fingerprinting

Total DNA was extracted from individual feces as previously described [23]. Afterwards, the V6-V8 region of 16S rRNA gene was amplified using the HotStar Master Mix Kit (Qiagen, Courtaboeuf, France). The Dcode universal mutation detection system (BioRad, Paris, France) was used to separate PCR products according to their specific sequence (GC percentage) by TTGE. Electrophoresis was performed as described earlier [23]. Gels were stained with SYBR green I nucleic acid gel stain (Roche Diagnostics, GmbH, Mannheim, Germany) and read on the Storm system (Molecular Dynamics). TTGE profiles were analyzed with the Gelcompar software program (version 2.2, Applied Maths, Kortrijk, Belgium). The analysis took into account the number of bands, their position on the gel, and their intensity. Similarity coefficients (Pearson correlation method) were calculated for each pair of profiles, yielding a similarity matrix. A dendrogram was constructed

from this matrix by performing an unweighted pair group method using arithmetic averages (UPGMA) algorithm [23, 24].

2.5 Quantification of specific antibodies in serum and feces

Purified CMP were passively coated on plates (Nunc Immunoplate Maxisorp) and incubated overnight with appropriate dilutions of sera in EIA buffer (0.1 M phosphate buffer, 0.1% BSA, 0.15 M NaCl). Allergen-specific IgG1, IgG2a, IgA, and IgE responses were evaluated as previously described [25]. Specific IgA responses were measured in fecal samples [26]. Results are reported as absorbance units (AU) at 414 nm.

2.6 Spleen and MLN cell culture and cytokine production

At the end of experiments, four pools of two spleens were constituted per group of eight mice. Spleens were homogenized in RPMI-10 (RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U penicillin, 100 µg/mL streptomycin). After lysis of red blood cells (180 mM NH₄Cl, 17 mM Na₂EDTA), splenocytes were resuspended in RPMI-10. In the second experiment, *in vitro* reactivation was also performed on MLN cells in order to compare systemic and local immune responses. Therefore, MLN from each group of mice were pooled and cells were resuspended in RPMI-10. Cells from spleen (10⁶ cells/well) and MLN (5.10⁵ cells/well) were incubated for 60 h at 37°C (5% CO₂) 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, Paisley, UK) as a negative control. Cytokine levels were assayed using Cytosets™ Kit (BioSource International Europe, Nivelles, Belgium) or BioPlex technology according to the manufacturer's recommendations (BioRad, Hercules, CA, USA). In the second experiment, in addition to Th1 and Th2 cytokines, the profile of cytokines measured was extended to IL-10 and IL-17. Results from splenocyte reactivation are expressed as mean ± SD.

2.7 Statistical analyses

Data were analyzed using the nonparametric Mann–Whitney test to compare two different groups or the nonparametric Kruskal–Wallis test and Dunn's multiple comparison test to compare three different groups. Statistical analyses were performed with GraphPad Prism 5.01 software and $p < 0.05$ was considered significant (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). Data on cytokine secretions were analyzed using the unpaired *t*-test.

3 Results

3.1 Oral sensitization of GF and CV BALB/c mice to cow's milk

3.1.1 Antibody responses

Production of BLG-specific IgE and IgG1 was statistically significant in GF mice after the third milk administration (Fig. 1A). In contrast, BLG-specific IgE and IgG1 responses in CV mice were significant only after the fourth milk administration and were lower than those measured in GF mice. BLG-specific IgG2a and IgA antibodies were detected in GF mice whereas none was observed in CV mice. At the end of the experiment, BLG-specific IgE, IgG1, IgG2a, and IgA responses were all significantly higher in GF mice than in CV mice. No production of specific antibodies was observed in sera from GF and CV control mice (data not shown).

In contrast, differences between GF and CV mice for CAS-specific antibody responses did not reach statistical significance (Fig. 1B). Compared to the BLG-specific IgE response, production of CAS-specific IgE antibodies was delayed in GF mice since it increased only after the fourth milk administration. It is also noteworthy that production of CAS-specific IgG2a and IgA was significant in GF mice only.

The final IgE and IgG1 responses against the four fractions of CAS were then determined (Fig. 2). No significant difference between GF and CV mice was observed for CAS-specific IgE responses, which were directed only against α S1- and α S2-caseins (Fig. 2A). Production of IgG1 antibodies specific to α S1- and α S2-caseins was also not significantly different between GF and CV mice ($p = 0.13$ and 0.23 , respectively). No significant production of β -casein-specific IgG1 was detected (Fig. 2B) but a significant production of κ -casein-specific IgG1 was evidenced in GF mice.

