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Oral exposure to the free amino acid glycine inhibits the acute allergic response in a model of cow's milk allergy in mice

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ABSTRACT

The conditionally essential amino acid glycine functions as inhibitory neurotransmitter in the mammalian central nervous system. Moreover, it has been shown to act as an anti-inflammatory compound in animal models of ischemic perfusion, post-operative inflammation, periodontal disease, arthritis and obesity. Glycine acts by binding to a glycine-gated chloride channel, which has been demonstrated on neurons and immune cells, including macrophages, polymorphonuclear neutrophils and lymphocytes. The present study aims to evaluate the effect of glycine on allergy development in a cow's milk allergy model. To this end, C3H/HeOuJ female mice were supplemented with glycine by oral gavage (50 or 100 mg/mouse) 4 hours prior to sensitization with cow's milk whey protein, using cholera toxin as adjuvant. Acute allergic skin responses and anaphylaxis were assessed after intradermal allergen challenge in the ears. Mouse mast cell protease-1 (mMCP-1) and whey specific IgE levels were detected in blood collected 30 minutes after an oral allergen challenge. Jejunum was dissected and evaluated for the presence of mMCP-1-positive cells by immunohistochemistry. Intake of glycine significantly inhibited allergy development in a concentration dependent manner as indicated by a reduction in; acute allergic skin response, anaphylaxis, serum mMCP-1 and serum levels of whey specific IgE. In addition, in-vitro experiments using rat basophilic leukemia cells (RBL), showed that free glycine inhibited cytokine release but not cellular degranulation. These findings support the hypothesis that the onset of cow's milk allergy is prevented by the oral intake of the amino acid glycine. An adequate intake of glycine might be important in the improvement of tolerance against whey allergy or protection against (whey-induced) allergy development.

Abbreviations: mMCP-1, Mouse mast cell protease-1; CMA, cow's milk allergy; IEC, Intestinal epithelial cells; Ig, immunoglobulin; AA, amino acid; CEAA, conditional essential amino acid; RBL, rat basophilic leukemia cells; GlyR, glycine receptor; DC, dendritic cell; Th2, T helper type 2.

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1. Introduction

Food allergy is defined as an "adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food" [1]. Genetic predisposition, environmental factors, timing, duration and extent of exposure are important determining factors of allergic sensitization [2]. In the past decades, the prevalence of food allergies has strongly increased resulting in a major public health issue worldwide [3-5]. Different, challenge-confirmed studies show that up to 5% of the children in the Western world are affected by cow's milk allergy (CMA) [6].

Although it is not completely understood how allergic sensitization to food antigens occurs, it is generally accepted that the condition of the intestinal immune system plays an important role [7]. Intestinal epithelial cells (IEC) are pivotal in maintaining intestinal immune homeostasis. IECs provide a physical and biochemical barrier and release conditioning factors, which regulate the cells of both the innate and adaptive immune system [8]. Loss of IEC function and consequently an increase in intestinal permeability is thought to contribute to both the sensitization and the anaphylactic effector phase of food allergy [9]. Indeed, both clinical observations and in-vivo experimental evidence demonstrate that intestinal barrier dysfunction contributes to the severity of food allergy-induced symptoms [9].

Amino acids (AA) can be classified into essential, non-essential and conditionally essential AA (CEAA) [10]. CEAA are those AAs from which the rate of utilization is greater than the rate of synthesis under certain conditions (e.g. pregnancy, lactation, early life weaning, infection and inflammation). Examples of CEAA in humans are glycine, glutamine, arginine, proline and taurine, which have been shown to play important roles in establishing or maintaining intestinal barrier integrity as well as regulating intestinal inflammatory responses [11].

Glycine is important for growth in early life and well known for its role as an inhibitory neurotransmitter in the central nervous system. In addition, accumulating evidence suggests that glycine is also an effective immunomodulatory, anti-inflammatory and cytoprotective agent [12]. Glycine has been shown to protect IECs from inflammatory oxidative stress through maintenance of epithelial antioxidant defenses [13] and protect the intestine from ischemia–reperfusion injury [14] or chemical-induced colitis [15]. Furthermore, oral intake of glycine suppresses zymosan-induced inflammation of the joints [16], reduces intestinal injury following endotoxemia [17], attenuates the oxidative and inflammatory burden in a mouse model of cancer cachexia [18] and reduces the severity of pancreatic damage in a model of acute pancreatitis [19].

Under normal conditions, mucosal contact with innocuous antigenic molecules induces non-inflammatory responses and oral tolerance [20]. However, in case of food allergies, mucosal homeostasis is disturbed, resulting in an inflammatory condition leading to food hypersensitivity.

Therefore, based on the previous studies, we hypothesized that the free amino acid glycine prevents the development of food allergy. This hypothesis will be tested in a cow's milk allergy mouse model [21]. We examined the effects of oral supplementation of free glycine on the whey-induced allergic response, measured as changes in the acute and systemic parameters. To determine a potential mechanism, the effect of glycine on IgE-mediated crosslinking-induced degranulation and cytokine production was studied in rat basophilic leukemia cells (RBL).

2. Methods and materials

2.1. *Diet, oral sensitization and challenge of mice*

All experimental procedures using laboratory animals were approved by an independent ethics committee for animal experimentation (DEC Consult, Soest, The Netherlands). Four-week-old female, specific pathogen-free (SPF) C3H/HeOuJ mice, bred for at least two generations on a cow's milk-free diet, were purchased from Charles River Laboratories (Maastricht, The Netherlands). All mice were fed the semi-purified AIN-93G soy-based diet (SSniff Spezialdiäten GmbH, Soest, Germany) [22].

In total 38 mice were randomly distributed into 4 groups: Negative control (NC, n = 8, sham sensitized), positive control (PC, n = 10, whey-sensitized) and PC plus two levels of oral glycine supplementation (PC+ 50 mg glycine and PC or 100 mg glycine, respectively; n = 10 for each group). Mice were orally sensitized with 20 mg of homogenized whey (WPC60, Milei, Friesland Campina, Amersfoort, The Netherlands) mixed with 10 μg of the adjuvant cholera toxin (List Biological Laboratories, Campbell, CA, USA) in 0.5 mL PBS, once weekly, for a period of 5 weeks [21]. The sham-sensitized mice received cholera toxin in PBS. At 24 and 2–4 hours before each sensitization, the two glycine treatment groups received either 50 or 100 mg glycine (ICN Biomedicals, Aurora, OH, USA) dissolved in 200 μL water by oral gavage. All sham-sensitized mice received a sham gavage with water.

