The protective farm effect against allergies in a holoBLG based FSMP (food for specific medical purposes)-lozenge confers immune resilience in BALB/c mice

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Abstract

Background: We previously proposed the whey protein beta-lactoglobulin (BLG) loaded with iron-siderophore complexes as the active principle in the farm protective effect against allergies. A lozenge as food for specific medical purposes (FSMP) was formulated to assess its therapeutical efficacy in BALB/c mice and in-vitro experiments. Methods: Binding of iron-catechin into BLG was confirmed by spectroscopy and docking calculations. Serum IgE binding of children allergic to milk, or tolerating milk, was assessed to loaded (holo-) versus empty (apo-) BLG and for human mast cell degranulation. BLG and Bet v 1 double-sensitized mice were orally treated with the lozenge or placebo, and immunologically analysed after systemic allergen challenge. Human PBMCs of pollen allergic subjects were flow cytometrically assessed after stimulation with holoBLG in conjugation with catechin-iron complexes as ligands in a dietary supplement or with the apoBLG. Results: One major IgE- and T cell epitope were masked by catechin-iron complexes, which impaired IgE binding of milk allergic children and degranulation of mast cells. In mice, only supplementation with the lozenge reduced clinical reactivity to BLG and Bet v 1, promoted Tregs, and suppressed antigen presentation. In allergic subjects, stimulation of PBMCs with holoBLG led to a significant increase of intracellular iron in circulating CD14+ cells with significantly lower expression of HLADR and CD86 compared to their stimulation with apoBLG. Conclusion: The FSMP lozenge targeted antigen presenting cells and dampened immune activation in human immune cells and allergic mice in an antigen nonspecific manner, thereby conferring immune resilience against allergic symptoms.

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Background: We previously proposed the whey protein beta-lactoglobulin (BLG) loaded with iron-siderophore complexes as the active principle in the farm protective effect against allergies. A lozenge as food for specific medical purposes (FSMP) was formulated to assess its therapeutical efficacy in BALB/c mice and *in vitro* experiments.

Methods: Binding of iron-catechin into BLG was confirmed by spectroscopy and docking calculations. Serum IgE binding of children allergic to milk, or tolerating milk, was assessed to loaded (holo-) versus empty (apo-) BLG and for human mast cell degranulation. BLG and Bet v 1 double-sensitized mice were orally treated with the lozenge or placebo, and immunologically analysed after systemic allergen challenge. Human PBMCs of pollen allergic subjects were flow cytometrically assessed after stimulation with holoBLG in conjugation with catechin-iron complexes as ligands in a dietary supplement or with the apoBLG.

Results: One major IgE- and T cell epitope were masked by catechin-iron complexes, which impaired IgE binding of milk allergic children and degranulation of mast cells. In mice, only supplementation with the lozenge reduced clinical reactivity to BLG and Bet v 1, promoted Tregs, and suppressed antigen presentation. In allergic subjects, stimulation of PBMCs with holoBLG led to a significant increase of intracellular iron in circulating CD14+ cells with significantly lower expression of HLADR and CD86 compared to their stimulation with apoBLG.

Conclusion: The FSMP lozenge targeted antigen presenting cells and dampened immune activation in human immune cells and allergic mice in an antigen nonspecific manner, thereby conferring immune resilience against allergic symptoms.

Introduction

The prevalence of allergies is rising in the westernized world and is associated with urban life-style [1].

This is in contrast to children growing up in a farm environment and drinking farm milk who suffer less frequently from allergies and asthma [2]. This effect is referred to as the "protective farm effect". Exposure to cow sheds [3] and raw milk consumption are two independent factors contributing to the protective farm effect. In raw milk, in particular the whey protein content has been linked to protection against allergies [4]. Notably, beta-lactoglobulin (BLG) is a major whey compound, with a great similarity to the human lipocalin-2 (LCN-2) with innate immunoregulatory potency. In allergics LCN-2 levels are reduced and can be corrected by allergen immunotherapy [5]. Hence, interference with the regulatory lipocalin system with an exogenous lipocalin could be beneficial in allergy. We also detected BLG as a major compound in cow stable dust and aerosolized around cattle farms [6].

In recent studies, we demonstrated that BLG prevented allergic sensitization, when fulfilling its innate function shuttling ligands to immune cells: when BLG carried micronutrients within its protein pocket - such as iron-siderophore complexes [7], or retinoic acid [8-10] - it acted as a tolerogen preventing antigen presentation and activating anti-inflammatory pathways [11]. Importantly, transport of these micronutrient resulted in immune resilience in an antigen-unspecific manner and prevented the onset of allergy also to other allergens [12].

Here, we went one step further seeking clinical translation for the new principle in the protective farm effect. Considering that farm dust and raw milk exposure need thorough time for mucosal absorption, we developed a dietary supplement as a lozenge from compounds compatible with EU regulations for food safety [13]. Besides zinc [6] and retinoic acid, the flavonoids catechin and epicatechin from cocoa [14,15] was chosen for complexing iron and combined with whey protein BLG derived from organic farms in EU and Switzerland.

Here, we demonstrate that the combination of the dietary components in the lozenge activated the anti-inflammatory aryl hydrogen receptor pathway (AhR) similar to the single purified components. IgE of children allergic to milk bound less to the ligand-filled, holo-form of BLG and also antigen-specific degranulation of human mast cells was significantly diminished. The impact of the lozenge was further tested in double-sensitized mice resulting in decreased antigen presentation, promotion of regulatory T cells and reduced proliferation that was accompanied by reduced allergic symptoms to both allergens. The results identify the new lozenge as a successful translation of the protective farm effect transporting micronutritional ligands to immune cells that initiate tolerogenic pathways in mice. The beneficial innate and allergen-nonspecific effect of the lozenge has been clinically confirmed in house dust mite allergic rhinoconjunctivitis patients [16].

Materials and Methods

Ethical approval

Sera of milk allergic patients (10 patients positive and 10 patients negative to oral cow's milk allergen challenge) were retrospectively collected in accordance with the Helsinki Declaration of 1975 and under approval of the ethical committee of the Bambino Gesù Pediatric Hospital, Rome; individual informed consent from all donors was collected by Dr. Alessandro Fiocchi, Children's hospital Bambino Gesú, Rome, Italy. Open food challenges as described in the AAAAI/Europrevall protocol[47]were performed to confirm milk allergy. Children who had no symptoms with the cumulative administration of the entire milk dose corresponding to 144 mL were considered negative.

Blood cells from sixteen birch and/or grass pollen allergic volunteers (ethical approval number 1370/2018) were stimulated, as described below, with apoBLG or holoBLG and analysed by flow cytometry. All subjects provided their written informed consent under institutional review board of the Medical University approved protocols before participation. This study was approved by the institutional ethics committee of Vienna and conducted in accordance with the Helsinki Declaration of 1975.

Animals

Female BALB/c mice, 5–7 weeks of age, were obtained from Charles River Laboratories and kept under conventional housing and treated according to European Union rules of animal care, with the permission of the Austrian Ministry of Sciences (BMWF-66.009/0133-WF/V/3b/2016).

Supplement/Placebo:

The lozenge ImmunoBON® was produced and provided by Biomedical International R+D GmbH, Austria containing active ingredients (5mg holoBLG using iron-catechin$_3$ complexes as ligand; molar ratio BLG: iron: catechine= 1:1:3, 10µg retinol and 176µg zinc). The placebo lozenge formulated without active ingredients, consisted of sorbitol, mountain herbs flavour and karo-coffee providing a similar appearance and taste like the verum lozenge.

Allergic therapeutic model.

Sample size for the mouse experiments were based on the literature. No randomization was performed. Female BALB/c mice, 5–7 weeks of age were split in 3 groups: (n=8 for lozenge, n=8 for placebo, n=6 for naïve). Animals were immunized three times with 5µg BLG and 5µg Bet v 1 in conjunction with Al(OH)3 in biweekly intervals, thereafter mice received oral gavages of aliquots of crushed lozenges containing active ingredients (5mg holoBLG using iron-catechin$_3$ complexes as ligand; molar ratio BLG: iron: catechin= 1:1:3, 10µg retinol and 176µg zinc) or not (placebo-group), 6 times on 3 consecutive days in weekly intervals. Subsequently, all mice were challenged intraperitoneally with 50µg BLG, followed one week later by 50µg Bet v 1.

Body temperature and movements were monitored for 20 minutes after i.p. challenge using an Imaging system [17]. The allergic symptoms of challenged animals were scored as previously described [18]; 0 points for no symptoms; 1 for scratching and rubbing around the nose and head, 2 for puffiness around the eyes and mouth, diarrhea, pilar erection, reduced activity, and/or

decreased activity with increased respiratory rate; 3 wheezing, labored respiration, cyanosis around the mouth and the tail and 4 for no activity after prodding, or tremor and convulsion. After euthanasia with CO_2, blood was collected by cardiac puncture and sera were stored at −80°C until further processing. Spleens were collected. Results of two separate, independent experiments were compared.

