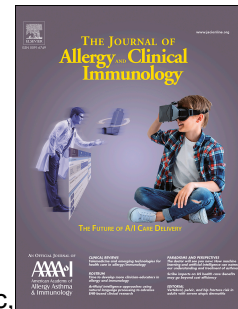


Journal Pre-proof

Cow milk protein beta-lactoglobulin confers resilience against allergy by targeting complexed iron into immune cells

Franziska Roth-Walter, PhD, Sheriene Moussa Afify, MD, Luis F. Pacios, PhD, Bart R. Blokhuis, Ing, Frank Redegeld, PhD, Andreas Regner, MSc, Lisa-Marie Petje, BSc, Alessandro Fiocchi, MD, Eva Untersmayr, MD, Zdenek Dvorak, PhD, Karin Hufnagl, PhD, Isabella Pali-Schöll, PhD, Erika Jensen-Jarolim, MD



PII: S0091-6749(20)30742-9

DOI: <https://doi.org/10.1016/j.jaci.2020.05.023>

Reference: YMAI 14589

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 12 January 2019

Revised Date: 5 May 2020

Accepted Date: 7 May 2020

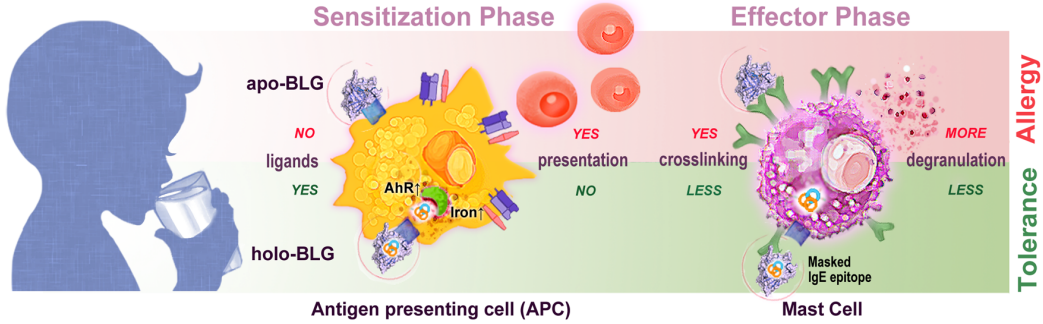
Please cite this article as: Roth-Walter F, Afify SM, Pacios LF, Blokhuis BR, Redegeld F, Regner A, Petje L-M, Fiocchi A, Untersmayr E, Dvorak Z, Hufnagl K, Pali-Schöll I, Jensen-Jarolim E, Cow milk protein beta-lactoglobulin confers resilience against allergy by targeting complexed iron into immune cells, *Journal of Allergy and Clinical Immunology* (2020), doi: <https://doi.org/10.1016/j.jaci.2020.05.023>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology.



How beta-lactoglobulin with ligands confers immune resilience



Abbreviations

BLG Beta(β)-lactoglobulin

- apo-BLG: β -lactoglobulin without ligand
- holo-BLG: β -lactoglobulin with ligand

AhR Arylhydrocarbon receptor
APC Antigen presenting cell
IgE Immunoglobulin E

Key Legend



Cow milk protein beta-lactoglobulin confers resilience against allergy by targeting complexed iron into immune cells

Franziska Roth-Walter PhD^{1,2*}, Sheriene Moussa Afify MD^{1,3}, Luis F Pacios PhD⁴, Bart R. Blokhuis Ing⁵, Frank Redegeld PhD⁵, Andreas Regner MSc¹, Lisa-Marie Petje BSc¹, Alessandro Fiocchi MD⁶, Eva Untersmayr MD², Zdenek Dvorak PhD⁷, Karin Hufnagl PhD^{1,2}, Isabella Pali-Schöll PhD^{1,2}, Erika Jensen-Jarolim MD^{1,2*}

1. The Interuniversity Messerli Research Institute of the University of Veterinary Medicine Vienna, Medical University Vienna and University Vienna, Vienna, Austria.
2. Institute of Pathophysiology and Allergy Research, Center of Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria.
3. Laboratory Medicine and Immunology Department, Faculty of Medicine, Menoufia University, Egypt.
4. Biotechnology Department, ETSIAAB, Center for Plant Biotechnology and Genomics, CBGP (UPM-INIA), Technical University of Madrid, Madrid, Spain.
5. Faculty of Science, Division of Pharmacology, Department of Pharmaceutical Sciences, Utrecht University, The Netherlands
6. Childrens Hospital Bambino Gesù, Rome, Italy.
7. Department of Cell Biology and Genetics, Faculty of Science, Palacky University, Olomouc, Czech Republic

*Corresponding authors:

Franziska Roth-Walter, PhD

Comparative Medicine, The interuniversity Messerli Research Institute, University of Veterinary Medicine Vienna, Medical University of Vienna and University of Vienna

Veterinärplatz 1, A-1210 Vienna, Austria; Email: franziska.roth-walter@meduniwien.ac.at

and

Erika Jensen-Jarolim, MD

Inst. of Pathophysiology and Allergy Research, Center of Pathophysiology, Infectiology and Immunology, Medical University Vienna

Waehringer G. 18-20, 1090 Vienna, Austria; Email: erika.jensen-jarolim@medunwien.ac.at

Disclosure of potential conflicts of interest:

E.J-J., L.F.P. and F.R-W. 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. The other authors declare no relevant conflict of interest in relation to this publication.

Funding

The study was supported by the Austrian Science Fund FWF, grant SFB F4606-B28, and in part by Biomedical International R+D GmbH, Vienna, Austria and by Bencard Allergie GmbH, Munich, Germany. SMA was supported by a grant of the Ministry of Higher Education, Egypt.

Abstract

Background: Beta-lactoglobulin (BLG) is a bovine lipocalin in milk with innate defense function. It is not understood under which circumstances BLG is associated with tolerance or allergy towards milk.

Objective: Here we assessed the capacity of ligand-free (apo-) versus -loaded (holo-) BLG to protect mice against allergy using iron-quercetin as exemplary ligand, and studied the molecular mechanisms.

Methods: Binding of iron-quercetin into BLG was modeled and confirmed by spectroscopy and docking calculations. Serum IgE binding of milk allergic and tolerant children to apo- and holo-BLG was assessed. Mice were intranasally treated with apo- versus holo-BLG and after systemic challenge immunologically analysed. AhR activation was evaluated with reporter cells and Cyp1A1 expression. Treated human peripheral blood mononuclear cells and human mast cells were assessed by FACS and degranulation, respectively.

Results: Modelling predicted masking of major IgE and T cell epitopes of BLG by ligand-binding. In line, IgE binding of milk allergic children was reduced towards holo-BLG which also impaired degranulation of mast cells. In mice, only treatments with holo-BLG prevented allergic sensitization and anaphylaxis, while sustaining regulatory T cells. BLG facilitated quercetin-dependent AhR-activation and, downstream AhR, lung Cyp1A1 expression. Holo-BLG shuttled iron into monocytic cells and impaired their antigen-presentation.

Conclusion: The cargo of holo-BLG is decisive in preventing allergy *in vivo*. BLG without cargo acted as allergen *in vivo* and further primed human mast cells for degranulation in an antigen-independent fashion. Our data provide mechanistic explanation why the same proteins can either act as tolerogen or allergen.

Key Messages

- Ligands of beta-lactoglobulin, such as iron-quercetin complexes, are decisive in preventing allergic sensitization *in vitro* and *in vivo*.
- Ligand-binding mask major IgE- and T cell epitopes of BLG with milk-allergic patients' IgE showing reduced binding to the ligand-loaded holo-form of beta-lactoglobulin.
- Delivery of iron-quercetin complexes by BLG to antigen presenting cells, increased intracellular iron levels while decreasing their antigen presentation capacities
- The released complexes activated the aryl hydrocarbon receptor pathway sustaining the numbers of regulatory T cells *in vivo*.
- Immune reactions to the empty, unloaded beta-lactoglobulin were in significant contrast to the observations with the loaded holo-form, and resulted in specific allergy

Capsule summary

The ligands of beta-lactoglobulin are decisive in preventing milk sensitization *in vitro* and *in vivo* by synergistic molecular mechanisms causing a non-response.

Key Words: allergen, allergy, beta-lactoglobulin, BLG, Bos d 5, cow milk, iron, ligand, lipocalin, milk, quercetin, tolerance

List of abbreviations:

BLG, beta-lactoglobulin; DFO, deferoxamine; AhR, aryl hydrocarbon receptor; LCN, lipocalin; PR10-family, pathogen-related 10 family; FeQ2, iron-quercetin complexes; Fe, iron; UV-VIS, ultraviolet-visible light spectroscopy

99 Introduction

100 The prevalence of allergies is rising in the westernized world, which is partly attributed to living
 101 in a “too clean” environment and which is also affected by life-style factors¹. Numerous studies
 102 have shown that exposure to farms², as well as consumption of unprocessed farm milk during
 103 the first years of life protects against atopic eczema, hay fever and asthma³. For farm milk the
 104 reduced allergy risk⁴⁻⁶ is particularly associated with the whey protein content: the more native,
 105 undestroyed whey proteins are present the more it is considered protective^{7,8}. Notably, roughly
 106 half of the whey proteins are constituted by beta-lactoglobulin. It seems a paradox that at the
 107 same time beta-lactoglobulin is considered a major milk allergen, to which the majority of milk
 108 allergic individuals are sensitized to^{9,10}. Importantly, despite comparable milk consumption only
 109 certain individuals will become milk allergic, strongly suggesting that co-factors such as their
 110 ligands might be decisive in the sensitization process.

