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Extensively hydrolyzed casein formula alone or with L. rhamnosus GG reduces β-lactoglobulin sensitization in mice

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Running title: Hydrolyzed casein formula with LGG against CMA

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Abstract page

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Extensively hydrolyzed casein formula alone or with L.rhamnosus GG reduces β-lactoglobulin sensitization in mice

Pediatr Allergy Immunol

Background. Extensively hydrolyzed casein formula (EHCF) has been proposed for the prevention and is commonly used for the treatment of cow's milk allergy (CMA). The addition of the probiotic *Lactobacillus rhamnosus* GG (LGG) to EHCF may induces faster acquisition of tolerance to cow's milk. The mechanisms underlying this effect are largely unexplored. We investigated the effects of EHCF alone or in combination with LGG on β-lactoglobulin (BLG) sensitization in mice.

Methods. Three-weeks old C3H/HeOuJ mice were sensitized by oral administration of BLG using cholera toxin as adjuvant at weekly intervals for 5 weeks (sensitization period). Two experimental phases were conducted: 1. EHCF or EHCF+LGG given daily, starting 2 weeks before the sensitization period and then given daily for 5 weeks, and 2. EHCF or EHCF+LGG given daily for 4 weeks, starting 1 week after the sensitization period. Diet free of cow's milk protein was used as control. Acute allergic skin response, anaphylactic symptom score, body temperature, intestinal permeability, anti-BLG serum IgE and interleukin (IL)-4, IL-5, IL-10, IL-13, IFN-γ mRNA expression were analysed. Peptide fractions of EHCF were characterized by reversed phase (RP)-HPLC, MALDI-TOF mass spectrometry and nano-HPLC ESI MS/MS.

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Results. EHCF administration before or after BLG-induced sensitization significantly reduced acute allergic skin reaction, anaphylactic symptom score, body temperature decrease, intestinal permeability increase, IL-4, IL-5, IL-13 and anti-BLG IgE production. EHCF increased expression of IFN -γ and IL-10. Many of these effects were significantly enhanced by LGG supplementation. The peptide panels were similar between the two study formulas and contained sequences that could have immunoregolatory activities.

Conclusions. The data support dietary intervention with EHCF for CMA prevention and treatment through a favorable immunomodulatory action. The observed effects are significantly enhanced by LGG supplementation.

Keywords: **bovine casein, food allergy, hypoallergenic formula, intestinal permeability, probiotics**

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Introduction

Extensively hydrolyzed casein formula(EHCF) has been proposed for CMA prevention in at-risk infants [1] and it is commonly used as a first line option for CMA treatment[2,3]. The efficacy of extensively hydrolyzed formulas on food allergy prevention is still controversial [4]. Conversely, in a prospective study of infants with confirmed CMA, treatment with EHCF induced faster tolerance acquisition compared with other available formulas (hydrolyzed rice formula, soy formula and amino acid-based formula) [5]. This effect was potentiated by the addition of the probiotic *Lactobacillus rhamnosus* GG (LGG) [5-7]. Apart from being hypoallergenic, little is known regarding a possible immunomodulatory role of the casein hydrolysis-derived peptides against CMA, whether alone or in combination with LGG. Immunomodulatory effects of EHCF have been demonstrated in a rat model of type1 diabetes[8], suggesting possible similar effects against other immune-mediated conditions, such as food allergy. Murine models have been adopted to investigate the immune mechanisms elicited by different dietary interventions in CMA [9-12]. In this study, we investigated the mechanisms elicited by EHCF, either alone or in combination with LGG, against β-lactoglobulin(BLG) sensitization in mice. Peptide fractions within EHCF were also characterized.

Methods

Murine model experiments

Animals

Three-week-old female C3H/HeOuJ mice were used for all experiments(Charles River Laboratories, Calco, Lecco, Italy). Mice were housed in the animal facility under a 12L:12D lighting cycle, 20-24ºC ambient temperature range and 40%-70% relative humidity. The mice were acclimated to their environment for 1week before the experiments began. Each treatment group

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received an isocaloric diet during all experimental procedures (0.5kcal/gr of body weight/day). All procedures involving the animals were carried out in accordance with the Institutional Guidelines and complied with the Italian D.L. no.116 of January 27, 1992 of the Italian Ministry of Health and associated guidelines in the European Communities Council Directive of November 24, 1986 (86/609/ECC) and were approved by the Institutional Committee on the Ethics of Animal Experiments(CSV) of the University of Naples "Federico II" and by the Ministero della Salute (Protocol no. 2012-0024683).

