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► To cite this version:

Stéphanie S. Morin, Hervé Bernard, Laetitia L. Przybylski-Nicaise, Gerard G. Corthier, Sylvie S. Rabot, et al. Allergenic and immunogenic potential of cow's milk beta-lactoglobulin and caseins evidenced without adjuvant in germ-free mice. *Molecular Nutrition and Food Research*, 2011, 55 (11), pp.1700-1707. 10.1002/mnfr.201100024 . hal-01001247

HAL Id: hal-01001247

<https://hal.science/hal-01001247v1>

Submitted on 1 Sep 2023

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This is the peer-reviewed version of the following article: “Allergenic and immunogenic potential of cow's milk β -lactoglobulin and caseins evidenced without adjuvant in germ-free mice.”, which has been published in final form at

<https://doi.org/10.1002/mnfr.201100024>

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Allergenic and immunogenic potential of cow's milk β -lactoglobulin and caseins evidenced without adjuvant in germ-free mice.

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Abbreviations: **BLG**, β -lactoglobulin; **BLGn**, native BLG; **BLGd**, denatured BLG, **CAS**, whole casein fraction; **CMA**, cow's milk allergy, **CMP**, caseinomacropptide; **GF**, germ-free; **CV**, conventional

Keywords: Allergen/ β -lactoglobulin/Casein/Germ-free/Heat-treatment/Immunogenicity

Abstract

Scope: In most animal models of allergy, the development of an IgE response requires the use of an adjuvant. Germ-free (GF) mice exhibit Th2-polarized antibody responses combined with defective immunosuppressive mechanisms. The sensitizing potential of milk proteins was investigated in GF mice in the absence of adjuvant.

Methods and results: β -lactoglobulin (BLG) and caseins (CAS) allergenicity was evaluated by means of intraperitoneal injections without adjuvant. Injections of BLG induced significant IgE and IgG1 responses in GF mice while CAS injections provoked the production of IgG1 toward κ - and α S1-caseins. No significant antibody response was evidenced in conventional (CV) mice. After *in vitro* BLG-reactivation, IL-4, IL-5, IL-13 and IFN- γ productions by splenocytes were higher in GF mice than in CV mice. Heat-treatment decreased BLG allergenicity as indicated by the absence of IgE production in GF mice. However, heat-treatment increased protein immunogenicity and led to the production of anti-BLG and anti- κ -casein IgG1 in both GF and CV mice. This correlated with enhanced productions of IL-4, IL-5 and IL-13 in BLG-reactivated splenocytes from CV mice.

Conclusions: Gut colonization by commensal bacteria appeared then to significantly reduce the susceptibility of mice toward the intrinsic allergenic and immunogenic potential of milk proteins.

1 Introduction

Cow's milk allergy (CMA) affects about 2.5% of infants during their first years of life and is the most common food allergy in early childhood [1]. Although most children outgrow CMA by 3 years of age, CMA increases the risk of developing persistent CMA and other atopies, such as allergic asthma, atopic eczema, rhinoconjunctivitis or egg allergy [2]. Patients suffering from CMA develop an IgE response toward different cow's milk proteins. They are mainly sensitized to β -lactoglobulin (BLG) and α -lactalbumin from the whey fraction and to α S1-, α S2-, β - and κ -caseins from the whole casein (CAS) fraction [3]. BLG is susceptible to heat denaturation that leads to the exposure of new antigenic sites and thus enables the production of antibodies specific to non-conformational, *i.e.* sequential epitopes [4]. In contrast, caseins are natively unfolded proteins [5, 6]. Their antigenicity and allergenicity are not affected by heat-treatment [7-9].

In most murine models of allergy, an adjuvant is used in order to enhance IgE responses and to elicit allergic reactions [10-13]. However, the use of adjuvant may induce specific IgE responses against proteins that are usually not considered as allergenic [14, 15]. Thereby, protocols without adjuvant have been proposed to study the intrinsic allergenic potential of a protein. As oral administration leads generally to the induction of oral tolerance, other routes of administration have been considered such as transdermal applications or intraperitoneal (i.p.) injections. These strategies have been particularly tested on BALB/c mice that present a profile of high IgE responders equivalent to an atopic phenotype [16]. Transdermal sensitization to milk proteins led to the induction of an IgE response toward BLG and α -lactalbumin while after i.p. injections of BLG at different doses, most of the evaluated mouse models required adjuvant to get IgE [15, 17, 18].