3.1.2 Cytokine production

After BLG reactivation of splenocytes, no significant difference between GF and CV mice was observed for the secretions of Th2-cytokines IL-4 and IL-5 or Th1-cytokine IFN- γ (Fig. 3A). In contrast, secretion of IL-4 and IL-5 after CAS-reactivation was significantly higher in CV mice than in GF mice (Fig. 3B). Secretion of IFN- γ after BLG or CAS reactivation was statistically significant only in CV mice (Fig. 3).

3.2 Impact of delayed gut colonization on the susceptibility of BALB/c mice to develop oral sensitization against cow's milk

3.2.1 Conventionalization of GF mice

As shown in Fig. 4, colonization of GF mice was successful as CV and CVd mice displayed comparable gut bacterial

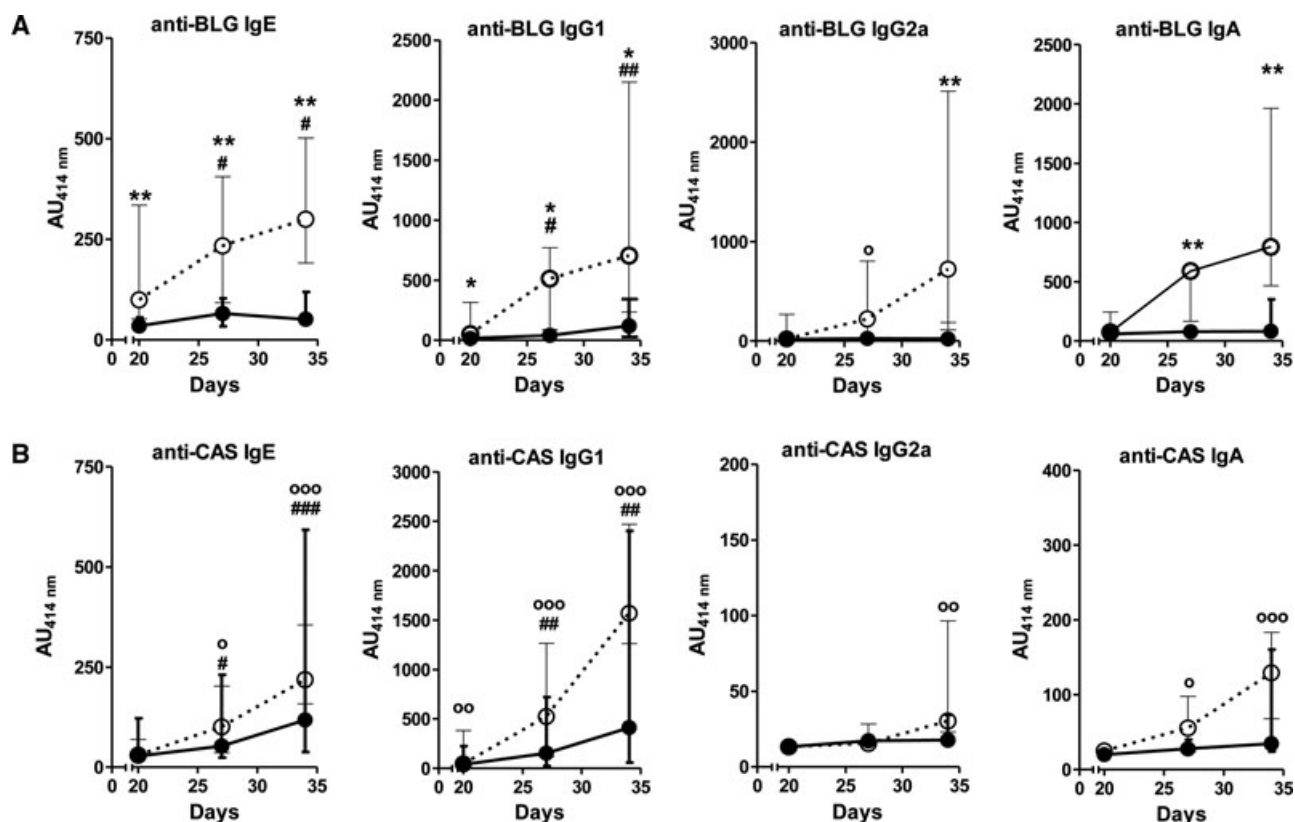


Figure 1. Time-course of specific antibody responses toward β -lactoglobulin (BLG) (A) and whole casein fraction (CAS) (B) in germ-free (GF) mice (open circles) and conventional (CV) mice (closed circles) orally sensitized to cow's milk. Assays were performed as duplicate on sera from eight mice/group. Sera were diluted 1:50 for IgE determination, 1:100 for IgG2a and IgA determination and 1:1000 for IgG1 determination. No specific antibody response was detected in control mice (see Section 2). Specific Ig levels were reported as Absorbance Units (AU) at 414 nm. Median and interquartile range are shown. GF Milk significantly different from CV Milk (Mann–Whitney test, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$), GF Milk from GF Ctrl (° symbol) and CV Milk from CV Ctrl (# symbol).

diversity. The TTGE-banding patterns were compared in a single dendrogram that did not reveal any clustering according to the timing of gut bacterial colonization.

