Immunoreactive properties of α -casein and κ -casein: Ex vivo and in vivo studies

Ewa Fuc, Dagmara Złotkowska, Emilia Stachurska, and Barbara Wróblewska¹

Department of Immunology and Food Microbiology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima 10, Olsztyn, Poland

ABSTRACT

The aim of this study was to evaluate the ex vivo and in vivo studies immune potential of α - and κ -casein. Ex vivo, naïve mouse splenocytes were stimulated with α - or κ -casein. After 120 h of culture, the proliferation index (PI), determined by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) staining, did not vary for either antigen, suggesting similar ex vivo immunogenic potential of both casein fractions. In vivo, BALB/cmmdb mice were sensitized with α - or κ -casein and then gavaged with primary antigen. Mice immunized with α -casein had higher levels of IgG ($2^{16.33}$) and IgA ($2^{10.22}$) in serum at the end of the experiment compared with mice immunized with κ -casein (2^{15} and $2^{9.3}$ for IgG and IgA, respectively). The use of α -casein for mouse immunization and ex vivo lymphocyte stimulation resulted in higher concentrations of secreted cytokines (IL-4, IL-10) compared with κ -casein stimulation. This is consistent with increasing regulatory T cell (Treg) lymphocyte populations, independent of the antigen used for stimulation. In summary, the immunogenic potential of α - and κ -casein was similar. Humoral and cellular immune responses confirmed their strong, independent potential to induce B and T cells. We propose that the lymphocyte proliferation index be used as an initial screening for protein immunogenicity.

Key words: immune potential, α -casein, κ -casein, immunoreactivity, lymphocyte proliferation index

INTRODUCTION

Cow milk allergy (CMA) is the most common type of food allergy, affecting 0.6 to 2.5% of preschool children,

0.3% of teens, and 0.5% of adults. The occurrence of allergy to cow milk proteins is highest in early childhood but later subsides (Jo et al., 2014). The high incidence of milk allergy in children can be attributed to the fact that cow milk proteins are often the first foreign food antigens introduced into a child's diet (Rangel et al., 2016).

Milk proteins (at concentrations of 30–35 g/L) are a heterogeneous group with various structures and properties (Barłowska et al., 2011; Tsabouri et al., 2014). The 2 main groups of milk proteins are whey and casein. Casein, accounting for 80% of total milk proteins, consists of 4 fractions: α_{S1} - (39–46% of total caseins), α_{S2} - (8–11%), β - (25–35%), and κ -CN (8–15%). Additionally, γ -CN is the result of β -CN degradation (Kamínski et al., 2007). Caseins create ordered aggregates dispersed in colloids (micelles), with diameters ranging between 100 and 300 nm and a molecular weight from 19 to 25.2 kDa. These micelles are resistant to high temperature but sensitive to digestive enzymes (Hochwallner et al., 2014; Matsuo et al., 2015). κ -Casein, a calcium-insensitive protein, consists of hydrophobic (AA 1–105) and hydrophilic (AA 106–169) segments, resulting in stable casein micelles (Creamer et al., 1998).

Milk proteins after enzymatic digestion are a main source of bioactive peptides that positively affect body function, support the growth of *Bifidobacterium* species, inhibit the adhesion of bacteria and viruses in the jejunum mucus, and act as anti-inflammatory and immunomodulatory factors (Jiehui et al., 2014; Ferrario et al., 2015; Wróblewska et al., 2018). However, approximately 20 proteins in cow milk can cause strong allergic reactions (El-Agamy, 2007). It is assumed that caseins are responsible for the strongest systemic allergic reactions. A study of 80 children suffering from CMA revealed that the percentage of children reacting to milk allergens in the skin prick test was 87.5% for β -LG, 88.8% for α -LA, and 90% for total caseins. By the immunoCAP test (CAP test against α -LA, β -LG, caseins, and BSA), 72.5% reacted to β -LG, 76.3% to α -LA, and 77.5% to total caseins. By immunoblotting, 35% reacted to β -LG, 62.5% to α -LA, 63.8% to total

Received April 12, 2018.

Accepted August 21, 2018.

¹Corresponding author: b.wroblewska@pan.olsztyn.pl

caseins (61.3% to α -CN and 45% to β -CN), and 61.3% to BSA (Restani et al., 2009). In other studies, milk protein epitopes have been identified by tests on serum from individuals with CMA. Among 29 epitopes on cow milk proteins, 23 were found on caseins (Vila et al., 2001; Järvinen et al., 2002). The AA sequences that react with IgE antibodies are AA 19–30, 93–98, 123–132, and 141–150 for α_{S1} -CN, and AA 16–35 and 34–53 for κ -CN, the major milk allergens (Vila et al., 2001; Järvinen et al., 2002; Elsayed et al., 2004; Matsuo et al., 2015). Individuals with persistent CMA and high IgE levels have significantly higher specific IgG levels (Høst et al., 1992). Regardless of the method of analysis, casein was the most immunodominant allergen. Our previous study confirmed that some technological modifications of casein (e.g., hydrolysis) reduce its immunoreactivity compared with whey proteins (Wróblewska et al., 2007). The reaction with human serum showed that the low molecular fraction (molecular weight <6 kDa) obtained from sodium caseinate hydrolysate produced with alcalase, pepsin, and lactozyme was less immunoreactive than those obtained after hydrolysis of whey proteins.

Polysensitization due to different fractions of caseins is often observed and is caused by cross-reactivity associated with epitope similarity. In CMA cases, sensitization against α - and κ -CN is frequently observed, in 100 and 91.7% of cases, respectively (Fiocchi et al., 2011). However, few reports have compared the effect of these 2 proteins on the immune system response. According to the scientific opinion of EFSA experts on the production of infant and follow-on formulas from protein hydrolysates (EFSA, 2017), it should be required of manufacturers to characterize the immunomodulatory effect of each milk protein and its potential for cross-reactivity (EFSA Panel on Dietetic Products et al., 2017). The precise characterization of the effect of proteins on the host's immune system is the basis of the personalized diet for individuals with CMA or infants with the risk of developing CMA.

