

Invariant Natural Killer T Cells Contribute to the Allergic Response in Cow's Milk Protein-Sensitized Mice

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Key Words

Invariant natural killer T cells • Cow's milk allergy •
 α -Galactosylceramide

Abstract

Background: Little is known about the contribution of the invariant natural killer T (iNKT) cells in the onset of food allergy. Using a mouse model for cow's milk allergy the function of iNKT cells was investigated. **Methods:** Mice were sensitized orally with casein or whey proteins. One hour before the sensitizations the mice were injected intraperitoneally with α -galactosylceramide (α GalCer) or control. One week after the last sensitization acute allergic skin reactions were measured. Furthermore, in the liver, spleen and mesenteric lymph nodes (MLN) percentages of iNKT cells were analyzed and liver lymphocyte restimulation assays were performed. **Results:** Whey- or casein-sensitized mice treated with α GalCer showed enhanced acute allergic skin reactions. The percentage of iNKT cells in the liver of sensitized mice was reduced compared to sham-sensitized mice. α GalCer treatment was found to deplete iNKT cells in the liver of sensitized as well as sham-sensitized mice, and these hepatocytes did not respond to ex vivo restimulation with α GalCer. α GalCer

treatment did not reduce iNKT cell percentages in the spleen and MLN of sham-sensitized mice but abrogated the increase in iNKT cell percentage in the spleen upon whey sensitization, whereas it enhanced the iNKT cell percentage in the MLN of casein-sensitized mice. Due to the repeated application of α GalCer, livers were functionally depleted of iNKT cells. This resulted in an increased allergic effector response which was most pronounced in whey-sensitized mice and associated with enhanced whey-specific immunoglobulin levels. **Conclusion:** iNKT cells may suppress cow's milk allergic symptoms in mice and may differentially regulate oral sensitization for casein and whey.

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Introduction

Cow's milk allergy (CMA) affects 3–5% of infants and is outgrown in most cases within 3–5 years [1, 2]. IgE-mediated CMA predisposes infants to other food allergies and even asthma later in life [3, 4]. In most individuals the condition comprises skin, respiratory tract and gastrointestinal tract symptoms and in some cases, although rarely, even systemic anaphylaxis [5, 6]. Oral

tolerance is believed to be induced in the mesenteric lymph nodes (MLN) and Peyer's patches by regulatory T cells [7, 8]. However, invariant natural killer T (iNKT) cells also play a pivotal role in oral tolerance by inducing regulatory T cells and in addition by clonally deleting antigen-specific CD4⁺ T cells [9]. Natural killer T (NKT) cells are a subset of cells that express a T cell receptor, which makes them T lymphocytes. There are several subsets of NKT cells. However, the most studied NKT cell is the iNKT, or type I NKT cell, which expresses an invariant V α 14J α 18 T cell receptor chain in combination with V β 2, V β 7 and V β 8 in mice [10]. iNKT cells, T cells and NK cells share cell surface markers like CD161 and NKR-P1 [11]. T cells interact with peptides presented on MHC class I or II molecules by antigen-presenting cells, whereas iNKT cells do not interact with MHC class I or II molecules. However, iNKT cells recognize glycolipids presented by CD1d, a nonclassical antigen-presenting molecule [11, 12]. iNKT cells, therefore, can generate an adaptive immune response towards a class of antigens which is not covered by conventional T cells. These antigens include mycobacterial cell wall antigens, gangliosides, tumor-derived phospholipids and nonlipidic molecules [13, 14]. The ability of iNKT cells to show flexibility in their cytokine response has led to the proposition that iNKT cells have regulatory properties and might be involved in homeostasis of the mucosal immune system [12, 15, 16]. In mice these cells are present at low frequencies in the thymus, bone marrow, spleen, MLN and Peyer's patches (<2.0%) [17] (Schouten et al., unpubl data). In contrast, hepatic lymphocytes consist of approximately 20% iNKT cells. The best-characterized and potent-specific iNKT cell agonist is a glycolipid isolated from the marine sponge *Agelas mauritianus*, α -galactosylceramide (α GalCer) [18–20]. The complex of CD1d plus α GalCer binds to the iNKT cell receptor and activates the cell. After iNKT stimulation interleukin (IL)-4 and interferon (IFN)- γ are rapidly produced and secreted. More recently it has been shown that iNKT cells also produce T_H17 cytokines, both IL-17 and IL-22 [21, 22].

The function of iNKT cells has been studied in several diseases including diabetes [23], multiple sclerosis [23], colitis [24] and cancer [25]. However, the contribution of iNKT cells, using α GalCer, in the onset of allergy is, to our knowledge, only studied in allergic asthma models [26–28]. With regard to food allergy, and in particular CMA, so far no data are available on the role of iNKT cells in the induction or maintenance of allergy to food ingredients and/or tolerance. Therapeutic approaches include the elimination of cow's milk proteins

from the diet and several forms of immunotherapy [29–31]. To assess the mechanisms underlying CMA, the present study focused on the contribution of iNKT cells in casein and whey protein-induced hypersensitivity. The effects of α GalCer application as an iNKT-specific stimulus were studied during oral sensitization in a murine CMA model.