3.2.2 Antibody responses

At first, CVd and CV mice seemed to be equally responsive to the protocol of sensitization in terms of BLG-specific antibody responses. Indeed, anti-BLG IgE, IgG1, IgG2a, and IgA responses in CVd mice were similar to those found in CV mice and significantly lower than those in GF mice after the fourth milk administration (Fig. 5A). However, after the last milk administration, BLG-specific antibody responses in CVd mice were not anymore significantly different from those observed in GF mice. IgG1 and IgA responses in CVd were even significantly higher than in CV mice. In addition, IgG1 and IgG2a responses against α -lactalbumin, another whey protein, were higher in CVd than in CV mice (Supporting Information Fig. S1) but no IgE response was detected against α -lactalbumin in any group of mice.

Production of α S1- and κ -casein-specific IgG1 antibodies was significantly higher in CVd mice than in CV mice (Fig. 5B). Using the Mann–Whitney test, production of α S1-casein-specific IgG1 in GF mice was also significantly higher than in CV mice ($p = 0.038$). No significant difference was evidenced between CAS-specific IgE responses from GF, CVd, and CV mice (data not shown).

Fecal BLG- and κ -casein-specific IgA responses remained significantly lower in CVd and CV mice compared to GF mice, even after the last milk administration (Fig. 5C). On the contrary, fecal α S1- and α S2-casein-specific IgA responses in CVd mice were similar to those measured in GF mice and remained significantly higher than in CV mice.

3.2.3 Cytokine production

As observed previously, CAS reactivation of splenocytes resulted in a higher production of Th2 cytokines (IL-5 and IL-13) and IL-10 in CV mice than in GF mice while BLG reactivation did not reveal any significant difference, except for IL-13 production (Fig. 6A). In this case, reactivated splenocytes