Immune reactions against individual cow milk proteins can vary, and the host organism can react in an allergenic or tolerogenic way. Therefore, the immune potential of casein fractions and their influence on humoral and cellular responses and T lymphocyte activation should be determined. Various lymphocytes, such as CD4⁺, CD8⁺, and natural killer (NK) cells, are involved in the immune system response to allergens; however, the predominant population is the CD4 T lymphocytes, which are the key regulators (Woodfolk, 2007). The result of inflammatory reactions associated with allergy is determined by the ability of cells to differentiate into T helper (Th)1, Th2, and Th17

pro-inflammatory lymphocytes or anti-inflammatory T regulatory cells (Treg; Jo et al., 2014).

The aim of this study was to evaluate the immunoreactive properties of 2 fractions of milk casein, α - and κ -CN. We verified their immune potential in 2 experiments, *in vivo* and *ex vivo*.

MATERIALS AND METHODS

Proteins used for mice immunizations and cell stimulation, α -casein, and κ -casein were purchased from Sigma-Aldrich (Poznan, Poland). The experimental scheme is presented in Figure 1.

In Vivo Experiment

Female BALB/Ccmdb mice (6 to 8 wk old) were obtained from the Center of Experimental Medicine in Białystok (Poland). The mice were kept in individual ventilated cage systems in the animal facility of the Institute of Animal Reproduction and Food Research, Polish Academy of Sciences (Olsztyn). Mice were fed a maintenance diet for rat and mice (#1320, totally pathogen free, Altromin International, <https://altromin.com>) free from milk proteins. Water and food were provided *ad libitum*. Animals were randomly assigned to 2 groups ($n = 10/\text{group}$), the α -casein-sensitized group (group A) and the κ -casein-sensitized group (group K). Mice were immunized intraperitoneally (i.p.) with 100 μg of antigen with Freund's adjuvant on d 0, 7, and 14. From d 48 to 62, 200 μg of antigen was administered intragastrically to mice (with cholera toxin as mucosal adjuvant on d 48, 55, and 62). Fecal and blood samples were collected once a week, starting on d 14 after immunization. All procedures used in the experiment were approved by the Local Ethics Committee in Olsztyn (43/2015).

Lymphocyte Isolation

Spleen and mesenteric lymph nodes (MLN) were isolated from mice. Tissues were dounced in incomplete medium (RPMI 1640 supplemented with 10 mM HEPES and 10 units/mL penicillin-streptomycin solution) and filtered through an 80- μm nylon filter to remove cell debris. Additionally, splenocytes were incubated for 5 min with red blood cell lysis buffer (Sigma) to remove the erythrocytes. Then, cells were washed and centrifuged at $413 \times g$ (5418R, Eppendorf, Hamburg, Germany; Mierzejewska et al., 2008) at 10°C for 10 min and suspended in 1 mL of incomplete medium. The number of lymphocytes was calculated using Bürker Cell Counter (Sigma) after Trypan Blue staining.

Lymphocyte Culture

Lymphocytes were plated on 96-well microplates at a concentration of 1×10^6 cells/100 μ L in complete medium [CM: RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1 mM nonessential amino acids, 1 mM HEPES, 1 mM sodium pyruvate, 10 units/mL penicillin-streptomycin solution]. After 12 h of culture at 37°C and 5% CO₂, cells were stimulated with antigens (α -CN or κ -CN) at a concentration of 200 μ g/mL. Concanavalin A was used as a positive control and medium (unstimulated cells) as a negative control. Af-

ter 120 h, cells were collected, centrifuged at 10°C and $413 \times g$ for 10 min and the supernatant was collected and stored at -80°C until analysis. Cells were subject to lymphocyte phenotyping.

Lymphocyte Proliferation Assays

The lymphocyte proliferation index (PI) was determined by using cytometric and colorimetric methods. For the cytometric method, carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE, Sigma) was dissolved in dimethyl sulfoxide to obtain a final stock solution of

Figure 1. Scheme of ex vivo experiment, where lymphocytes from naive mice were isolated from spleens and cultured with α -CN or κ -CN stimulation. After 5 d, proliferation index were determined by 2 independent assays. In the in vivo experiment, 2 groups of mice ($n = 10$ each) were immunized and boosted on d 0, 7, and 14 by i.p. injection of 100 μ g of α -CN or κ -CN with Freund's adjuvant. From d 48 to 62, 200 μ g of antigen was administered intragastrically to mice. All mice were killed on d 67. MTT = 3-(4,5 dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assay; CFSE = carboxyfluorescein diacetate *N*-succinimidyl ester (flow cytometry); CFA = complete Freund adjuvant; IFA = incomplete Freund adjuvant; o = oral gavage; TNF = tumor necrosis factor. Color version available online.

5 mM and stored at -20°C . For dye labeling, lymphocytes were resuspended in 1 mL of RPMI-1640 medium with 5% heat-inactivated FBS and carefully transferred to fresh tubes. Subsequently, 1.1 μL of the CFSE stock solution was added to the cells and incubated for 5 min at 20°C in the dark. Cells were washed twice in PBS (0.1 M PBS, pH = 7.2) supplemented with 5% FBS, and once in PBS supplemented with 1% FBS followed by centrifugation at $300 \times g$ for 5 min at 20°C . Cells were plated on 96-well microplates at a concentration at 1×10^6 cells/100 μL in CM and stimulated with 200 $\mu\text{g}/\text{mL}$ of antigen. After 120 h of incubation at 37°C and 5% CO_2 , lymphocytes were washed and stained with rat anti-mouse CD4 antigen (PerCP-Cy 5.5, Clone RM4-5, BD Pharmingen, San Diego, CA) and propidium iodide (BD Pharmingen). Fifty thousand events were collected from each sample, and results were analyzed using FlowJo software (FlowJo LLC, Ashland, OR) with proliferation platform.