One week after the last sensitization the acute allergic skin reaction was measured after intradermal (id) injection of 10 μL of homogenized whey protein (1 mg/mL in PBS) in the ear pinnae. An overview of the model is shown in Fig. 1.

The body temperature was monitored 15, 30, 45 and 60 min after intradermal challenge using a programmable temperature transponder (IPTT-300, Biomedical data systems, Delware, USA) which was subcutaneously implanted in all mice; and the anaphylactic shock severity was scored with the scoring table from Li et al. [23] (Table 1). Animals reaching the humane endpoint, set at a shock score of 4, were sacrificed and not considered for further analysis. Ear thickness was measured before and 1 hour after the injection using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands). The whey-induced ear swelling (μm) was defined as the difference between ear-thickness before and 1 hour after injection. Two days after the acute allergic skin testing, the mice were orally challenged with whey (100 mg whey in 0.5 mL PBS). After 30 minutes, mice were bled under terminal anesthesia (isoflurane/air) followed by cervical dislocation, dissection of the jejunum was performed for immunohistochemistry. Success of the allergic sensitization was determined by comparing the data from PC animals to the NC animals. To test whether glycine supplementation

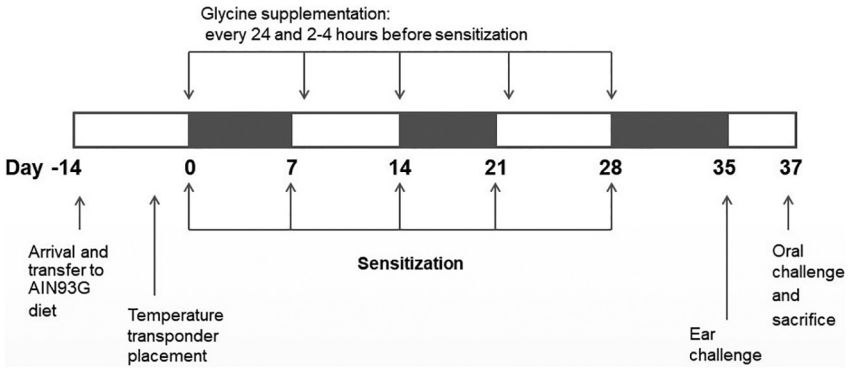

Fig. 1 – A schematic representation of the experimental model.

attenuated the allergic response, data from the glycine supplemented animals were compared to PC animals. To test whether the highest dose of glycine could fully mitigate the allergic response, data from the PC+ 100 mg/glycine animals were compared to the NC animals.

2.2. *Blood collection*

Blood was collected by eye-extraction in MiniCollect tubes (Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands) and centrifuged for 5 minutes at 14000 rpm in an Eppendorf centrifuge. The serum was stored at –80 for immunoglobulin and mMCP-1 analysis.

2.3. *Measurement of mMCP-1 and whey-specific serum immunoglobulins*

Serum levels of mucosal mast cell protease-1 (mMCP-1) were analyzed using a commercially available ELISA (eBioscience, Vienna, Austria), according to the manufacturer's protocol. Serum levels of whey-specific IgE, IgG_1 and IgG_{2a} were analyzed by means of ELISA. Microlon plates (Greiner, Alphen aan de Rijn, The Netherlands) were coated with whey (20 μg/well in coating buffer) (Sigma, Zwijndrecht, The Netherlands) for 18 h at 4°C. After washing the plates were blocked with 2% HSA (Human Serum Albumin, Sigma) in wash-buffer (PBS with 0.05% Tween-20) and serum samples were incubated for 2 h at room temperature (RT). After washing, the samples were incubated with 1 μg biotin-labeled rat anti-mouse IgE, IgG_1 or IgG_{2a} (Pharmingen) for 1.5 h at RT, washed and incubated with streptavidin-horseradish peroxidase (Sanquin, Amsterdam, The Netherlands) for 1 h at RT. The plates were washed and developed with TMB 1-step ULTRA substrate (Pierce, Fisher Scientific, Landsmeer, The Netherlands). The reaction was stopped after 10 min with 4 mol/L H_2SO_4 and absorbance was measured at 450 nm (BioTek, Powerwave HT, BioTek, Bad Friedrichstall, Germany).

Table 1 – scoring system for anaphylactic symptoms

Score	Symptoms
0	No symptoms
1	scratching and rubbing around the nose and head
2	puffiness around the eyes and mouth, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate
3	wheezing, labored respiration, and cyanosis around the mouth and the tail
4	no activity after prodding or tremor and convulsion
5	death

2.4. *Immunohistochemistry*

The proximal, middle and distal parts of the jejunum were fixed in 4% formaldehyde in PBS for 24 h at RT, dehydrated and embedded in paraffin. Cross-sections of 5 μm were prepared (6–8 animals/ group) and cellular localization of mMCP-1 was assessed by an immunohistochemical staining as described previously [24]. Briefly, paraffin sections were deparaffinized and endogenous peroxidase activity was blocked with 0.3% H_2O_2 (Merck, Darmstadt, Germany) in methanol. For antigen retrieval, the slides were boiled in10mM citrate buffer (pH 6.0) and after blocking with 5% serum, the slides were incubated (overnight) with anti-mouse mMCP-1 (1:50, 14–5503, e-Biosciences). After washing, the tissue sections were incubated with biotinylated rabbit-anti rat (1:200, Dako, Glostrup, Denmark) followed by streptavidin-biotin complex/horseradish peroxidase (Vectastain Elite ABC, Vector Laboratories, Peterborough, UK). Staining was visualized using 0.05% diaminobenzidine (DAB) solution for 10 min, and sections were counterstained with Mayers' hematoxylin (Merck Millipore, Amsterdam, The Netherlands). Photomicrographs were taken with an Olympus BX50 microscope equipped with a Leica 320 digital camera.