111 Beta-lactoglobulin belongs to the lipocalin, LCN, protein family, to which nearly all major
 112 allergens from mammals belong, and which show a highly conserved fold despite having very
 113 low amino acid sequence homology¹¹.

114 In humans BLG is found immune-cell associated¹², implying that transport of BLG occurs via
 115 the lymphatics rather than the blood vessels. Cellular BLG uptake in humans occurs via specific
 116 receptors¹³⁻¹⁶ that are originally tailored for endogenous human LCN proteins such as LCN2¹⁷,
 117 ¹⁸ or LCN1¹⁹. We have previously demonstrated the high molecular similarity of human LCN2
 118 and BLG²⁰, implying receptor sharing.

119 Similarly to LCN2²¹ and LCN1²², BLG can bind to catechol-type siderophores^{18,23}, small
 120 molecules with a strong affinity to ferric iron, thereby withholding iron and impeding bacterial
 121 growth²⁴.

Other BLG ligands that have been described include fatty acids²⁵, retinoic acid²⁶ and polyphenols/flavonoids,²⁷ such as quercetin²⁸ and catechin^{25, 27, 29, 30}, which also have a very strong affinity to iron at physiological pH^{31, 32}. The reported complex stability constant $\log \beta$ of these polyphenols range from 44-47³¹. which is comparable to the iron-sequestering abilities of siderophores of bacterial origin^{20, 33, 34}.

In the present study, quercetin was chosen as exemplary ligand for BLG as: 1) quercetin can complex iron with a similarly high affinity^{23, 31, 35} to bacterial siderophores, thus better mimicking the natural innate function of BLG compared to fatty acids only; 2) it is a ligand described to bind to BLG^{36, 37} and other allergens³⁸⁻⁴⁰; 3) it is present in milk, as it is transferred via the cow's plant chow to the milk⁴¹ and consequently may represent a natural interaction partner of BLG in milk; and 4) quercetin *per se* has known anti-inflammatory properties⁴².

As siderophore-binding of human LCN2^{17, 18, 43-46} is decisive in activating or suppressing our immune system²³, we sought to investigate whether BLG, that already exploits LCN-pathways, may similarly modulate our immune response by ligand-binding.

In previous work we have already demonstrated that the loaded holo-form of BLG is immunosuppressive and that only the ligand-free apo-form was able to evoke an immune response *in vitro*²³. Consequently, we address here the *in vivo* relevance as well as mechanistic explanations for the observed "immune shut-down".

We show that the immunosuppressive tolerogenic properties of BLG are closely linked with shuttling the iron-quercetin complex to innate cells. Ligand-binding masked a major B cell epitope on BLG, affected specific antigen recognition and both iron and quercetin *per se* promoted a resilience to immune activation by impairing antigen presentation, via transport of iron and activating the aryl hydrocarbon receptor pathway. The results identify the transport of

145 environmental or nutritional ligands by BLG to immune cells to be pivotal in the induction of
146 immune tolerance, and underscore the potential in preventing and treating allergic disorders.

Journal Pre-proof

Materials and Methods

Ethical approval

Volunteers donated blood after providing written informed consent. The study was approved by the ethic committee (#1972/2017) of the Medical University of Vienna and conducted in accordance with the Helsinki Declaration of 1975. Sera of milk allergic patients (20 patients positive and 20 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 Gesù, Rome, Italy. Open food challenges as described in the AAAAI/Europrevall protocol⁴⁷ 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.

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).

Structural and docking analysis

BLG structure was taken from the complex with retinoic acid, PDB entry 1GX9⁴⁸. The geometries of quercetin were obtained upon energy minimization with the MM2 force field of initial structures drawn using the ChemBioDraw/ChemBio3D Ultra 12.0 package. Docking input

files for protein and ligands were prepared with AutoDockTools⁴⁹. A grid box of 22 Å x 22 Å x 22 Å with origin at a point corresponding to the C6 atom in the retinoic acid molecule in the 1GX9 structure was used for docking calculations, which were performed with AutoDock Vina⁵⁰. The docking geometry with the lowest affinity energy E_{aff} was selected. Estimates of dissociation equilibrium constants K_d were then calculated for the protein-ligand complexes by assuming $E_{\text{aff}} \sim \Delta G$ with $K_d = \exp(-\Delta G/RT)$ at $T = 298.15$.

Protein structural diagrams were prepared by UCSF Chimera and Pymol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC, 2015^{51, 52}). Surface area, solvation energy and solvation effect calculations were performed using PISA module of CCP4 package⁵³. Ligand Explorer was used to analyze the ligand-protein interaction and to measure distances, which was further validated by NCONT module of CCP4⁵⁴.

Spectral analysis

For spectral analysis 0.9% NaCl (for Fig 1C) or deionized water (Fig 1D) was used as buffer to minimize iron-contamination from the air. The pH was kept constant at pH 7 by addition of NaOH. Fluorescence was measured by exciting samples at 450nm and measuring emission in 20nm steps starting from 480 to 800nm, using a Tecan InfiniteM200 PRO reader. Optical density was measured at a constant concentration of 100µM quercetin or 50µM iron, BLG or deferoxamine and combinations thereof. All measurements were repeated at least three times with similar results.

Generation of apo- and holo BLG

Commercially available bovine beta-lactoglobulin (L0130, Sigma) was dissolved in deionised water (20 mg/ml) and dialyzed three-times against 10 μ M deferoxamine mesylate salt. Further dialyzation against deionized water was performed (*apo*-BLG d 5). *Holo*-BLG was generated by forming iron-quercetin complexes (FeQ2) with quercetin (Sigma 1592409) and iron (Iron-standard AAS, Sigma 16596) at a ratio 2:1 and adjusting the pH to 7.2 with 10mM NaOH and adding *apo*-BLG to a final concentration of 1 μ M BLG, 2 μ M quercetin and 1 μ M iron.

Allergic sensitization and challenge of mice.

Sample size for the mouse experiments were based on the literature. No randomization was performed. We estimated natural exposure to BLG for a 20kg child consuming daily one 200ml glass cow milk being roughly 1g BLG, corresponding to 1mg BLG/20 g mouse. In our settings, we estimated a 100fold lower local mucosal concentration by applying 10 μ g BLG (5 μ g/nostril) to the respected groups. As such, mice were split into groups of 5-7 animals and treated six times on two consecutive days intranasally with 10 μ l/mouse (5 μ l per nostril) containing 1) apoBLG: 10 μ g apoBLG ($\approx$ 0.5nM) and 0.3 μ g deferoxamine ($\approx$ 0.5nM, DFO); 2) holoBLG: 0.5nM BLG with 0.5nM iron (=28ng, Fe) and 1nM quercetin (=338ng, Q2); 3) FeQ2: 0.5nM iron (=28ng, Fe) and quercetin (=338ng, Q2); or 4) DFO. One week later, mice were intranasally challenged with 20 μ g BLG on two consecutive days and challenged 24 hours later intraperitoneally with 50 μ g BLG in 0.9% NaCl. Four mice from different groups were challenged at each cycle. An investigator that challenged and scored the animals, was blinded to which group the mice belonged to. The animals were scored as previously described¹² giving 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 erecti, 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. Body temperature and movements were
 monitored for 20 minutes using an Imaging system (Biomedical Int. R+D GmbH, Austria)⁵⁵.
 After euthanasia with CO₂, blood was collected by cardiac puncture and sera were stored at
 -80°C until further processing. Spleens and lungs were collected. Lungs were perfused with
 0.9% NaCl, before fixation in 3.7 % neutral paraformaldehyde and paraffin-embedding. For
 histological analysis, sections of 5 µm thickness were taken. Results of three separate,
 independent experiments were compared.

Measurement of mouse serum antigen-specific antibodies by ELISA

BLG-specific IgG1, IgA, IgG2a and IgE levels were measured by ELISA. Briefly, BLG
 (1µg/well) or serial dilutions of mouse IgG1, IgG2a, IgE, and IgA standards (highest
 concentration for IgG1, IgG2a and IgA 1000 ng/ml, for IgE standard 100ng/ml) were coated,
 blocked with 1% BSA in phosphate buffer saline, PBS, and incubated with diluted sera (1: 100
 for IgG1, IgG2a, IgA and 1:15 for IgE) overnight at 4°C. Specific antibodies were detected with
 monoclonal rat anti-mouse antibodies IgG1 (clone A85-1), IgG2a (clone R19-15), IgA (clone
 c10-1), 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.

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

5µg/ml apoBLG, BLG with equimolar concentration of deferoxamine or holoBLG diluted in 0.89% NaCl were coated (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, HRP,- conjugated goat anti-human IgE antibody (Invitrogen A18793) diluted at 1:4000 in 0.89% NaCl/0.05% Tween-20 , using tetramethylbenzidine (e Bioscience) 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 cytokine response of stimulated splenocytes and human peripheral blood mononuclear cells

Isolated splenocytes of individual mice were plated at a density of 5×10^6 cells/ml and cultured with 5 and 25 µg/ml BLG or 5µg/ml holoBLG, 2.5 µg/ml concanavalin A (Sigma) or medium alone for 72 h at 37°C/5% CO₂.

Blood cells were lysed for 10 min with red blood cell lysis buffer (eBioscience) and washed before peripheral blood cells were incubated with BLG (10µM), iron, quercetin and combinations thereof in media containing neither phenol red nor fetal calf serum, FCS, for 18h³⁵. Secreted mouse and human cytokines were measured with the corresponding commercial ELISAs (Invitrogen/eBioscience, for human or mouse IL10, IL13 and IFNγ) according to the manufacturer's instructions.