Measurement of antigen-specific antibodies in mouse serum by ELISA

BLG/Bet v 1 specific IgG1 and IgE levels were measured by ELISA. Briefly, BLG or Bet v 1 (1µg/well) serial dilutions of mouse IgG1 and IgE standards (highest concentration for IgG1, 1000 ng/ml, for IgE standard 100pg/ml) were coated, blocked with 1% BSA in PBS, and incubated with diluted sera (1: 100 for IgG1 and 1:15 for IgE) overnight at 4°C. Specific antibodies were detected with monoclonal rat anti-mouse antibodies IgG1 (clone A85–1), or IgE (clone R35-72) followed by polyclonal peroxidase-labeled goat anti-rat IgG (GE Healthcare). Tetramethylbenzidine (eBioscience) was used as substrate and 1.8 M sulfuric acid was used as stop solution followed by optical density measurement at 450 nm.

Spectral analysis

For spectral analysis, deionized water was used as buffer to minimize iron-contamination from the air. The pH was kept constant at pH 7 by addition of NaOH. Optical density was measured at a constant concentration of 100µM quercetin or 300µM catechin with incremental increase of iron. All measurements were repeated at least three times with similar results.

Measurement of BLG-specific antibodies of milk-sensitized children by ELISA

5µg/ml apoBLG, or holoBLG diluted in 0.89% NaCl were coated on 96 well plates (100 µl/well) overnight at 4 °C. After 2 h blocking at room temperature with 200 µl/well 0.89% NaCl containing + 0.05% Tween 20 and 0.05% albumin (20%, 200g/L Biotest, CSL Behring), wells were incubated with 100 µl of human serum diluted 1:10 in 0.89% NaCl/0.05% Tween-20 overnight at 4 °C. Detection was performed using horseradish peroxidase-conjugated goat anti-human IgE antibody (Invitrogen A18793) diluted at 1:4000 in 0.89% NaCl/0.05% Tween-20, using tetramethylbenzidine (eBioscience) as a substrate and 1.8 M sulfuric acid to stop color development. The optical density was measured at 405 nm using an Infinite M200Pro microplate reader (Tecan, Austria). Between the steps rigorous washing was performed with 0.89% NaCl/0.05% Tween-20.

***In vitro* stimulation of splenocytes**

Isolated splenocytes of individual mice were plated at a density of 5×10^6 cells/ml and cultured with 25 µg/ml BLG or Bet v 1 (Sigma) or medium alone for 72 h at 37°C/5% CO_2.

Secreted mouse cytokines were measured with the corresponding commercial ELISAs (Invitrogen/eBioscience, for mouse IL-5, IL-10, IL-13 and IFNγ) according to the manufacturer's instructions.

AZ-AhR reporter assay

AZ-AhR cells were incubated at 37°C, 5% CO2 on 96-well plates at a density of 2 x 10^4 cells/ well for 18h. Subsequently cells were stimulated for 18h in triplets with 90µM

catechin/epicatechin alone or in complex with iron and increasing concentrations of BLG (10µM). Compounds were initially incubated together for 15 minutes and the pH was adjusted to pH 7 prior addition of BLG. The positive control cells were treated with 20nM indirubin. Cells were washed once with 0.89% NaCl and lysis buffer was added. After a single freeze-thaw cycle, 20 µl/well of lysates were transferred into a black 96-well flat-bottom plate (Thermo Scientific) and bioluminescent reaction were started with addition of 100 µl/well of luciferase assay reagent (Promega). Chemiluminescence was measured (10 sec/well) using a spectrophotometer (Tecan InfiniteM200 PRO).

Flow cytometric analyses of murine cells

For the evaluation of Treg cells, single-cell suspensions of murine splenocytes (0.5 million cells) were stained for CD4+CD25+Foxp3+ T-regulatory cells using anti-FOXP3 PerCP-Cyanine5.5 (clone FJK-16s), anti-CD4 FITC (clone GK1.5) and anti-CD25 PE (clone PC61.5) antibodies according to the manufacturer's instructions (eBioscience). Doublets were excluded before gating the lymphocytic population, followed by consecutive gating for CD4+, CD25+ and Foxp3+.

For staining of co-stimulatory molecules on dendritic cells, splenocytes were incubated for 30min with Calcein Violet 450 AM (Thermo-Fisher), anti-CD11c PE (clone N418), anti-MHC II I-Ad APC (clone AMS-32.1) and anti-CD86 FITC (clone GL1) in staining buffer (eBioscience) before cells were washed twice in Hepes-buffer (20 mM Hepes, 150 mM NaCl, pH 7.2). Cells were acquired on a flow cytometer, gating on CD11c+ in the living monocytic population, before plotting on MHC Class II I-Ad+CD86+ cells.

A third set was stained for CD71+ as a marker for proliferation using anti-CD71 PE (eBioscience, clone R17217) in combination with anti-CD19 APC (Biolegend, clone 6D5) and using calcein-AM (Thermo-Fisher), as a living marker. Here, first doublets were excluded, before gating on the living cells as Calcein+. Then cells were gated on the lymphocytic population on the FSC/SSC plot, followed by gating on the CD19+ and CD71+ populations.

***In vitro* stimulation of peripheral blood mononuclear cells**

Peripheral blood mononuclear cells (PBMCs) from sixteen birch and/or grass pollen allergic subjects were isolated by Ficoll-Paque (GE Healthcare) and washed with 0.9% NaCl, before cells were incubated with apoBLG (5 µM) or holoBLG (5 µM BLG plus 15 µM catechine and 5 µM iron) in media neither containing phenol red nor fetal calf serum for 18 hours. Only a single time-point was measured due to the technical limitations that were encountered when working with iron, as media and buffers had to be iron-free, though iron is essential for cell survival.

Flow cytometric analyses of human cells

To determine the intracellular iron content and the expression of co-stimulatory molecules on CD14+ monocytic cells, cells were stained with combinations of Calcein Violet 450 AM (Thermo-Fisher), CD14-APC (Biolegend, clone M5EZ), HLADR-PE (Biolegend, San Diego, Calif, clone L243PC), and CD86-PE-CY7 (Biolegend, clone IT2.2). Doublets were excluded before gating on the CD14+ in the living monocytic population, followed by consecutive gating

for HLADR+, CD86+ and calcein+ and geometric mean fluorescence intensity (MFI) were calcutated for each fluorochrome.
For Treg cells staining, a combination of CD3-APC-Cy7 (Biolegend, clone SK7), CD4-PE-Cy7 (Biolegend, clone SK3), CD25-APC (Biolegend, clone BC96), CD127-PE (Biolegend, clone A019D5) and Calcein Violet 450 AM (Thermo-Fisher) were used for flow cytometric analysis. Doublets were excluded, before gating on the living lymphocytic population for CD3+ and CD4+ cells and plotting for the corresponding CD25+ and CD127- cells. Tregs is presented as fold expression normalized to medium and to the relative number of CD3+CD4+ T-cells.
All acquisition was performed on a FACS Canto II machine (BD Bioscience, San Jose, CA, USA). Recorded events were analyzed with the FlowJo software version 10.3 (FlowJo, LLC, Ashland, OR, USA).
For cytokine analyses, supernatants of stimulated PBMCs were flowcytometrically assessed for IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, TNF-α and IFN-γ using multiplex system according to the manufacture's instruction (LEGENDplex™ Human Th1/Th2 Panel 8-plex, Biolegend).

Human mast cell generation

Human peripheral blood mononuclear cell-derived mast cells were generated as previously described by Folkerts et al [19]. Briefly, peripheral blood mononuclear cells were obtained from buffy coats of healthy blood donors and CD34+ precursor cells were isolated using the EasySep Human CD34 Positive Selection Kit (STEMCELL Technologies). CD34+ cells were maintained for 4 weeks under serum-free conditions using StemSpan medium (STEMCELL Technologies) supplemented with recombinant human IL-6 (50 ng/ml; Peprotech), human IL-3 (10 ng/ml; Peprotech) and human Stem Cell Factor (100 ng/mL Peprotech, Rocky Hill, NJ). After 4 weeks, the cells were cultured in Iscove's modified Dulbecco's medium/ 0.5% bovine serum albumin with human IL-6 (50 ng/mL, Peprotech, Rocky Hill, NJ), and 3% supernatant of Chinese hamster ovary transfectants secreting murine stem cell factor (a gift from Dr P. Dubreuil, Marseille, France). The mature MCs were identified by flow cytometry based on positive staining for CD117 (eBioscience) and FcεRIa (eBioscience) using BD FACS Canto II (approximately 90%).