For the quantification of mMCP-1 positive cells, sections were also stained as described before, except the Mayers' hematoxylin counterstaining. All stained slides were scanned at 100 × magnification using a virtual BX61VS microscope (Olympus). For automated morphometric analysis, digital photomicrographs of the jejunal tissue sections (proximal, middle, distal) were taken with VS-ASW WSI software (version 2.7, Virtual Slide System multi slide) (Olympus Soft Imaging Solutions GmbH, Münster, Germany) and saved into TIFF images without noticeable loss of image resolution. Images were viewed in ImageJ (version 1.50i, National

Institutes of Health, Bethesda, MD, USA) and the mMCP-1 positive cells were blindly analyzed on entire section scans using macro-commands. Briefly, the staining intensity for DAB was assessed using color threshold analysis. Threshold color was converted to black-white, such that positive staining appeared black, and likewise absence of stain appeared white. Once images were black and white, the average number of positive DAB-stained cells in the complete jejunum was calculated based on the positive DBA-stained cells per tissue section.

2.5. Cross-linking induced RBL degranulation and cytokine production

RBL-2H3 cells were primed by 100 ng/mL mouse IgE α-dinitrophenyl in culture medium (DMEM with 10% FBS and 1% penicillin/streptavidin) and transferred to a 96-well flat bottom plate (100 μL /well). After 18 h of culturing, the cells were washed 4 times with 200 μL Tyrode's buffer (130 mM NaCl, 190 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES, and 0.1% bovine serum albumin, pH 7.4). Glycine (25 μL) in a concentration series starting form 500 μg/mL in Tyrode's buffer or Tyrode's buffer (control) was added to the cells and incubated for 1 hour. Thereafter, 25 μL of DNP-BSA (100 ng/mL) and 50 μL Tyrode's buffer were added. After 1 h supernatants were collected for analyzing degranulation. Maximum (100%) degranulation was measured after adding 10 μL 1% v/v Triton X-100 (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). Background release was measured in a control without adding the allergen (not activated, NA). Cell-supernatant for cytokine analysis was collected after 20 h culturing and stored at –80°C. Metabolic activity of RBL-2H3 cells was measured as an indicator of cell viability using a WST-1 assay (WST-1, Roche Diagnostics, Almere, The Netherlands) according to the manufacturers' protocols. Briefly, RBL-2H3 cells were incubated for 20 h with or without glycine. Absorbance was measured directly after WST-1 addition (T = 0) and after another 4 h (T = 4) incubation period. The difference in absorbance between T = 4 and T = 0 of the unstimulated control was set at 100%.

2.6. Degranulation assay

As a readout of degranulation, β-hexosaminidase release was measured by using the fluorescent substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma-Aldrich) as described previously [25]. Shortly, 50 μL of the substrate (60.6 μg/mL 0.1 mol/L citric acid, pH 4.5) was added to each well and incubated for 1 h. The reaction was stopped by the addition of 100 μL glycine buffer (0.2 mol/L glycine, 0.2 mol/L NaCl, pH 10.7). Fluorescence intensity was detected, excitation 360 and emission 460 nm, using Fluorstar (BMG Labtech GmbH, Offenburg, Germany).

2.7. Cytokine detection

Cytokine levels in RBL-2H3 culture supernatants were detected using a commercial Multiplex Bead immunoassay (BioRad, Veenendaal, The Netherlands), including TNFα, IL-4, IL-10 and IL-13 according to the manufacturers' protocol. The analyses were performed by using the Bio-Plex system (BioRad) and results were calculated using Bio-Plex Manager Software 3.1 (BioRad).

2.8. cDNA synthesis and real-time PCR

RBL-2H3 cells were taken from routine cultures (T = 0) or after 24 hours of culturing in Tyrode's buffer (T = 24) and lysed in Trizol (Thermo Fisher Scientific). Brain tissue dissected from a rat was homogenized in Trizol using mechanical disruption in a MPbio Fast-Prep homogenizer (MP Biomedicals). After addition of chloroform, RNA was isolated from the aqueous fraction using Qiagen RNeasy Mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturers' protocol. RNA quantity and quality was determined with the Nanodrop (Thermo Fisher Scientific). RNA of sufficient quality was transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturers' protocol. Real-time PCR was performed on a 7900HT Fast real-time PCR machine (Thermo Fisher Scientific) using SYBR select Master Mix (Thermo Fisher Scientific) and custom RT2 qPCR Primer assays (Qiagen). PPR06760A rat Glra1, PPR06773F rat Glra2, PPR06755E rat Glra3, PPR68961A rat Glra4, PPR06818A rat Glrb, PPM03562A rat B2M (reference gene). Relative target mRNA abundance was calculated by: relative mRNA abundance = $100 \times 2^{Ct[\text{reference gene}] - Ct[\text{target gene}]}$.

2.9. Statistical analyses

All data are expressed as means ± standard error of the mean (SEM) unless otherwise indicated. The number of animals in each of the groups in the experiments was based on the sealed envelope™ software (https://www.sealedenvelope.com/). Power calculations were based on testing for continuous outcome (Superiority Trial). Sample size calculations were based on previous obtained results of the primary outcome, the ear-swelling response after antigen challenge. Additional animals were added to the whey-sensitized groups to correct for previously experienced anaphylaxis induced death. After drop-outs (anaphylaxis), the total number of mice per group of which the data were used for statistical analysis was: N = 8 sham-sensitized mice, N = 6 whey-sensitized sham treated mice, N = 8 whey-sensitized glycine 50 mg treated mice, N = 8 whey-sensitized glycine 100 mg treated mice. All readout parameters were tested for normal distribution using D'Agostino & Pearson normality test. If the parameters were normally distributed, PC was compared to NC and PC+ 100Gly to NC using either a parametric T-test or a nonparametric Mann–Whitney test. The effect of glycine supplementation was analyzed using either one-way ANOVA with a post hoc Dunnett's test (normally distributed) or Kruskal-Wallis with a post hoc Dunn's test to test for overall significance of differences. Correlations among IgE and mMCP-1 levels, using data from all groups, were calculated using the Pearson's linear regression model. *P* values were calculated using GraphPad Prism 7 and differences considered significant if $P < .05$.