Lymphocyte proliferation was also determined by a colorimetric 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (MTT Cell Proliferation Assay Kit, Cayman Chemicals, Ann Arbor, MI). Lymphocytes were cultured in 96-well microplates at a concentration of 1×10^6 cells/100 μL of CM. After 12 h of culture stabilization at 37°C and 5% CO_2 , cells were stimulated with α - or κ -CN antigen at 200 $\mu\text{g}/\text{mL}$. After 120 h of incubation, 10 μL of MTT solution was added to each well and the plates were incubated for 4 h. During incubation, formazan crystals appeared on the bottom of the wells. Next, 100 μL of crystal-dissolving solution was added and incubated for 4 h. Finally, absorbance was measured at 570 nm using a Jupiter UVM 340 spectrophotometer (ASYS, Hitech GmbH, Eugendorf, Austria).

Lymphocyte Phenotyping

Lymphocytes were stained with fluorescein isothiocyanate (FITC) rat anti-mouse CD4 (553929, clone H129.19; BD Biosciences, San Diego, CA), PerCP-Cy 5.5 rat anti-mouse CD25 (551071, clone PC61; BD Biosciences), Alexa Fluor rat anti-mouse CD8a (557959, clone 53-6.7; BD Biosciences), phycoerythrin (PE) rat anti-mouse CD3 (555275, clone 17A2; BD Biosciences) and Alexa Fluor 647 rat anti-mouse forkhead box P3 (Foxp3) (560401, clone MF23; BD Pharmingen). Cells were incubated at 4°C for 15 min and washed with fluorescence-activated cell sorting (FACS) buffer (PBS supplemented with 5% FBS). For intracellular marker Foxp3 staining, cells were fixed in 2% paraformaldehyde, washed with FACS buffer, permeabilized using ice-cold methanol for 20 min at room temperature, and stained the next day. The samples were analyzed

using a BD LSR Fortessa Cell Analyzer (Erembodegem, Belgium) with FACS Diva software, version 6.2 (BD Biosciences). Fifty thousand events were collected from each sample. The gating tree was set as follows: FSC/SSC (forward and side scatter; representing cell distribution by size and intracellular composition) lymphocytes were gated in the range from 100 to 150 kDa, then CD4^+ and CD8^+ were selected. The CD4^+ T cells were additionally gated for CD25^+ , and double positive $\text{CD4}^+\text{CD25}^+$ was gated for Foxp3^+ .

Serum and Fecal Sample Collection

After coagulation, blood samples were centrifuged at $16,900 \times g$ and 10°C for 10 min using an Eppendorf 5418R centrifuge (Eppendorf; Mierzejewska et al., 2008). Fresh fecal pellets were solubilized in 0.01% NaN_3 and extracted by vortexing at 4°C for 10 min (Fugamix, ELMI Ltd. Laboratory Equipment, Riga, Latvia). After centrifugation, supernatants were collected and frozen at -20°C .

Determination of Specific Immunoglobulin Classes

Standard direct ELISA was used to determine the titer of specific immunoglobulins (IgG, IgG_1 , IgG_{2b} , IgG_3 , and IgA). Briefly, 96-well plates were coated with antigen (α -CN or κ -CN) and incubated at 37°C for 1.5 h. Then, the remaining protein-binding sites were blocked with 1% BSA in PBS (10 mM PBS, pH = 7.4), incubated, and washed 3 times in PBS + 0.5% Tween 20 (PBST). After washing, serum samples or fecal extracts in suitable solutions were added to the plate and incubated overnight at 8°C . After another washing step, horseradish peroxidase (HRP)-labeled secondary antibodies to specific immunoglobulins (G, G_1 , G_{2b} , G_3 , or A) were added to each well and incubated for 1 h. For visualization, enzyme substrate (3,3',5,5'-tetramethylbenzidine, TMB) was added (Millipore, Billerica, MA) and absorbance was measured at 405 nm after 1 h on a Jupiter UVM spectrophotometer. Endpoint titer (EPT) values were expressed as the reciprocal dilution of the last sample dilution of 0.1 OD (optical density) above the negative control.

Cytokine Profile

The levels of cytokines IL-2, IL-4, IL-6, tumor necrosis factor (TNF), $\text{IFN-}\gamma$, IL-10, and IL-17A were evaluated using a BD Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine Kit (560485, BD Biosciences) and analyzed on a BD LSR Fortessa Cell Analyzer. Data were analyzed with FCAP Array 3.0 software (BD Biosciences).

Statistical Analysis

Results are presented as the mean $\pm$ standard deviation. Statistical significance was determined by the Mann-Whitney test when the levels of antibodies in humoral response were compared. The Student's *t*-test was used to compare percentage of lymphocyte subpopulations in MLN after different treatments. One way-ANOVA with Tukey's post hoc tests was used for proliferation index, splenocyte T cell profile, and comparison of cytokine secretions. Statistical analysis was performed by using Statistica software (StatSoft Corp., Kraków, Poland); *P*-values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Humoral Response Against Casein Fractions (In Vivo Study)

Mouse reactions to antigens were determined in vivo (Figure 2). Mice were immunized and gavaged with α - or κ -CN (groups A and K, respectively). A developing humoral response was observed in both experimental groups. We found significantly higher ($P < 0.05$) IgA levels in terminal serum samples (at d 68) in group A ($2^{10.22 \pm 0.83}$) compared with group K ($2^{9.3 \pm 0.82}$; Figure 2A). A similar trend was observed for serum-specific IgG at the same time point: $2^{16.33 \pm 0.82}$ versus $2^{15 \pm 1}$ for groups A and K, respectively ($P < 0.05$). Fecal-specific IgA remained at a low level during the experiment for both groups (Figure 2A). It has been shown previously that individuals with CMA have high levels of IgG (Høst et al., 1992). A high level of IgG was also observed in mice immunized with bovine lactoferrin (Negaoui et al., 2016). Our experiment showed that both casein fractions induced a high level of serum-specific IgG and IgA and similar levels of fecal IgA. α -Casein is classified as the strongest allergen present in cow milk. Our experiment showed that κ -CN has a high immunogenic potential, suggesting that it may also have strong allergenic properties.