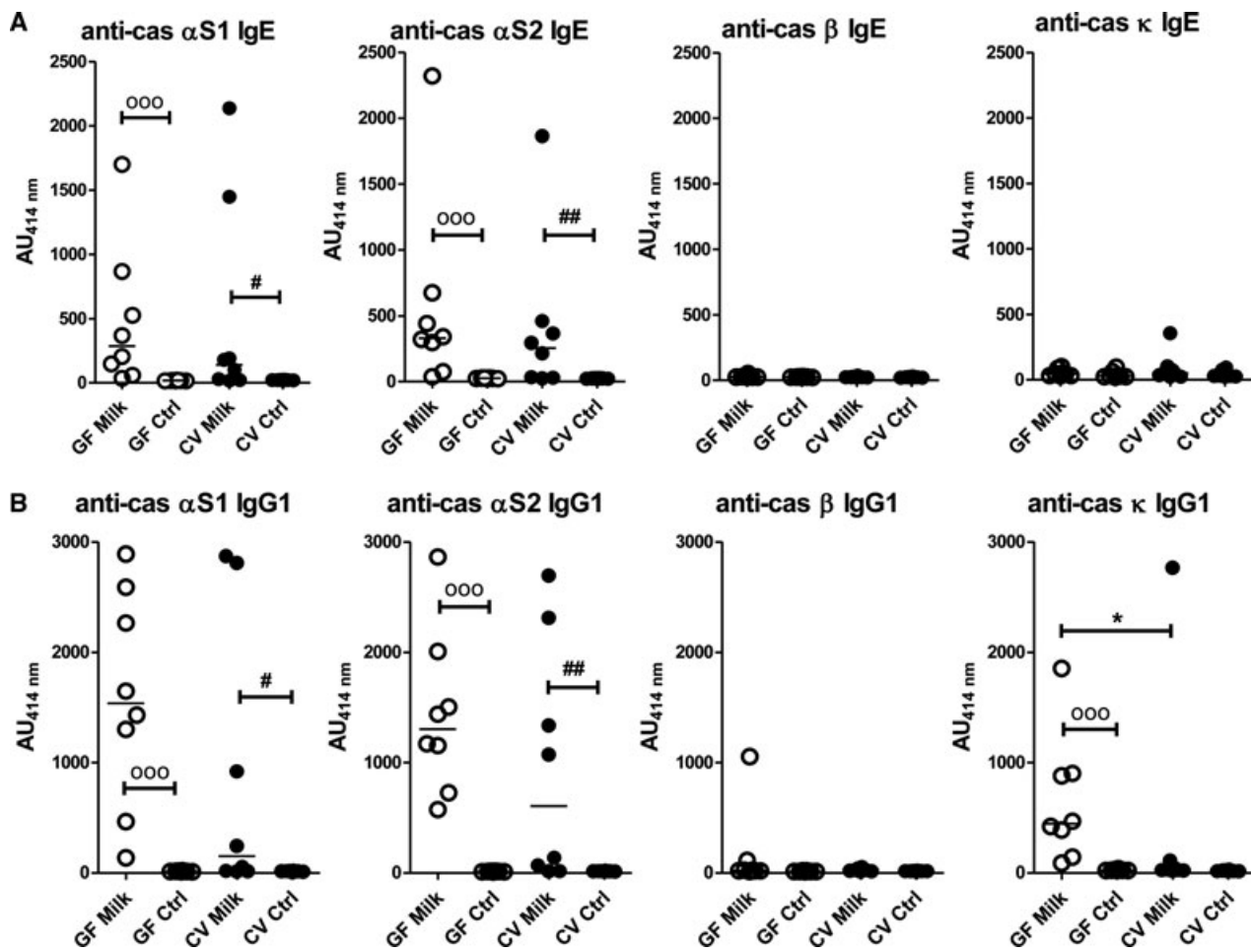


Figure 2. Specific IgE (A) and IgG1 (B) responses toward the different fractions of caseins in GF mice (open symbols) and CV mice (black symbols) after the last administration of cow's milk (see Section 2). Assays were performed as duplicate on sera from eight mice/group. Sera were diluted 1:1000. Specific antibody responses were reported as AUs at 414 nm and median is shown. GF Milk significantly different from CV Milk (Mann–Whitney test, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$), GF Milk from GF Ctrl (° symbol) and CV Milk from CV Ctrl (# symbol).

from CVd mice displayed a pattern of cytokine secretion very similar to the one observed in GF mice. No significant differences were evidenced for the production of IFN- γ and IL-17. In contrast, reactivation of pooled MLN cells resulted in a totally different profile of cytokine production (Fig. 6B). Secretion of Th2 cytokines was higher in GF mice than in CV mice, after both BLG and CAS reactivation. Reactivated MLN cells from CVd mice exhibited a pattern of cytokine secretion comparable to the one observed in CV mice. Production of IL-17 and IFN- γ was also higher in CV and CVd mice than in GF mice.

4 Discussion

In contrast to BLG, intraperitoneal administrations of CAS without adjuvant did not induce specific IgE antibodies in GF BALB/c mice [17]. In this work, GF mice were sensi-

tized via the oral route with cow's milk in order to compare the immunogenic and allergenic potential of BLG, CAS, and α -lactalbumin in presence of the whole food matrix.

Despite the use of CT as an adjuvant, specific IgE responses were restricted to a limited number of CMP. In addition to BLG, specific IgE antibodies were produced only against α S1- and α S2-caseins whereas no IgE response was detected toward α -lactalbumin, β -, and κ -caseins. The susceptibility of GF mice to the allergenic properties of BLG and α S1-casein is in good agreement with prevalence data since BLG and α S1-casein have been reported to induce the highest specific IgE response in CM-allergic infants [27]. It is nevertheless noteworthy that the production of specific IgE antibodies was detected only against caseins that share the lowest level of identity with their murine counterparts. Indeed, sequences of bovine α S1-, α S2-, β -, and κ -caseins display a percentage of identity of 19%, 21%, 36%, and 33% with the corresponding murine homologues.