Germ-free (GF) mice are reared in a sterile environment and thereby, exhibit an immature immune system because of the absence of viable bacteria [19]. We recently

investigated the influence of the gut microbiota on the sensitization level of BALB/c mice after i.p. injection of BLG with incomplete Freund's adjuvant. GF mice were shown to develop a higher BLG-specific IgE response than conventional (CV) mice [20]. The immunosuppressive mechanisms observed in CV mice, presumably through the activation of Treg cells, were also reported to be defective in GF mice [21-23]. In this regard, the absence of efficient immunosuppressive mechanisms in GF BALB/c mice could be of interest in order to avoid the use of adjuvant for evaluating the sensitizing potential of a protein.

In the present work, we investigated whether the intrinsic allergenic and immunogenic potential of two major milk allergens, namely BLG and CAS, could be evidenced in GF mice in the absence of adjuvant. The capacity of BLG, α S1-, α S2-, β - and κ -caseins, to induce systemic antibody responses was first determined. The cellular response was also evaluated by performing *in vitro* splenocytes reactivation. In addition, we tested whether heat-treatment of BLG and CAS mixed together, and the resulting intermolecular rearrangements, could affect the humoral and cellular response induced by i.p. injections of these proteins.

2 Materials and Methods

2.1 Reagents

Caseins and BLG were isolated, purified and characterized, as previously described [24]. In order to inhibit proteolytic degradation by plasmin, the purification of caseins involved heat-treatment (20 min at 100°C, [25]). Endotoxin levels in purified milk proteins were below the detection limit of 0.1 EU/mg (QCL-1000 kit, Lonza Walkersville, Walkersville, Md, USA).

The BLG purified from cow's milk is considered to be BLG in the native conformation (BLG_n). Reduced and alkylated BLG, prepared as described by Negroni *et al.* [4], is considered to be BLG in a denatured conformation (BLG_d). A solution containing CAS (2.34 mg/mL) and BLG (0.33 mg/mL) in PBS (100 μ L) was heated in an Eppendorf Biopur tube for

10 min at 95°C before being rapidly cooled in an ice-water mixture. Quantification of the residual native BLG was performed and showed that more than 94% of the BLG was heat-denatured [4].

2.2 Mice

Conventional SOPF female BALB/cByJ mice (Charles River Laboratories, L'Arbresle, France), were used after having checked the absence of milk protein in their diet. Germ-free BALB/cByJ mice were bred in the germ-free animal facilities (ANAXEM platform, INRA, Jouy-en-Josas, France) and housed in sterile Trexler-type isolators (Getinge-La Calhène, Vendôme, France). Autoclaved tap water and a sterilized pelleted standard chow deprived of cow's milk proteins (R03, SAFE, Augy, France) were given ad libitum. The germ-free status was monitored by aerobic and anaerobic culture of feces and by microscopic examination of fecal preparations. Animals were 7-8 weeks old at the start of the experiments. All experiments were performed with permission 91-493 of the French Veterinary Services.

2.3 Experimental protocols

In a first set of experiments, GF and CV mice (n=8/group) were injected intraperitoneally with 5 µg of filter-sterilized BLG (GF-BLG and CV-BLG) or CAS (GF-CAS and CV-CAS) dissolved in D-PBS (Gibco, Invitrogen) on day 1 and day 18. Blood samples from the retro-orbital venous sinus and fecal samples were collected on day 11 and 31. Control GF and CV mice injected with D-PBS (GF-PBS and CV-PBS, n=8/group) were bled on the same days. On day 36, spleens were collected for *in vitro* reactivation. In a second set of experiments, GF and CV mice (n=8/group) were injected intraperitoneally with a heated (GF-HP and CV-HP) or non-heated (GF-NP and CV-NP) mix containing 5 µg of CAS and 0.7 µg of BLG on day 1 and 19. Blood samples were collected on day 32 and spleens were collected on day 36.