Phase 1

The experimental design is reported in Figure 1Sa. Briefly, starting from two weeks prior to sensitization, mice were given three different experimental diets: solid diet free of cow's milk protein (Harlan Laboratories, Udine, Italy), EHCF (Nutramigen, Mead Johnson Nutrition, Evansville, IN, USA), or EHCF+LGG (Nutramigen LGG1,Mead Johnson Nutrition, Evansville, IN, USA). EHCF and EHCF+LGG formulas were prepared daily by dissolving the powder in distilled water, as indicated by the manufacturer, and were provided to the mice in sterile bottles. The daily amount of formula consumed by the mice was measured by subtracting the final bottle weight from the baseline weight. The composition of the three experimental diets is presented in Table1 (supplementary information). We used 4/5 mice per group in two independent experiments. After 14days, mice were sensitized orally using a blunt needle on day 0, 7, 14, 21, 28 with 20mg of β-lactoglobulin (BLG) (Sigma-Aldrich, Steinheim, Germany) in PBS (200µl) mixed with 10µg cholera toxin (CT) (Sigma-Aldrich, Steinheim, Germany) as adjuvant [13]. One week after the final sensitization, acute allergic skin response was assessed. On next day rectal temperature was measured, then mice were challenged by gavage with BLG (50mg in 200µl of PBS). After 1h, the anaphylaxis score was assessed and rectal temperature was measured again. After 24h , mice were sacrificed, blood samples were collected, and mesenteric lymph nodes (MLN)were aseptically excised and cryopreserved.

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Phase 2

The experimental design is reported in Figure 1Sb. Briefly, mice fed with a solid cow's milk protein-free diet were sensitized orally as described above. One week after the final sensitization, mice were given three different experimental diets: solid diet free of cow's milk protein, EHCF, or EHCF+LGG. We used 4/5 mice per group in two independent experiments. At the end of the 5-week treatment, mice were challenged and sacrificed as described above.

Acute allergic skin response, anaphylaxis symptom score and body temperature

Acute allergic skin response was evaluated according to a previously described procedure [14]. The ear swelling was calculated by correcting the allergen-induced ear thickness with the basal ear thickness. The delta ear swelling was expressed as μm.

Hypersensitivity symptoms were scored by an investigator blind to the study group assignment 1 hour after oral BLG challenge, as previously described [12].

Body temperature was measured by rodent thermometer(Bioseb, Valbonne, France).

Serum anti-BLG immunoglobulin E

Blood samples obtained from mice by intracardiac puncture were collected in serum separator tubes. The serum portion was separated by centrifugation at 10,000 x g for 5min at 20ºC. Serum samples were then aliquoted into Eppendorf tubes and stored at -20ºC until analysis. The level of anti-BLG serum IgE was detected by ELISA. Ninety-six-well plates (Sigma-Aldrich, Steinheim, Germany) were coated with 100μg/mL of BLG in 0.1mol/L Na-bicarbonate/carbonate coating buffer (pH 9.6). After overnight incubation at 4°C, plates were washed 3 times with 150μl of PBS plus 0.05% Tween-20 (PBS-T) and blocked with 100μl of 2% BSA in PBS-T for 2h at 37 °C. Subsequently, the plates were washed 3 times, and 100μl of serially diluted serum samples were added to the wells and incubated at 37°C for 90min. Plates were then washed 3 times and 100μl of Biotin Rat-anti-mouse IgE (BD Biosciences, Milano, Italy) were added to each well. The plates

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were incubated at 37°C for 2h and washed 5 times. Then, 100µl of horseradish peroxidase-conjugated anti-mouse antibodies were added to each well and the plates were again incubated at 37°C for another 60 min and washed 3 times. Then, 100µl of TMB (3, 3', 5, 5' tetramethyldiaminebenzidine) was added to each well and 15min were allowed for the development of colorimetric reactions. Absorbance was read at a wavelength of 450nm in a microplate reader.

Intestinal permeability in vivo

From each study group of both experimental phases, 4 animals were used to investigate intestinal permeability with 4,000 Da FITC-labeled dextran as previously described [15]. FITC-dextran concentration was determined by spectrophotometry (HTS-7000 Plus-plate-reader; Perkin Elmer, Wellesley, Massachusetts, USA).