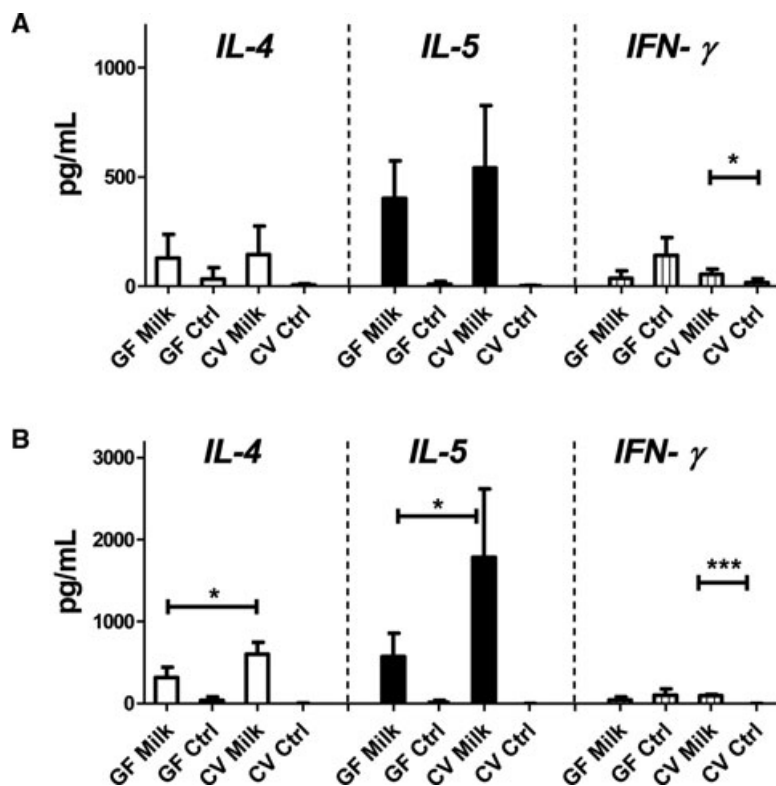


Figure 3. Cytokine secretions by (A) BLG- or (B) CAS-reactivated splenocytes from mice orally sensitized to cow's milk (see section 2). Results are represented as cytokine secretions in supernatants of reactivated splenocytes after subtraction of cytokines assayed in supernatants of D-PBS-reactivated splenocytes, thus corresponding to specific production. Data were analyzed using the unpaired *t*-test (**p* < 0.05, mean + SD was shown).

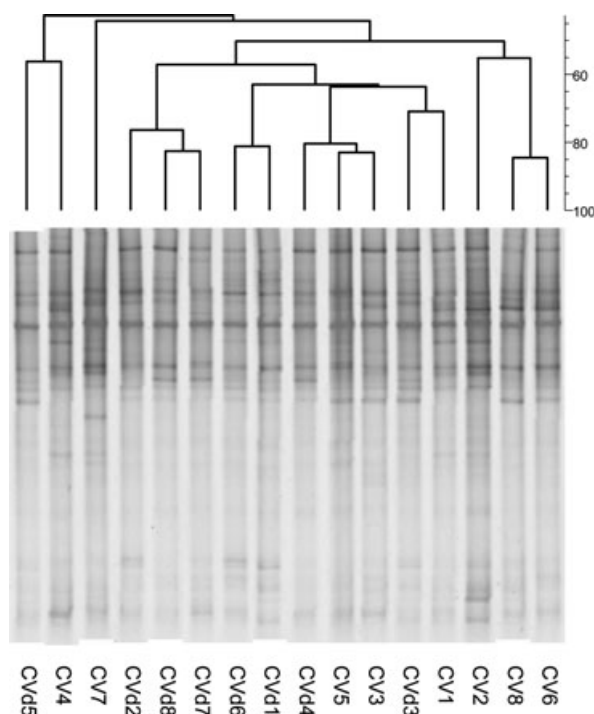


Figure 4. Temporal temperature gradient gel electrophoresis (TTGE) fingerprinting profiles of 16S rRNA gene amplicons (obtained using primers for the V6-V8 region) of individual feces from CVd and CV mice on the day preceding oral sensitization to cow's milk (see Section 2).

As expected, GF mice exhibited Th2-biased antibody responses with higher BLG-specific IgE and IgG1 levels than CV mice. However, in contrast with previous results obtained after intraperitoneal injections of BLG [12, 17], oral administrations of cow's milk to GF mice also led to higher levels of BLG-specific IgG2a than in CV mice. In the same way, production of α -lactalbumin-specific IgG2a antibodies was higher in GF mice while CAS-specific IgG2a response was significant only in GF mice. Thus, whereas sensitization via intraperitoneal route showed Th2-biased antibody responses in GF mice, sensitization via the oral route evidenced a general hyperresponsiveness of GF mice with both excessive Th2 and Th1 serum antibody responses to dietary antigens. Similarly, Tsuda et al. reported the role of intestinal commensal bacteria in transgenic BALB/c OVA23–3 mice for the down-regulation of both Th2 and Th1 antibody productions in response to an ovalbumin-containing diet [14].