Human mast cell degranulation assay

Sera of children allergic or tolerant to milk were preincubated with BLG, catechin, iron alone or combination thereof, or with dietary surrogates with whey, cocoa and iron before adding to primary human mast cells (0.8 Mio/ml, 40 000 cells/well) overnight. Subsequently, supernatants were collected and incubated with 200 µM 4-methylumbelliferyl-β-d-glucosaminide (4-MUG) in 100mM citric acid, pH4.5 for 1 h at 37 °C. Enzymatic reaction was then terminated by adding 0.1 M glycine buffer, pH 10.7. As positive control, cells were lysed with 0.2% Triton X-100. The β-hexosaminidase content was quantified by measuring fluorescence at ex360/em452 nm. Degranulation was assessed by measurement of released ß-hexosaminidase in the supernatant and of unreleased enzyme in the respective cell lysate. The presented results were calculated as percentage release of total ß-hexosaminidase content, with a release from unstimulated controls of 0.041 %, from positive controls with anti-human IgE only of 35 % and with ionomycin of 94 %.

Statistical analyses

Mice groups and cellular studies were compared by ANOVA following Tukey's multiple comparisons test or using mixed-effects analysis following Sidak's multiple comparisons test, when data points were missing. For analyzing cell phenotypes of lozenge- or placebo- treated patients Mann-Whitney U test was used, for binding of patient sera to different BLG forms Wilcoxon matched-pairs signed test was applied and when testing antigen-specific degranulation of mast cells preincubated to different stimuli one-way ANOVA following Tukey's multiple comparisons test were applied. All tests were two-sided and considered significant when $p < 0.05$.

Results

BLG binds to iron-catechin complexes

Catechin with a complex stability constant log ß of 47.4 at physiological pH [20,21] binds strongly to ferric iron. Indeed, binding to ferric iron leads to color formation and can be monitored by UV-VIS spectroscopy **(Fig. 1 B).** Importantly, BLG binds strongly to these iron(catechin)$_3$ and iron(epicatechin)$_3$ complexes with calculated affinity constants reaching the lower nM-range with 16.7 and 46.1nM respectively **(Fig. 1A+C)**. This leads to a discoloration, which is visible macroscopically and spectroscopically. The data confirm that the flavonoid catechin at physiological aerobic conditions is usually present in complex with iron and that BLG is able to bind these compounds.

Ligand binding masks B and T cell epitopes of BLG affecting IgE binding and antigen-specific mast cell degranulation

As previously published with quercetin as ligand[11], also catechin **(Fig. 2)** and epicatechin **(Fig. E1)** clearly affected the experimentally deduced epitope regions of BLG. When we compared the ligand-binding site with the two described dominant IgE epitope regions at residues K75-D85 located in the loop and E127-P144 at the site of the alpha helix [26] and the "weaker" IgE epitopes described at segments L31-P48, K47-K60, and L57-I78, it became apparent that also the iron(catechin)3 complex was in near proximity to the major B cell epitope 1 **(Fig. 2A)**, but not of epitope 2, which is located at the opposite site of epitope 1 on the alpha-helix. Importantly, we determined the clinical relevance of epitope masking by comparing IgE-binding to BLG with or without catechin-iron ligands, in milk sensitized children who reacted positive (n=10) and negative (n=10) to oral cow milk challenge, termed milk allergic and milk tolerant respectively **(Fig. 2B+C)**. Interestingly, the milk-allergic subjects had reduced IgE-binding to the holo-form of BLG, but not the milk-tolerant individuals, emphasizing that children tolerant to milk recognize different epitopes than children allergic to milk.

Ligand binding also affected the immune dominant T cell epitope at residues 97-117 of BLG as also described for retinoic acid [8] and for quercetin [11] with the most important core residues spanning from Y102-E112 (YLLFCMENSAE) [22,23]. As depicted in **Fig. 2A**, catechin-iron complexes co-localized with the T cell epitope residues at position L39 and F105 being within a 3Å proximity, and L32, V41, I56, K70, I71, N88, M107, A118 and Q120 being within 3.5 Å. The T cell epitope masking may alter T cell activation as described for the major birch pollen allergen Bet v 1 in two studies[24,25] as endolysosomal enzymes are hindered in generating

antigenic peptides for antigen presentation. Among endolysosomal proteases, cathepsin S, predominantly expressed in antigen presenting cells, has two predicted cleavage sites, at BLGp99-105 and BLGp109-115[8]. Both are located within the minimum essential region of BLG (YLLFCMENSAE) suggesting that also here the position F105 is occupied by the ligand and hinders cleavage.

We also addressed whether the ligands had an impact on the effector phase, using human mast cells. Pooled sera of children allergic to milk were preincubated with BLG alone or in combination with iron-catechin to form IgE-antigen complexes prior to addition to human mast cells. Similarly, we used dietary agents such as whey as a source BLG, cocoa for catechin as well as iron to form IgE-antigen complexes prior addition to the cells. Antigen-specific degranulation was significantly reduced when crosslinking was conducted using BLG in conjunction with iron-catechin implying again that the holo-form of BLG filled with ligand was less allergenic than the empty apo-form. Also, when using dietary agents as surrogate for BLG, catechin and iron, a similar and significant reduction in mast cell degranulation was observed.

Hence, B cell epitope masking resulted in reduced IgE binding and mast cell degranulation, whereas T cell epitope masking interferes with Cathepsin S cleavage upon endosomal entry and alters antigen processing and in consequence proper antigen presentation and potentially T cell activation.

BLG facilitates AhR activation by (epi)catechin-iron complexes

As the dietary supplement contained cocoa extract as a source for BLG ligands which is rich of the flavonoids epicatechin and catechin, we assessed whether anti-inflammatory pathways such as the cytoplasmic arylhydrocarbon receptor (AhR) were activated by these flavonoids. AhR is described to interact with many exogenous ligands [26] and to mediate primarily anti-inflammatory feedback mechanisms[27] that promote regulatory T cells[26,28,29], while preventing Th2-skewing[30] [31] and impairing antigen presentation [32]. Using reporter cells, we could demonstrate that catechin and epicatechin could concentration-dependently activate AhR and interestingly activation was significantly enhanced when they were present as a complex with iron **(Fig. 3A)** implying active transport of the complex. Similarly, to our previous report, addition of BLG significantly enhanced (epi)catechin-dependent activation of the AhR-pathway suggesting synergistic pathways which directed targeting to the BLG receptors **(Fig. 3B)**. We also tested whether the single constituents of the lozenge and combinations thereof were able to activate the AhR pathway **(Fig. 3C)**. Indeed, similar to other reports [33], the whey concentrate alone was able to activate AhR, pointing towards already present ligands in the whey concentrate, whereas neither cocoa extract, iron or the cocoa-iron combinations were able to initiate this pathway. Importantly, combination of all mentioned constituents again significantly enhanced activation of the AhR-pathway compared to whey alone, confirming that BLG serves as a carrier for flavonoid-iron complexes able to activate AhR.

Dietary supplementation with verum lozenge containing holoBLG reduced clinical reactivity *in vivo*

We analysed whether holoBLG with catechin-iron complexes as ligands in a dietary supplement could be used therapeutically. A lozenge containing BLG, iron-catechins as well as zinc and vitamin A were formulated to be suitable also for human use.
Mice were simultaneously sensitized with alum against BLG and Bet v 1, before treating them orally with crushed aliquots of verum lozenges containing 5mg BLG with iron-catechin complexes, 10µg retinol and 175µg zinc, or placebo lozenges devoid of active ingredients. Allergic reactivity was analysed by systemic challenges first with BLG and in a second step also with Bet v 1 **(Fig. 4A)**.
Treatment in the verum lozenge group resulted in a significant decrease of BLG-specific IgG1 and Bet v 1-specific IgG1 and IgE antibodies **(Fig. 4B+C)**. Importantly, allergic mice treated with holoBLG, but not with the placebo substances, were protected against anaphylaxis to both antigens BLG and Bet v 1 **(Fig. 4D)**.

Lozenge treatment decreased immune reactivity by promoting tolerance: less lymphocytic and B cell proliferation, more Tregs and decreased antigen presentation *in vivo*

In line with a suppressed immune response, treatment with the verum lozenge rendered comparable splenocyte numbers as the naïve group **(Fig. 5A)**. Also, the expression of the proliferation marker CD71 on lymphocytes and B cells did not significantly differ from the naïve group **(Fig. 5B+C)**, in contrast to the group treated with the placebo lozenge. However, splenocytes of double-sensitized mice still had the capacity to respond similarly to *in vitro* stimulation with BLG and Bet v 1, irrespective whether the groups were treated with the verum or placebo lozenges **(Fig. E2)**. Regulatory T cells were significantly elevated after verum lozenge treatment compared to the placebo lozenge-treated group **(Fig. 5D)**. Verum lozenge treatment was accompanied by impaired antigen presentation in line with a decreased expression of costimulatory markers **(Fig. 5E)**. Hence, supplementation with the verum lozenge increased immune resilience in an ongoing Th2-response.