2.4 Quantification of BLG-specific antibodies in serum and in feces.

Epitope specificity of BLG-specific antibodies was determined using two different assays. The detection of antibodies recognizing both sequential and conformational epitopes of BLG_n was performed by using biotinylated BLG immobilized on neutravidin-coated plates. The detection of antibodies recognizing only the denatured form of BLG was performed by using BLG_d-coated plates. Purified caseins were as well directly coated. Plates were then incubated overnight with diluted sera (1:50 unless otherwise noted) in EIA buffer (0.1 M phosphate buffer, 0.1% BSA, 0.15 M NaCl). Allergen-specific IgG1, IgG2a, IgA and IgE binding and measurement of total IgE levels were performed as previously described [10]. Results are reported as absorbance units (AU) at 414 nm. Non-specific binding (NSB) was determined using sera from PBS-injected mice. A response was considered significant when higher than $NSB + 3\sigma_{n-1}$. Secretion of specific IgA and IgG1 in fecal samples was also measured [26].

2.5 Spleen cell culture and cytokine productions

At the end of the experiments, spleens from each group of mice were pooled 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. Cells were incubated for 60h at 37°C (5 % CO₂) in 96-well culture plates (10⁶ cells/well) in the presence of BLG (20 µg/mL), CAS (20 µg/mL) or D-PBS (negative control). The IL-4 and IFN-γ cytokines level was assayed in duplicate using CytoSets™ kit (BioSource International Europe, Nivelles, Belgium). IL-5 and IL-13 were assayed using the BioPlex technology according to the manufacturer's recommendations (BioRad, Hercules, CA, USA). No statistical analysis was performed as results are expressed as mean ± SD of duplicate determination on pools.

2.6 Statistical analyses.

Data were analyzed using the non-parametric Mann-Whitney test to compare the different groups with the control group. Statistical analyses were performed with GraphPad Prism 5.01 software and a $P < 0.05$ was considered significant (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

3 Results

3.1 Susceptibility of GF and CV BALB/c mice to the intrinsic allergenicity and immunogenicity of BLG and CAS

3.1.1 Antibody responses

BLG and CAS were first separately injected in GF and CV mice. After the first i.p. injection, no antibody response was detected in GF and CV mice (data not shown). After the second injection, a significant production of BLG-specific IgE ($P = 0.03$, 5/8 mice), in correlation with a significant increase of total IgE production (Spearman non parametric correlation, $r = 0.903$, $P = 0.002$), and IgG1 ($P = 0.002$, 6/8 mice) was evidenced in GF mice only (Fig. 1A and 1B). Of note, the basal level of total IgE was significantly lower in GF mice compared to CV mice, as expected since GF mice exhibit a biased systemic immunity with low immunoglobulin levels [19]. Injections of CAS led also to the production of specific IgG1 in GF mice only ($P = 0.001$, 8/8 mice, Fig. 1C) but no CAS-specific IgE response was detected (data not shown). Among CAS constituents, only κ - and α S1-caseins were immunogenic ($P = 0.001$, 8/8 GF mice and $P = 0.009$, 6/8 GF mice, respectively, Fig. 1C) whereas IgG1 response to α S2- and β -caseins was not significant (data not shown). No specific IgG2a and IgA response in sera and no specific antibody response in fecal extracts were observed (data not shown).

3.1.2 Cytokine productions

After BLG-reactivation, secretion of Th2 cytokines (IL-4, IL-5 and IL-13) by splenocytes from BLG-injected GF mice was at least four-fold enhanced compared to CV mice (Fig. 2A). Production of Th1 cytokine IFN- γ was also two-fold higher in GF than in CV mice. After CAS-reactivation, production of IL-4, IL-5 and IL-13 cytokines was comparable between GF and CV mice. Only IFN- γ production could distinguish the GF mice from the CV mice since CAS-specific secretion of IFN- γ was null in GF mice (Fig. 2B).

3.2 Influence of heat-treatment on the immunogenic and allergenic potential of BLG and CAS.

In order to take into account the formation of intermolecular disulfide bridges during heat-treatment between BLG and CAS, the proteins were mixed together with respect to the ratio BLG/CAS found in cow's milk and then heated for 10 minutes at 95°C.