Induction of IgG₁ production is characteristic of the allergic Th2 response (Adel-Patient et al., 2000). We found κ -CN to be less effective than α -CN in inducing IgG₁ (Figure 2B). Specific IgG₁ EpT was significantly higher in mice after exposure to α -CN than κ -CN ($2^{16.86 \pm 0.35}$ vs. $2^{16.2 \pm 0.45}$ respectively; $P < 0.05$). Similar tendencies were found for IgG_{2b} and G₃ after immunization with α - and κ -CN. Mice immunized with α -CN had statistically higher specific IgG_{2b} (EpT $2^{12.5 \pm 0.53}$) compared with the group immunized with κ -CN (EpT $2^{10.2 \pm 1.14}$; $P < 0.01$). Statistically significant differences were also observed for specific IgG₃, where in A group

EpT was $2^{11.29 \pm 1.25}$ and in K group EpT was $2^{9.9 \pm 0.88}$ ($P < 0.05$). The results suggest that α -CN induced a strong Th2 type response and κ -CN a slightly weaker allergenic potential. An increased IgG₁ level was found in serum of IgE-mediated CMA patients compared with tolerized patients (Sletten et al., 2007), which suggests a possible role of κ -CN in inducing tolerance mechanisms.

Cellular Response to α - and κ -CN (In Vivo and Ex Vivo Study)

Proliferation Potential. We performed 2 parallel proliferation assays (MTT and CFSE) to estimate the response of in vivo naïve lymphocytes under stimulation with α - or κ -CN. In the MTT assay, the lympho-

Figure 2. Terminal specific antibodies endpoint titers (EpT) in (A) blood serum (IgA and IgG) and fecal extracts (secretory IgA), and (B) IgG subclasses in blood serum. Mice were administered with α -CN (patterned bars; group A) or κ -CN (solid bars; group K). Results are presented as mean of the group $\pm$ SD. Statistical differences between groups are indicated as * $P < 0.05$ and ** $P < 0.01$.

cyte PI were 1.25 and 1.31 after stimulation with α -CN and κ -CN, respectively (Figure 3), and these results were confirmed by the CFSE assay. Naïve lymphocytes had similar PI levels independent of the stimulating antigen, which might suggest that α -CN and κ -CN have similar immunoreactive potential and, probably, similar epitope structures, which could explain their cross-sensitivity (Fiocchi et al., 2011). Figure 4 shows the PI of lymphocytes from group A (Figure 4A) and group K (Figure 4B) during stimulation with α - or κ -CN, with concanavalin A as a positive control. We compared PI of lymphocytes stimulated with antigens and PI of lymphocytes grown in medium only (negative control). We found statistically significant differences in group K, 1.41 and 1.63 for α - and κ -CN stimulation, respectively ($P < 0.05$ and $P < 0.01$; Figure 4B). Surprisingly, group A lymphocytes stimulated with α -CN did not vary significantly compared with cells growing in medium only (Figure 4A). This might suggest a predominant immunogenic potential of κ -CN over α -CN during in vitro stimulation. We found no significant differences between groups A and K lymphocyte PI values after stimulation with α -CN or κ -CN (additional T test analysis). This indicates that both proteins have similar immunogenic potential. Their effects on lymphocytes during in vitro culture probably arise through different mechanisms; this finding requires further examination in additional experiments. Kapila et al. (2013) showed that lymphocytes stimulated with whole casein had a significantly different PI from that of the negative control. Ruiter et al. (2006) found that the PI of peripheral blood lymphocytes in individuals with CMA is highest after α -CN and κ -CN stimulation, which is in agreement with our results demonstrating similar immunogenic potential of those 2 casein fractions.

Figure 3. Lymphocyte proliferation index (PI) in splenic lymphocytes of naïve mice stimulated with 200 μ g/mL α -CN or κ -CN determined by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (white bars) and carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) staining assay (gray bars). Concanavalin A (ConA) was used as a positive control.

Contribution of Lymphocytes. Food components can modulate the immune system response and some components can induce specific cell populations that can drive the immune system response to tolerance, inflammation, or allergy. The T lymphocytes play a crucial role in cell-mediated immunity. The antigen administration route and types of adjuvant used influence T-lymphocyte profiles (Zlotkowska et al., 2012); T cells differentiate into subpopulations such as helper ($CD3^+CD4^+$), cytotoxic ($CD3^+CD8^+$), and Treg ($CD4^+CD25^+FoxP3^+$) cells that modulate the immune response (Stock et al., 2004; Leonardi et al., 2007).

We determined the contribution of $CD4^+$, $CD8^+$, $CD4^+CD25^+$, and $CD4^+CD25^+Foxp3^+$ T cells in the lymphocyte population from splenic cultures stimu-

Figure 4. Lymphocyte proliferation index (PI) of splenocytes from mice immunized with (A) α -CN (group A), and (B) κ -CN (group K). Cultures were stimulated with 200 μ g/mL α -CN or κ -CN. Concanavalin A (ConA) was used as positive control, and medium alone as a negative control. Results are presented as mean of the group $\pm$ SD. Statistical differences between groups are indicated by * $P < 0.05$; ** $P < 0.01$.

lated with antigens (Figure 5). Compared with α -CN stimulation, stimulating group A splenocytes with κ -CN significantly induced $CD4^+$ T cells, with levels of 42.62 and 23.54% ($P < 0.01$), respectively. Group K splenocytes were less reactive but significant differences were found in $CD4^+$ induction: 33.2 and 23.25% ($P < 0.05$) for κ - or α -CN stimulation, respectively. Compared with ex vivo antigen stimulation, we did not find significant differences between groups (about 20% and 35 to 40% $CD4^+$ during α - and κ -CN stimulation, respectively), but group A lymphocytes clearly reacted upon changing the stimulation antigen. Induction of $CD8^+$ T cells in ex vivo stimulation is shown in Figure 5C. Stimulation with α - or κ -CN induced a significantly higher number of $CD8^+$ lymphocytes in group A (32.53%) than in group K (23.7%; $P < 0.05$). A similar trend was observed with κ -CN stimulation. The contribution of $CD8^+$ lymphocytes in group A was statistically higher (37.18%) compared with group K (27.98%; $P < 0.01$). Significant differences ($P < 0.01$) were also found in group A during stimulation of lymphocytes with α - or κ -CN (Figure 5C).