Gene expression analysis by quantitative Real Time PCR

Total RNA was isolated from mesenteric lymph nodes (MLN) from mice by solubilization in Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA) and quantified using the Nanodrop 2000c spectrophotometer(Thermo Scientific, Waltham, MA, USA). For cDNA synthesis, 1µg total RNA was transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Quantitative Real Time PCR (qRT-PCR) analysis of IL-4, IL-5, IL-13, IFN-γ and IL-10 was performed using TaqMan specific probes (Applied Biosystems, Grand Island, NY, USA) (Table2, supplementary information). Samples were run in triplicate at 95°C for 30sec followed by 40 cycles of 95°C for 10s and 60°C for 30s, using a Light Cycler 7900HT (Applied Biosystems). Data analysis was performed by comparative threshold cycle (CT) method. The quantitative gene expression was calculated with the comparative CT method and normalized against the CT of Glucuronidase (GUS) messenger as a reference gene.

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Characterization of peptides from study formula

Peptide extraction

The EHCF powders (1.0g)were defatted with hexane (10mL, twice), air-dried and finally reconstituted in distilled water. Peptides were purified by solid-phase extraction using a Sep-pak® pre-packed C18 reversed-phase cartridge (Waters, Milford, MA, USA), according to the manufacturer's instruction. Peptides were eluted with 3mL of 70% acetonitrile/0.1%TFA, concentrated with a speed-vac and finally lyophilized.

Reversed-phase (RP)-HPLC

Purified peptides were re-dissolved in 1.5mL of 0.1%TFA and fractionated by HPLC using a modular system HP 1100 (Agilent, Palo Alto, CA, USA)equipped with a 2.0 mm i.d. x 250 mm, C18, 5 µm reversed-phase column (Phenomenex, Torrance, CA, USA). Solvent A was 0.1% TFA in water, and solvent B was 0.1% TFA in acetonitrile. Separations were performed with a 5–65% gradient of solvent B over 60 min, after a 5 min isocratic elution at 5% B at a constant flow rate of 0.2 mL/min. The column effluents were monitored by UV detection at wavelengths λ = 214 and 280 nm. Approximately 40 µg of peptides were injected for each run. Post-run elaboration was carried out using the HPLC off-line software purchased with the instrument(Agilent, Palo Alto, CA, USA).

MALDI-TOF mass spectrometry

The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) analyses were performed on a PerSeptiveBiosystems Voyager DE-PRO instrument (Foster City, CA, USA), using α-cyano-4-hydroxycynnamic acid matrix (10mg/mL aqueous 50% acetonitrile/0.1%TFA) as the matrix, as previously described [16].The spectra were analyzed with the Data Explorer software (version 4.0) provided with the instrument (Applied Biosystems, Foster City, CA, USA).

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Nano-HPLC ESI MS/MS

Nanoflow HPLC separations were carried out with an Ultimate 3000 nano-HPLC system (Dionex, Sunnydale, CA, USA) equipped with a FAMOS Autosampler (Dionex). Peptide extracts were re-dissolved in 0.1% (v/v) TFA at the approximate concentration of 0.5µg/µL and 2.5µL was loaded onto a C_{18} trapping cartridge (LC Packings, Dionex, USA) before separation on a capillary column (PepMap, C18, 5µm, 300 A, 75 i.d.µm x 15cm, LC Packings, Dionex). Peptides applying a linear gradient from 2% to 45% of solution B over 90 min, following 10 min of isocratic elution at 2% of solution B, at a constant flow rate of 300nL/min. Solvent A was H_2O containing 0.1% formic acid, and solvent B was 0.08% formic acid in 80% acetonitrile (v/v).

Effluents were analyzed on-line using an electrospray hybrid quadrupole/time-of-flight (ESI-Q-TOF) mass spectrometer (Q-Star Pulsar, Applied BioSystems, Foster City, CA, USA) and peptides were identified according to a previously described procedure [16]. Samples were analyzed in triplicate, and peptides were included in the identification list if they occurred in all replicates.

Statistical analysis

The Kolmogorov-Smirnov test was used to determine whether continuous variables were normally distributed. All tests of significance were two-sided. A *p* value of 0.05 was considered significant. All analyses were conducted by a statistician blinded to mice group assignment using SPSS release 20.0.0 (IBM SPSS Statistics) and GraphPad Prism 5.0.