Materials and Methods

Chemicals

Casein and whey proteins were obtained from DMV International (Veghel, The Netherlands). Cholera toxin (CT) was purchased from Quadratech Diagnostics (Epsom, UK), PBS from Cambrex Bio Science (Verviers, Belgium) and biotin-labeled rat anti-mouse IgE, IgG1, IgG2a and IgA from BD Biosciences (Alphen aan den Rijn, The Netherlands). α GalCer was a kind gift from Kyowa Hakko Kirin Co., Ltd (Japan). All other chemicals were obtained from Sigma-Aldrich-Chemie, Zwijndrecht, The Netherlands.

Oral Sensitization, Treatment and Challenge of Mice

Three-week-old specific pathogen-free female C3H/HeOuJ mice were purchased from Charles River Laboratories (Maas-tricht, The Netherlands), bred and raised on a cow's milk protein-free diet and housed in the animal facility. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. The mice were sensitized intragastrically with 0.5 ml homogenized casein or whey (40 mg/ml PBS) with CT (20 μ g/ml PBS) as an adjuvant, using a blunt needle. Control mice received CT alone. The mice were boosted weekly for a period of 5 weeks as described previously [32]. One hour before every sensitization the mice were intraperitoneally treated with 100 μ l α GalCer (20 μ g/ml PBST) or control (PBS and 0.5% Tween). One week after the last sensitization feces were collected and the mice were challenged intradermally in the ear pinnae to determine the acute allergic skin reaction and intragastrically with 0.5 ml homogenized casein or whey (100 mg/ml PBS). After 16 h blood samples were collected in Minicollect[®] Z Serum Sep vials (Greiner Bio-One) and the mice were sacrificed by cervical dislocation. The collected blood was centrifuged for 15 min at 16,000 g and sera were stored at –70°C.

Acute Allergen-Specific Skin Reaction

The acute allergen-specific skin reaction was measured after intradermal injection with 20 μ l homogenized casein or whey protein (0.5 mg/ml in PBS) in the ear pinnae. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands) at t = 0 and 1 h after challenge. The ear swelling (μ m) was calculated by subtracting the basal (t = 0 h) from the specific casein- or whey-induced ear swelling after 1 h.

Measurement of Specific Serum Immunoglobulins

Concentrations of casein- and whey-specific IgE, IgG1 and IgG2a were determined in the serum, whereas specific IgA was measured in the feces by means of ELISA as described previously [32]. In short, plates were coated with 100 μ l casein or whey (20

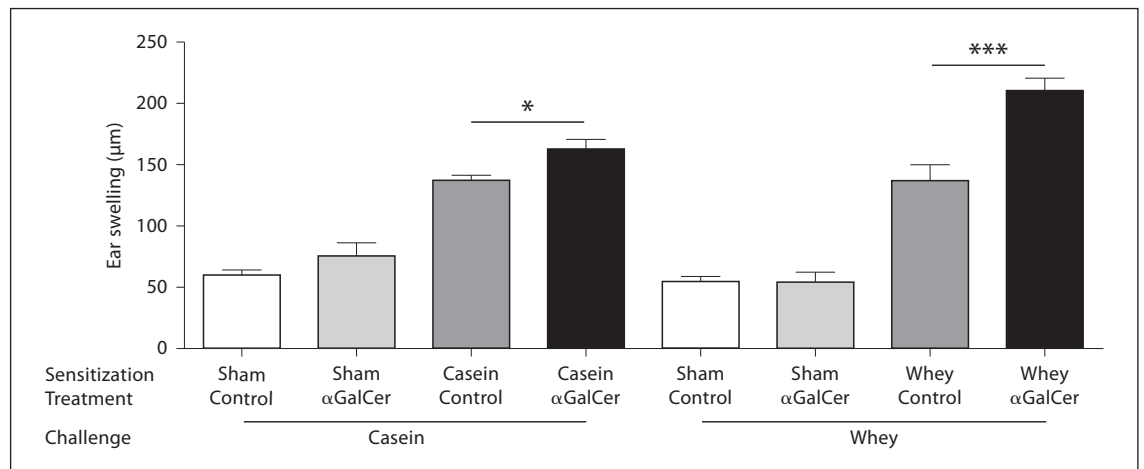


Fig. 1. The acute allergic skin reaction of sham-, casein- or whey-challenged mice, control or α GalCer-treated. Mice were sensitized intragastrically with CT and whey or casein 5 times during weekly intervals. CT alone was used for sham sensitization. Before each sensitization, the mice were injected intraperitoneally using α GalCer or control. Mean $\pm$ SEM; * $p < 0.05$, *** $p < 0.001$; $n = 5-6$ per group.

mg/l) and, after washing, several serum or fecal water dilutions were added. Bound whey- or casein-specific antibodies were detected using biotin-labeled rat anti-mouse IgE, IgG1, IgG2a or IgA (1 mg/l) and streptavidin-horseradish peroxidase. Results were expressed as arbitrary units and calculations were based on titration curves with pooled reference sera from mice which had been immunized intraperitoneally with casein or whey alum.