Surprisingly, the early and strong BLG-specific IgE response in GF mice was not accompanied with higher levels of CAS-specific IgE antibodies in GF mice compared to CV mice. This fact suggests that IgE responses toward BLG or CAS resulted from two different mechanisms of sensitization, the one involved in BLG sensitization being more efficient in GF mice than in CV mice. In this regard, Roth-Walter et al. reported that soluble BLG can be transcytosed through enterocytes while uptake of insoluble CAS or aggregated BLG occurs in Peyer's patches [28]. Although UHT milk was used in order to preserve the sterility of GF mice,

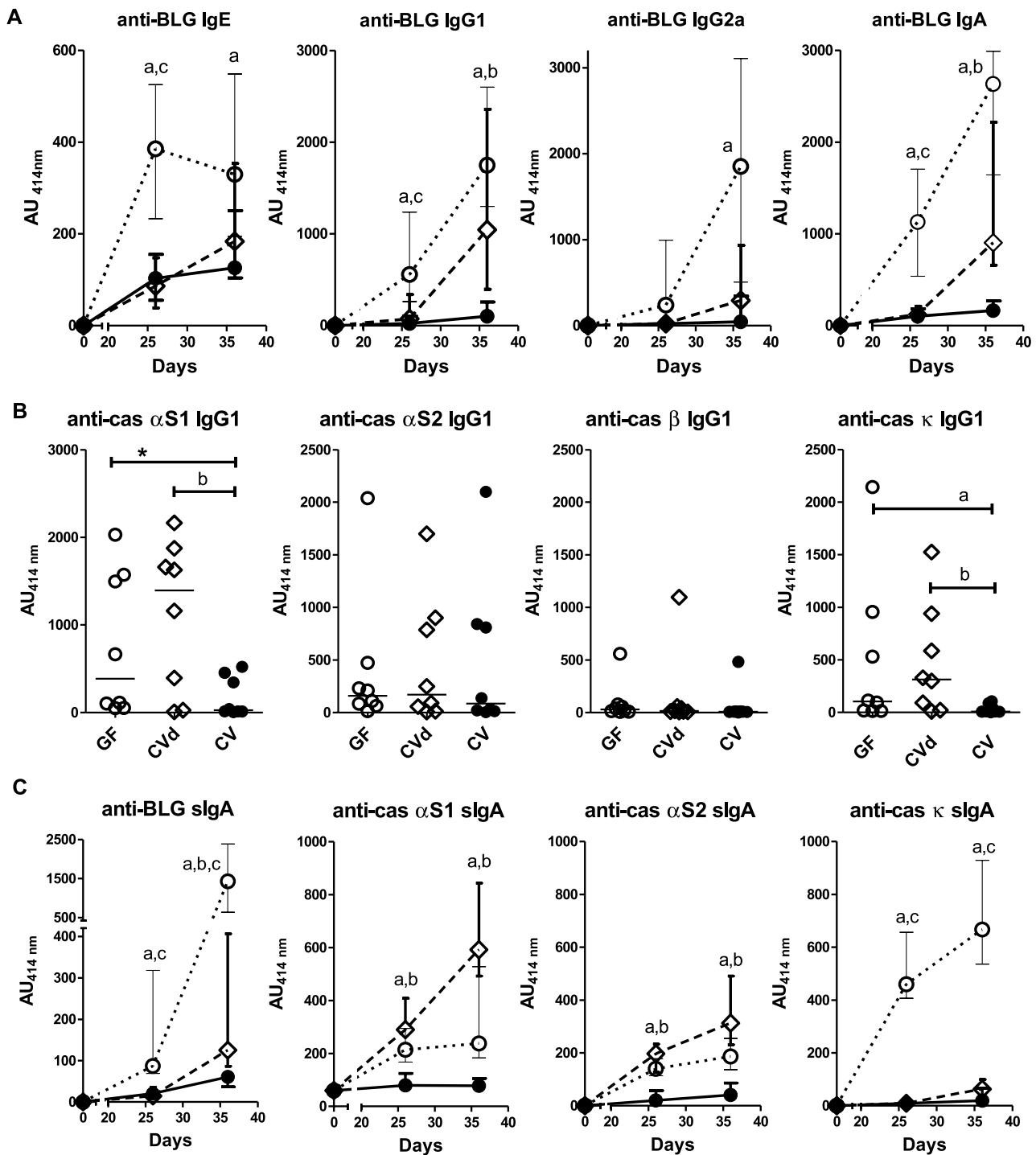


Figure 5. Impact of delayed gut colonization on serum antibody responses toward BLG (A, on day 26 and 36) and α S1-, α S2-, β - and κ -caseins (B, on day 36) and on fecal IgA responses toward BLG, α S1-, α S2- and κ -caseins (C, on day 26 and 36). GF mice (open circle), CVd mice (open diamonds), and CV mice (closed circles) were orally sensitized to cow's milk (see Section 2). Assays were performed as duplicate on sera from eight mice/group. Specific antibody responses were reported as AUs at 414 nm. Median and interquartile range are shown. Data were analyzed using Kruskal–Wallis test and Dunn's multiple comparison test. Significant differences are indicated for GF Milk versus CV Milk (^a $p < 0.05$), for CVd Milk versus CV Milk (^b $p < 0.05$) and for GF Milk versus CVd Milk (^c $p < 0.05$).