Splenocytes from groups A and K induced a similar percentage of $CD4^+CD25^+$ T cells after κ -CN stimulation. Significant differences ($P < 0.05$) in $CD4^+CD25^+$

population induction, 4.97% for group A and 8.25% for group K, were observed only after α -CN stimulation (Figure 5B). Regulatory T cells are essential for immune response and allergy development (Zheng, 2013; Noval Rivas and Chati, 2016). Therefore, $CD4^+CD25^+$ T cells were additionally phenotyped to determine the presence of the transcription factor Foxp3 (Figure 5D). The lymphocytes from group A showed statistically higher contribution of $CD4^+CD25^+Foxp3^+$ cells after κ -CN stimulation, 65.1%, compared with α -CN, 38.9% ($P < 0.05$). The group K lymphocytes showed a similar contribution of Treg cells regardless of the antigen used for stimulation. The numbers of Treg were 20.75 and 22.8%, respectively, after α - or κ -CN stimulation. Splenocyte K group stimulated with α - or κ -CN was characterized by a similar contribution of Treg lymphocytes. Additionally, in vitro stimulation of splenocytes with κ - or α -CN showed that group A lymphocytes were more reactive against other casein fractions than the primary antigen. Induction of $CD3^+CD4^+$ cells was varied and increased to $98.25 \pm 0.49\%$ when cells were stimulated with κ -CN and decreased to $54.7 \pm 6.22\%$ ($P < 0.01$) after stimulation with α -CN. Lymphocytes from group K (mice immunized with κ -CN) were less sensitive to antigen stimulation in vitro; the percentage

Figure 5. T cell profiles after stimulation of mice splenocytes with 200 μ g/mL of antigen or concanavalin A (ConA) as a positive control. (A) $CD4^+$ T cells (as % of total lymphocyte population); (B) $CD4^+CD25^+$ T cells (as % of total lymphocyte population); (C) $CD8^+$ T cells (as % of total lymphocyte population); (D) forkhead box P3 (Foxp3 $^+$) T cells (as % of $CD4^+CD25^+$ population). Mice were previously immunized with α -CN (patterned bars; group A) or κ -CN (solid bars; group K). Results are presented as mean of the group $\pm$ SD. Statistical differences are indicated by * $P < 0.05$; ** $P < 0.01$.

of CD3⁺CD4⁺ cells was similar ($63.55 \pm 6.2\%$ and $64.55 \pm 4.45\%$) upon κ - or α -CN stimulation, respectively. The highest expression of CD4 on CD3⁺CD4⁺ cells was found in atopic infants at birth and 3 mo of life and not later (at 6, 12, and 18 mo; Zdolsek et al., 1999). We observed significant differences in CD3⁺CD4⁺ percentage between experimental groups, suggesting that α -CN is more immunogenic than κ -CN.

Mesenteric lymph nodes play a key role in the development of immune responses as part of the gut-associated lymphoid tissue (GALT), which is a component of the mucosal immune system (Ruth and Field 2013). Figure 6 shows CD4⁺, CD8⁺, and CD4⁺CD25⁺ subpopulations of lymphocytes isolated from MLN. Immunizing mice with α -CN (group A) induced more CD4⁺ T cells in MLN than immunizing mice with κ -CN (group K): $34.7 \pm 6.8\%$ versus $10.6 \pm 2.7\%$, respectively ($P < 0.01$). Statistically significant differences were found in the

number of CD8⁺ T cells, with $18.3 \pm 2.9\%$ and $8.15 \pm 0.49\%$ ($P < 0.05$) in group A and K, respectively. The contribution of CD4⁺CD25⁺ in CD4⁺ lymphocytes was similar in both groups: $12.4\% \pm 5.9$ and $13.7\% \pm 7.8$ in groups A and K, respectively. The percentage of Treg was higher but not significantly different in MLN collected from group A mice than group K mice. The effect of the tested proteins on individual lymphocyte subpopulations was different. The contribution of the Treg cells, which play a significant role in controlling and modifying the allergic response by suppressing effector Th2 cells and IgE secretion (Akdis et al., 2005; Palomares et al., 2010), was higher in group A. The results of the present study indicated that α -CN-treated mice developed higher levels of Treg, which might suggest the immune response was directed to Th1-type response. Additionally, group A was characterized by a higher contribution of CD8⁺. The CD8⁺ lymphocytes

Figure 6. T cell profiles of lymphocytes isolated from mesenteric lymph nodes (MLN) of mice immunized with α -CN (group A; patterned bars) or κ -CN (group K; solid bars). (A) CD4⁺ and CD8⁺ T cells as a % of total lymphocyte population; and (B) CD4⁺CD25⁺ cells as a % of the CD4⁺ T cell subset. Statistical differences are indicated by ** $P < 0.01$. In panels A, B, and C, the dot plots indicate frequency of CD4⁺, CD8⁺, and CD4⁺CD25⁺ T cells in mesenteric lymph nodes, respectively. Color version available online.

may participate in allergen-related airway reactions and in production of type 2 cytokines (IL-4, IL-5, and IL-13). Studies have shown that transferring CD8⁺ and CD4⁺ cells from sensitized to naïve mice was connected with transferring the allergic response and induction of secretion of inflammatory cytokines in airway hyper-reactivity (Schaller et al., 2005). Other studies indicated that CD8⁺ lymphocytes have a protective role in the early stages of allergic sensitization, which is related to increasing production of IFN- γ (Stock et al., 2004). We found α -CN to be more effective in inducing humoral response and inducing CD4⁺, CD8⁺, and CD4⁺CD25⁺FoxP3⁺ T cells, inducing after in vivo immunization and ex vivo stimulation. In conclusion, α -CN appears to present a higher allergenic potential than κ -CN.