3. Results

3.1. Glycine attenuates acute allergic skin response and symptoms of anaphylaxis in whey-sensitized mice

To determine the allergic response, mice were challenged with whey and the acute allergic skin reaction was measured.

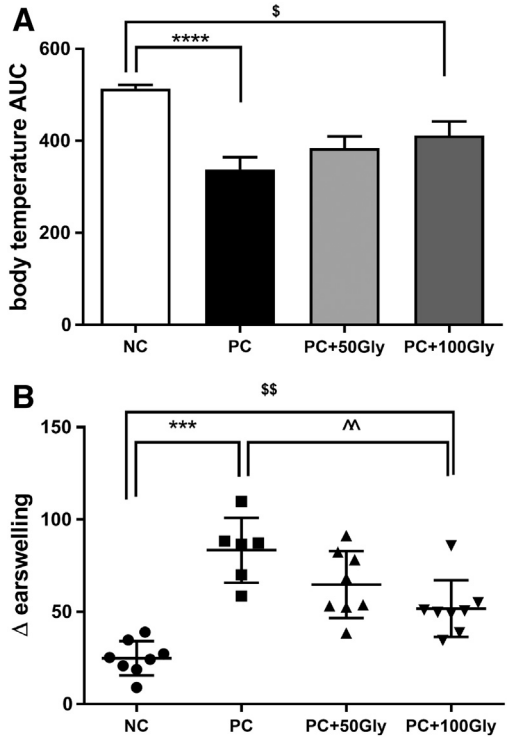

Fig. 2 – The effect of glycine intake before sensitization on body temperature (A) and the acute allergic skin response (B).The acute ear swelling was measured before and 1 hr. after the i.d. challenge with whey. The systemic anaphylaxis reaction was assessed by monitoring the body temperature before and 15 and 30 minutes after i.d. challenge with whey. Values are means ± SEM, n = 6–8. Comparisons between the positive control (PC) and glycine supplemented groups (PC + 50Gly, PC+ 100Gly) were performed using one-way analysis of variance (ANOVA) (A) or Kruskal-Wallis test (B). When there was an interaction, Dunnett's multiple comparisons test (A) or Dunn's multiple comparisons test (B) was used to determine differences among groups. Comparisons between negative control (NC) and PC or PC + 100Gly were performed using unpaired 2-tailed Student t test (A) or Mann–Whitney test (B). Significant differences between NC and PC control are indicated by $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. Differences between PC and positive control +50 mg/day glycine (PC+ 50Gly) or positive control +100 mg/day glycine (PC+ 100Gly) are indicated by $^{\wedge}P < .05$, $^{\wedge\wedge}P < .01$, $^{\wedge\wedge\wedge}P < .001$. Differences between NC and PC + 100Gly are indicated by $^{\$}P < .05$, $^{\$\$}P < .01$, $^{\$\$\$}P < .001$.

Four animals from the PC group and two animals from both the PC+ 50 mg glycine and PC+ 100 mg glycine groups reached the humane endpoint based on shock score and were sacrificed. Samples from these animals were excluded from further analysis.

Temperature responses were collected at intervals of 15 minutes for 1 hour and the area under the curve (AUC) was calculated. PC mice showed a significant decline in body temperature compared to NC mice ($P < .01$, Fig. 2A). PC mice receiving the highest dose of glycine showed a reduction in body temperature drop, however, this did not reach significance (PC + glycine 100 mg, Fig. 2A).

Upon i.d. challenge, the ear swelling in the PC animals was increased (83.4 ± 7.2 μm) compared to the NC animals (24.9 ±

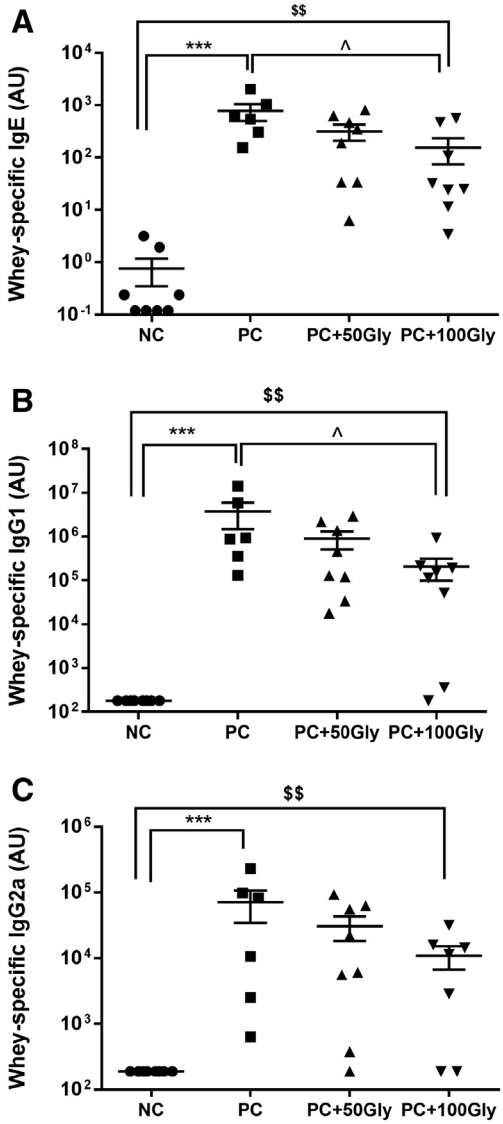

Fig. 3 – Effect of glycine intake before whey sensitization on serum IgE (A), IgG1 (B) and IgG2a (C) levels in arbitrary units (AU). Values are means ± SEM, n = 6–8. Comparisons between the positive control (PC) and glycine supplemented groups (PC+ 50Gly, PC+ 100Gly) were performed using Kruskal-Wallis test. When there was an interaction, Dunn's multiple comparisons test was used to determine differences among groups. Comparisons between negative control (NC) and PC or PC+ 100Gly were performed using Mann–Whitney test. Significant differences between NC and PC control are indicated by $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. Differences between PC and positive control +50 mg/day glycine (PC+ 50Gly) or positive control +100 mg/day glycine (PC+ 100Gly) are indicated by $^{\wedge}P < .05$, $^{\wedge\wedge}P < .01$, $^{\wedge\wedge\wedge}P < .001$. Differences between NC and PC+ 100Gly are indicated by $^{\$}P < .05$, $^{\$\$}P < .01$, $^{\$\$\$}P < .001$.