AZ-AhR cells

AZ-AhR cell line are human hepatoma HepG2 cells transfected with pGL-4.27-DRE construct containing several AhR binding sites upstream of luciferase reporter gene, as previously described^{56, 57}. AZ-AHR were cultured in Dulbecco's modified Eagle's medium (Gibco) without phenol red, supplemented with 10% of fetal calf serum, 100 U/ml streptomycin, 100 µg/mL penicillin, 4 mM L-glutamine, 1% nonessential amino acids, and 1mM sodium pyruvate. In biweekly intervals 0.2mg/ml hygromycin B (Sigma) was added to the culture. Cells were maintained at 37 °C and 5% CO₂ in a humidified incubator. During stimulation experiments, the same medium as described above, but without 10% FCS, was used. Cells were routinely tested for mycoplasma contamination.

AZ-AhR reporter assay

AZ-AhR cells were incubated at 37°C, 5% CO₂ on 96-well plates at a density of 2×10^4 cells/well for 18h. Subsequently cells were stimulated for 18h in triplets with 90µM quercetin alone or in complex with iron and increasing concentrations of BLG (5µM, 10µM). Compounds were first incubated together for 15 minutes and the pH was adjusted to pH 7 before addition of BLG. The positive control was 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

Peripheral blood mononuclear cells were isolated by Ficoll-Paque (GE Healthcare)^{23, 35, 57}, washed with 0,9% NaCl, before peripheral blood cells were incubated with BLG (10 μ M), iron, quercetin and combinations thereof in media containing neither phenol red nor fetal calf serum for 18h³⁵. We only measured a single time-point due to the technical limitations that we encounter when working with iron. All media and buffers have to be iron-free and devoid of potential interaction partners e.g. phenol red, though it is essential for cell survival. As such, PBMCs had to be cultivated without any FCS and longer time points would have overshadowed the “immediate” impact of iron.

Subsequently, cells were stained with combinations of Calcein-AM (ThermoFisher), CD3-APC-Cy7 (eBioscience, clone SK7), CD14-APC-Cy7 (Biolegend, clone M5EZ), HLADR-PE (Biolegend, clone L243PC), CD86-PE-CY7 (Biolegend, clone IT2.2) for flow cytometric analysis.

For murine splenocytes, single-cell suspensions (0.5 million cells) were stained for CD4+Foxp3+ T-regulatory cells using anti-forkhead-box-protein 3 (FOXP3) PE (eBioscience, clone FJK-16s) and anti-CD4-FITC (eBioscience, clone RM4-5) antibodies, according to the manufacturer's instructions (eBioscience, #88-8111). Doublets were excluded before gating to the lymphocytic and plotting to CD4+Foxp3+ cells. A second set of cells were stained for CD71+ as a marker for proliferation using anti-CD71 PE (eBioscience, clone R17217) and using Calcein as a living

marker. Here, first doublets were excluded, before gating on the living cells as Calcein+, gating on the lymphocytic population on the FSC/SSC plot, followed by gating on the CD71+ population. Cells were acquired by flow cytometry (BD Bioscience Canto II machine). Acquired cells were analyzed using the FACSDiva Software 6.0. Figure preparation for the PBMCs was conducted with cytoflow 1.0.

Immune-histochemical staining

Lung sections were stained for Cyp1A1. Briefly, unlabeled CYP1A1 (clone B-4, Santa Cruz Biotechnology) was conjugated to alkaline phosphatase using a commercial kit (Lightning-Link® Alkaline Phosphatase Conjugation Kit, Innova Biosciences, Cambridge UK) according to the manufacturer's recommendation. After deparaffinization, heat-mediated antigen retrieval (30min in 1mM Tris- EDTA, pH9) and blocking with anti-mouse CD16/CD32 (1:100, in 2% FCS/tris buffered saline,TBS, 30min), slides were incubated overnight at 4°C with alkaline-phosphatase conjugated anti-mouse Cyp1A1-antibody (1:50 in TBS), before adding BCIP-NBT as a substrate. Slides were counterstained with methyl green and mounted with resinous mounting medium (Sigma). Between each step, vigorous washing was performed with TBS. Images were acquired and manually quantified with a Zeiss Axio Imager Z1 microscope at 20x magnification.

Human mast cell generation

CD34+ derived human mast cells were generated from surplus autologous stem cell concentrates as previously described⁵⁸. Briefly, frozen stem cell concentrates were rapidly thawed at 37°C

under sterile conditions and poured into a large cell culture flask (Greiner). 20% human serum albumin clinical solution (HSA) (Sanquin), 6% hydroxyethyl starch clinical solution (Braun) and RPMI 1640 containing 10 U/ml Heparin (LEO pharma) were then added slowly and consecutively to the cell concentrate. Cells were then filtered through a cell dissociation sieve (Sigma) and incubated with DNase (200 U.I./ml, Roche) for 15min. After washing, cells were re-suspended in PBS containing 4% HSA and then incubated with Fc-Block (Miltenyi) for 15min, CD34⁺ positive selection cocktail (StemCell) for 15min and nanoparticles for 10min. Subsequently, CD34⁺ cells were sorted with an EasySep R Magnet (StemCell) according to the manufacturer's protocol. Finally, sorted cells were re-suspended in serum-free expansion medium (SFEM) (StemCell) supplemented with human low-density lipoprotein, LDL, (50µg/ml, StemCell). On day 1, human recombinant IL-3 (100 ng/ml, Biolegend), and SCF (100 ng/ml, Miltenyi) were added. Every three to four days, IL-3 and SCF were added at a final concentration of 20 ng/ml. At the end of the second week, mast cells were maintained under 20 ng/ml SCF with the withdrawal of IL-3. 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 FcεRIα (eBioscience) using BD FACSCanto II (approximately 90%).

Human mast cell degranulation assay

Antigen-independent approach: primary human mast cells (0.8 Mio/ml) were sensitized with 10% mouse hybridoma IgE supernatant (clone 26-82) overnight, washed with RPMI 1640

without phenol red/1%FCS/1mM Glutamax, counted and a 50 μ l cell suspension (40 000 cells/well) was incubated for 2 hours without or with a 5, 50, 500, 5000nM BLG, holoBLG, quercetin2, iron-quercetin2 , before cross-linking with rat anti-mouse IgE (1ug/ml, BD Pharmingen) for 60 min at 37°C. For beta-hexosaminidase (β -hex) assay, cell-free supernatants were collected after 60 min and incubated with 200 μ M 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 in order to quantify the total β -hex content. The β -hex content was quantified by measuring fluorescence at ex360/em452 nm. For the antigen-dependent approach, human mast cells were incubated with serum pools from milk -allergic (n=10) and milk-tolerant (n=10) donors, followed by incubation with *apo*- or *holo*-BLG (5 nM). Degranulation was assessed by measurement of released β -hexosaminidase in the supernatant and of unreleased enzyme in the respective cell lysate. The presented results were calculated as percentage release of total β -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 some data points were missing. When comparing different treatment on primary cells or binding of patient sera. Wilcoxon matched-pairs signed test for comparing two and RM-one-way ANOVA following Tukey's multiple comparisons test for more than three groups, were applied. All test were two-sided and considered significant, when $p < 0.05$.

Results

Quercetin-iron, but not ferrioxamine complexes are bound by BLG

Quercetin with a complex stability constant $\log \beta$ of 44.2 at pH 7.4^{31, 59} binds strongly to ferric iron at physiological pH. Importantly, BLG bound very strongly to iron(quercetin)₂ complexes (FeQ2, Fig.1A), with calculated affinities in the nM range (Fig. 1B), but not to non-catechol-based siderophores such as ferrioxamine. Complex formation with iron led to concentration-dependent quenching of quercetin fluorescence, (Fig.1C) and induced a visible color change (Fig. 1D). In spectral analysis, addition of BLG led to a marked decrease of the absorption peak at 330nm and seemed to “revert” the induced color change upon complex formation of quercetin-iron (Fig.1D).

These data confirmed that quercetin at physiological pH is usually present in a complex with iron and that BLG can absorb these complexes

Holo-BLG prevents antibody-formation and reduces splenocytic cytokine release *in vivo*.

Subsequently, we assessed whether ligand loading with FeQ2 to BLG had biological implications *in vivo*, by intranasally applying apo- or holoBLG to BALB/c mice. We thus applied in the mice 100fold less than an equivalent human dose for instance of BLG naturally contained in a 200ml glass of milk. The respiratory nasal mucosal surfaces are chronically exposed to myriad of antigens, commensal bacteria, but also iron. We needed to ensure that these local factors would not interfere with the apoBLG protocol so that during the sensitization process the calyx of apo-BLG would remain empty. Therefore, for the intranasal apoBLG treatment, equimolar concentration of the iron-chelator deferoxamine (DFO⁶⁰), a chemical approved by the Food and Drug Administration for treating iron overload, was added to BLG to

guarantee its apo-form upon mucosal application; a second group of mice were treated with “prefilled” holoBLG and the control groups were sham-treated with DFO or FeQ2 alone.

After 6 nasal applications (Fig. 2A) in the absence of any added adjuvants, only mice treated with the apo-form of BLG had significantly elevated serum-levels of BLG-specific IgG1, IgE, IgG2a and IgA antibodies, whereas nasal exposure of mice to holoBLG prevented production of all BLG-specific immunoglobulins classes (Fig. 2B). In fact, levels of antibodies in mice sensitized with holoBLG did not statistically differ from the control groups treated with DFO or FeQ2 alone.