Flow Cytometry

Single cell suspensions were prepared from mouse livers (liver lymphocytes were isolated using 40% Percoll gradient), spleens and MLN and blocked for 30 min in PBS containing 1% BSA and 5% FCS. Cells were counted using a Coulter Z1 particle counter (Beckman Coulter) and diluted to a concentration of 2×10^6 cells/ml. For identification of iNKT cells, cells were incubated for 30 min at 4°C with anti-CD3-PerCP-CY5.5 (1:100), anti-CD4-FITC (1:100), anti-CD19-APC and CD1d tetramer loaded with α GalCer (1:100). Antibodies were purchased from BD, except for CD1d tetramer preloaded with α GalCer, which was purchased from ProImmune Ltd. (Oxford, UK). iNKT cells were gated using CD3+CD19-CD4+CD1d/ α GalCer tetramer+. Flow-cytometric analysis was performed using FACSCalibur and CellQuest Pro Analysis software (BD).

Restimulation Assay

In hepatocyte culture, supernatants of single cell suspensions (1×10^6 /ml) restimulated using α GalCer (100 ng/ml) or control were harvested at 48 h and concentrations of granulocyte macrophage colony-stimulating factor, IFN- γ , tumor growth factor (TNF)- α , IL-2, IL-4, IL-5, IL-10 and IL-12p70 were determined by multiplex cytokine bead assay (Bio-Rad, Venendaal, The Netherlands) using a Luminex 200 Analyzer (Nuclilab, Ede, The Netherlands). Data were analyzed using xPonent software 3.0.380.0 (Luminex Corp., Austin, Tex., USA).

Statistics

All data were analyzed using one-way ANOVA and post hoc Bonferroni's comparison test or Mann-Whitney test. Analyses of the immunoglobulin levels and cytokine concentrations were performed on log-transformed data. Statistical analyses were conducted using GraphPad Prism software (version 4.03). $p < 0.05$, $p < 0.01$ and $p < 0.001$ were indicated when analyses reached statistical significance. Data are represented as mean $\pm$ SEM.

Results

Acute Allergic Skin Reaction

One week after the last sensitization the mice were challenged intradermally in the ear with casein or whey. After 1 h the acute allergic skin reaction was determined. α GalCer treatment did not affect the ear swelling reaction of sham-sensitized mice (60.7 ± 3.3 vs. 76.0 ± 10.1 μ m for casein challenge and 53.7 ± 4.6 vs. 54.5 ± 8.0 μ m for whey challenge). However, both casein- and whey-sensitized mice developed an increased acute allergic skin reaction after α GalCer treatment (137.2 ± 4.5 vs. 162.5 ± 7.5 μ m for casein-sensitized mice, $p < 0.05$, and 136.4 ± 12.7 vs. 209.9 ± 10.2 μ m for whey-sensitized mice, $p < 0.001$; fig. 1).

Allergen-Specific Immunoglobulin Levels

Sixteen hours after oral challenge, serum was obtained from all mice. Casein-specific IgE, IgG1 and IgA levels were enhanced in casein- and whey-sensitized mice when