3.2.1 Antibody responses

Despite a lower dose of BLG (0.7 μ g), IgE production was still significant in sera from GF mice injected with the mix of native BLG and CAS (P=0.04, 5/8 mice, Fig. 3A). However, production of BLG-specific IgE was not significant after injection of heated proteins. As no IgE to denatured BLG (BLGd) was detected in sera from any group of mice, IgE observed in the GF-NP group were thereby directed against BLG conformational epitopes (Fig. 3B). In contrast, heat-treatment enhanced BLG immunogenicity. Indeed, production of IgG1 specific to BLGd was significant in both GF mice and CV mice (P=0.01, 6/8 GF mice and P=0.03, 6/8 CV mice) whereas, without heat-treatment, BLGn-specific IgG1 response was significant only in GF mice (P=0.01, 6/8 mice).

As previously observed, no CAS-specific IgE was detected in any group of mice (data not shown) while production of IgG1 specific to κ -casein (P=0.001, 8/8 mice) and α S1-casein

($P=0.01$, 6/8 mice) was still significant in the GF-NP group. Injection of heated proteins induced as well an IgG1 response toward κ - and α S1-caseins ($P=0.01$, 6/8 mice, Fig. 3C) but responses against β - and α S2-caseins remained not significant (data not shown). Remarkably, the IgG1 response to κ -casein was significant in CV-HP mice ($P=0.009$, 6/8 mice).

As the IgG1 response to κ -casein was particularly strong, immunogenicity of the para κ -casein (1-105) and the caseinomacropeptide (CMP, 106-169), was further compared. The IgG1 response in the GF-NP group appeared then to be mainly directed toward the CMP (Fig.4), as similarly observed in the GF-HP and CV-HP groups (data not shown).

3.2.2 Cytokine productions

After BLG-reactivation of splenocytes from mice injected with non-heated proteins, secretion of IL-4, IL-5, IL-13 and IFN- γ was still higher in GF mice than in CV mice (Fig. 5 and 2). The pattern of secreted cytokines was substantially different in mice injected with heated proteins since productions of IL-4, IL-5 and IL-13 were 2 to 3-fold higher in CV mice than in GF mice. After CAS-reactivation, secretion of IL-5 or IL-13 was not affected by heat-treatment while IL-4 production tended to increase. Heat-treatment had more effect on IFN- γ production, which was reduced in CV mice and enhanced in GF mice.

4 Discussion

In the present study, the propensity of GF mice to develop excessive humoral and cellular responses compared to CV mice was investigated in order to evidence the intrinsic allergenic and immunogenic potential of BLG and CAS.

First, the Th2-biased humoral response previously reported in GF BALB/c mice was confirmed since only IgE and/or IgG1 were detected after injection of BLG or CAS [20, 23]. Contrarily to BLG, no CAS-specific IgE was detected. This result is in accordance with

another adjuvant-free mouse model aiming to evaluate the allergenicity of milk proteins through the transdermal route. In that work, IgE have been evidenced only against proteins from the lactosera but not against β - or κ -casein [17]. The absence of CAS-specific IgE response suggest that establishment of an allergic response to CAS may require a higher number of i.p. injections since frequencies of administration are critical [15]. On the other hand, the absence of CAS-specific IgE could also indicate that the role of the food matrix, the digestive processes and other environmental factors are important for CAS allergenicity as previously described for purified peanut allergens [27]. In this case, evaluation of intrinsic properties through i.p. injections of purified proteins could not be used to predict accurately their potential allergenicity, as suggested by Ladics *et al.*[15].