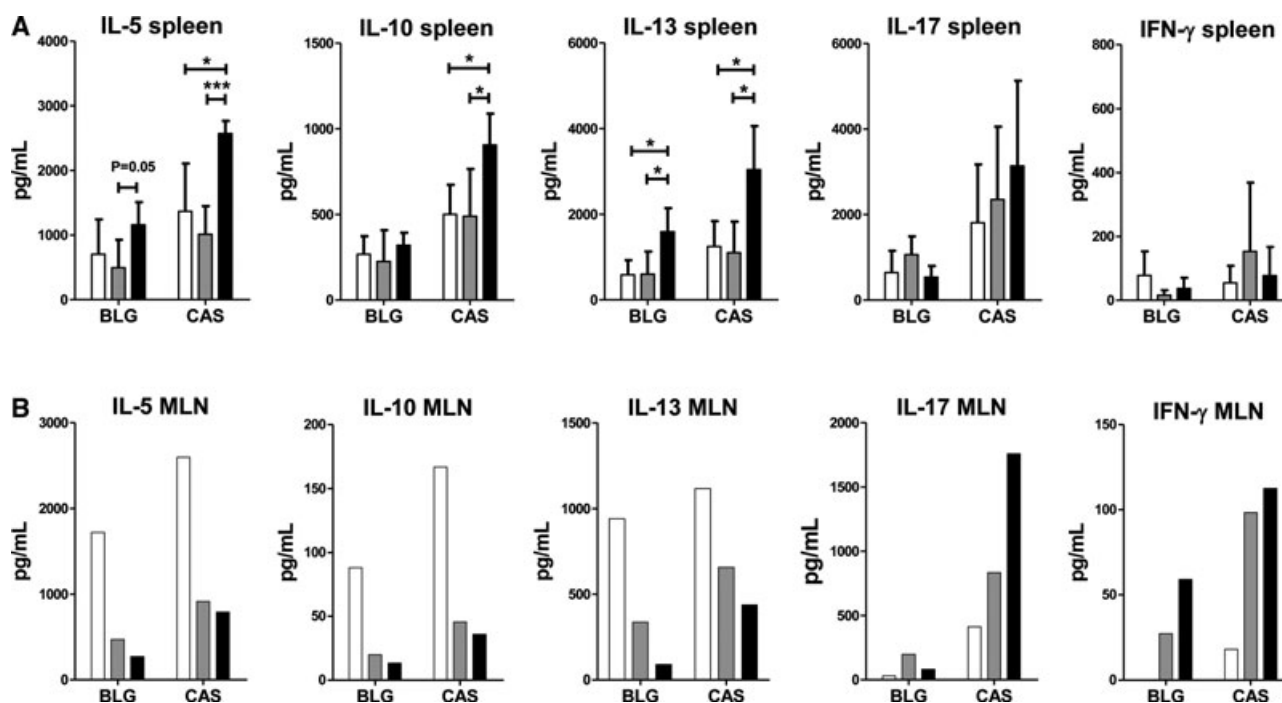


Figure 6. Cytokine secretions by BLG- or CAS-reactivated splenocytes (A) and MLN cells (B) from mice orally sensitized to cow's milk (see Section 2). Results are represented as cytokine secretions in supernatants of reactivated cells after subtraction of cytokines assayed in supernatants of D-PBS-reactivated cells, thus corresponding to specific production. Data on reactivated splenocytes were analyzed using the unpaired *t*-test (**p* < 0.05, mean + SD was shown). No statistical analysis was performed on data obtained with pooled MLN cells of eight mice/group.

2 to 10% of the BLG retained its native conformation and thereby its solubility [29, 30]. The allergenic potential of BLG may thus be enhanced in GF mice because of the capacity of the residual soluble BLG to be transcytosed through enterocytes. Furthermore, as development of Peyer's patches in GF mice is impaired because of the absence of gut microbiota [31], sensitization to CAS via uptake through Peyer's patches may be less efficient in GF mice than in CV mice. Accordingly, production of Th2 cytokines by CAS-reactivated splenocytes was significantly higher in CV mice than in GF mice. However, Th2-cytokine secretion in reactivated splenocytes was associated with a higher level of IgE antibodies in GF mice than in CV mice. Indeed, similar IL-4 and IL-5 production in BLG-reactivated splenocytes of GF and CV mice correlated with higher BLG-specific IgE responses in GF mice compared to CV mice. In the same way, equivalent CAS-specific IgE responses in GF and CV mice correlated with lower IL-4 and IL-5 production in CAS-reactivated splenocytes of GF mice. Nevertheless, IgG1 responses toward κ - and α S1-caseins remained significantly higher in GF mice than in CV mice, thus confirming their strong intrinsic immunogenicity as previously reported after intraperitoneal administrations without adjuvant [17].