Iron transport by holoBLG into CD14+ monocytic cells, is accompanied by decreased antigen presentation

In a next step, we assess whether similar results could be obtained when incubating PBMCs from pollen allergic patients with apoBLG or holoBLG for 18h. Co-stimulatory molecules as well as intracellular iron content of CD14+ cells were assessed flow cytometrically. Similar to previous published data using iron-quercetin complexes as BLG's ligand [11,12], here we demonstrated that transport of iron-catechine complexes by BLG increased the intracellular iron content of CD14+ populations, reduced relative numbers of CD14+ monocytic cells **(Fig. 6A)** and significantly suppressed the expression of the co-stimulatory molecules, HLADR+ and CD86+ **(Fig. 6B+C)** compared to cells stimulated with apoBLG. The data further support our therapeutic murine model showing that dietary supplementation with holoBLG and catechin-iron complexes was sufficient to ameliorate clinical reactivity *in vivo* irrespective to which allergens mice were sensitized to. It emphasizes the function of BLG in providing antigen presenting cells with micronutrients and thereby blocking immune activation. Mechanistically targeting antigen presenting cells and shuttling anti-inflammatory ligands into

these cells, lead to reduced antigen presentation, less proliferation in an antigen-nonspecific manner.

Cytokine analysis revealed that, in particular **(Fig. E3)**, the release of pro-inflammatory cytokine TNF-α was significantly reduced upon overnight stimulation with holoBLG, but not with apoBLG. Additionally, released IL-4 and IL-6 levels tended to be lower when cells were incubated with holoBLG. Whereas IFN-γ, IL-10 and IL5 levels did not differ in cells treated either with apo- or holoBLG.

Moreover, in line with previous reports[7], the relative number of CD3+CD4+ T-cells was significantly downregulated compared to cells stimulated with apoBLG **(Fig. 6D),** while holoBLG treatment led to promotion of regulatory T cells **(Fig. 6F)** whereas their iron content was comparable between different treatments **(Fig. 6E)**. Summing up, the increased iron-levels in antigen presenting cells went along with decreased expression of costimulatory markers, as well as an increase of T cells with a supposed regulatory phenotype upon treatment with holoBLG. In this study, iron-catechine complexes were used as ligand for BLG to provide antigen presenting cells with micronutrients and to promote an immune resilience state.

Discussion

We have previously shown that sensitization to BLG can be prevented when BLG is filled with ligands, as they initiate anti-inflammatory pathways leading to tolerance induction and immune resilience [11].

Here, we went a step further and addressed whether the protective farm effect may be applied and exploited for already allergic subjects by using BLG as a carrier for anti-inflammatory ligands to antigen presenting cells. We formulated a lozenge containing food and dietary supplements approved for human use: whey concentrate as a source of BLG, cocoa as a source rich of epicatechin and catechin, furthermore ferric iron, retinoic acid and zinc.

We confirm here that catechins are strong iron-chelators, activate the AhR-pathway and most importantly that BLG can bind to these complexes and facilitate activation of AhR pathway, irrespective of whether it co-applied with whey concentrate or applied in pure form. Similarly, as with other ligands, we show for the holo-form of BLG reduced IgE binding and mast cell degranulation with serum from children allergic to milk. Importantly, the ligands mask a major IgE epitope [34], as well as a well described T cell epitope [8,22,34].This suggests that children allergic to milk at one point were sensitized to BLG-devoid of any ligands, the only setting when IgE can be generated to a region which otherwise would be masked by the ligand.

Oral supplementation of allergic mice with the lozenge containing holoBLG led to reduced clinical reactivity in an antigen-unspecific manner as clinical symptoms improved not only against BLG, but also towards Bet v 1 and was accompanied by reduced proliferation, antigen presentation and promotion of regulatory T cells. Importantly, this is in agreement with our previous results, when the single component BLG complexed with iron-quercetin applied via the nasal route prevented allergy development in an antigen-unspecific manner *in vivo* [12]. The data are also in line with our *in vitro* generated data with human immune cells [11] showing that holoBLG shuttled catechin-iron complexes specific into monocytic cells and thereby hindered their activation and maturation. It provides also a rational for the antigen-unspecific effect against allergies as the monocytic cells provided with micronutrients are the antigen-presenting cells that link the innate with the adaptive immune branch.

Particularly the provision of iron seems pivotal in modulating an immune response. The iron status deeply affects immunity, with iron-deficiency known to cause immune hyperactivity [35,36] prone initially towards Th2 [37-39] and being associated with atopic dermatitis [40], allergic rhinitis [41,42] and asthma [43-45]. In contrast, in iron depleted conditions, immune cells seem to shift towards a more resilient state with macrophages polarizing towards an anti-inflammatory M2 sub-type *in vivo* and *in vitro* [46], antigen presentation being decreased [11,46], CD4+ counts [47], and to a lesser extent CD8+ cells being negatively affected [48].

The lozenge was also spiked with vitamin A and zinc, essential contributors to immunity: Vitamin A supports mucosal regulation and predominantly anti-inflammatory pathways [10], whereas zinc deficiencies potentially cause lymphopenia, as zinc functions as a second messenger for innate immunity. Zinc efflux is required for dendritic cell maturation and zinc influx for macrophage activation [49]. Importantly, vitamin A as well as zinc have been described as natural ligands for BLG before [6,8].

Similarly, numerous studies have shown that initiation of the aryl hydrocarbon receptor - highly expressed in immune cells capable of antigen presentation such as monocytes [50], dendritic cells, macrophages [51] and B cells [52,53], impedes allergic sensitization [54], reduces antigen presentation [27], while promoting regulatory cells [26,55-57].

AhR activation represses differentiation of B cells into plasmablasts *ex vivo* and antibody-secreting plasma cells *in vivo* [52], which may also account for the observed decrease in antigen-specific antibody levels in the verum lozenge group *in vivo.*

The AhR pathway may also be an underappreciated contributor to inhibit class switch and may also contribute to the protective farm effect, where specifically the class switch along the IgG1/IgG4/IgE-Th2 axis seems to be suppressed [58].

Based on the preclinical data, several clinical studies are underway with a recent published clinical trial showing that supplementation with holoBLG lozenges for 3 months resulted in a significant (antigen-nonspecific) reduction of the symptom burden in house dust mite allergic patients whose symptoms were tested before and after supplementation in an allergen exposure chamber[16].

Conclusion

Based on our previous findings, we generated a lozenge as FSMP (food for the specific medical purpose) of allergies, exploiting the molecular mechanism of targeted micronutrition via holoBLG. The lozenge caused immune resilience by shuttling micronutrients to antigen presenting cells in vitro and in vivo in BALB/c mice. Thereby antigen presenting cells were redirected and modulated an ongoing Th2-response resulting in reduced clinical reactivity in an antigen-unspecific manner. In line with previous clinical trials [16], our findings propose that indeed the farm effect can be exploited in form of an FSMP lozenge for therapeutical or prophylactic approaches against the allergy epidemic.

Disclosure of potential conflicts of interest:

F.R-W., E.J-J. and L.F.P. are inventors of EP2894478, LCN2 as a tool for allergy diagnostic and therapy. EP 14150965.3, Year: 01/2014; US 14/204,570, owned by Biomedical International R+D GmbH, Vienna, Austria, underlying the ImmunoBON® lozenge. S.M.A has been employed by a research grant supported by Biomedical Int. R+D GmbH and Bencard Allergie GmbH, Germany, directed by F.R-W. The other authors declare no relevant conflict of interest in relation to this publication.

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Author contributions: S.M.A. conducted all mouse experiments and analysis, provided support and contributed to the other experiments and writing; A.R conducted AhR and spectroscopic experiments, provided support and contributed to the writing; L.F.P. performed the in silico analysis and contributed to the writing; B.R.B. and F.R. conducted mast cell experiments, provided material and contributed to the writing; S.A.J, acquired blood samples, and contributed to the writing; I.P-S. contributed in the mouse experiments, provided support and contributed to manuscript editing; R.B. contributed to flow cytometric experiments, writing and provided support; K.H. conducted experiments, provided support and contributed to writing; A.F. and Z.D. provided samples, support and contributed to the writing; S.G. and M.F.K. provided support and contributed in manuscript writing; E.J-J. directed research and development of the lozenge, and contributed in manuscript writing; F.R-W. conceived, directed the research, designed the experiments, interpreted the data and wrote the manuscript.