However, GF mice appeared to be particularly susceptible to CAS immunogenicity since all GF mice and no CV mice were found to be responsive. The ratio of α S1-, α S2-, β - and κ -caseins in the micelle (3:1:3:1, respectively) does not account for their relative immunogenicity since only α S1- and κ -caseins were immunogenic in GF mice. In CM-allergic patients, comparison of the humoral responses to the four caseins reveals that allergenicity of cow's milk proteins is positively correlated with their immunogenicity and that α S1-casein is often the most allergenic and immunogenic casein [28-30]. In this regard, the development of an IgG1 response to α S1-casein in GF mice is in good agreement with the prevalence data. The fact that κ -casein was the most immunogenic casein in GF mice was more surprising. However, κ -casein, and particularly its hydrophilic C-terminal half containing the CMP, was previously reported to be highly immunogenic in mice immunized with whole casein [5]. The high immunogenicity of the CMP is indeed consistent with the general conception that κ -casein is localized at the surface of the micelle [31]. Thus, although κ -casein is not described as the most immunogenic or allergenic casein, we may wonder

whether the development of an early and rapid IgG1 response toward κ -casein could promote the subsequent development of IgG and IgE responses toward other caseins.

We further investigated the impact of heat-treatment on the allergenic potential of BLG and CAS. Heat-treatment denatured BLG and inhibited in GF mice the IgE response that was shown to be directed against the conformational epitopes of BLG. Moreover, the specificity of the IgG1 response reflected properly the conformational state of the BLG injected in GF mice. Indeed, the IgG1 response against BLG_n in mice injected with heat-denatured protein or against BLG_d in mice injected with non-heated protein was not significant. In contrast, only heat-denatured BLG was able to induce a BLG-specific IgG1 response in CV mice. Previous reports using adjuvant showed that heat-denaturation of allergens could reduce their sensitising potential while triggering their immunogenicity [32]. The greater immunogenicity of heated proteins is then probably due to the generation of aggregates which are known to be more immunogenic than soluble proteins [33]. Pasteurization of milk proteins was also shown to improve BLG immunogenicity, but in this case, IgE response was significantly increased [7]. This discrepancy could be due to the lower temperature used during pasteurization, around 63-72°C, which could have just partially denatured BLG. But, in agreement with our data, reactivation of splenocytes by BLG led to increased secretions of IL-5, IL-13 and IFN- γ in mice sensitized with pasteurized BLG. In GF mice, secretion of Th2 cytokines by splenocytes tended to decrease after heat-treatment, thus reflecting the absence of IgE response toward BLG_d.

While the humoral CAS-specific response in GF mice was not markedly affected by heat-treatment, the most striking effect was the production of IgG1 toward κ -casein in CV mice. As this was correlated with the production of BLG-specific IgG1, the concomitant enhanced immunogenicity of BLG and κ -casein may result from the formation of disulfide bridges between the two proteins during heat-treatment [34]. The formation of aggregates

between BLG and κ -casein may thus improve the immunogenicity of both proteins in CV mice. Remarkably, after heat-treatment, reactivation of splenocytes by BLG or by CAS resulted in very similar profile of cytokine secretion. This may reveal a possible effect of milk protein aggregation that conferred to BLG and CAS a comparable potential for *in vitro* stimulation of splenocytes.

In conclusion, GF mice exhibited an enhanced susceptibility to the allergenic and/or immunogenic potential of BLG and CAS. Only the native form of BLG, in comparison with BLGd, could induce an IgE response. The specificity of the IgG1 response reflected also the conformational structure of the BLG injected into mice. In addition, only α S1- and κ -caseins were immunogenic while no response was evidenced toward α S2- and β -caseins. As CAS injections did not lead to the production of specific IgE, this approach remains inadequate for predicting the potential allergenicity of proteins. Nevertheless, the higher susceptibility of GF mice could certainly be advantageously used to study the impact of the gut microbiota on the development of an allergic response and to investigate the relative importance of the food matrix and other environmental factors during the sensitization process to cow's milk proteins. In this regard, other routes of allergen exposure are currently under investigation.

We thank Benoit Lignon-Couffon and Pascal Guillaume for careful breeding of germ-free mice; Anne Foussier and Sandrine Ah-Leung for skilled technical assistance. Stephanie Morin is recipient of a grant from the department of Alimentation Humaine (AlimH, INRA, France). Catherine Hazebrouck is remembered with affection.

The authors have declared no conflict of interest.