Conventionalization of GF mice was performed 3 weeks after weaning in order to determine whether hyperresponsiveness of GF BALB/c mice to oral sensitization could be

modulated after a delayed acquisition of gut microbiota. Indeed, Herbst et al. reported that exposure of adult GF C57BL/6 mice to a complex commensal microbiota is effective at reversing elevated IgE production induced by intraperitoneal injections of ovalbumin [32]. Similarly, Rodriguez et al. described that GF C3H mice colonized with an infant gut microbiota at weaning age were protected from excessive BLG-specific IgG1 response although no difference in the BLG-specific IgE response was evidenced between GF, CVd, and CV mice [33]. These results suggest that even late acquisition of a complex gut microbiota could be sufficient to protect mice from the development of allergic responses. However, bacterial stimulation of the immune system during the early postnatal life is considered to be critical for the proper development of immunoregulatory mechanisms, as postulated by the hygiene hypothesis. In this regard, two recent studies showed that the absence of proper host–microbe interaction during the first weeks of life can permanently change the immune profiles of mice and may thus alter the immune system reactivity [34, 35]. Accordingly, in the present work, CVd mice were likely affected by the lack of bacterial stimulation during postnatal period since they remained more responsive to experimental sensitization than CV mice. Time course of antibody responses revealed nevertheless that CVd mice seemed at first more resistant than GF mice to the development of antibody responses against CMP. Moreover, some

local immune responses in CVd mice such as the fecal BLG- and κ -casein-specific IgA responses were similar to those measured in CV mice. Cytokine productions in reactivated MLN cells of CVd and CV mice were also similar although they were significantly different in reactivated splenocytes. Interestingly, Hansen et al. reported that distribution of several mononuclear cell populations was differentially altered in MLN and spleen by a GF postnatal period [34]. In that study, higher relative amounts of NK cells, NKT cells, or IFN- γ -producing T cells were detected in spleens, but not in MLN, of both GF and CVd mice compared to CV mice.

In conclusion, GF mice orally sensitized to CMP were significantly more responsive to the allergenic potential of BLG than CV mice. In contrast, no significant difference was observed between GF and CV mice for the CAS-specific IgE response, although α S1- and κ -caseins were more immunogenic in GF mice. The mechanisms leading to oral sensitization to BLG and CAS are thus probably differentially affected by the absence of gut microbiota. In addition, the delayed acquisition of gut microbiota by GF BALB/c mice persistently affected the reactivity of their immune system and altered systemic and local responses toward the major CM allergens. Further studies are required to elucidate the mechanisms by which gut microbiota affects the proper maturation of the host immune system. In this regard, studies based on the use of gnotobiotic mice should take carefully into consideration the timing of bacterial inoculation, at birth, weaning or during adulthood.

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Supporting Information Figure S1:

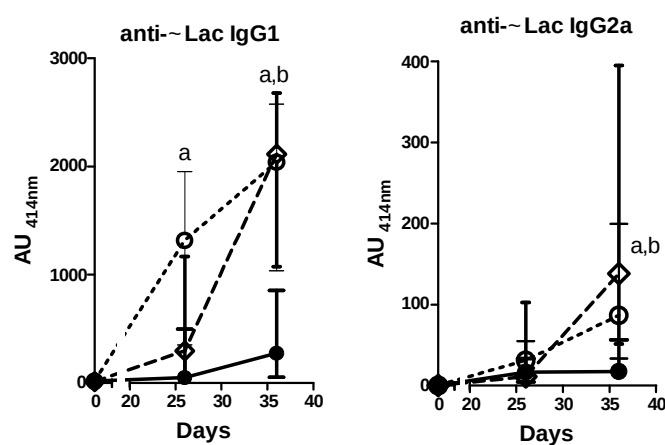


Figure S1: Impact of delayed gut colonization on IgG1 and IgG2a responses toward α -lactalbumin. GF mice (open circle), CVd mice (open diamonds) and CV mice (closed circles) were orally sensitized to cow's milk (see Section 2). Assays were performed as duplicate on sera from 8 mice/group. Specific antibody responses were reported as AUs at 414 nm. Median and interquartile range were shown. Data were analysed using Kruskal-Wallis test and Dunn's multiple comparison test. Significant differences were indicated for GF Milk vs CV Milk (^a $p < 0.05$), for CVd Milk vs CV Milk (^b $p < 0.05$) and for GF Milk vs CVd Milk (^c $p < 0.05$).