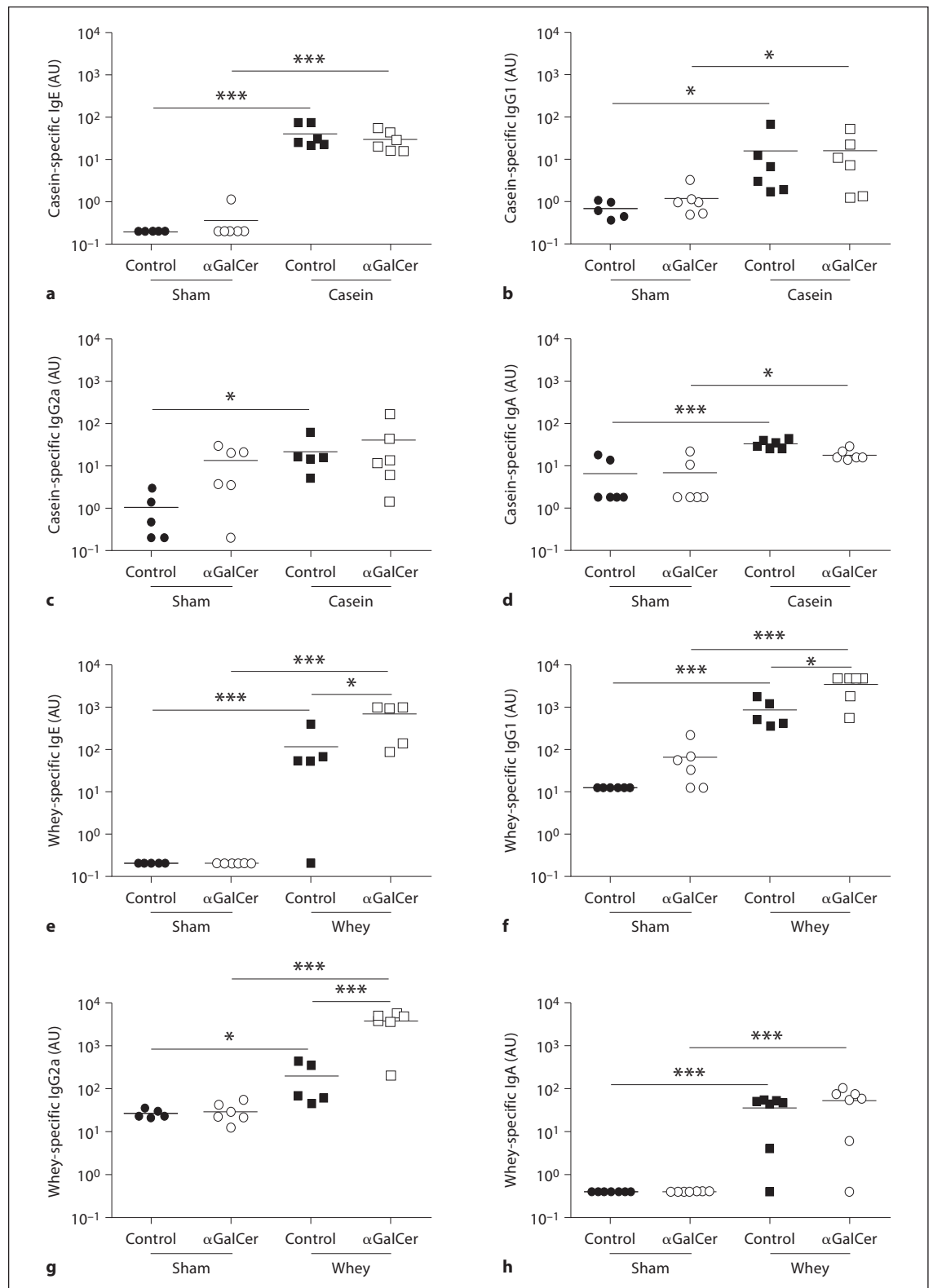


Fig. 2. Serum-specific immunoglobulins and fecal-specific IgA in control or α GalCer-treated casein- or whey-sensitized mice. Casein-specific immunoglobulins (a–d) and whey-specific immunoglobulins (e–h). AU = Arbitrary units. Mean $\pm$ SEM; * $p < 0.05$, *** $p < 0.001$; $n = 5$ –6 per group.

compared to sham sensitization (fig. 2a, b, d–f, h). Specific IgG2a was only enhanced in whey-sensitized mice and tended to be enhanced in casein-sensitized mice (fig. 2c, g). α GalCer treatment did not affect immunoglobulin levels in casein-sensitized mice but further enhanced whey-specific IgE, IgG1 and IgG2a, but not IgA, in whey-sensitized mice (fig. 2e–h).

Flow Cytometry

The effect of sensitization and α GalCer treatment on iNKT cell frequency was determined using flow cytometry. Casein sensitization ($14.6 \pm 2.5\%$) and whey sensitization ($14.0 \pm 2.8\%$) were found to reduce the percentage of hepatic iNKT cells in comparison with sham-sensitized mice ($22.9 \pm 0.6\%$, $p < 0.05$; fig. 3a). In addition, α GalCer treatment was found to deplete hepatic iNKT cells in both sham- and casein-sensitized and whey-sensitized mice in comparison with control-treated mice ($p < 0.001$; fig. 3a). Low percentages of iNKT cells were found in the spleen and MLN possibly as a consequence of migration. Whey sensitization was found to enhance iNKT cell percentages in the spleen of whey-sensitized mice ($2.4 \pm 0.2\%$) compared to sham-sensitized (1.4 ± 0.2) and casein-sensitized ($1.2 \pm 0.1\%$) mice and α GalCer treatment was found to abrogate this ($1.5 \pm 0.1\%$, $p < 0.001$; fig. 3b). In addition, whey sensitization was found to enhance iNKT cell percentages in the MLN of whey-sensitized mice ($0.92 \pm 0.04\%$) compared to sham-sensitized ($0.4 \pm 0.0\%$) and casein-sensitized mice ($0.47 \pm 0.02\%$; $p < 0.05$ and $p < 0.01$, respectively; fig. 3c). The frequency of MLN iNKT in whey-sensitized mice was higher than in casein-sensitized mice ($p < 0.05$). α GalCer treatment was not found to affect the percentage of NKT cells in whey-sensitized mice but did increase MLN iNKT cells of casein-sensitized mice versus control-treated casein-sensitized mice (0.7 ± 0.09 vs. $0.5 \pm 0.02\%$, $p < 0.05$; fig. 3c).