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Figure legends

Figure 1. BLG-specific IgE responses, total IgE and correlation between specific and total IgE in the GF-BLG group (A), specific IgG1 responses toward BLG (B), and CAS, α S1- and κ -caseins (C). GF mice (open symbols) and CV mice (black symbols) were intraperitoneally injected with D-PBS (control, GF-PBS and CV-PBS), BLG (5 μ g, GF-BLG and CV-BLG) or CAS (5 μ g, GF-CAS and CV-CAS), without adjuvant (see section 2). Assays were performed as duplicate on sera from 8 mice/group. Sera were diluted 1:500 for the BLG-specific IgG1 determination and 1:50 otherwise. Specific Ig levels were reported as absorbance units at 414 nm and means are indicated. Significantly different from the control group (Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

Figure 2. Cytokines secretions by (A) BLG- or (B) CAS-reactivated splenocytes from mice intraperitoneally injected with BLG or CAS (see section 2). Cells were incubated for 60h at 37°C (5% CO₂). Results are represented as cytokines secretions in supernatants of reactivated splenocytes after subtraction of cytokines assayed in supernatants of D-PBS-reactivated splenocytes, thus corresponding to specific production.

Figure 3. Specific IgG1 and IgE responses toward BLG (A), denatured BLGd (B), α S1- and κ -caseins (C). GF mice (open symbols) and CV mice (black symbols) were intraperitoneally injected with D-PBS (control, GF-PBS and CV-PBS), a non-heated mix of BLG + CAS (0.7 μ g + 5 μ g, GF-NP and CV-NP) or a heated mix of BLG + CAS (0.7 μ g + 5 μ g, GF-HP and CV-HP, see section 2). Sera were diluted 1:50. Specific Ig levels were reported as absorbance units at 414 nm and means are indicated. Significantly different from the control group (Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

Figure 4. Specificity of the IgG1 response toward κ -casein in GF mice intraperitoneally injected with a non-heated mix of BLG + CAS (0.7 μ g + 5 μ g, GF-NP). The IgG1 response is mainly directed toward the C-terminal part of κ -casein (CMP) as compared with the N-terminal part (para κ -casein). Wilcoxon signed rank test, * P < 0.05,** P<0.01.

Figure 5. Cytokines secretions by (A) BLG- or (B) CAS-reactivated splenocytes from mice intraperitoneally injected with D-PBS (control, GF-PBS and CV-PBS), a non-heated mix of BLG + CAS (0.7 μ g + 5 μ g, GF-NP and CV-NP) or a heated mix of BLG + CAS (0.7 μ g + 5 μ g, GF-HP and CV-HP) (see section 2). Cells were incubated for 60h at 37°C (5% CO₂). Results are represented as cytokines secretions in supernatants of reactivated splenocytes after subtraction of cytokines assayed in supernatants of D-PBS-reactivated splenocytes, thus corresponding to specific production.

Figure 1.