In accordance with the failure to produce BLG-specific antibodies in the holoBLG-treated mice, their splenocytes, stimulated with BLG, secreted significantly lower levels of Th2- associated but also regulatory cytokines (Fig. 2C), showed significantly less proliferation, and had significantly more regulatory T cells, Tregs, compared to mice treated with apoBLG (Fig.3A+B).

In line with the observed suppressed immunological profile, allergen challenge led to systemic anaphylaxis only in the apoBLG treated mice, whereas mice treated with the ligand-loaded holoBLG were protected from body temperature drop, physical impairment and anaphylaxis symptoms (Fig. 3C).

As such, transport of FeQ2 ligands by BLG prevented specific immune responses that could lead to allergic symptoms or anaphylaxis.

The ligand quercetin provides an anti-inflammatory stimulus by activating the arylhydrocarbon receptor in vitro and in vivo.

As the sole difference in the BLG treatment protocols was the addition of ligands, we investigated the underlying molecular mechanisms for the quercetin-iron cargo of BLG. Among receptor candidates, activation of the cytoplasmic promiscuous arylhydrocarbon receptor (AhR) has been described to mediate anti-inflammatory feedback mechanisms⁶¹, to promote Tregs^{57, 62}⁶³ and to suppress Th2-skewing^{64 65} and antigen presentation.⁶⁶ AhR interacts with a plethora of exogenous ligands such as acrolein⁵⁷ and importantly also with plant flavonoids such as quercetin⁶⁷, emphasizing the participation of environmental or nutritional factors in immune regulation. Using reporter cells, we could demonstrate quercetin-dependent activation of the AhR-pathway (Fig. 4A), which was significantly enhanced upon addition of BLG, implying synergy with directed targeting to the BLG receptors (Fig 4B). Iron import is tightly regulated and requires active transport. As such, formation of iron complexes inhibited quercetin transport and consequently quercetin-dependent AhR-activation, which could - to a certain extent- be restored by addition of BLG. In line with the *in vitro* data, prominent CYP1A1 expression, a transcription factor downstream of AhR, was present in the lungs of holoBLG-treated mice compared to mice treated with apoBLG or controls (Fig.4 C and D). Hence, BLG is capable of transporting its cargo to, and releasing it into cells, leading to AhR-activation which contributes to the observed immune resilience.

Transport of quercetin-iron complexes increases intracellular iron in monocytes and leads to impaired antigen presentation.

The cargo of BLG also included the essential trace element iron. The iron status *per se* is critical for the activation status of antigen presenting cells, in particular of macrophages, with a decrease resulting in immune activation, whereas an increase of “complexed iron” leading to

immunosuppression⁶⁸⁻⁷³. This is in contrast to “free” non-complexed, not protein-bound iron, which leads to the formation of reactive oxygen species. As depicted in Figure 5 and Figure S1, in primary human monocytic cells, incubation with the ligand-loaded holo-form resulted in an increase of intracellular iron, as measured by the quenching of the calcein-signal compared to incubation with apo-BLG. Interestingly, this phenomenon was restricted to the monocytic CD14⁺ compartment (Fig.5A) and did not extend to CD3⁺ T cells, in which no increase in the intracellular iron-levels and no changes in the relative CD3⁺ numbers were observed (Fig.5 B, Fig. S3). Holo-BLG treatment did not result in increased cell death in the general cellular population (Fig. S2) but affected, as previously reported²³, the relative abundance of CD3⁺CD4⁺ subsets (Fig. S3) and of the CD14⁺ population (Fig. S1). Further analyses of the CD14⁺ population revealed that holo-BLG treatment decreased HLADR expression, resulting in lower relative numbers of CD14⁺HLADR⁺CD86⁺ cells (Fig.5C, Fig S1). In its apo-form, BLG was a potent immune stimulator and led to secretion of Th2-associated cytokines even in iron-deficient conditions (Fig.5 D and E). In contrast, uptake of BLG with ligands led to an increase of intracellular iron in monocytes and impaired antigen presentation by downregulating MHC class 2 and co-stimulatory molecules, thereby preventing immune activation and cytokine secretion.

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

As antigen presentation was clearly impaired, we further analyzed the possible direct impact of FeQ2 ligands on BLG epitope regions. We compared the ligand-binding site with known IgE and T cell epitopes of BLG. For BLG, two experimentally deduced dominant IgE epitope regions

were reported at residues K75-D85 in a loop and E127-P144 at the site of the alpha helix²⁶, with “weaker” IgE epitopes described at segments defined by residues L31-P48, K47-K60, and L57-I78. As depicted in Fig. 6A, the ligand binding site was in near proximity of the IgE epitope 1, which was described to be relevant in subjects with persistent milk-allergy⁷⁴, but did not affect epitope 2, which is located at the opposite site of epitope 1 on the alpha helix. To determine whether ligand-binding had any biological consequence in humans, we determined IgE-binding to BLG with or without ligands, in milk sensitized children who reacted positive (n=20) and negative (n=20) to oral cow milk challenge, termed milk allergic and milk tolerant respectively (Fig 6B and patients’ characteristics in Table S1). Indeed, the milk-allergic subjects had reduced IgE-binding to the holo-form of BLG, loaded with FeQ2 complexes, compared to apoBLG or when using an irrelevant chelator such as deferoxamine. Importantly, IgE-binding of milk-tolerant children was not affected by the ligands of BLG, emphasizing that IgE of milk tolerant children recognize different epitopes that are not in close proximity to the ligand-binding site. It also underscores that IgE in milk allergic children is formed under conditions in which BLG is devoid of ligands and thus fulfilling the requirements for allergenicity.

Next, we addressed whether ligand-binding also affected the immune dominant T cell epitope for BLG, located at residues 97-117, with the most important core residues Y102-E112 (YLLFCMENSAE)^{26, 75, 76}.

As clearly shown in Fig.6C, ligand-binding co-localized in particular with the T cell epitope residues F105, C106 and M107. Antigen stability is crucial for antigen processing and can determine the sensitizing potential of allergens by altering epitope specific T-cell activation as described for the major birch pollen allergen Bet v 1 in two studies^{77, 78}. Endolysosomal enzymes such as Cathepsin S process antigens to peptides for presentation. Interestingly, two of its

predicted cleavage sites are located within the dominant T cell epitope at BLGp99-105 and BLGp109-115²⁶, suggesting that also here ligands masking the position F105 prevent proper endolysosomal processing and antigen processing.

The co-localization strongly suggests that ligand-binding impaired the T-cell stimulatory capacity of the holoBLG in our *in vivo* murine model as well as in human peripheral blood mononuclear cells *in vitro* (Fig3A and 5D, respectively).

As FeQ2 binding to BLG masked IgE and T cell epitopes and affected antigen presentation, we also addressed whether the ligands alone had an impact in the effector phase, using primary human mast cells in an antigen-independent approach (Fig. 6D). Here, IgE sensitized mast cells were preincubated with BLG, quercetin or FeQ2 complexes, alone and in combinations thereof, before being crosslinked via anti-IgE. Also in this setting, only apoBLG and quercetin, which are able to sequester iron, promoted mast cell degranulation. In contrast, holoBLG did not further sensitize these cells, suggesting a role for iron in this resilience state (Fig.6D). This is in line with literature showing that dependent on the degree of iron saturation - lactoferrin and transferrin can inhibit the degranulation capacity of mast in vitro and in vivo⁷⁹⁻⁸¹.

Discussion

Here we revealed a previously unrecognized function of the lipocalin beta-lactoglobulin, the major whey compound of milk, to provide immune tolerance when loaded with FeQ2. Our data suggest that proper loading prevents the antigenicity of the cow milk allergen itself, by masking important immune epitopes on the one hand, but also by shuttling ligands to immune cells that down-tune their antigen presentation skills. HoloBLG shuttled complexed iron into antigen presenting cells but not into lymphocytes, and reduced the readiness of mast cells to degranulate.

Our data suggest that the nasal mucosa may represent a natural route for tolerance induction to airborne BLG from cattle farms, explaining the allergy-protective effect of farm living. We propose that BLG could be exploited for mucosal tolerance induction in preventive or therapeutic settings.

Molecular studies also showed that the tolerogenic aryl hydrocarbon receptor pathway was activated. AhR-expression levels vary in immune cells, with high expression levels found in monocytes⁸², dendritic cells, macrophages⁸³ and B cells^{84,85}. Within the T cell compartments, Th17 cells and, to a lesser extent Treg cells, express AhR⁸⁶. Importantly AhR activation has been reported to reduce inflammation^{87,88}, abrogate Th2 cell differentiation, downregulate FcεRI expression on antigen presenting cells⁶¹ and promote Tregs^{89,90}. In line with literature, we show concentration-dependent AhR activation by quercetin, which was significantly enhanced by the addition of BLG *in vitro* in a reporter cell system. Moreover, Cyp1A1 expression was significantly upregulated in the lungs of holoBLG-treated mice, which is associated with protection to allergic sensitization, a sustainment of Tregs and reduced clinical immediate type reactivity. As we were not able to detect Cyp1A1 expression in the controls, in which only iron-quercetin complexes were applied without BLG, we propose that BLG is needed for effective ligand transport and activation of the AhR pathway *in vivo*.