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Figure 1

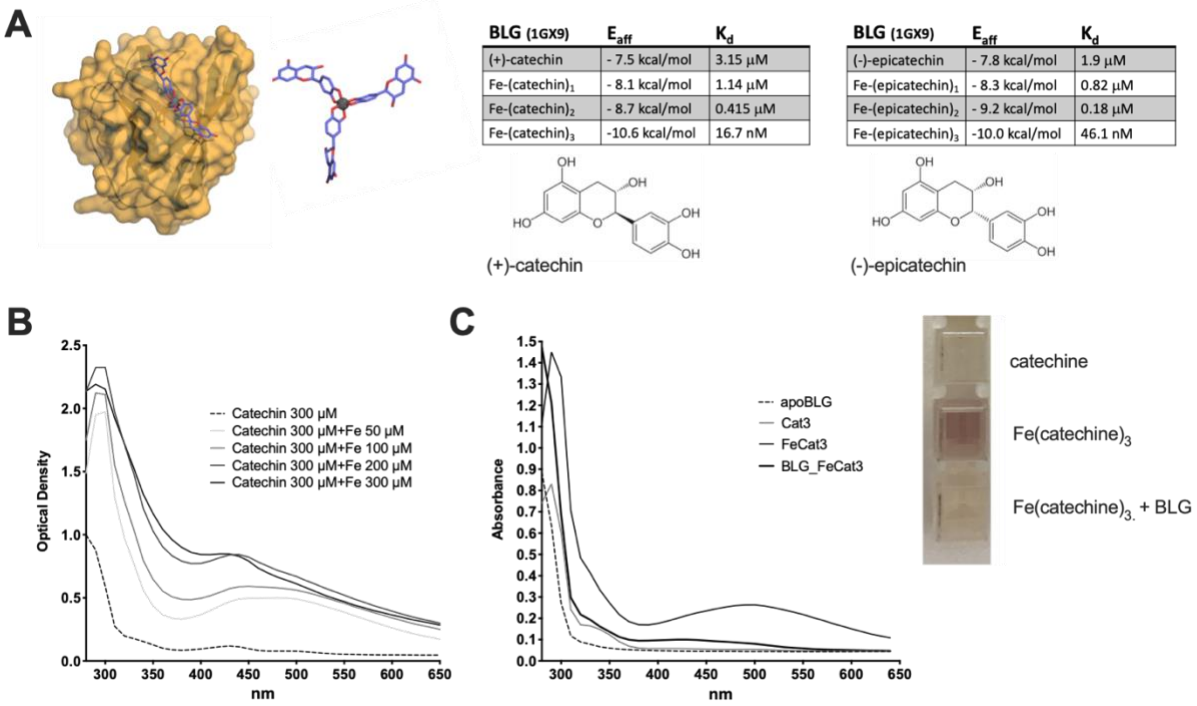


Figure 1: BLG binds to iron-catechin complexes. A, Protein surface of beta-lactoglobulin, BLG, incorporating iron(catechin)$_3$ (FeCat$_3$) (sticks with carbons in deep blue, oxygens in red, and iron shown as a grey sphere) and calculated affinities of catechin/epicatechin in conjunction with iron to BLG. **B,** Optical spectra of 300µM catechin with increasing concentration of ferric iron at pH 7.3. **C,** Optical spectra and color change of 150µM catechin, 50µM iron, BLG and combinations thereof.

Figure 2

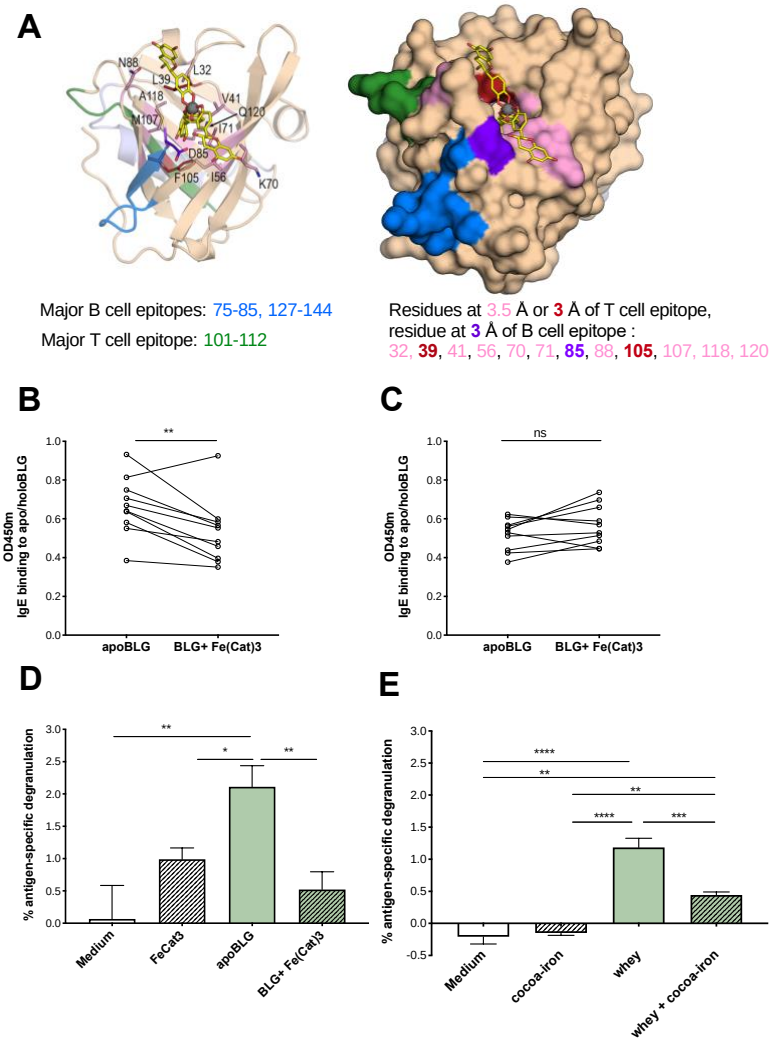


Figure 2: Ligand binding masks B cell and T cell epitopes of BLG affecting IgE binding and mast cell degranulation. A, Structure of BLG- Fe(catechin)$_3$ complex shown as cartoon (left) and as molecular surface (right). Fe(catechin)$_3$ is depicted as sticks with carbons in cyan and oxygens in red. Iron atom is represented as a grey sphere. Major B-cell epitope 1 (75-85) and epitope 2 (127-144) are marked in marine blue and light blue, respectively. The T-cell epitope (101-112) is marked in green. Residues within a 3.5 Å distance from any atom of Fe(epicatechin)$_3$ are shown as sticks with carbons in pink. Two residues within a 3.0 Å distance from any atom of Fe(catechin)$_3$ (39 and 105) are shown as sticks with carbons in deep red. The third residue within a 3.0 Å distance from the ligand, D85, is also part of the B-cell epitope 1 and is shown as sticks with carbons in violet purple. **B**, IgE binding to BLG alone or in combination with iron-catechin in milk allergic (n=10) and **C**, milk tolerant children (n=10) respectively. Degranulation of human mast cells sensitized with pooled milk allergic sera and **D,** crosslinked with BLG alone or in combination with iron-catechin complexes or **E** crosslinked with combinations of dietary agents such as whey containing BLG, cocoa containing catechin and iron alone, IgE binding were compared in B and C by Wilcoxon matched-pairs signed test and for D and E by one-way ANOVA following Tukey's multiple comparisons test. Mean ± SEM; **P* < 0.05, ***P* < 0.01.