3.3 μm, $P < .01$). Glycine intake of 100 mg/day before sensitization significantly inhibited the increase in ear swelling ($P < .01$ PC+ 100Gly, Fig. 2B). However, glycine was not able to fully mitigate the allergic response as the ear swelling remained significantly different from NC animals ($P < .01$ PC+ 100Gly, Fig. 2B).

3.2. *Glycine reduces serum levels of whey-specific Ig and mMCP-1*

After the mice were euthanized, whey-specific antibody responses were measured in serum. The whey-specific IgE levels increased from 0.7 ± 0.4 (AU) in the NC mice to 784 ± 280 (AU) in the PC mice (PC, $P < .01$). Glycine intake before sensitization inhibited the production of whey-specific IgE at a dose of 100 mg/day (PC+ 100Gly, $P < .05$, Fig. 3A). Moreover, the whey-specific increases in IgG_1 were also counteracted by glycine intake (PC+ 100 mg glycine, $P < .05$, Fig. 3B). Whey-specific IgG2a levels were attenuated by glycine supplementation but this did not reach significance (Fig. 3C).

None of the measured responses could be completely prevented by glycine intake as there remained a significant difference in whey-specific antibody levels between PC + 100Gly and NC animals. mMCP-1, a protease present in mouse mucosal mast cells, appears in the circulation after mast cell degranulation. Hence, serum mMCP-1 levels were analyzed 30 mins after the oral whey challenge. The mMCP-1 levels in the PC animals increased compared to the NC animals from 16.6 ± 7.7 to 1244 ± 144 ng/mL (PC, $P < .01$). Glycine intake before sensitization attenuated the increase in mMCP-1 release (PC+ 50Gly, $P < .05$ and PC+ 100Gly, $P < .01$, Fig. 4A). Whey challenge-induced levels of IgE and mMCP-1 were significantly correlated ($P < .0001$, $r^2 = 0.429$, n = 30, Fig. 4B).

3.3. *Glycine counteracts the decline of mMCP-1-positive cells in the jejunum of whey-sensitized mice*

To investigate the effect of glycine on mast cell frequency in the intestines, the jejunum was dissected and mMCP-1-positive cells were stained, which indicates the presence of mucosal mast cells. mMCP-1-positive cells could be detected across the entire length of the jejunum and were quantified as described in the materials and methods (Fig. 5A). Compared to the NC mice, a significant reduced number of mMCP-1-positive cells could be detected in the PC animals (76 ± 17 versus 55 ± 22 mMCP-1-positive cells per slice, respectively $P < .01$) (Fig. 5B). Mice receiving glycine showed significantly more mMCP-1 positive cells in the jejunum (PC + 100Gly, $P < .01$) compared to PC animals. Glycine intake at 100 mg glycine was able to fully mitigate the challenge induced decrease in mMCP-1 positive cells as there was no significant difference between the NC and PC+ 100Gly animals.

3.4. *Glycine attenuates cytokine release but not cellular degranulation by RBL cells*

In vitro effects of glycine on IgE-crosslinking-induced degranulation and cytokine release were studied in RBL-2H3 cells. Addition of glycine, up to 500 μg/mL to the culture medium, did not change

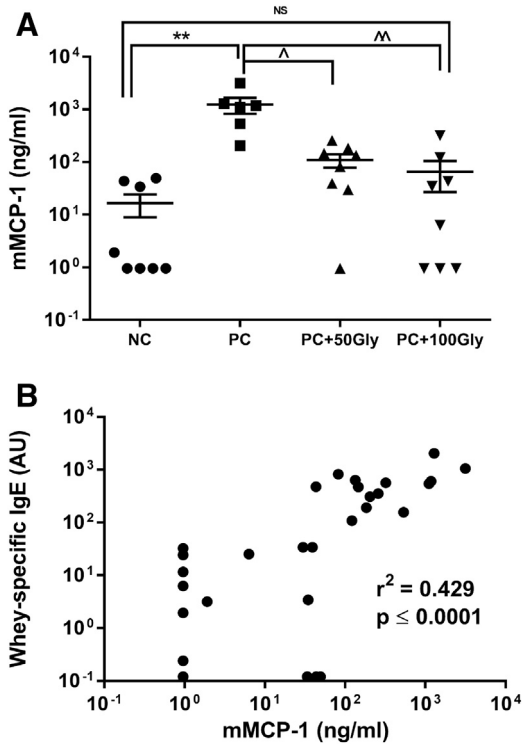

Fig. 4 – Effect of glycine on serum mMCP-1 levels (A) detected 30 min after oral challenge with whey. Values are means ± SEM, n = 6–8. Comparisons between the positive control (PC) and glycine supplemented groups (PC+ 50Gly, PC+ 100Gly) were performed using Kruskal-Wallis test. When there was an interaction, Dunn's multiple comparisons test was used to determine differences among groups. Comparisons between negative control (NC) and PC or PC+ 100Gly were performed using Mann–Whitney test. Significant differences between NC and PC control are indicated by *P < .05, **P < .01, ***P < .001. Differences between PC and positive control +50 mg/day glycine (PC+ 50Gly) or positive control +100 mg/day glycine (PC+ 100Gly) are indicated by ^P < .05, ^^P < .01, ^^^P < .001. Differences between NC and PC + 100Gly are indicated by $P < .05, $$P < .01, $$$P < .001. The Pearson r regression analysis (B) between whey-specific IgE and mMCP1 levels 30 minutes after oral-challenge with whey ($P < .0001$, $r^2 = 0.429$, n = 30).

the metabolic activity of RBL-2H3 cells, indicating that the tested glycine levels are not cytotoxic (Fig. 6A). Cross-linking-induced degranulation, which was detected by β-hexosaminidase analyses, was not altered by the tested glycine concentrations (data not shown). Cytokine release of TNFα, IL-4 and IL-13 increased strongly after crosslinking-induced cell activation, whereas the increase of IFNγ and IL-10 was less pronounced. Glycine attenuated the increased release of all tested cytokines (Fig. 6B-F).