Results

Murine model experiments

Phase 1

BLG-sensitized mice showed a significantly higher acute allergic skin response, anaphylactic symptom score and body temperature decrease compared to control animals. All of these effects were significantly reduced by pre-treatment with EHCF. The presence of LGG significantly

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increased the effect of EHCF on allergic skin response. Similarly, BLG-sensitized animals showed a significant increase in serum BLG-specific IgE and intestinal permeability compared to control animals (Figure 1, A-E). These effects were significantly inhibited by EHCF. In addition, EHCF pre-treatment stimulated a significant increase in IL-10 and IFN-γ expression correlated with reduction in IL-4, IL-5 and IL-13 expression (Figure 2, A-E). The presence of LGG significantly potentiated EHCF activities.

Phase 2

EHCF treatment significantly reduced the acute allergic skin response, anaphylactic symptom score and body temperature decrease compared to sensitized animals. The presence of LGG induced more pronounced effects on all these variables. Dietary treatment with EHCF induced a significant reduction in anti-BLG IgE serum levels and intestinal permeability increase (Figure 3, A-E). EHCF stimulated a significant reduction in IL-4, IL-5 and IL-13 expression and an increase in IFN-γ and IL-10 expression (Figure 4, A-E). The presence of LGG significantly enhanced all of these effects.

Characterization of peptides from study formulas

The RP-HPLC-UV comparison demonstrated similar peptide profiles for EHCF and EHCF+LGG (Figure 2Sa). This observation was confirmed by MALDI-TOF MS and nano-HPLC ESI-Q-TOF MS/MS analyses (not shown). Figure 2Sb shows the MALDI-TOF MS-based assignment of EHCF peptides. No peptides longer that 12 residues were detected.. Including a previous chromatographic step, the nano-HPLC ESI-Q-TOF MS/MS analysis validated the MALDI-TOF MS results and resulted in a more detail characterization including peptide sequences. A comprehensive list of the peptides identified in the two formulas is reported in Table3 (supplementary information). The highest number of peptides (30 out 42, 71.5%) arose from β-casein, followed by αs1-casein (8 out of 42, 19.0%) and κ-casein (4 out of 42, 9.5%) in line with previous observations [17]. No fragments of whey proteins were detected in the current study.

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Discussion

The results of this study provide evidence that casein hydrolysis not only deletes allergenic epitopes but produces immunoregulatory peptides that counteract allergic response. The results of acute allergic skin response, anaphylactic symptoms, body temperature and intestinal permeability demonstrate that EHCF inhibits oral BLG sensitization, at least partly through a modulation of Th1/Th2 cytokine expression. We observed an enhancement in IL-10 expression, a major immune-regulatory cytokine that maintains mucosal homeostasis and limit excessive immune responses against dietary and bacterial antigens present in the intestinal lumen [18]. These findings are in keeping with the results of previous clinical studies demonstrating a preventive effect against CMA elicited by the same formula [19].

Similar effects were obtained in mice treated with EHCF after sensitization with BLG. Previous studies have demonstrated that casein hydrolysis-derived peptides can inhibit allergen uptake across the intestinal mucosa by inhibiting either the production of specific IgE or the antigen binding to IgE on mast cells [20-22]. Moreover, these peptides stimulate production of IL-10 by human lymphocytes [23] and improve gut barrier function both *in vivo* and *in vitro* [8,24]. The largest number of EHCF peptides (71.5%) arose from β-casein. Unlike in other investigations on casein-based formulas, we did not detect any fragments of whey proteins originating from contaminating BLG [16,25]. Several EHCF peptides were detected that derived from two specific β-casein domains, the C-terminal and β–casomorphin domains, with a previously demonstrated immunoregulatory role [26,27]. Interestingly, many peptides shared the common amino acid motif Pro-Phe-Pro (PFP) that occurs at the positions 61-63, 110-112 and 204-206 of β-casein. The PFP motif, along with its peptide precursors, has been previously indicated as the determinant of the immunoregulatory activity of β-casein regions [28]. Finally, β-casomorphin 7, detected in EHCF, stimulates intestinal mucin production [29]. Mucus lubricates the intestinal surface and functions as a dynamic defensive barrier limiting the access of luminal molecules into the mucosa [30].