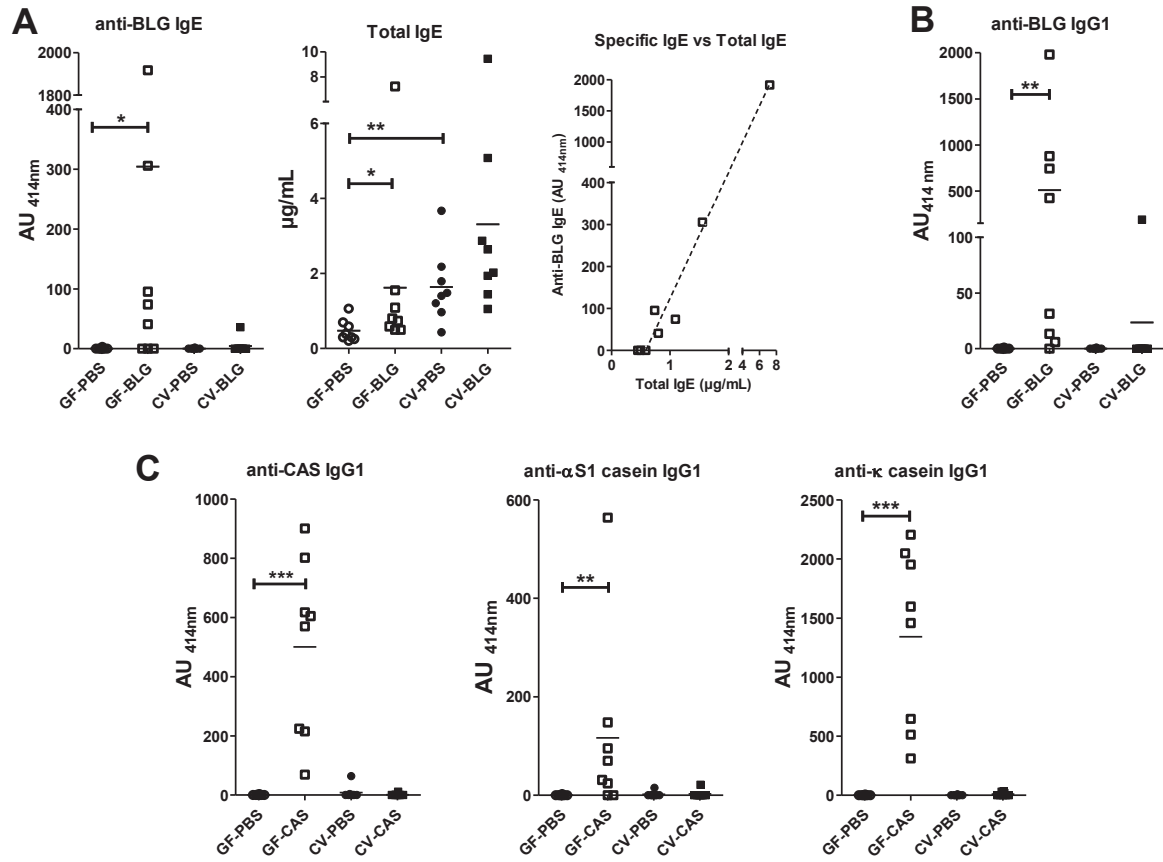


Figure 2.

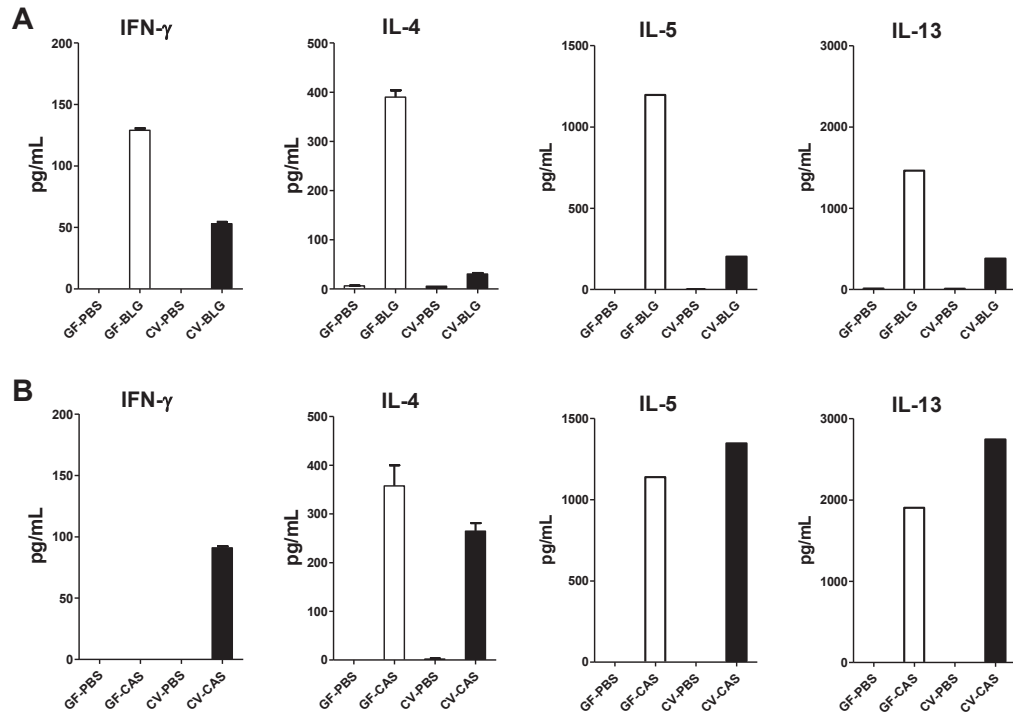


Figure 3.