We observed a general non-responsiveness when applying BLG with its ligands *in vivo* in terms of humoral and cellular responses, with reduced release of cytokines including immune suppressive cytokine IL10. This resilience to immune activation resembles findings observed in not-yet allergic subjects, that neither possess more Tregs nor secrete more IL10 than allergic subjects⁹¹. This is in contrast to former allergic subjects with secondarily acquired tolerance, who clearly show increased Tregs and IL10 levels⁹².

We also identified iron as an underappreciated contributor to immune tolerance. Indeed, iron deficiency favours a Th2 response⁹³⁻⁹⁵ and allergy⁹⁶⁻⁹⁸, whereas too much iron leads to immunosuppression⁹⁹, partly by increasing the ratio of Tregs in relation to effector T cells^{100, 101}.

An increase in intracellular iron has been linked to impaired antigen presentation and is associated with a suppressive phenotype in macrophages *in vitro* and *in vivo*⁶⁹. In contrast, reduced cellular iron content in macrophages enhances the generation of pro-inflammatory cytokines⁷¹. Similarly, increased iron-levels negatively affect CD4+ counts¹⁰², and to a lesser extent CD8+ cells⁷². Accordingly, our *in vitro* evidence suggests that holoBLG is primarily taken up by antigen presenting cells rather than by T cells. Additionally, epitope masking, simultaneous increase of intracellular iron and initiation of the AhR cascade prevents effective antigen presentation¹⁰³. Our data also show an impact of iron in the effector phase of allergy, where it directly acts on mast cells in an antigen-unspecific manner and lowers mediator release. In contrast, iron-free apoBLG as well as quercetin, as an iron-chelator, alone or in combination with BLG, increased the degranulatory ability of these mast cells. Similarly, studies have shown that histamine release from mast cells can be inhibited *in vitro*⁷⁹ and *in vivo*⁷⁹ - dependent on their degree of iron saturation- with iron-laden lactoferrin and transferrin^{79, 81}.

The loading state of BLG could indeed be the nexus between the allergenic or tolerogenic state of this major whey compound and likely expand to other ligands such as retinoic acid that mask the immunodominant T-cell epitope of BLG²⁶.

This is also hinted by the reduced IgE recognition of the loaded BLG molecule by IgE of milk allergic children, implying that allergic children at a certain time point generated IgE against the “naked” epitopes of an apoBLG form. It is also in contrast to children sensitized but tolerating

milk, who did not discriminate between the filled or empty proteins, which may reflect IgE binding to minor BLG epitopes not affected by ligand binding.

The immune system has evolved to survey and respond to environmental stimuli. In this regard, beta-lactoglobulin as an innate defense protein seems to hunt for ligands and nutrients and in humans confer protection by calming immune cells, via its ability to transport ligands to our immune cells.

The study may also have implications for understanding the allergy- and asthma-protective effects of farm milk and living in a farm environment, as the holoBLG loaded with its ligands might preferentially occur in non-processed, non-defatted milk.

Indeed, binding of polyphenols (catechins^{104, 105}, quercetin^{36, 106}, luteolin¹⁰⁷, rutin³⁶, etc.) has been reported to increase the anti-oxidant activity of BLG^{105, 108, 109} and lead to enhanced intestinal uptake¹¹⁰. In contrast, BLG depletion reduces the antioxidant activities of milk by 50%, while purified BLG is only considered a mild antioxidant¹¹¹. Studies have also shown that the ability of BLG to bind to ligands such as retinol and palmitic acid is lost upon heating¹¹², similar the antioxidant activity in milk is reduced upon heating^{111, 113}, while at the same time its antigenicity increases^{26, 112}.

Collectively, we have discovered a previously unrecognized role of the major milk protein beta-lactoglobulin to transport ligands to immune cells, thereby providing an immune-regulatory effect resulting in resilience to immune activation and protection against allergic sensitization and symptoms. Our findings propose that spiking beta-lactoglobulin with natural ligands like quercetin or retinoic acid may pave the path towards a new generation of prophylactic or therapeutic approaches against the allergy epidemic.

Acknowledgments:

We thank Anton Vidovic, Manuela Czernohaus and Katharina Kienast for help in the experimental set-up and technical assistance. We thank Galateja Jordakieva for designing the Graphical Sketch and proof-reading the manuscript.

Author contributions: F.R-W. conceived, directed the research, conducted and designed the experiments, analyzed and interpreted the data and wrote the manuscript; S.M-A. conducted all mouse experiments and analysis, 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; A.R. and L.M.P. performed experiments, analyzed data and contributed to writing; A.F., E.U. and Z.D. provided samples, support and contributed to the writing, I.P-S. and K.H. conducted experiments, provided samples, support and contributed to the writing, E.J-J. financed and directed research, , and contributed to design of study and manuscript writing.

Supplementary Materials

Materials and Methods

Table S1. Characteristics of milk allergic and milk tolerant children

Fig. S1. Intracellular iron levels, CD14, HLADR, CD86 expression of different treated primary cells.

Fig. S2. No significant differences in cell death in the gated cell population.

Fig S3. HoloBLG treatment reduced intracellular iron and relative numbers of CD3+CD4+ cells

References

1. Jordakieva G, Kundi M, Untersmayr E, Pali-Scholl I, Reichardt B, Jensen-Jarolim E. Country-wide medical records infer increased allergy risk of gastric acid inhibition. *Nat Commun* 2019; 10:3298.
2. Stein MM, Hrusch CL, Gozdz J, Igartua C, Pivniouk V, Murray SE, et al. Innate Immunity and Asthma Risk in Amish and Hutterite Farm Children. *N Engl J Med* 2016; 375:411-21.
3. Ege MJ, Mayer M, Normand AC, Genuneit J, Cookson WO, Braun-Fahrlander C, et al. Exposure to environmental microorganisms and childhood asthma. *N Engl J Med* 2011; 364:701-9.
4. Waser M, Michels KB, Bieli C, Floistrup H, Pershagen G, von Mutius E, et al. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. *Clin Exp Allergy* 2007; 37:661-70.
5. Brick T, Schober Y, Bocking C, Pekkanen J, Genuneit J, Loss G, et al. omega-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. *J Allergy Clin Immunol* 2016; 137:1699-706 e13.
6. Jonsson K, Barman M, Moberg S, Sjoberg A, Brekke HK, Hesselmar B, et al. Fat intake and breast milk fatty acid composition in farming and nonfarming women and allergy development in the offspring. *Pediatr Res* 2016; 79:114-23.
7. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, et al. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. *J Allergy Clin Immunol* 2011; 128:766-73 e4.
8. Abbring S, Kusche D, Roos TC, Diks MAP, Hols G, Garssen J, et al. Milk processing increases the allergenicity of cow's milk-Preclinical evidence supported by a human proof-of-concept provocation pilot. *Clin Exp Allergy* 2019; 49:1013-25.
9. Lam HY, van Hoffen E, Michelsen A, Guikers K, van der Tas CH, Bruijnzeel-Koomen CA, et al. Cow's milk allergy in adults is rare but severe: both casein and whey proteins are involved. *Clin Exp Allergy* 2008; 38:995-1002.
10. Kaczmarek M, Wasilewska J, Cudowska B, Semeniuk J, Klukowski M, Matuszewska E. The natural history of cow's milk allergy in north-eastern Poland. *Adv Med Sci* 2013; 58:22-30.
11. Jensen-Jarolim E, Pacios LF, Bianchini R, Hofstetter G, Roth-Walter F. Structural similarities of human and mammalian lipocalins, and their function in innate immunity and allergy. *Allergy* 2016; 71:286-94.
12. Roth-Walter F, Berin MC, Arnaboldi P, Escalante CR, Dahan S, Rauch J, et al. Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. *Allergy* 2008; 63:882-90.
13. Fluckinger M, Merschak P, Hermann M, Haertle T, Redl B. Lipocalin-interacting-membrane-receptor (LIMR) mediates cellular internalization of beta-lactoglobulin. *Biochim Biophys Acta* 2008; 1778:342-7.
14. Mansouri A, Gueant JL, Capiaumont J, Pelosi P, Nabet P, Haertle T. Plasma membrane receptor for beta-lactoglobulin and retinol-binding protein in murine hybridomas. *Biofactors* 1998; 7:287-98.
15. Flower DR. Beyond the superfamily: the lipocalin receptors. *Biochim Biophys Acta* 2000; 1482:327-36.
16. Hesselink RW, Findlay JB. Expression, characterization and ligand specificity of lipocalin-1 interacting membrane receptor (LIMR). *Mol Membr Biol* 2013; 30:327-37.
17. Devireddy LR, Gazin C, Zhu X, Green MR. A cell-surface receptor for lipocalin 24p3 selectively mediates apoptosis and iron uptake. *Cell* 2005; 123:1293-305.
18. Roth-Walter F, Schmutz R, Mothes-Luksch N, Lemell P, Zieglmayer P, Zieglmayer R, et al. Clinical efficacy of sublingual immunotherapy is associated with restoration of steady-state serum lipocalin 2 after SLIT: a pilot study. *World Allergy Organ J* 2018; 11:21.
19. Wojnar P, Lechner M, Redl B. Antisense down-regulation of lipocalin-interacting membrane receptor expression inhibits cellular internalization of lipocalin-1 in human NT2 cells. *J Biol Chem* 2003; 278:16209-15.
20. Roth-Walter F, Jensen-Jarolim E, Gomez-Casado C, Diaz-Perales A, Pacios LF, J S. LCN2 as a tool for allergy diagnostic and therapy. . EP 14150965.3, US 14/204,570 2014.