Restimulation Assay

Single cell suspensions which were used for flow-cytometric analysis were restimulated using α GalCer and analyzed. α GalCer was used to specifically target the iNKT cells and to investigate their cytokine response. Restimulation of splenocytes using casein or whey did not result in detectable levels of cytokines. Cytokine production of control-stimulated hepatocytes was below detection limit (data not shown). Ex vivo restimulation of hepatocytes from sham-, casein- or whey-sensitized mice with α GalCer resulted in the secretion of all eight measured cytokines (granulocyte macrophage colony-stimulating fac-

tor, IFN- γ , TNF- α , IL-2, IL-4, IL-5, IL-10 and IL-12p70) which showed similar patterns. iNKT-produced IL-4 (T_H2) and IFN- γ (T_H1) are known to influence T_H1/T_H2 balance and allergy development; therefore, IL-4 and IFN- γ are shown (fig. 4). No differences were detected between the groups. However, no correction for the percentage of iNKT cells in the liver was performed and these were significantly lower in casein- and whey-sensitized mice in comparison with sham-sensitized mice. In vivo α GalCer treatment rendered the hepatocytes unresponsive to ex vivo α GalCer restimulation and no cytokine production was detected.

Discussion

Recent evidence implies that iNKT cells are involved in oral tolerance induction [33]. iNKT cells can be found in large numbers in the liver but can also be found within the intestine, since 2% of the mouse intestinal lamina propria lymphocyte population consists of iNKT [34]. The contribution of iNKT cells in the pathophysiology of CMA is unknown. Therefore, the effect of oral sensitization on iNKT cell populations in the liver, spleen and MLN was investigated. Furthermore, α GalCer, a specific iNKT agonist, was used to intervene in the development of allergy during oral sensitization with casein or whey in mice.

This study shows for the first time that oral sensitization of mice with either casein or whey dramatically decreases iNKT cell percentage in the liver in association with the establishment of CMA. Although the percentage of iNKT cells was reduced in the liver typically in whey-sensitized mice, it was increased in the spleen and MLN, which was not observed in casein-sensitized mice. This suggests that sensitization, differentially affects the distribution of iNKT depending on the protein provided. Since the iNKT population is affected during sensitization, this may imply that these cells contribute to the development of allergic disease or are involved in the suppression of (food) allergic disorders. iNKT cells have been shown to induce or break oral tolerance [27, 33] and may suppress or enhance allergic disease in mice [18, 35]. These dual mechanisms may partly be explained by the existence of different subsets of iNKT. In humans it was shown that part of the population of α GalCer-reactive iNKT cells produced high amounts of IL-4, IL-10 and IFN- γ and induced IL-12-producing dendritic cells in vitro, resulting in T_H1 skewing of the effector response, while another subset produced relatively low amounts of