Cytokine Profiles. Food antigens can trigger an inflammatory or allergy immune system response through different mechanisms involving different T-cell subsets and cytokines secreted. Cytokines are reliable markers to describe the immune system response. After antigen stimulation of lymphocytes from groups A and K, the concentrations of IL-2, IL-4, IL-6, TNF, IFN- γ , and IL-10 secreted into the medium were determined. Splenocytes from group A secreted more cytokines from the analyzed panel after stimulation with primary antigen than splenocytes from group K. The concentrations of IL-6 and TNF in group A cultures were similar, regardless of the antigen used for stimulation, which was in contrast to group K lymphocytes, where different levels of secreted cytokines were observed depending on the used antigen.

Interleukin-10 is the main regulatory cytokine of inflammation and plays the key role in anergic states (Alonso et al., 2007). Secretion of IL-10 (Figure 7) was significantly higher for group A splenocytes than for group K splenocytes, 6,358.6 $\pm$ 1,671.6 pg/mL versus 704.3 $\pm$ 123.4 pg/mL with α -CN stimulation and 4,324.6 $\pm$ 609.4 pg/mL versus 908.2 $\pm$ 208.4 pg/mL ($P < 0.05$) with κ -CN stimulation. These results also indicated differences in the immunogenic and immunoreactive potential of the α - and κ -CN fractions. The literature reports the immunomodulatory function of IL-10. Its concentration changes during induction of tolerance in individuals with CMA and increases after additional stimulation with a priming antigen in T-lymphocyte culture (Frossard et al., 2004; Sommanus et al., 2014).

Interleukin-4 participates in many immune regulatory mechanisms, in allergic inflammation, humoral immunity, induction of B lymphocytes, differentiation of naïve T helper cells into Th2 cells, and IgE secretion (Steinke and Borish, 2001). In our study, the concentration of IL-4 was significantly higher in group A splenocyte cultures stimulated with α -CN than in group K:

Figure 7. Concentration of IL-10 in culture medium of mice splenocytes stimulated with α -CN or κ -CN. Mice were immunized with α -CN (patterned bars; group A) or κ -CN (solid bars; group K). Results are represented as mean of the group $\pm$ SD. Statistical differences between groups are indicated by * $P < 0.05$; ** $P < 0.01$.

200.5 $\pm$ 47.7 pg/mL versus 72.9 $\pm$ 4.7 pg/mL ($P < 0.05$; Figure 8). Stimulating cells with κ -CN resulted in a low concentration of secreted IL-4 (16.6 $\pm$ 7.3 and 27.5 $\pm$ 10.7 pg/mL for groups A and K, respectively). Other studies in a CMA mouse model reported an increase in IL-4 secretion in splenocyte cultures after stimulation with cow milk proteins (without differentiation into the fractions; Li et al., 1999; Lara-Villoslada et al., 2004). Our results complement the earlier research and take into account the immunogenic potential of individual casein fractions.

We found α -CN to be more immunogenic than κ -CN and it was able to initiate inflammation with increasing secretion of pro-inflammatory cytokines. We showed that food components can modulate the immune system response at various points within humoral and cellular reactions.

Figure 8. Concentration of IL-4 in culture medium of mice splenocytes stimulated with α -CN or κ -CN. Mice were immunized with α -CN (patterned bars; group A) or κ -CN (solid bars; group K). Results are represented as mean of the group $\pm$ SD. Statistical differences between groups are indicated by * $P < 0.05$; ** $P < 0.01$.

CONCLUSIONS

Our results indicated that both α - and κ -casein are immunogenic proteins that induce a similar level of humoral response, although differences are apparent at the cellular level. A Th2 response was induced by α -CN, and large amounts of IL-10 were secreted by α -casein-sensitized splenocytes. Therefore, we can assume that an allergy to κ -CN leads to CMA in the long term. The differences in response at the cellular level suggested unique immunomodulatory properties for each type of casein fraction, which should be considered when designing diet formulas for individuals with CMA or gastrointestinal problems.

ACKNOWLEDGMENTS

This study was funded by KNOW (Leading National Research Centre) Scientific Consortium: “Healthy Animal—Safe Food” (decision of Ministry of Science and Higher Education, Warsaw, Poland, No. 05-1/KNOW2/2015).