21. Bao G, Clifton M, Hoette TM, Mori K, Deng SX, Qiu A, et al. Iron traffics in circulation bound to a siderocalin (Ngal)-catechol complex. *Nat Chem Biol* 2010; 6:602-9.
22. Fluckinger M, Haas H, Merschak P, Glasgow BJ, Redl B. Human tear lipocalin exhibits antimicrobial activity by scavenging microbial siderophores. *Antimicrob Agents Chemother* 2004; 48:3367-72.
23. Roth-Walter F, Pacios LF, Gomez-Casado C, Hofstetter G, Roth GA, Singer J, et al. The major cow milk allergen Bos d 5 manipulates T-helper cells depending on its load with siderophore-bound iron. *PLoS One* 2014; 9:e104803.
24. Chaneton L, Perez Saez JM, Bussmann LE. Antimicrobial activity of bovine beta-lactoglobulin against mastitis-causing bacteria. *J Dairy Sci* 2011; 94:138-45.
25. Kanakis CD, Hasni I, Bourassa P, Tarantilis PA, Polissiou MG, Tajmir-Riahi HA. Milk beta-lactoglobulin complexes with tea polyphenols. *Food Chem* 2011; 127:1046-55.
26. Hufnagl K, Ghosh D, Wagner S, Fiocchi A, Dahdah L, Bianchini R, et al. Retinoic acid prevents immunogenicity of milk lipocalin Bos d 5 through binding to its immunodominant T-cell epitope. *Sci Rep* 2018; 8:1598.
27. Stojadinovic M, Radosavljevic J, Ognjenovic J, Vesic J, Prodic I, Stanic-Vucinic D, et al. Binding affinity between dietary polyphenols and beta-lactoglobulin negatively correlates with the protein susceptibility to digestion and total antioxidant activity of complexes formed. *Food Chem* 2013; 136:1263-71.
28. Rawel HM, Rohn S, Kroll J. Influence of a sugar moiety (rhamnosylglucoside) at 3-O position on the reactivity of quercetin with whey proteins. *Int J Biol Macromol* 2003; 32:109-20.
29. Zhang L, Wang Y, Xu M, Hu X. Galloyl moieties enhance the binding of (-)-epigallocatechin-3-gallate to beta-lactoglobulin: A spectroscopic analysis. *Food Chem* 2017; 237:39-45.
30. Keppler JK, Martin D, Garamus VM, Schwarz K. Differences in binding behavior of (-)-epigallocatechin gallate to beta-lactoglobulin heterodimers (AB) compared to homodimers (A) and (B). *J Mol Recognit* 2015; 28:656-66.
31. Perron NR, Brumaghim JL. A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. *Cell Biochem Biophys* 2009; 53:75-100.
32. Perron NR, Hodges JN, Jenkins M, Brumaghim JL. Predicting how polyphenol antioxidants prevent DNA damage by binding to iron. *Inorg Chem* 2008; 47:6153-61.
33. Cook GM, Loder C, Soballe B, Stafford GP, Membrillo-Hernandez J, Poole RK. A factor produced by *Escherichia coli* K-12 inhibits the growth of *E. coli* mutants defective in the cytochrome bd quinol oxidase complex: enterochelin rediscovered. *Microbiology* 1998; 144 (Pt 12):3297-308.
34. Khodr HH, Hider RC, Duhme-Klair AK. The iron-binding properties of aminochelin, the mono(catecholamide) siderophore of *Azotobacter vinelandii*. *J Biol Inorg Chem* 2002; 7:891-6.
35. Roth-Walter F, Gomez-Casado C, Pacios LF, Mothes-Luksch N, Roth GA, Singer J, et al. Bet v 1 from birch pollen is a lipocalin-like protein acting as allergen only when devoid of iron by promoting Th2 lymphocytes. *J Biol Chem* 2014; 289:17416-21.
36. Mirpoor SF, Hosseini SMH, Nekoei AR. Efficient delivery of quercetin after binding to beta-lactoglobulin followed by formation soft-condensed core-shell nanostructures. *Food Chem* 2017; 233:282-9.
37. Sahihi M, Heidari-Koholi Z, Bordbar A-K. The Interaction of Polyphenol Flavonoids with β -lactoglobulin: Molecular Docking and Molecular Dynamics Simulation Studies. *Journal of Macromolecular Science, Part B* 2012; 51:2311-23.
38. Plundrich NJ, Bansode RR, Foegeding EA, Williams LL, Lila MA. Protein-bound Vaccinium fruit polyphenols decrease IgE binding to peanut allergens and RBL-2H3 mast cell degranulation in vitro. *Food Funct* 2017; 8:1611-21.
39. Jacob T, von Loetzen CS, Reuter A, Lacher U, Schiller D, Schobert R, et al. Identification of a natural ligand of the hazel allergen Cor a 1. *Sci Rep* 2019; 9:8714.
40. Garrido-Arandia M, Silva-Navas J, Ramirez-Castillejo C, Cubells-Baeza N, Gomez-Casado C, Barber D, et al. Characterisation of a flavonoid ligand of the fungal protein Alt a 1. *Sci Rep* 2016; 6:33468.

41. Besle JM, Viala D, Martin B, Pradel P, Meunier B, Berdague JL, et al. Ultraviolet-absorbing compounds in milk are related to forage polyphenols. *J Dairy Sci* 2010; 93:2846-56.
42. Yu ES, Min HJ, An SY, Won HY, Hong JH, Hwang ES. Regulatory mechanisms of IL-2 and IFN γ suppression by quercetin in T helper cells. *Biochem Pharmacol* 2008; 76:70-8.
43. Tong Z, Wu X, Ovcharenko D, Zhu J, Chen CS, Kehrer JP. Neutrophil gelatinase-associated lipocalin as a survival factor. *Biochem J* 2005; 391:441-8.
44. Kehrer JP. Lipocalin-2: pro- or anti-apoptotic? *Cell Biol Toxicol* 2010; 26:83-9.
45. Shields-Cutler RR, Crowley JR, Hung CS, Stapleton AE, Aldrich CC, Marschall J, et al. Human Urinary Composition Controls Antibacterial Activity of Siderocalin. *J Biol Chem* 2015; 290:15949-60.
46. Devireddy LR, Hart DO, Goetz DH, Green MR. A mammalian siderophore synthesized by an enzyme with a bacterial homolog involved in enterobactin production. *Cell* 2010; 141:1006-17.
47. Grabenhenrich LB, Reich A, McBride D, Sprickelman A, Roberts G, Grimshaw KEC, et al. Physician's appraisal vs documented signs and symptoms in the interpretation of food challenge tests: The EuroPrevall birth cohort. *Pediatr Allergy Immunol* 2018; 29:58-65.
48. Kontopidis G, Holt C, Sawyer L. The ligand-binding site of bovine beta-lactoglobulin: evidence for a function? *J Mol Biol* 2002; 318:1043-55.
49. Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, et al. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J Comput Chem* 2009; 30:2785-91.
50. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* 2010; 31:455-61.
51. Carson M. Ribbons. *Methods Enzymol* 1997; 277:493-505.
52. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* 2004; 25:1605-12.
53. Krissinel E, Henrick K. Inference of macromolecular assemblies from crystalline state. *J Mol Biol* 2007; 372:774-97.
54. Moreland JL, Gramada A, Buzko OV, Zhang Q, Bourne PE. The Molecular Biology Toolkit (MBT): a modular platform for developing molecular visualization applications. *BMC Bioinformatics* 2005; 6:21.
55. Manzano-Szalai K, Pali-Scholl I, Krishnamurthy D, Stremnitzer C, Flaschberger I, Jensen-Jarolim E. Anaphylaxis Imaging: Non-Invasive Measurement of Surface Body Temperature and Physical Activity in Small Animals. *PLoS One* 2016; 11:e0150819.
56. Novotna A, Pavek P, Dvorak Z. Novel stably transfected gene reporter human hepatoma cell line for assessment of aryl hydrocarbon receptor transcriptional activity: construction and characterization. *Environ Sci Technol* 2011; 45:10133-9.
57. Roth-Walter F, Bergmayr C, Meitz S, Buchleitner S, Stremnitzer C, Fazekas J, et al. Janus-faced Acrolein prevents allergy but accelerates tumor growth by promoting immunoregulatory Foxp3⁺ cells: Mouse model for passive respiratory exposure. *Sci Rep* 2017; 7:45067.
58. Yu Y, Blokhuis BR, Garssen J, Redegeld FA. A Transcriptomic Insight into the Impact of Colon Cancer Cells on Mast Cells. *Int J Mol Sci* 2019; 20.
59. El Hajji H, Nkhili E, Tomao V, Dangles O. Interactions of quercetin with iron and copper ions: complexation and autoxidation. *Free Radic Res* 2006; 40:303-20.
60. Fine JM, Baillargeon AM, Renner DB, Hoerster NS, Tokarev J, Colton S, et al. Intranasal deferoxamine improves performance in radial arm water maze, stabilizes HIF-1 α , and phosphorylates GSK3 β in P301L tau transgenic mice. *Exp Brain Res* 2012; 219:381-90.
61. Koch S, Stroisch TJ, Vorac J, Herrmann N, Leib N, Schnautz S, et al. AhR mediates an anti-inflammatory feedback mechanism in human Langerhans cells involving Fc ϵ RI and IDO. *Allergy* 2017; 72:1686-93.