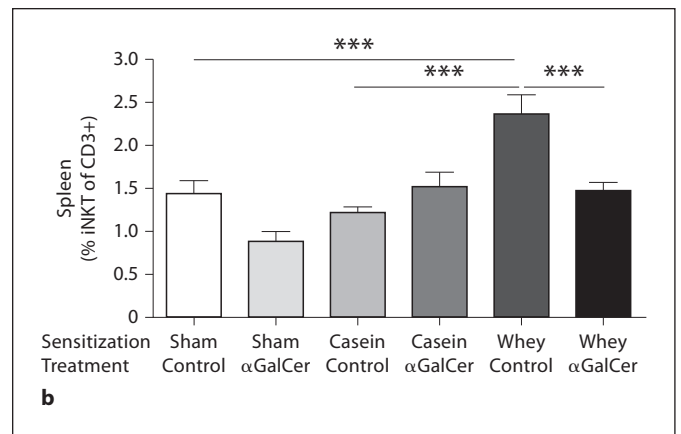
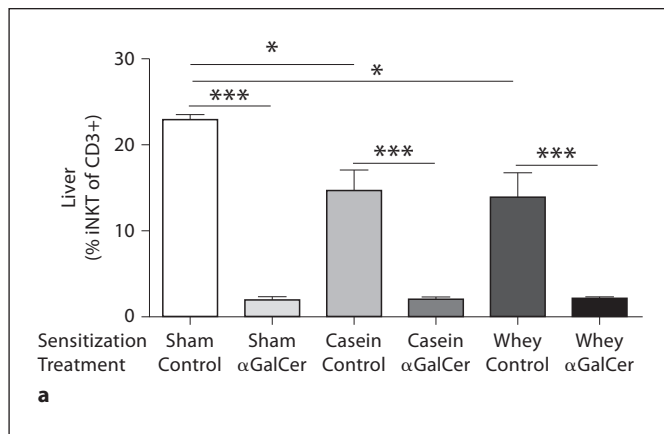
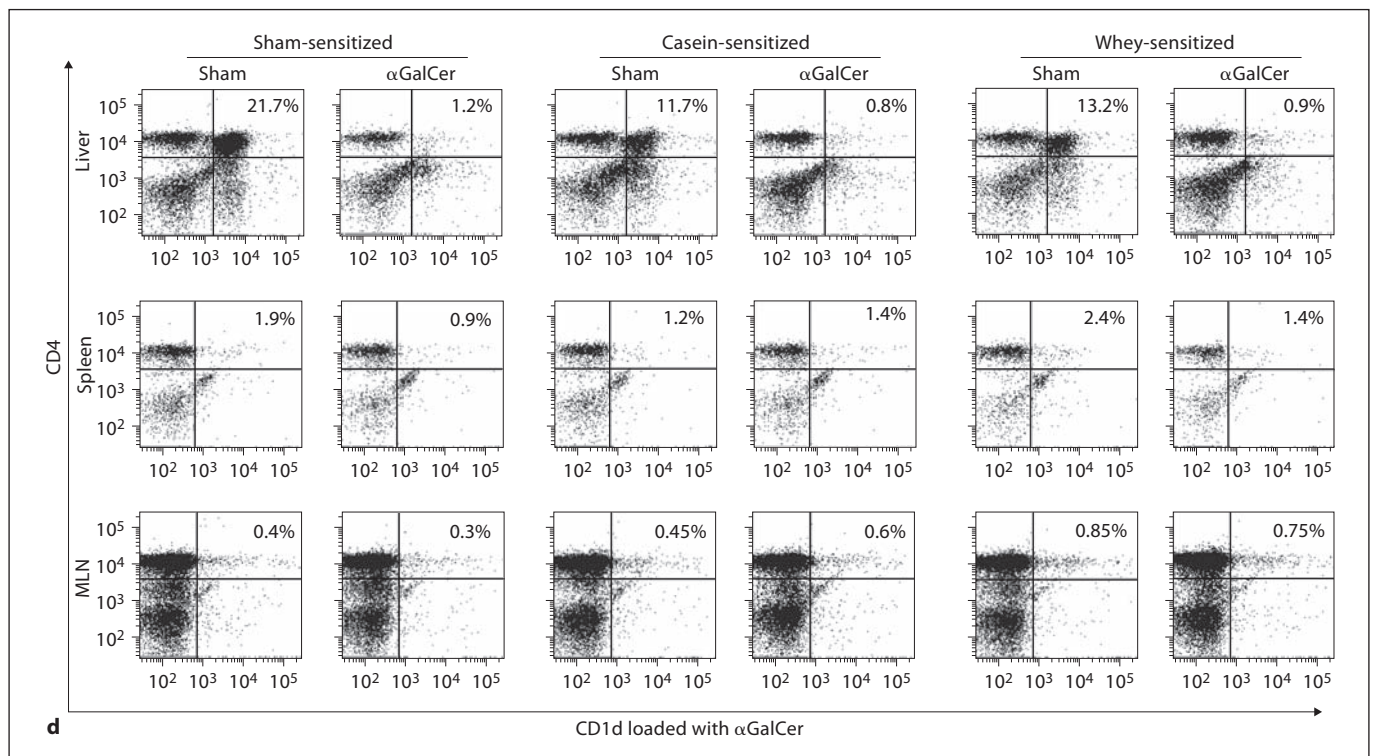
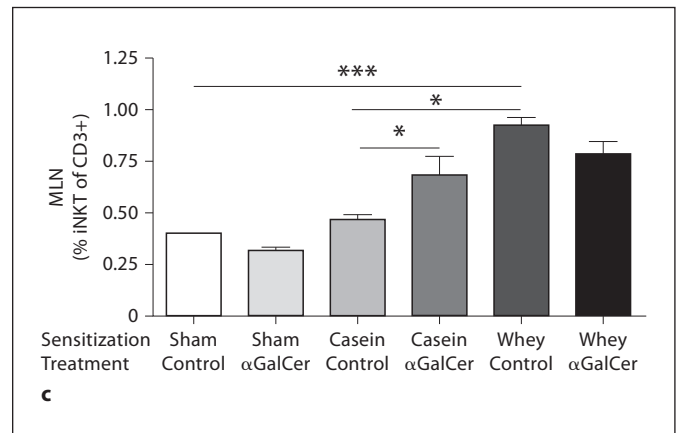


Fig. 3. Flow-cytometric analysis of iNKT cells in control or α GalCer-treated sham-, casein- or whey-sensitized mice. Cells were isolated and stained with CD3, CD4 and α GalCer-loaded CD1d tetramers. Percentages are calculated within the CD3⁺ population and representative dot plots are shown. iNKT in the liver (**a**), in the spleen (**b**) and in the MLN (**c**). **d** Representative dot plots of the CD4⁺CD1d tetramers loaded with α GalCer⁺ in the CD3⁺ population. Mean $\pm$ SEM; * $p < 0.05$, *** $p < 0.001$; $n = 5-6$ per group.