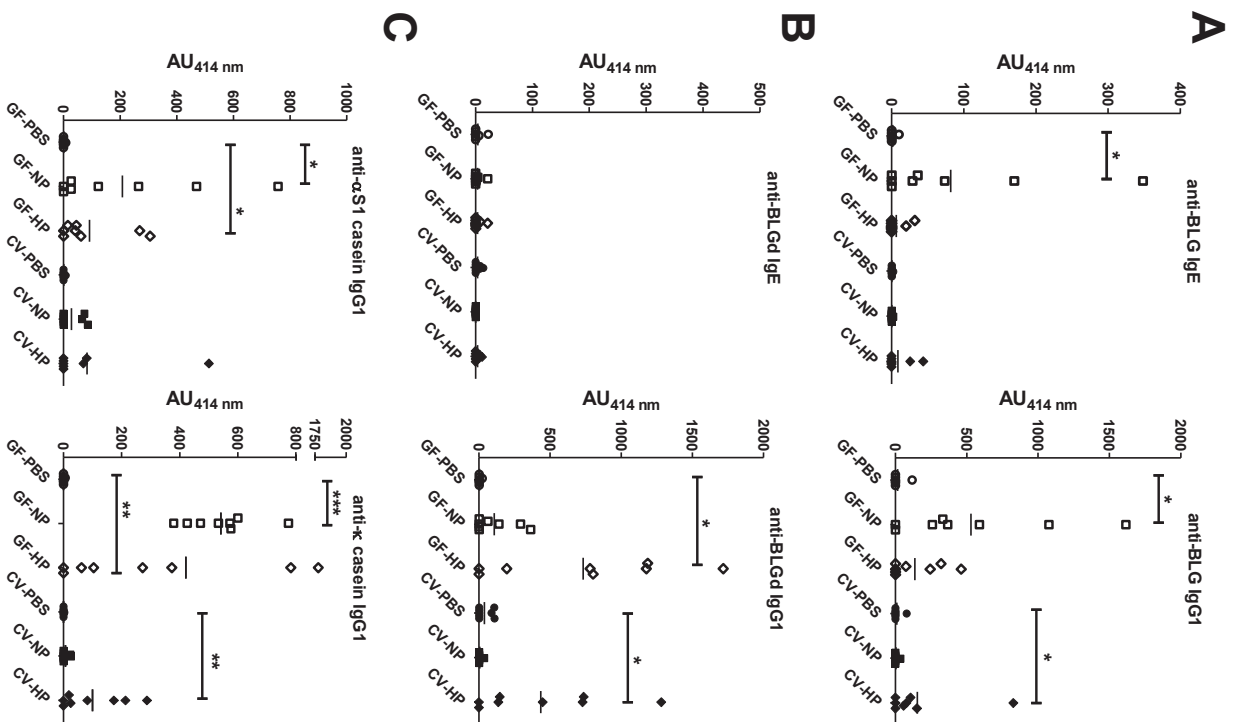


Figure 4.

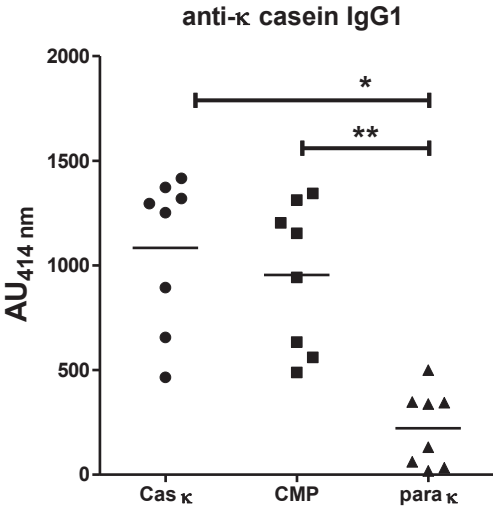


Figure 5.