REFERENCES

- Adel-Patient, K., C. Créminon, H. Bernard, G. Clément, L. Négroni, Y. Frobert, J. Grassi, J. M. Wal, and J. M. Chatel. 2000. Evaluation of a high IgE-responder mouse model of allergy to bovine β -lactoglobulin (BLG): Development of sandwich immunoassays for total and allergen-specific IgE, IgG1 and IgG2a in BLG-sensitized mice. *J. Immunol. Methods* 235:21–32. [https://doi.org/10.1016/S0022-1759\(99\)00210-0](https://doi.org/10.1016/S0022-1759(99)00210-0).
- Akdis, M., K. Blaser, and C. A. Akdis. 2005. T regulatory cells in allergy: Novel concepts in the pathogenesis, prevention, and treatment of allergic diseases. *J. Allergy Clin. Immunol.* 116:961–968. <https://doi.org/10.1016/j.jaci.2005.09.004>.
- Alonso, R., F. Pineda, E. Enrique, R. Tella, and A. Cistero-Bahima. 2007. Usefulness of serum interleukin-10 in determining food tolerance. *Allergy* 62:710–711. <https://doi.org/10.1111/j.1398-9995.2007.01333.x>.
- Barłowska, J., M. Sz wajkowska, Z. Litwińczuk, and J. Król. 2011. Nutritional value and technological suitability of milk from various animal species used for dairy production. *Compr. Rev. Food Sci. Food Saf.* 10:291–302. <https://doi.org/10.1111/j.1541-4337.2011.00163.x>.
- Creamer, L. K., J. E. Plowman, M. J. Liddell, M. H. Smith, and J. P. Hill. 1998. Micelle stability: κ -Casein structure and function. *J. Dairy Sci.* 81:3004–3012. [https://doi.org/10.3168/jds.S0022-0302\(98\)75864-3](https://doi.org/10.3168/jds.S0022-0302(98)75864-3).
- EFSA. 2017. Trusted science for safe food: Protecting consumers' health with independent scientific advice on the food chain. Programming document 2017–2019. European Food Safety Authority (EFSA). <https://doi.org/10.2805/918292>.
- EFSA Panel on Dietetic Products, D. Turck, J. Bresson, B. Burlingame, T. Dean, S. Fairweather-Tait, M. Heinonen, K. I. Hirsch-Ernst, I. Mangelsdorf, H. J. McArdle, A. Naska, M. Neuhauser-Berthold, G. Nowicka, K. Pentieva, Y. Sanz, A. Sjödin, M. Stern, D. Tomé, H. Van Loveren, M. Vinceti, P. Willats, M. Fewtrell, H. Przyrembel, A. Titz, and S. Valtueña-Martinez. 2017. Scientific and technical guidance for the preparation and presentation of an application for authorisation of an infant and/or follow-on formula manufactured from protein hydrolysates. *EFSA J.* 15:e04779. <https://doi.org/10.2903/j.efsa.2017.4779>.
- El-Agamy, E. I. 2007. The challenge of cow milk protein allergy. *Small Rumin. Res.* 68:64–72. <https://doi.org/10.1016/j.smallrumres.2006.09.016>.
- Elsayed, S., D. J. Hill, and T. V. Do. 2004. Evaluation of the allergenicity and antigenicity of bovine-milk α s1-casein using extensively purified synthetic peptides. *Scand. J. Immunol.* 60:486–493. <https://doi.org/10.1111/j.0300-9475.2004.01493.x>.
- Ferrario, C., S. Duranti, C. Milani, L. Mancabelli, G. A. Lugli, F. Turrone, M. Mangifesta, A. Viappiani, M. C. Ossiprandi, D. van Sinderen, and M. Ventura. 2015. Exploring amino acid auxotrophy in *Bifidobacterium bifidum* PRL2010. *Front. Microbiol.* 6. <https://doi.org/10.3389/fmicb.2015.01331>.
- Fiocchi, A., G. R. Bouygue, M. Albarini, and P. Restani. 2011. Molecular diagnosis of cow's milk allergy. *Curr. Opin. Allergy Clin. Immunol.* 11:216–221. <https://doi.org/10.1097/ACI.0b013e32834694ef>.
- Frossard, C. P., C. Hauser, and P. A. Eigenmann. 2004. Antigen-specific secretory IgA antibodies in the gut are decreased in a mouse model of food allergy. *J. Allergy Clin. Immunol.* 114:377–382. <https://doi.org/10.1016/j.jaci.2004.03.040>.
- Hochwallner, H., U. Schulmeister, I. Swoboda, S. Spitzauer, and R. Valenta. 2014. Cow's milk allergy: From allergens to new forms of diagnosis, therapy and prevention. *Methods* 66:22–33. <https://doi.org/10.1016/j.ymeth.2013.08.005>.
- Høst, A., S. Husby, B. Gjesing, J. N. Larsen, and H. Løwenstein. 1992. Prospective estimation of IgG, IgG subclass and IgE antibodies to dietary proteins in infants with cow milk allergy. *Allergy* 47:218–229. <https://doi.org/10.1111/j.1398-9995.1992.tb00654.x>.
- Järvinen, K. M., K. Beyer, L. Vila, P. Chatchatee, P. J. Busse, and H. A. Sampson. 2002. B-cell epitopes as a screening instrument for persistent cow's milk allergy. *J. Allergy Clin. Immunol.* 110:293–297.
- Jiehu, Z., M. Liuliu, X. Haihong, G. Yang, J. Yingkai, Z. Lun, D. Xi An Li, Z. Dongsheng, and Z. Shaohui. 2014. Immunomodulating effects of casein-derived peptides QEPVL and QEPV on lymphocytes in vitro and in vivo. *Food Funct.* 5:2061–2069. <https://doi.org/10.1039/c3fo60657k>.
- Jo, J., J. Garssen, L. Knippels, and E. Sandalova. 2014. Role of cellular immunity in cow's milk allergy: Pathogenesis, tolerance induction, and beyond. *Mediators Inflamm.* 2014:249784. <https://doi.org/10.1155/2014/249784>.
- Kamiński, S., A. Cieslińska, and E. Kostyra. 2007. Polymorphism of bovine beta-casein and its potential effect on human health. *J. Appl. Genet.* 48:189–198. <https://doi.org/10.1007/BF03195213>.
- Kapila, R., P. K. Kavadi, and S. Kapila. 2013. Comparative evaluation of allergic sensitization to milk proteins of cow, buffalo and goat. *Small Rumin. Res.* 112:191–198. <https://doi.org/10.1016/j.smallrumres.2012.11.028>.
- Lara-Villoslada, F., M. Olivares, J. Jiménez, J. Boza, and J. Xaus. 2004. Goat milk is less immunogenic than cow milk in a murine model of atopy. *J. Pediatr. Gastroenterol. Nutr.* 39:354–360. <https://doi.org/10.1097/00005176-200410000-00010>.
- Leonardi, S., N. Rotolo, G. Vitaliti, L. Spicuzza, and M. La Rosa. 2007. IgE values and T-lymphocyte subsets in children with atopic eczema/dermatitis syndrome. *Allergy Asthma Proc.* 28:529–534. <https://doi.org/10.2500/aap2007.28.3038>.
- Li, X. M., B. H. Schofield, C. K. Huang, G. I. Kleiner, and H. A. Sampson. 1999. A murine model of IgE-mediated cow's milk hypersensitivity. *J. Allergy Clin. Immunol.* 103:206–214. [https://doi.org/10.1016/S0091-6749\(99\)70492-6](https://doi.org/10.1016/S0091-6749(99)70492-6).
- Matsuo, H., T. Yokooji, and T. Taogoshi. 2015. Common food allergens and their IgE-binding epitopes. *Allergol. Int.* 64:332–343. <https://doi.org/10.1016/j.alit.2015.06.009>.
- Mierzejewska, D., P. Mitrowska, B. Rudnicka, E. Kubicka, and H. Kostyra. 2008. Effect of non-enzymatic glycosylation of pea albumins on their immunoreactive properties. *Food Chem.* 111. <https://doi.org/10.1016/j.foodchem.2008.03.046>.
- Negaoui, H., K. Eddine, E. Mecherfi, S. A. Tadjer, H. Grar, O. Kheroua, and D. Saidi. 2016. Bovine lactoferrin allergenicity as studied in murine model of allergy. *Food Agric. Immunol.* 27:711–723. <https://doi.org/10.1080/09540105.2016.1160365>.