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The peptide fractions of EHCF or EHCF+LGG were similar, demonstrating that LGG is unable to elicit any proteolytic action on immunoregulatory peptides. In addition, in both experimental phases, many EHCF effects were enhanced by the addition of LGG. An immunoregulatory action by LGG, skewing towards a Th1 response, has been already demonstrated in a murine model of CMA [31]. LGG is known to modulate immune functions via various pathways, including those involving enterocytes, monocytes, mast cells, dendritic cells, and regulatory T cells [32]. This probiotic reduces pro-inflammatory cytokine-induced alteration of intestinal permeability [32]. These data further support clinical evidence showing a more potent activity elicited by EHCF containing LGG on gut inflammation [33] and on oral tolerance acquisition in children affected by CMA [5-7].

In conclusion, the results of this study demonstrate that, in a mouse model, EHCF exerts multiple immunoregulatory activities that efficiently counteract several mechanisms involved in CMA pathogenesis and that the presence of LGG increases the magnitude of many of these effects. However, is important to underline that it is not possible to make general statements on the immunomodulatory effects of hydrolysates alone or in combination with probiotics. Every hydrolysate is unique because materials and production methods differ among available hypoallergenic formulas [34], and immunoregulatory effects elicited by probiotics are strain-specific [35]. This variability underscores the importance of specific peptides and probiotic strains present in hypoallergenic formulas.

Conflict of Interest:

The work was supported in part by the Italian Ministry of Health Grant PE-2011-02348447 and by an unrestricted grant from Mead Johnson Nutrition (Evansville, Indiana, USA) devoted to the Department of Translational Medical Science of the University of Naples “Federico II”. However, the Italian Ministry of Health and Mead Johnson Nutrition had no influence on: (1) the study design, (2) the collection, analysis, and interpretation of data; (3) the writing of the manuscript; and

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(4) the decision to submit the manuscript for publication. The authors have no other conflicts of interest that are directly relevant to the content of this paper, which remains their sole responsibility.

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Figure 1.

Phase 1 experiments. *In vivo* data. **(A)** acute allergic ear swelling response, **(B)** anaphylactic symptom score, **(C)** body temperature. *In vitro* data. **(D)** intestinal permeability to plasma DX-4000 FITC and **(E)** anti**-**BLG-serum IgE. Date were analyzed with independent sample t-test. *p<0.05.

Data represent pooled values from two separate experiments.

Figure 2.

Phase 1 experiments. Cytokines expression in MNL **(F)** IL-4 relative expression, **(G)** IL-5 relative expression**, (H)** IL-13 relative expression**, (I)** IL-10 relative expression and **(L)** IFN-γ relative expression. Date were analyzed with independent sample t-test. *p<0.05.

Data represent pooled values from two separate experiments.

Figure 3.

Phase 2 experiments. *In vivo* data. **(A)** acute allergic ear swelling response, **(B)** anaphylactic symptom score, **(C)** body temperature. *In vitro* data. **(D)** intestinal permeability to plasma DX-4000 FITC, **(E)** anti**-**BLG-serum IgE, Date were analyzed with independent sample t-test. *p<0.05.

Data represent pooled values from two separate experiments.

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Figure 4.

Phase 2 experiments. Cytokines expression in MNL **(A)** IL-4 relative expression, **(B)** IL-5 relative expression**, (C)** IL-13 relative expression**, (D)** IL-10 relative expression and **(E)** IFN-γ relative expression. Date were analyzed with independent sample t-test. *p<0.05.

Data represent pooled values from two separate experiments.