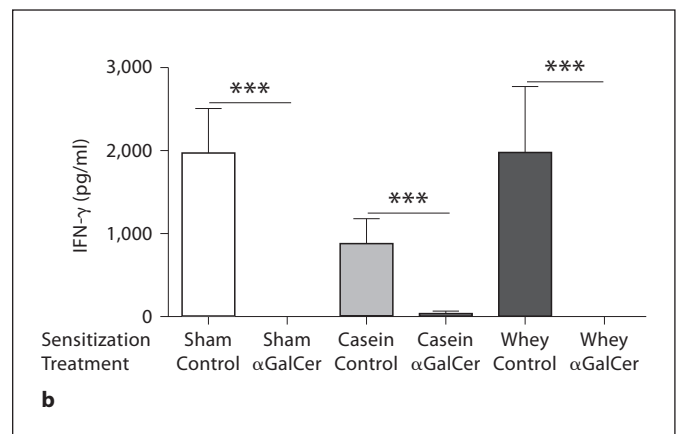
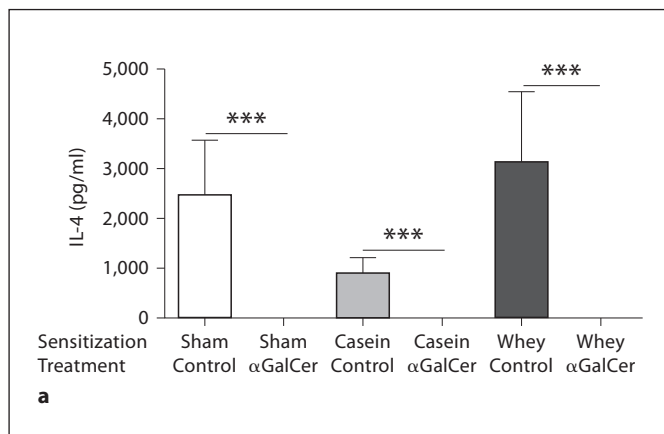
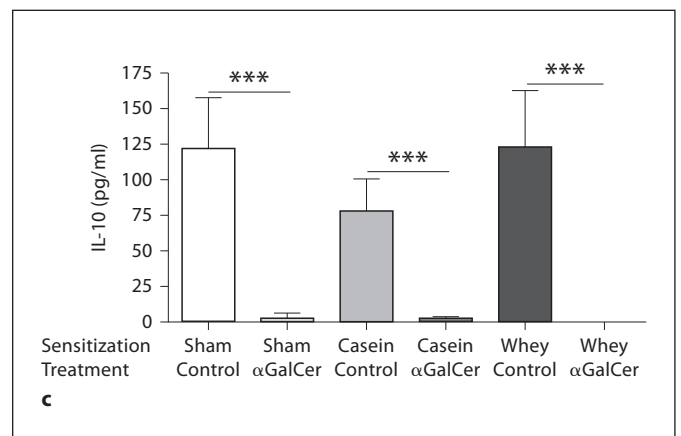


Fig. 4. Analysis of α GalCer-induced cytokine responses in control and α GalCer-treated sham-, casein- and whey-sensitized hepatocytes. IL-4 (a), IFN- γ (b) and IL-10 (c) secretion was measured 48 h after stimulation. Unstimulated cells did not secrete any cytokines. Mean $\pm$ SEM; *** $p < 0.001$; $n = 5-6$ per group.



IL-4 and IL-10 and induced dendritic cell cytotoxicity and a T_H2 -polarized immune response [36]. Typically, iNKT-derived IL-10 in particular was found to contribute to oral tolerance induction [33].

To further explore the involvement of iNKT in allergic sensitization for cow's milk proteins, agonist studies using α GalCer were performed. α GalCer is the best-characterized potent-specific iNKT cell agonist [18–20]. α GalCer treatment was intended to restore the frequency and/or activity of hepatic iNKT cells in casein- or whey-sensitized mice in order to inhibit the acute allergic skin reaction. However, multiple intraperitoneal injections of α GalCer resulted in an almost complete depletion of iNKT cells in the liver, both in sensitized and control mice, and an exacerbation of the allergic response in sensitized mice. A strong decrease in hepatic iNKT cells due to the α GalCer treatment could be explained by the migration of these cells towards other lymphoid organs or the periphery. However, FACS analysis did not show an equal enhancement of iNKT numbers in the spleen or in