- Noval Rivas, M., and T. A. Chati. 2016. Regulatory T cells in allergic diseases. *J. Allergy Clin. Immunol.* 138:639–652. <https://doi.org/10.1016/j.jaci.2016.06.003>.
- Palomares, O., G. Yaman, A. K. Azkur, T. Akkoc, M. Akdis, and C. A. Akdis. 2010. Role of Treg in immune regulation of allergic diseases. *Eur. J. Immunol.* 40:1232–1240. <https://doi.org/10.1002/eji.200940045>.
- Rangel, A. H. do N., D. C. Sales, S. A. Urbano, J. G. B. Galvão Júnior, J. C. de Andrade Neto, and C. de Souza Macêdo. 2016. Lactose intolerance and cow's milk protein allergy. *Food Sci. Technol. (Campinas)* 36:179–187. <https://doi.org/10.1590/1678-457X.0019>.
- Restani, P., C. Ballabio, C. Di Lorenzo, S. Tripodi, and A. Fiocchi. 2009. Molecular aspects of milk allergens and their role in clinical events. *Anal. Bioanal. Chem.* 395:47–56. <https://doi.org/10.1007/s00216-009-2909-3>.
- Ruiter, B., V. Trégoat, L. M'Rabet, J. Garssen, C. A. F. M. Bruijn-zeel-Koomen, E. F. Knol, and E. Van Hoffen. 2006. Characterization of T cell epitopes in α s1-casein in cow's milk allergic, atopic and non-atopic children. *Clin. Exp. Allergy* 36:303–310. <https://doi.org/10.1111/j.1365-2222.2006.02436.x>.
- Ruth, M. R., and C. J. Field. 2013. The immune-modifying effects of amino acids on gut-associated lymphoid tissue. *J. Anim. Sci. Biotechnol.* 4:27. <https://doi.org/10.1186/2049-1891-4-27>.
- Schaller, M. A., S. K. Lundy, G. B. Huffnagle, and N. W. Lukacs. 2005. CD8+ T cell contributions to allergen induced pulmonary inflammation and airway hyperreactivity. *Eur. J. Immunol.* 35:2061–2070. <https://doi.org/10.1002/eji.200425715>.
- Sletten, G. B. G., R. Halvorsen, E. Egaas, and T. S. Halstensen. 2007. Casein-specific immunoglobulins in cow's milk allergic patient subgroups reveal a shift to IgA dominance in tolerant patients. *Pediatr. Allergy Immunol.* 18:71–80. <https://doi.org/10.1111/j.1399-3038.2006.00489.x>.
- Sommanus, S., S. Kerddonfak, W. Kamchaisatian, S. Vilaiyuk, C. Sasisakulporn, W. Teawsomboonkit, and S. Benjaponpitak. 2014. Cow's milk protein allergy: Immunological response in children with cow's milk protein tolerance. *Asian Pac. J. Allergy Immunol.* 32:171–177. <https://doi.org/10.12932/AP0319.32.2.2013>.
- Steinke, J. W., and L. Borish. 2001. Th2 cytokines and asthma—Interleukin-4: Its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. *Respir. Res.* 2:66–70.
- Stock, P., T. Kallinich, O. Akbari, D. Quarcoo, K. Gerhold, U. Wahn, D. T. Umetsu, and E. Hamelmann. 2004. CD8+ T cells regulate immune responses in a murine model of allergen-induced sensitization and airway inflammation. *Eur. J. Immunol.* 34:1817–1827. <https://doi.org/10.1002/eji.200324623>.
- Tsabouri, S., K. Douros, and K. N. Priftis. 2014. Cow's milk allergenicity. *Endocr. Metab. Immune Disord. Drug Targets* 14:16–26.
- Vila, L., K. Beyer, K. M. Järvinen, P. Chatchatee, L. Bardina, and H. A. Sampson. 2001. Role of conformational and linear epitopes in the achievement of tolerance in cow's milk allergy. *Clin. Exp. Allergy* 31:1599–1606. <https://doi.org/10.1046/j.1365-2222.2001.01218.x>.
- Woodfolk, J. A. 2007. T-cell responses to allergens. *J. Allergy Clin. Immunol.* 119:280–294.
- Wróblewska, B., L. Jędrychowski, and M. Farjan. 2007. The allergenicity of a low molecular fraction of cow milk protein hydrolysates. *Milchwissenschaft* 62:375–379.
- Wróblewska, B., J. Juśkiewicz, B. Kroplewski, E. Wasilewska, D. Złotkowska, and L. Markiewicz. 2018. The effects of whey and soy proteins on growth performance, gastrointestinal digestion, and selected physiological responses in rats. *Food Funct.* 9:1500–1509. <https://doi.org/10.1039/c7fo01204g>.
- Zdolsek, H. A., J. Ernerudh, P. G. Holtd, J. Nilsson, and B. Björkstén. 1999. Expression of the T-cell markers CD3, CD4 and CD8 in healthy and atopic children during the first 18 months of life. *Int. Arch. Allergy Immunol.* 119:6–12. <https://doi.org/10.1159/000024169>.
- Zheng, S. G. 2013. Regulatory T cells vs Th17: Differentiation of Th17 versus Treg, are they mutually exclusive? *Am. J. Clin. Exp. Immunol.* 2:94–106.
- Złotkowska, D., E. Wasilewska, E. Kubicka, and B. Wróblewska. 2012. The effect of pea albumins on immune response in mice. *Centr. Eur. J. Immunol.* 37:232–236.