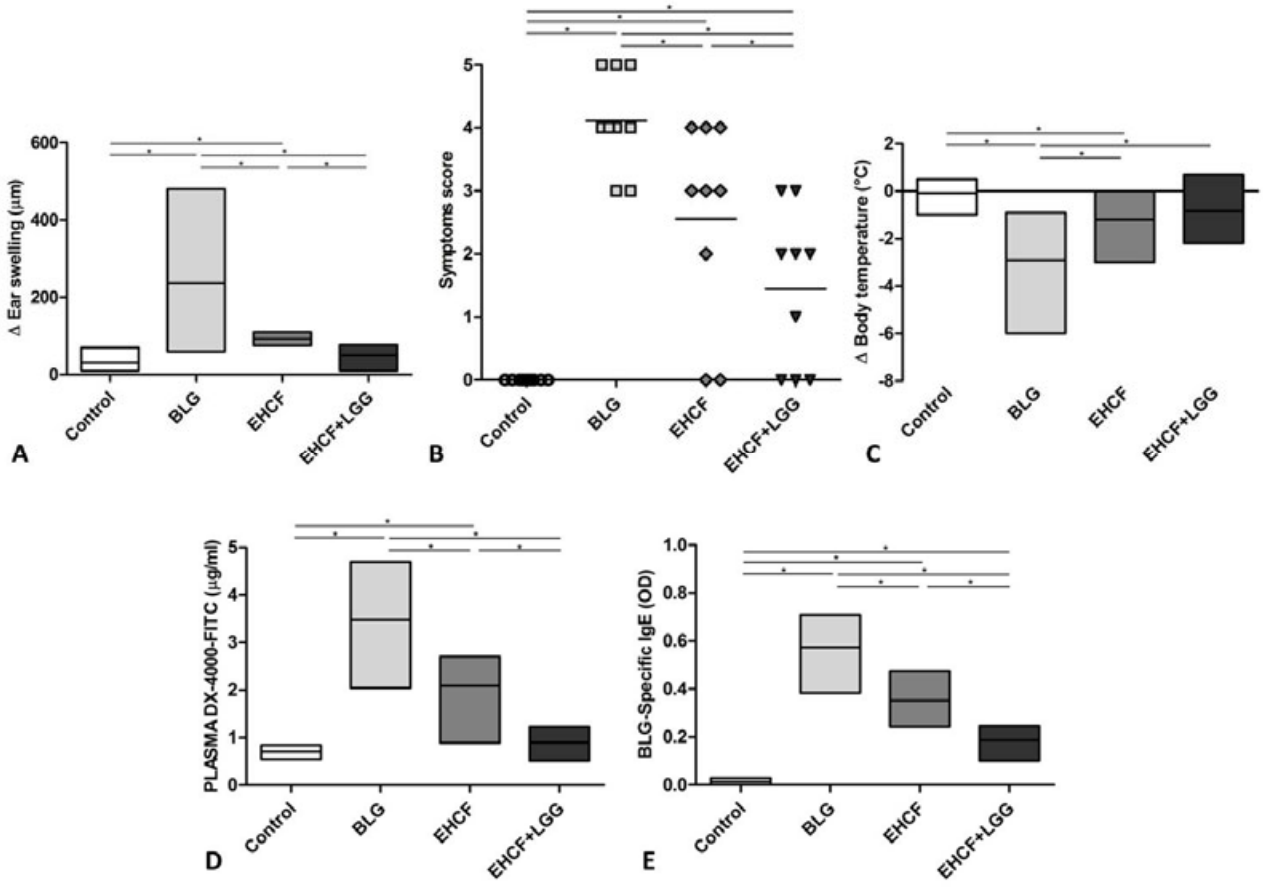

Figure 1. *In vivo* and *in vitro* parameters in Phase 1 experiments.

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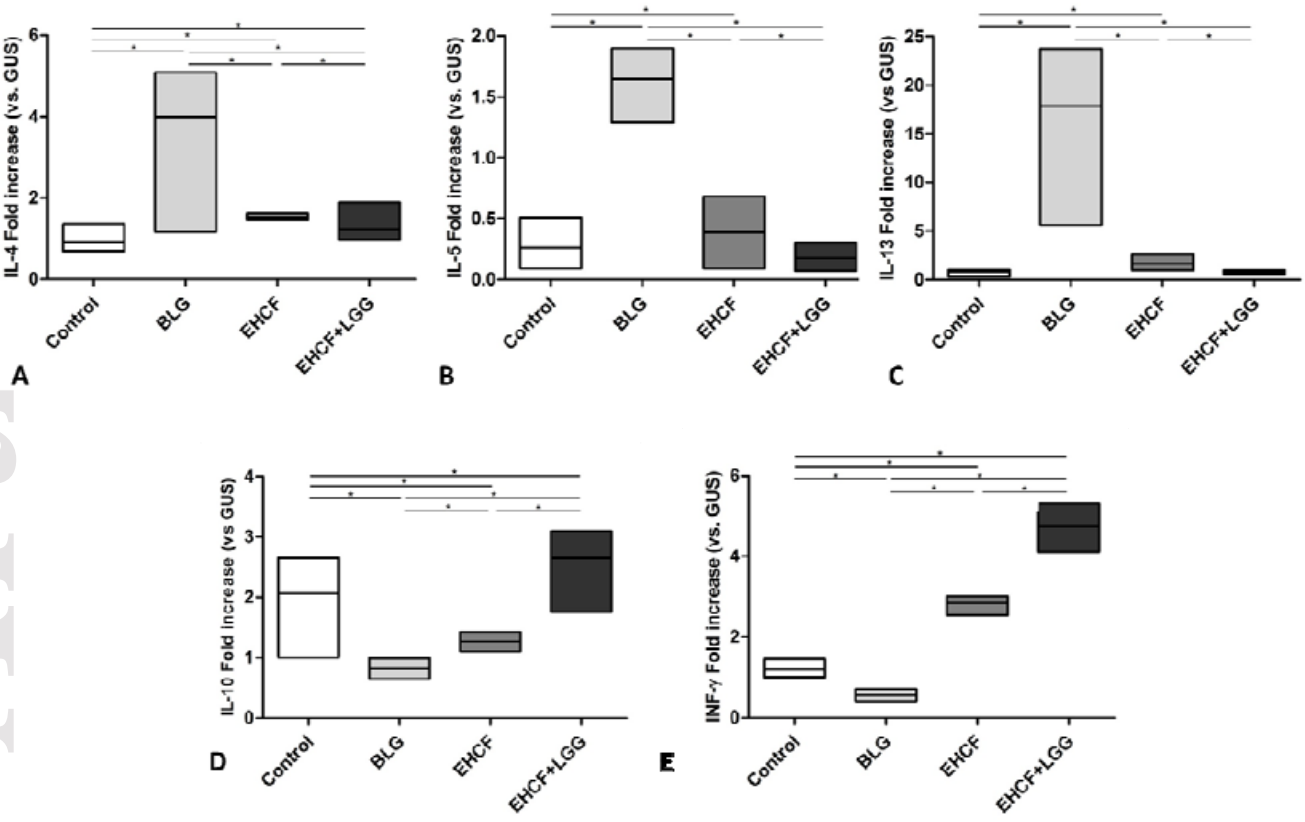