the MLN, suggesting that the iNKT cells were depleted instead of stimulated. Parekh et al. [37] and Sullivan and Kronenberg [38] demonstrated that a single high-dose administration of α GalCer (5 μ g/mouse) caused anergy of iNKT cells for at least 1 month. Matsuda et al. [20] and Hachem et al. [18] showed that a single lower dose of α GalCer (2 μ g/mouse) resulted in an inhibition of allergic airway inflammation in an ovalbumin model. More in line with our results are the studies by Meyer et al. [39] and Kim et al. [28], showing increased airway hyperreactivity after two doses of α GalCer (twice 2 μ g/mouse) or enhanced allergic asthma after intranasal application of α GalCer (3 times 1 μ g/mouse), respectively. The number of iNKT cells, however, was not analyzed in these studies.

Although oral sensitization resulted in reduced numbers of iNKT cells in the liver, these cells functionally reacted upon restimulation with α GalCer since several cytokines were produced, such as IFN- γ and IL-10, which are known to contribute to either T_H1 skewing or tolerance induction [33, 36]. This increase in cytokine produc-

tion upon ex vivo α GalCer restimulation is in agreement with other studies [26, 35, 40]. The produced cytokine concentrations in restimulated liver iNKT cells can be corrected for the percentage of iNKT cells in the liver. Lower frequencies of hepatic iNKT cells in control-treated whey-sensitized mice produced the same amounts of cytokines as sham-sensitized mice, whereas equal numbers would have produced higher levels of cytokines. Whey-sensitized mice, therefore, produced relatively more IL-4, IL-10 and IFN- γ , whereas the cytokine production per iNKT cell was probably not altered in casein-sensitized mice. iNKT cell depletion in the liver due to in vivo α GalCer treatment was confirmed in vitro, and the cytokine production of hepatocytes restimulated with α GalCer was almost completely lost compared to control-treated mice. α GalCer treatment in particular was found to enhance the acute allergic skin response in whey-sensitized mice. In these mice reduced iNKT numbers in the liver were compensated by enhanced cytokine production per cell. Although in the spleen and MLN of whey-sensitized mice the iNKT cell numbers were enhanced in vivo, α GalCer treatment only abrogated this in the spleen. This may imply a contribution of splenic iNKT in the suppression of CMA in particular in whey-sensitized mice.

Differences between casein- and whey-sensitized mice in cytokine production by liver iNKT cells and spleen and MLN iNKT frequencies indicate a different role for iNKT in casein and whey allergy in mice. The semi-invariant T cell receptor of NKT cells in particular recognizes CD1d-presented glycolipid antigens expressed by antigen-presenting cells or intestinal epithelial cells [34]. Casein and whey proteins differ with regard to physical, physiological and dietary properties [41–44]. The three-dimensional shape of the proteins differs. Individual caseins (11.6–25.2 kDa) are cross-linked and form aggregates. These nanoclusters form large structures called micelles, whereas whey (14.2–76.2 kDa) forms linear structures of mostly monomers and dimers. Furthermore, the glycolipid composition of whey and casein may differ. Antigen presentation therefore might be different for either casein or whey, thereby affecting iNKT-cell properties.

The dramatic augmentation of the acute allergic skin response in α GalCer-treated whey-sensitized mice was found to be associated with significantly enhanced whey-specific IgE, IgG1 and IgG2a when compared to control-treated whey-sensitized mice. In casein-sensitized mice, the enhancement of the acute skin response was much less severe and casein-specific immunoglobulins were not further enhanced upon α GalCer treatment. These

immunoglobulin data suggest an immunoglobulin-dependent mechanism for whey-sensitized mice, whereas for casein-sensitized mice another mechanism may underlie the increase in acute allergic skin response due to in vivo α GalCer treatment. Recently we have shown that the acute allergic skin response in casein-sensitized mice requires immunoglobulin-free light chains [45]. Immunoglobulin-free light chains are believed to form an alternative pathway to mast cell degranulation [46–49]. Although in several studies performed in the past casein-specific IgE remained undetectable, in the current study casein-specific IgE is detected in association with allergic symptoms. This may be explained by the biological diversity of the reaction to the casein protein. Indeed the allergic outcome is found to vary between the batch of mice, the laboratory setting, where the mice are housed (personal observations) and compromised immune function can change an IgE-mediated allergic reaction into immunoglobulin light chain dependency [50]. The α GalCer treatment may have increased the immunoglobulin light chain levels in the casein-sensitized mice, which may have caused the increased skin response in these mice.

It can be concluded that iNKT cells might play a regulatory role in the onset of CMA in mice. Oral sensitization lowers the percentage of iNKT cells in the liver. Multiple intraperitoneal injections of α GalCer at weekly intervals depleted hepatic iNKT cells and exacerbated the allergic response in mice orally sensitized with cow's milk proteins, a factor which was most pronounced for whey.

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