Figure 3

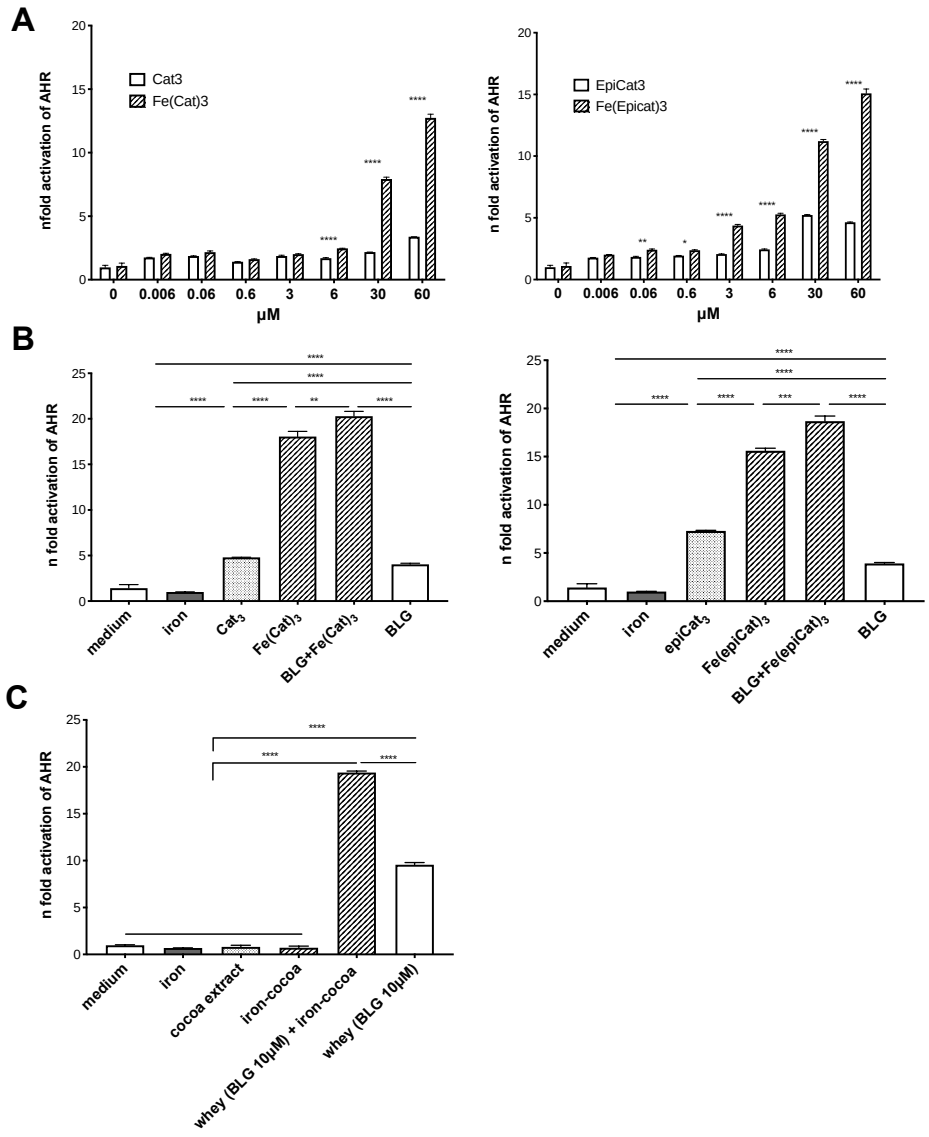
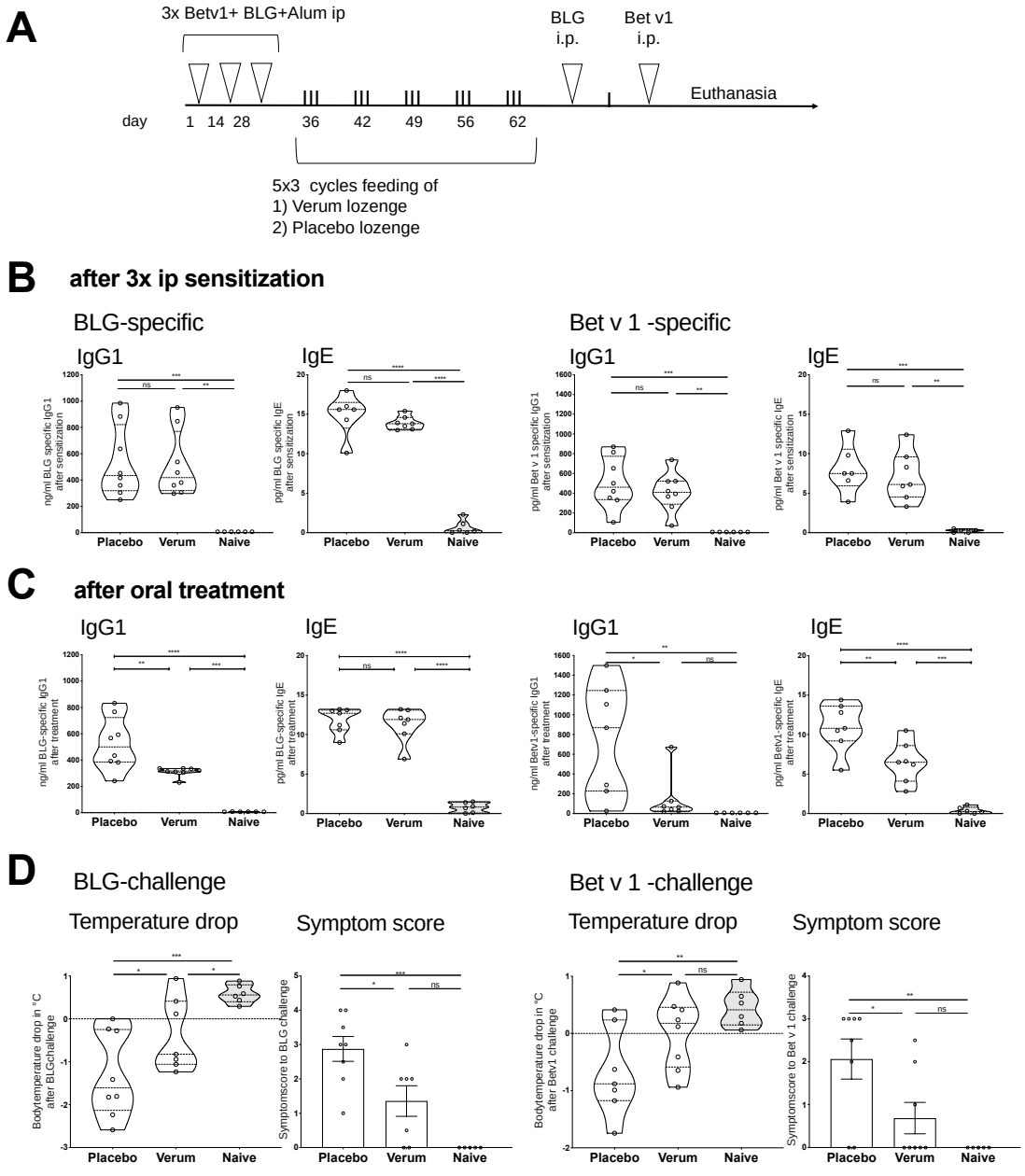


Figure 3: AhR activation by (epi)catechin-iron complexes is increased upon addition of BLG. AZ-AhR cells were treated with **A,** increasing concentrations of catechin (left graph) or epicatechin (right graph) alone (0 -60µM), or with addition of iron **B,** with 60µM of catechin (left) or epicatechin (right) in combination with 20µM iron and/or 2µM BLG, **C**, equivalent concentrations of dietary agents as source iron (iron citrate,10µM), epicatechin/catechin (cocoa extract, aprox. 30µM catechin/epicatechin) and BLG (whey concentrate containing approx.10µM BLG) for 18 h before luciferase-activity was measured in the supernatant. Representative data from two independent experiments normalized to medium alone. Concentration-dependent activation of AhR in graph A was compared with 2-way ANOVA, graph B and C with one-way ANOVA following Tukey's multiple comparisons test, Mean ± STD; **P* < 0.05***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

722 **Figure 4**



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725 **Figure 4: Oral supplementation with a lozenge containing holoBLG reduces clinical**
726 **reactivity in mice. A,** As depicted in the treatment scheme, mice were sensitized 3x with BLG
727 and Betv1 in combination with Alum, before receiving in 6 cycles aliquots of crushed lozenges
728 containing active ingredients (5mg holoBLG in verum lozenge) or not (placebo lozenge).
729 Subsequently, mice were challenged with BLG and Betv1 one week apart; **B,** BLG- and Bet
730 v1 specific antibodies before and **C,** after oral treatment; **D,** clinical response after BLG and
731 Bet v 1 challenge of double-sensitized mice after treatment with immune or placebo lozenges.
732 Data from one experiment are shown (n=8 for lozenge groups, n=6 for naïve group). Groups
733 were compared by one-way ANOVA followed by Tukey's multiple comparison, for symptom
734 score analyses ANOVA followed by Kruskal-Wallis test was used. *P < 0.05, ** P<0.01, ***
735 P<0.001, ** P<0.0001.
736