To determine whether RBL-2H3 cells express the glycine receptor (GlyR), RBL-2H3 cells were directly taken from culture and after 24 hours of culture in the absence of glycine and RNA was isolated. RT-PCR was performed to determine the presence of GlyR alpha subunits 1–4 and the GlyR beta subunit. Compared to rat brain cells, RBL-2H3 cells

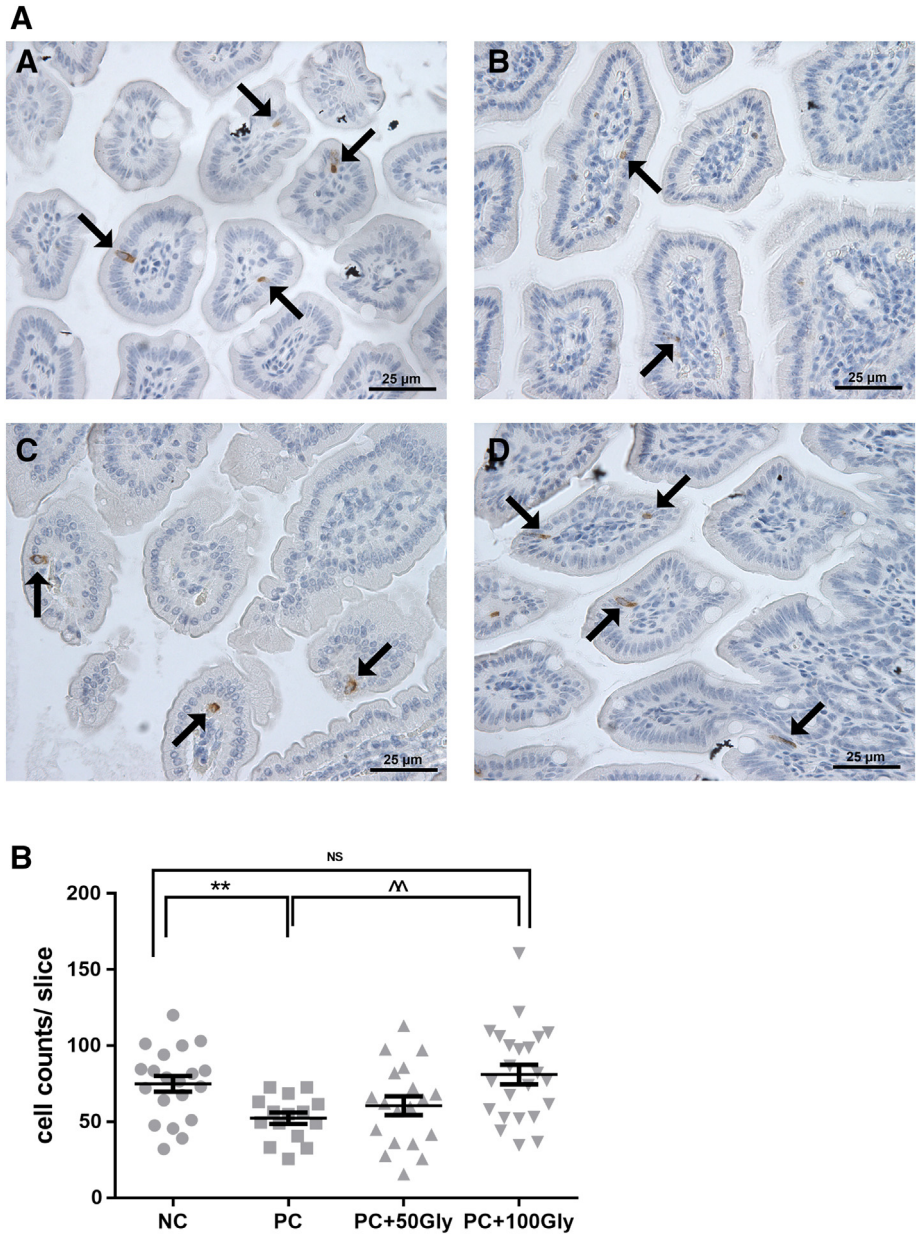

Fig. 5 – Effect of different glycine concentrations on mMCP-1 positive cells in jejunum. Parts of the jejunum (proximal, middle and distal) were collected, stained for mMCP-1 and mMCP-1 positive cells were automatically analyzed using ImageJ macro-commands. (A) Representative pictures of negative control (A), positive control (B), 50 mg/day glycine (C), 100 mg/day glycine (D). brown color (DAB staining): mMCP-1 positive cells. Magnification 400x. (B) mMCP-1 positive cell counts of the jejunum. Values are presented as means ± SEM, n = 6–8 and each point represents the cell count of mMCP-1 positive cells in the jejunum counting cells from the proximal, middle and distal part of the small intestine. Comparisons between the positive control (PC) and glycine supplemented groups (PC+ 50Gly, PC+ 100Gly) were performed using one-way analysis of variance (ANOVA). When there was an interaction, Dunnett's multiple comparisons test was used to determine differences among groups. Comparisons between negative control (NC) and PC or PC+ 100Gly were performed using unpaired 2-tailed Student t test. Differences between PC and positive control +50 mg/day glycine (PC+ 50Gly) or positive control +100 mg/day glycine (PC + 100Gly) are indicated by ^P < .05, ^^P < .01, ^^^P < .001. Differences between NC and PC+ 100Gly were not significant (NS).

were found to express very low, but detectable levels of GlyR alpha 1–3 and GlyR beta. GlyR alpha 4 had the highest relative expression (Fig. 7). Expression levels of the RBL GlyR subunits did not change after 24 h culture in the absence of glycine.