Figure 2. Cytokines expression in MNL in Phase 1 experiments.

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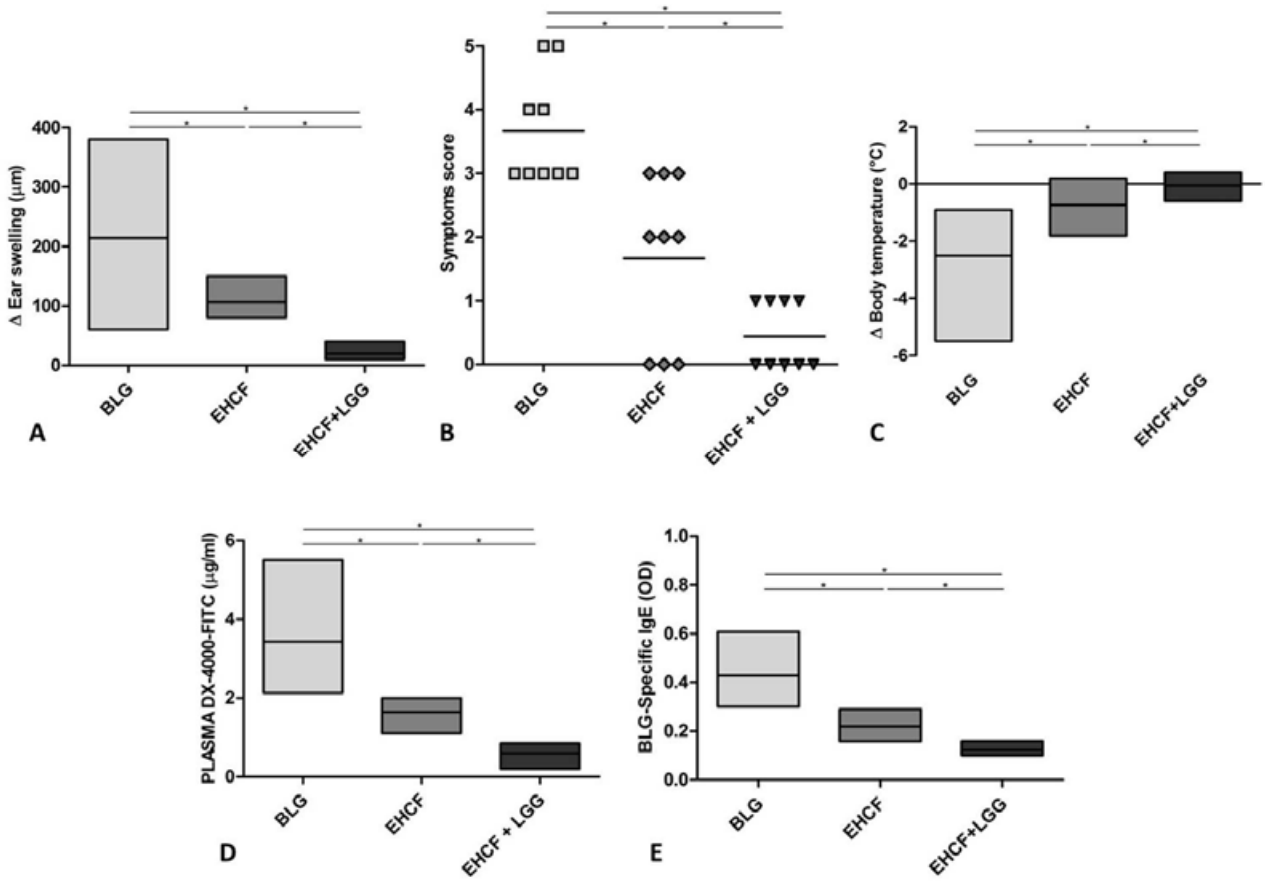