Figure 5

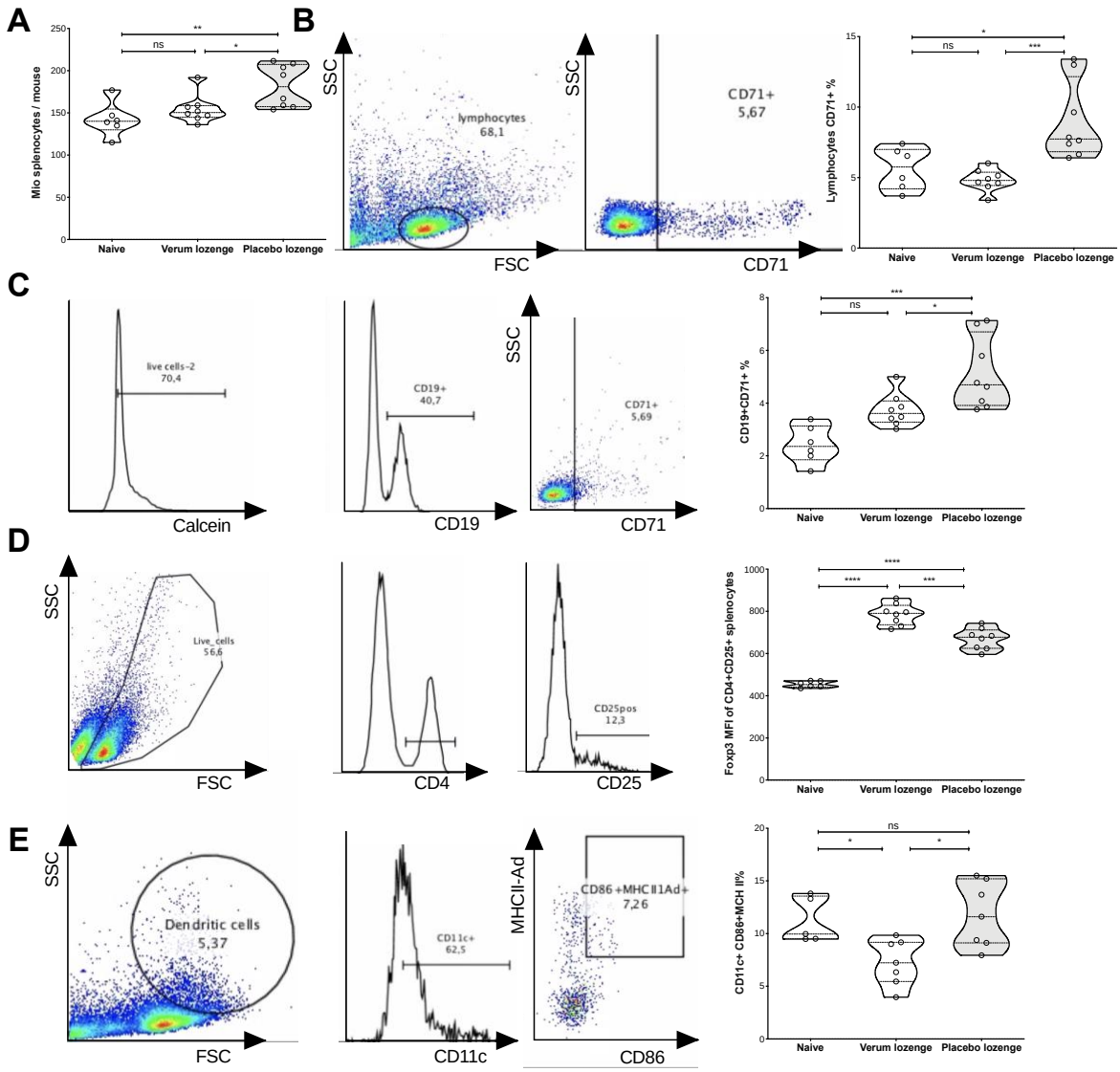

Figure 5: Treg promotion, decreased antigen presentation and decreased B cell proliferation upon treatment with the verum lozenge. A, Total splenocyte numbers; **B**, Lymphocyte proliferation using CD71 as proliferation marker **C,** B cell activation **D,** Foxp3 expression of CD4+CD25+ splenocytes; **E,** % of CD11c+CD86+MHCII+ splenocytes. Groups were compared by one-way ANOVA followed by Tukey's multiple comparison. *P < 0.05, ** P<0.01, *** P<0.001, ** P<0.0001.

747 **Figure 6**

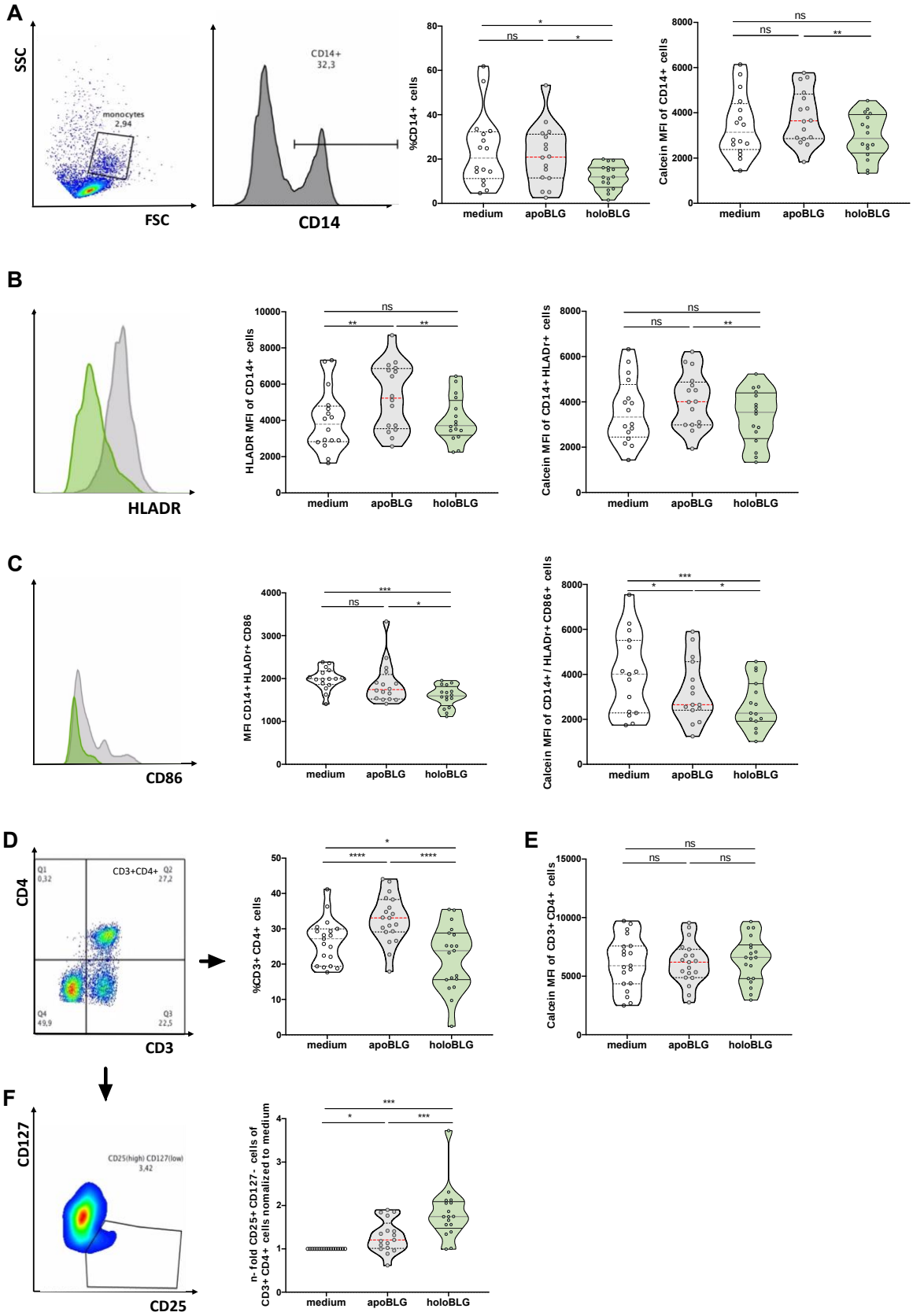

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Figure 6: holoBLG- stimulated PBMCs resulted in an increase of intracellular iron in circulating monocytes, impairment of antigen presentation and a decrease of the relative number of Th2 cells.

Flowcytometric analyses of stimulated human peripheral blood mononuclear cells of 16 allergic subjects with apoBLG or holoBLG and incubated overnight in iron-free media. **A,** CD14 positive cells were gated from the monocytic population and calcein MFI of CD14+ populations was compared, as iron quenches the calcein-signal. The intracellular iron content and expression of **B**, HLADR and **C**, CD86 were further analysed from CD14+ population. **D,** the relative number of CD3+CD4+ cells, **E,** Calcein MFI of CD3+CD4+ populations **F,** n-fold CD25+CD127-cells expression of CD3+CD4+ cells normalized to medium alone. Groups were compared by repeated measures 1-way ANOVA following the Tukey multiple comparisons test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns = non-significant.

Figure E1

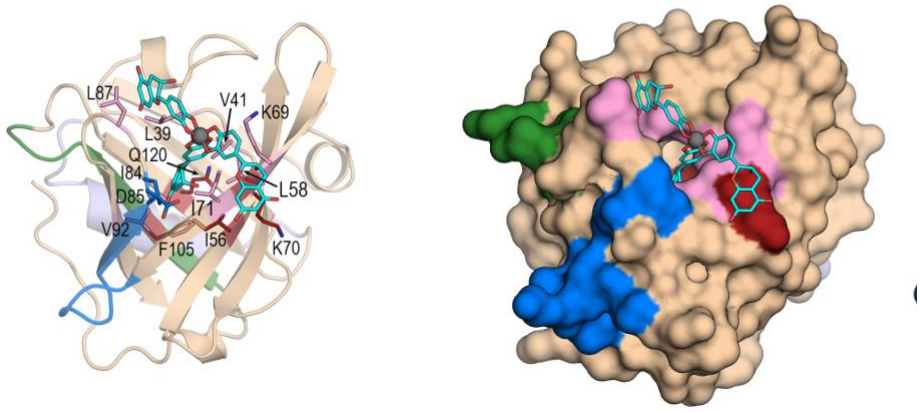

Figure E1: BLG binds iron(epicatechin)$_3$ complexes. Structure of BLG-with Fe(epicatechin)$_3$ complex as cartoon (left) and molecular surface (right) at the same orientation. Fe(epicatechin)$_3$ is depicted as sticks with carbons in cyan and oxygens in red. Iron atom is represented as a grey sphere. Major B-cell epitope 1 (75-85) and epitope 2 (127-144) are marked in marine blue and light blue, respectively. The T-cell epitope (101-112) is marked in green. Residues within a 3.5 Å distance from any atom of Fe(epicatechin)$_3$ are shown as sticks with carbons in pink. Four residues within a 3.0 Å distance from any atom of Fe(epicatechin)$_3$ (56, 70, 105, and 120) are shown as sticks with carbons in deep red. Two residues of B-cell epitope 1 that flank the entrance to the binding site (I84 and D85) are also shown as sticks with carbons in marine blue.