4. Discussion

Literature indicates that glycine levels between 10–1000 μmol/L can attenuate inflammatory responses and support intestinal

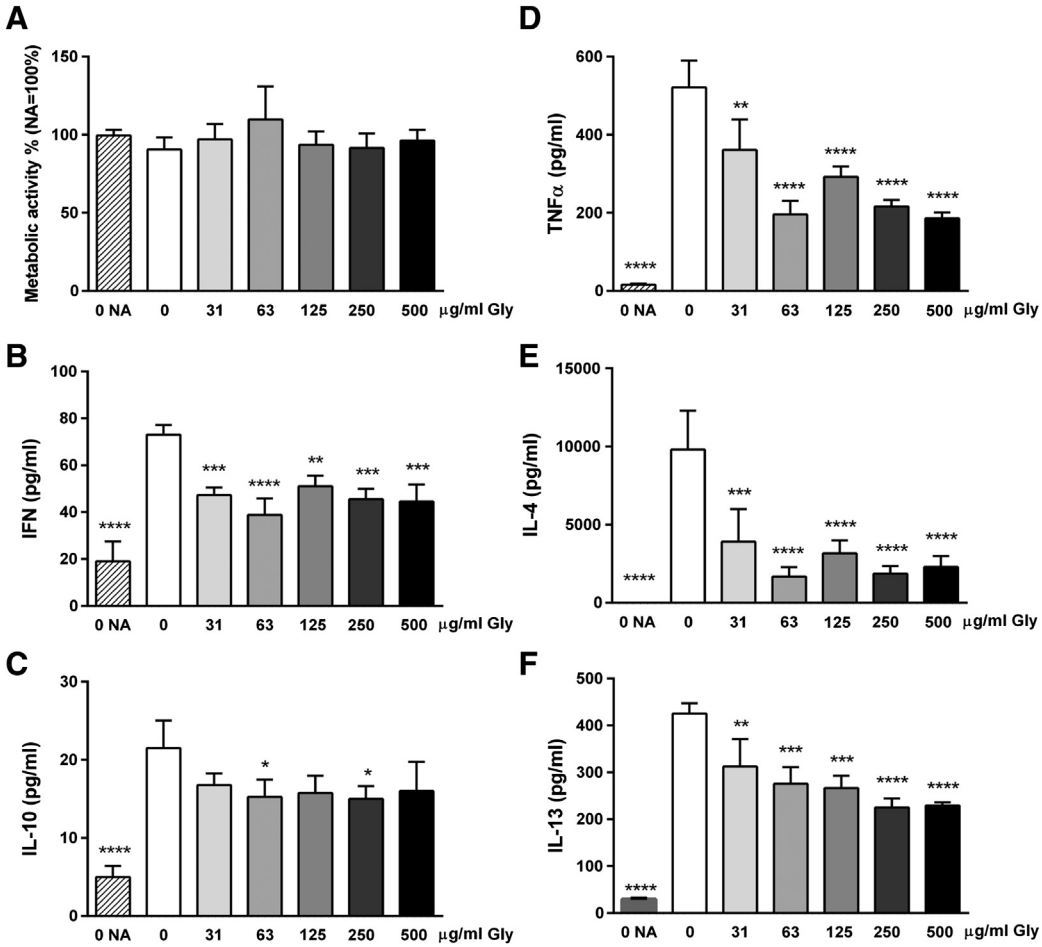

Fig. 6 – Effect of different concentrations of glycine on crosslinking induced metabolic activity (A) and cytokine production (B-F), 20 h after crosslinking-induced activation of the RBL-2H3 cells (0NA are not-activated control cells without glycine). Values are means ± SEM, n = 4. Comparisons between 0NA, activated cells without glycine (0) and with glycine were performed using one-way analysis of variance (ANOVA). When there was an interaction, Dunnett's multiple comparisons test was used to determine differences from activated cells without glycine (0). Significant differences are indicated by *P < .05, **P < .01, ***P < .001, ****P < .0001.

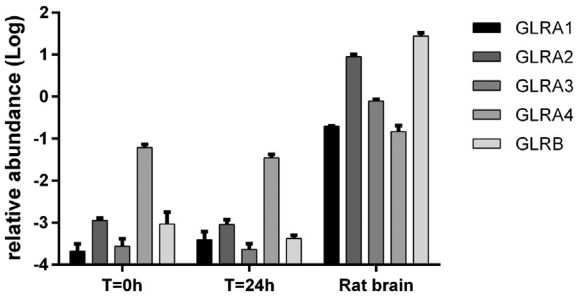

Fig. 7 – Glycine receptor subunit expression in RBL-2H3 cells and rat brain tissue. Data is represented as relative abundance directly from RBL culture (T = 0), after 24H of culture (T = 24) or from isolated rat brain tissue. Values are means ± SD from three pooled experiments (RBL cells). GLRA1 = Glycine receptor subunit Alpha 1, GLRA2 = Glycine receptor subunit Alpha 2, GLRA3 = Glycine receptor subunit Alpha 3, GLRA4 = Glycine receptor subunit Alpha 4, GLRB = Glycine receptor subunit Beta.

mucosal barrier function [26-31]. The present study shows that oral intake of the amino acid glycine inhibits allergic sensitization by a whey protein, resulting in a reduction of allergic responsiveness as confirmed by lower serum levels of whey specific IgE, a reduced acute skin reaction after i.d. whey-injection and a limited release of mMCP-1 following an oral whey-challenge.

Food allergy is characterized by an adverse immunologic reaction to proteins in food [32]. In the case of cow's milk allergy, the major allergens can be found in the casein and whey fraction with respectively αS1-casein, α-lactalbumin and β-lactoglobulin as the most important allergens [33]. In the current study, we investigated the effect of glycine on the whey-induced allergic response as a model for cow's milk allergy. Protective efficacy of glycine might already start during the allergic sensitization phase by counteracting the increased permeability and oxidative stress of the epithelial barrier induced by the adjuvant cholera toxin [31,34,35]. Glycine may further attenuate the anaphylactic effector

phase of the allergic response by inhibiting the inflammatory response following antigenic triggering. Glycine inhibits inflammatory responses and modulates cytokine production by various innate cells, including monocytes, intestinal- and alveolar- macrophages [26,28,30,36,37]. The best studied glycine signaling pathway involves the glycine receptor (GlyR). In the mammalian central nervous system, glycine mediates synaptic inhibition through activation of GlyR [38,39].