62. Gandhi R, Kumar D, Burns EJ, Nadeau M, Dake B, Laroni A, et al. Activation of the aryl hydrocarbon receptor induces human type 1 regulatory T cell-like and Foxp3(+) regulatory T cells. *Nat Immunol* 2010; 11:846-53.
63. Quintana FJ, Basso AS, Iglesias AH, Korn T, Farez MF, Bettelli E, et al. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* 2008; 453:65-71.
64. Xu T, Zhou Y, Qiu L, Do DC, Zhao Y, Cui Z, et al. Aryl Hydrocarbon Receptor Protects Lungs from Cockroach Allergen-Induced Inflammation by Modulating Mesenchymal Stem Cells. *J Immunol* 2015; 195:5539-50.
65. Negishi T, Kato Y, Ooneda O, Mimura J, Takada T, Mochizuki H, et al. Effects of aryl hydrocarbon receptor signaling on the modulation of TH1/TH2 balance. *J Immunol* 2005; 175:7348-56.
66. Aguilera-Montilla N, Chamorro S, Nieto C, Sanchez-Cabo F, Dopazo A, Fernandez-Salguero PM, et al. Aryl hydrocarbon receptor contributes to the MEK/ERK-dependent maintenance of the immature state of human dendritic cells. *Blood* 2013; 121:e108-17.
67. Jin UH, Park H, Li X, Davidson LA, Allred C, Patil B, et al. Structure-Dependent Modulation of Aryl Hydrocarbon Receptor-Mediated Activities by Flavonoids. *Toxicol Sci* 2018; 164:205-17.
68. Carrasco-Marin E, Alvarez-Dominguez C, Lopez-Mato P, Martinez-Palencia R, Leyva-Cobian F. Iron salts and iron-containing porphyrins block presentation of protein antigens by macrophages to MHC class II-restricted T cells. *Cell Immunol* 1996; 171:173-85.
69. Agoro R, Taleb M, Quesniaux VFJ, Mura C. Cell iron status influences macrophage polarization. *PLoS One* 2018; 13:e0196921.
70. Nairz M, Theurl I, Swirski FK, Weiss G. "Pumping iron"-how macrophages handle iron at the systemic, microenvironmental, and cellular levels. *Pflugers Arch* 2017; 469:397-418.
71. Nairz M, Schroll A, Haschka D, Dichtl S, Sonnweber T, Theurl I, et al. Lipocalin-2 ensures host defense against *Salmonella Typhimurium* by controlling macrophage iron homeostasis and immune response. *Eur J Immunol* 2015; 45:3073-86.
72. Hagag AA, Elgamasy MA, Abd Elbar ES. Study of Serum Immunoglobulin Levels and T lymphocyte Subsets in Children with Beta Thalassemia with Iron Overload in Egypt. *Egypt J Immunol* 2016; 23:97-105.
73. Morikawa K, Oseko F, Morikawa S. A role for ferritin in hematopoiesis and the immune system. *Leuk Lymphoma* 1995; 18:429-33.
74. Jarvinen KM, Beyer K, Vila L, Chatchatee P, Busse PJ, Sampson HA. B-cell epitopes as a screening instrument for persistent cow's milk allergy. *J Allergy Clin Immunol* 2002; 110:293-7.
75. Inoue R, Matsushita S, Kaneko H, Shinoda S, Sakaguchi H, Nishimura Y, et al. Identification of beta-lactoglobulin-derived peptides and class II HLA molecules recognized by T cells from patients with milk allergy. *Clin Exp Allergy* 2001; 31:1126-34.
76. Sakaguchi H, Inoue R, Kaneko H, Watanabe M, Suzuki K, Kato Z, et al. Interaction among human leucocyte antigen-peptide-T cell receptor complexes in cow's milk allergy: the significance of human leucocyte antigen and T cell receptor-complementarity determining region 3 loops. *Clin Exp Allergy* 2002; 32:762-70.
77. Freier R, Dall E, Brandstetter H. Protease recognition sites in Bet v 1a are cryptic, explaining its slow processing relevant to its allergenicity. *Sci Rep* 2015; 5:12707.
78. Machado Y, Freier R, Scheiblhofer S, Thalhamer T, Mayr M, Briza P, et al. Fold stability during endolysosomal acidification is a key factor for allergenicity and immunogenicity of the major birch pollen allergen. *J Allergy Clin Immunol* 2016; 137:1525-34.
79. Theobald K, Gross-Weege W, Keymling J, Konig W. Inhibition of histamine release in vitro by a blocking factor from human serum: comparison with the iron binding proteins transferrin and lactoferrin. *Agents Actions* 1987; 20:10-6.
80. Theobald K, Gross-Weege W, Keymling J, Konig W. Purification of serum proteins with inhibitory activity on the histamine release in vitro and/or in vivo. *Int Arch Allergy Appl Immunol* 1987; 82:295-7.

81. Mecheri S, Peltre G, Lapeyre J, David B. Biological effect of transferrin on mast cell mediator release during the passive cutaneous anaphylaxis reaction: a possible inhibition mechanism involving iron. *Ann Inst Pasteur Immunol* 1987; 138:213-21.
82. Skazik-Voogt C, Kuhler K, Ott H, Czaja K, Zwadlo-Klarwasser G, Merk HF, et al. Myeloid human cell lines lack functional regulation of aryl hydrocarbon receptor-dependent phase I genes. *ALTEX* 2016; 33:37-46.
83. Kreitinger JM, Beamer CA, Shepherd DM. Environmental Immunology: Lessons Learned from Exposure to a Select Panel of Immunotoxicants. *J Immunol* 2016; 196:3217-25.
84. Vaidyanathan B, Chaudhry A, Yewdell WT, Angeletti D, Yen WF, Wheatley AK, et al. The aryl hydrocarbon receptor controls cell-fate decisions in B cells. *J Exp Med* 2017; 214:197-208.
85. Villa M, Gialitakis M, Tolaini M, Ahlfors H, Henderson CJ, Wolf CR, et al. Aryl hydrocarbon receptor is required for optimal B-cell proliferation. *EMBO J* 2017; 36:116-28.
86. Esser C, Rannug A, Stockinger B. The aryl hydrocarbon receptor in immunity. *Trends Immunol* 2009; 30:447-54.
87. Bessede A, Gargaro M, Pallotta MT, Matino D, Servillo G, Brunacci C, et al. Aryl hydrocarbon receptor control of a disease tolerance defence pathway. *Nature* 2014; 511:184-90.
88. Goettel JA, Gandhi R, Kenison JE, Yeste A, Murugaiyan G, Sambanthamoorthy S, et al. AHR Activation Is Protective against Colitis Driven by T Cells in Humanized Mice. *Cell Rep* 2016; 17:1318-29.
89. Ye J, Qiu J, Bostick JW, Ueda A, Schjerven H, Li S, et al. The Aryl Hydrocarbon Receptor Preferentially Marks and Promotes Gut Regulatory T Cells. *Cell Rep* 2017; 21:2277-90.
90. Couroucl XI, Welty SE, Geske RS, Moorthy B. Regulation of pulmonary and hepatic cytochrome P4501A expression in the rat by hyperoxia: implications for hyperoxic lung injury. *Mol Pharmacol* 2002; 61:507-15.
91. Bacher P, Heinrich F, Stervbo U, Nienen M, Vahldieck M, Iwert C, et al. Regulatory T Cell Specificity Directs Tolerance versus Allergy against Aeroantigens in Humans. *Cell* 2016; 167:1067-78 e16.
92. Qamar N, Fishbein AB, Erickson KA, Cai M, Szychliński C, Bryce PJ, et al. Naturally occurring tolerance acquisition to foods in previously allergic children is characterized by antigen specificity and associated with increased subsets of regulatory T cells. *Clin Exp Allergy* 2015; 45:1663-72.
93. Thorson JA, Smith KM, Gomez F, Naumann PW, Kemp JD. Role of iron in T cell activation: TH1 clones differ from TH2 clones in their sensitivity to inhibition of DNA synthesis caused by IgG Mabs against the transferrin receptor and the iron chelator deferoxamine. *Cell Immunol* 1991; 134:126-37.
94. Jason J, Archibald LK, Nwanyanwu OC, Bell M, Jensen RJ, Gunter E, et al. The effects of iron deficiency on lymphocyte cytokine production and activation: preservation of hepatic iron but not at all cost. *Clin Exp Immunol* 2001; 126:466-73.
95. Leung S, Holbrook A, King B, Lu HT, Evans V, Miyamoto N, et al. Differential inhibition of inducible T cell cytokine secretion by potent iron chelators. *J Biomol Screen* 2005; 10:157-67.
96. Drury KE, Schaeffer M, Silverberg JI. Association Between Atopic Disease and Anemia in US Children. *JAMA Pediatr* 2016; 170:29-34.
97. Nwaru BI, Hayes H, Gambling L, Craig LC, Allan K, Prabhu N, et al. An exploratory study of the associations between maternal iron status in pregnancy and childhood wheeze and atopy. *Br J Nutr* 2014; 112:2018-27.
98. Shaheen SO, Macdonald-Wallis C, Lawlor DA, Henderson AJ. Haemoglobin concentrations in pregnancy and respiratory and allergic outcomes in childhood: Birth cohort study. *Clin Exp Allergy* 2017; 47:1615-24.
99. Shaheen SO, Newson RB, Henderson AJ, Emmett PM, Sherriff A, Cooke M, et al. Umbilical cord trace elements and minerals and risk of early childhood wheezing and eczema. *Eur Respir J* 2004; 24:292-7.
100. Zhang Q, Cui T, Chang Y, Zhang W, Li S, He Y, et al. HO-1 regulates the function of Treg: Association with the immune intolerance in vitiligo. *J Cell Mol Med* 2018; 22:4335-43.