Figure E2

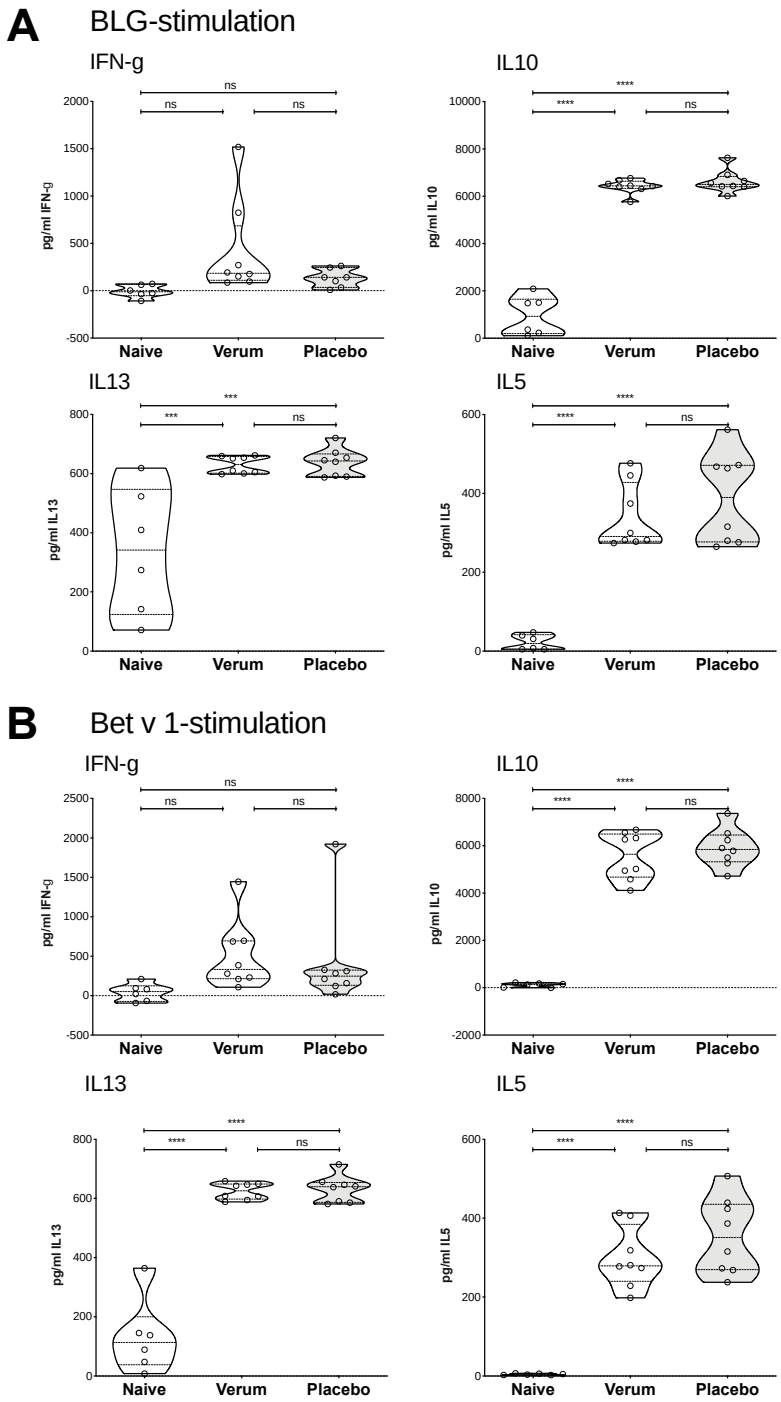

Figure E2: Released cytokines of stimulated splenocytes. Splenocytes of naïve (n=6), or Bet v 1- and BLG-double sensitized mice orally treated either with verum (n=8), or placebo lozenge (n=8), were stimulated for 72h with **A,** 25µg/ml BLG or **B,** 25µg/ml BLG before supernatants were assessed for IFN-γ, IL-10, IL-5 and IL-13. Representative data from two independent experiments are shown. Groups were compared by one-way ANOVA following Tukey's multiple comparisons test, Mean ± SEM; ***P<0.001, ****P<0.000.

Figure E3

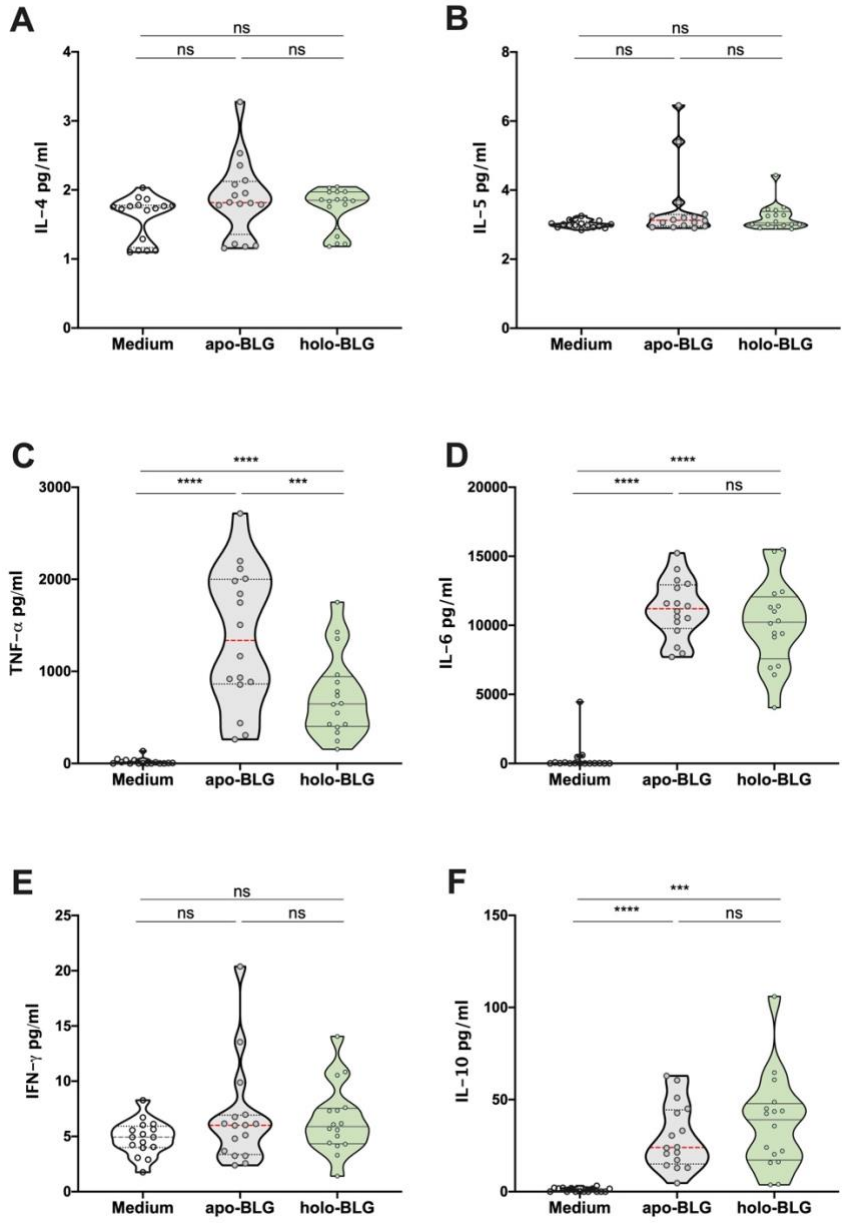

Figure E3: Released cytokines of stimulated PBMCs. PBMCs from 16 pollen allergic donors were incubated overnight in iron-free media after being stimulated with apo-BLG or holo-BLG. Multiplex-analysis of **A,** IL-4, **B,** IL-5, **C,** TNF-α, **D,** IL-6, **E,** IFN-γ and **F,** IL-10 concentrations in supernatants of stimulated PBMCs S. Groups were compared by repeated measures 1-way ANOVA following the Tukey multiple comparisons test. ***P < 0.001; ****P < 0.0001; ns = non-significant.