In the past years, accumulating evidence shows that glycine induces immunomodulatory effects via the GlyRs present on different immune cells, such as monocytes, macrophages and neutrophils, [40]. It should be noted, however, that glycine may utilize also GlyR independent pathways to protect cellular function, indicating that the presence of GlyR subunits is not a prerequisite for glycine's anti-inflammatory effect [41]. Glycine-induced modulation of cytokine production by mast cells, basophils, macrophages and dendritic cells (DCs) could be responsible for the detected decrease in whey-sensitization.

Cholera toxin induces intestinal CD103+ DCs to up-regulate OX40L and induce T helper type 2 (Th2) skewing from responder T cells [42]. An effect of glycine on cholera toxin-induced DC modulation could be another possible route by which glycine decreases whey-sensitizing effectiveness. Furthermore, our data indicates that glycine treatment attenuates the production of pro-inflammatory and Th2 skewing cytokines such as IL-13, TNFα and IL-4 respectively, in the basophilic cell line RBL-2H3. Basophils and mast cells express many similar receptors and perform overlapping functions in allergic inflammation [43]. Indeed, in mouse models basophils have been indicated to play an important role in the development of Th2-type immunity by acting as antigen presenting cell whilst producing Th2-type cytokines (IL-4 and IL-13) [44]. Therefore, the effects of glycine on the cytokine response following IgE-mediated crosslinking on RBL-cells may very well be comparable to effects on the mast cell.

In the IgE-mediated, classical hypersensitivity reaction (elicitation), food specific IgE antibodies bind to high affinity IgE receptors (FcεRI) on mast cells and basophils. At re-exposure the cell-bound IgE binds the allergen resulting in cross-linking of the IgE, cell activation and release of preformed and newly synthetized mediators of hypersensitivity.

The effect of glycine on crosslinking-induced degranulation (acute response) and cytokine production (newly synthesized) by RBL cells showed that glycine did not affect degranulation. Cytokine release of IL-4, IL13 and TNFα strongly increased after cross-linking induced RBL-activation. Glycine inhibited this cytokine increase, most likely via the GlyR, which was shown to be expressed by RBL cells. This inhibition of IL-4 and IL-13 is of particular relevance, since both cytokines are pivotal in the reactive allergic cascade [45].

IL-4 and IL-13 can be secreted by CD4+ Th2 cells, activated basophils and mast cells. They induce Th2 cell differentiation, B cell Ig class switch to IgE [45,46] and initiate mast cell mediated mucosal barrier disruption [47]. Although there is functional overlap between these cytokines, there is substantial evidence that IL-13 functions as a primary disease-inducing effector cytokine, whereas IL-4 amplifies type 2 immunity by facilitating the expansion of Th2 cell populations in the secondary lymphoid organs and by inducing mast cell amplification in food allergy [48,49]. Therefore, the glycine-induced inhibition of IL-4 and IL-13 production by basophils, as shown in the present study with RBL cells, might be followed by a reduced Th2 cell activation and improved intestinal integrity resulting in a decreased allergy development.

Immunohistochemical analysis of the jejunum showed a reduced frequency of mMCP-1 positive (mast) cells in whey-sensitized mice compared to the sham-sensitized mice. In contrast, significantly more mMCP-1 positive cells could be detected in the jejunum of whey-sensitized mice after supplementation with a high dose of glycine. After mast cell degranulation, the amount of mMCP-1 positive cells decreased in the small intestine as suggested earlier [21], which is in agreement with the increase in serum mMCP-1 levels following the oral challenge.

Glycine supplementation during sensitization caused a significantly lower mMCP-1 release following oral challenge. This can be explained by reduced levels of circulating whey-specific IgG1 and IgE antibodies, which are capable of inducing mast cell degranulation [50]. Another explanation might be that the jejunum of sham-sensitized animals shows similar numbers of mMCP-1 mast cells as glycine treated whey-sensitized mice, suggesting that glycine has a long-lasting protective effect of glycine on mast cell degranulation, potentially mediated via inhibition of IL-4 and IL-13 release during sensitization [48]. Moreover, in the gastrointestinal tract, mast cells regulate a plethora of events that are important in maintaining intestinal homeostasis, such as regulation of vascular and epithelial integrity, bacterial defense, innate and adaptive immunity, fibrosis, tissue repair and chemotaxis [51]. Therefore, glycine attenuation of dysregulated or uncontrolled mast cell activation may prevent loss of intestinal homeostasis which promotes inflammation and can lead to gastrointestinal diseases, such as food allergy [51,52].

The current study has a limitation. Whey was used to sensitize the mice thus we cannot comment on whether or not glycine is also protective for other milk-derived allergens.

In conclusion, to the best of our knowledge this is the first publication that demonstrates that the free CEAA glycine protects against (whey-induced) allergy development. These findings support the hypothesis that supplementation of glycine during the sensitization phase is capable of significantly reducing the acute and systemic allergic response. Glycine reduced whey-specific IgE levels and inhibited the anaphylactic effector phase of the allergic response upon antigen challenge. Although the exact working mechanism of glycine in this respect is still unknown, our data suggest that the effects of glycine on effector cells, such as basophils and mast cells, may play a key role. Both basophils and mast cells play a pivotal role in allergic inflammation and glycine-mediated inhibition of release of Th2 skewing factors or glycine-induced mast cell stabilization may contribute to the observed attenuation of the allergic response [53]. Mechanistic investigation is required for an in-depth understanding of the actions of glycine in allergic responses to food allergens. Therefore, future studies should aim to delineate the effects of glycine on mast cell function in the sensitization and effector phase of the allergic response. Overall, our findings may open new avenues for the management of allergy through the supplementation of free glycine.

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