Figure 3. In vivo and in vitro parameters in Phase 2 experiments.

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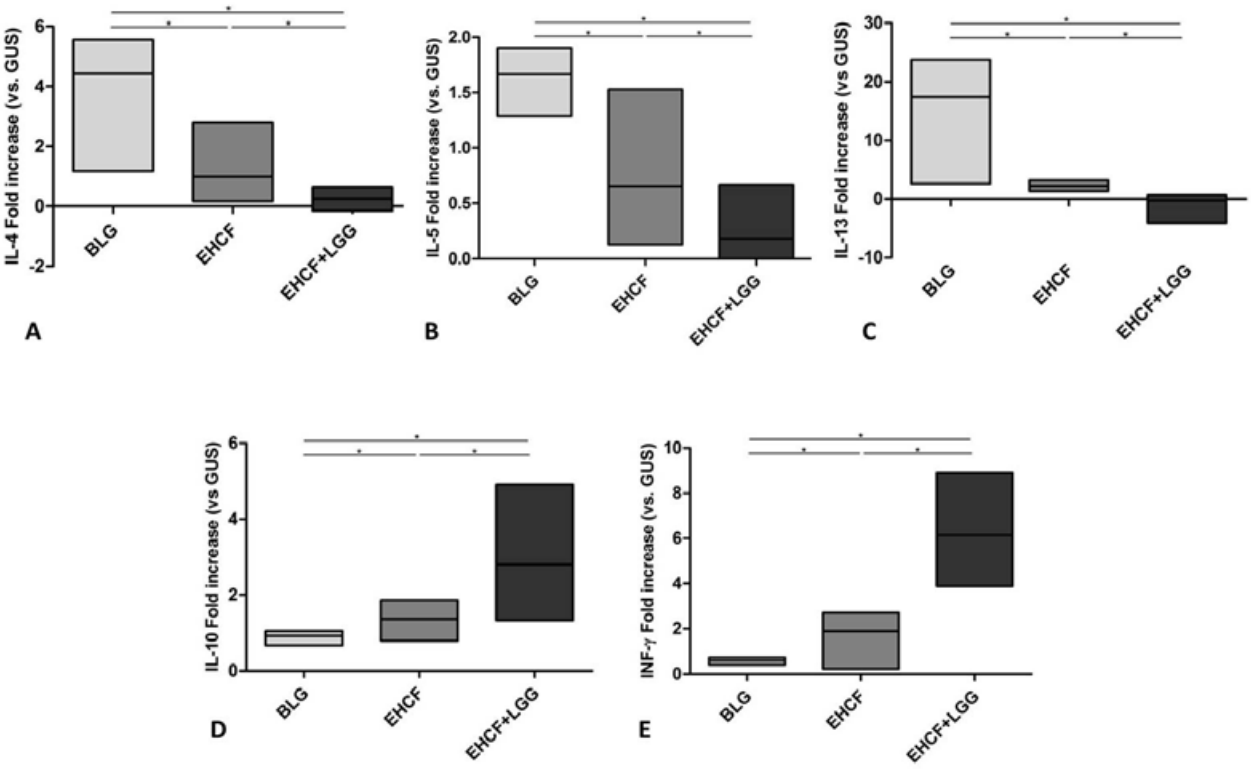

Figure 4. Cytokines expression in MNL in Phase 2 experiments.

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