101. Chen J, Lu WY, Zhao MF, Cao XL, Jiang YY, Jin X, et al. Reactive oxygen species mediated T lymphocyte abnormalities in an iron-overloaded mouse model and iron-overloaded patients with myelodysplastic syndromes. *Ann Hematol* 2017; 96:1085-95.
102. Cronin SJF, Seehus C, Weidinger A, Talbot S, Reissig S, Seifert M, et al. The metabolite BH4 controls T cell proliferation in autoimmunity and cancer. *Nature* 2018; 563:564-8.
103. Yang D, Shui T, Miranda JW, Gilson DJ, Song Z, Chen J, et al. Mycobacterium leprae-Infected Macrophages Preferentially Primed Regulatory T Cell Responses and Was Associated with Lepromatous Leprosy. *PLoS Negl Trop Dis* 2016; 10:e0004335.
104. Chen W, Wang W, Ma X, Lv R, Balaso Watharkar R, Ding T, et al. Effect of pH-shifting treatment on structural and functional properties of whey protein isolate and its interaction with (-)-epigallocatechin-3-gallate. *Food Chem* 2019; 274:234-41.
105. Tao F, Xiao C, Chen W, Zhang Y, Pan J, Jia Z. Covalent modification of beta-lactoglobulin by (-)-epigallocatechin-3-gallate results in a novel antioxidant molecule. *Int J Biol Macromol* 2019; 126:1186-91.
106. Salvi A, Carrupt P, Tillement J, Testa B. Structural damage to proteins caused by free radicals: assessment, protection by antioxidants, and influence of protein binding. *Biochem Pharmacol* 2001; 61:1237-42.
107. Li X, Lu Y, Deng R, Zheng T, Lv L. Chemical components from the haulm of *Artemisia selengensis* and the inhibitory effect on glycation of beta-lactoglobulin. *Food Funct* 2015; 6:1841-6.
108. Zommaro M, Toubro H, Sakono M, Imaizumi K. Prevention of peroxidative stress in rats fed on a low vitamin E-containing diet by supplementing with a fermented bovine milk whey preparation: effect of lactic acid and beta-lactoglobulin on the antiperoxidative action. *Biosci Biotechnol Biochem* 1998; 62:710-7.
109. Bartfay WJ, Davis MT, Medves JM, Lugowski S. Milk whey protein decreases oxygen free radical production in a murine model of chronic iron-overload cardiomyopathy. *Can J Cardiol* 2003; 19:1163-8.
110. Wang X, Ai T, Meng XL, Zhou J, Mao XY. In vitro iron absorption of alpha-lactalbumin hydrolysate-iron and beta-lactoglobulin hydrolysate-iron complexes. *J Dairy Sci* 2014; 97:2559-66.
111. Liu HC, Chen WL, Mao SJ. Antioxidant nature of bovine milk beta-lactoglobulin. *J Dairy Sci* 2007; 90:547-55.
112. Song CY, Chen WL, Yang MC, Huang JP, Mao SJ. Epitope mapping of a monoclonal antibody specific to bovine dry milk: involvement of residues 66-76 of strand D in thermal denatured beta-lactoglobulin. *J Biol Chem* 2005; 280:3574-82.
113. Kim YE, Kim JW, Cheon S, Nam MS, Kim KK. Alpha-Casein and Beta-Lactoglobulin from Cow Milk Exhibit Antioxidant Activity: A Plausible Link to Antiaging Effects. *J Food Sci* 2019; 84:3083-90.

Figure legends:

Fig. 1 Quercetin-iron complex formation and binding to BLG. **A**, Model of beta-lactoglobulin, BLG, incorporating iron(quercetin)₂ (FeQ2) or ferrioxamine complexes. **B**, Calculated affinities of quercetin as described by Roth-Walter F et al.²³ and desferrioxamine in the presence and absence of iron to BLG by AutoDock VINA **C**, Fluorescence quenching of quercetin upon addition of iron and **D**, optical spectra and color change of 100μM quercetin and 50μM desferrioxamine, iron, BLG and combinations thereof at pH 7.3.

Fig. 2 Holo-BLG treatment prevents antibody formation and reduces splenocytic cytokine-response. **A**, As depicted in the sensitization scheme, mice were intranasally sensitized 6 times in biweekly intervals with either apoBLG and equimolar concentrations of DFO in the apoBLG group, or apoBLG in combination with iron-quercetin complexes, representing the holoBLG group, or were sham-treated with iron-quercetin complexes alone (FeQ2) or deferoxamine alone (DFO). Subsequently, mice were challenged with BLG i.p and allergic response was monitored before euthanasia. **B**, BLG-specific IgG1, IgE, IgG2a, IgA-levels in serum as well as **C**, splenocytic cytokine response of mice intranasally treated with deferoxamine (DFO, n=5), FeQ2 (n=5), apoBLG (n=5), holoBLG (n=5). Representative data from three independent experiments are shown. Groups were compared by ANOVA following Tukey's multiple comparisons test, Mean ± SEM; ****P* < 0.001, *****P* < 0.0001.

Fig. 3 HoloBLG treatment prevents lymphocyte proliferation, promotes regulatory T cells and protects against allergic reactions. Splenocytes of the groups treated either with DFO, apoBLG, holoBLG or FeQ2 were analysed for **A**, CD71 expression and total numbers of splenocytes per spleen, **B**, for CD4 and Foxp3 coexpression. **C**, Body temperature drop, symptom score and representative images of body temperature (blue to red= low to high temperature) and movements (lines) recorded by the imaging cage after systemic challenge with BLG in the different treated groups. Representative data from three independent experiments are shown. Groups were compared by one-way ANOVA following Tukey's multiple comparisons test. Mean ± SEM; **P* < 0.05; ***P* < 0.01; ****P* < 0.001, *****P* < 0.0001.

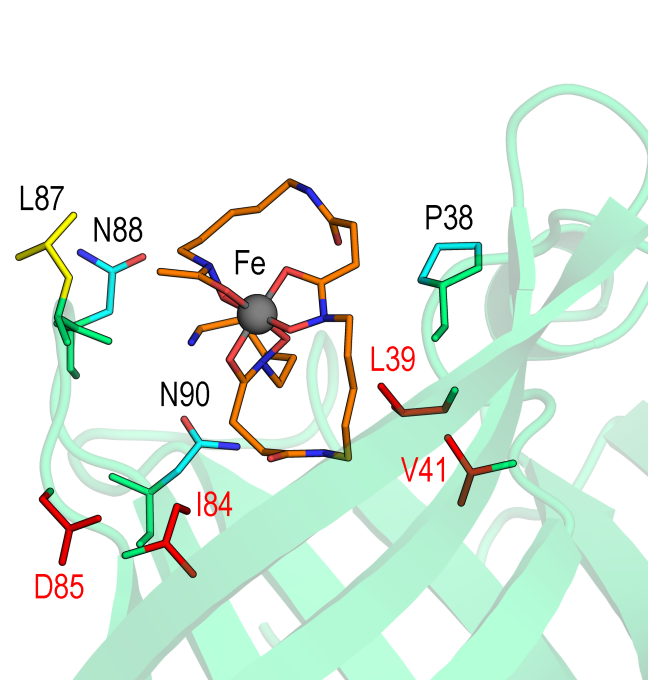
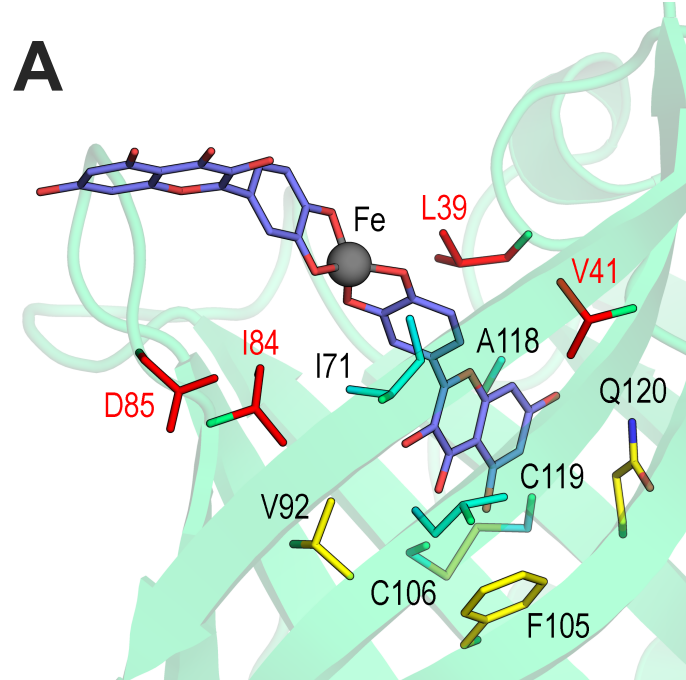
Fig. 4 BLG promotes quercetin-dependent AHR activation. AZ-AhR cells were treated with **A**, increasing concentrations of quercetin alone (0 /4.5/9/45/90μM), or quercetin and 2μM BLG **B**, with 90μM quercetin in combination with 45μM iron and/or 2μM BLG for 18 h, before luciferase-activity was measured in the supernatant. Representative data from three independent experiments normalized to medium alone. **C**, Representative regions of lung sections stained for Cyp1A1 and **D**, quantification of Cyp1A1 expression per image in the different treated mice. Concentration-dependent activation of AHR **A**, was compared using mixed-effects analysis following Sidak's multiple comparisons test. Differences of quercetin-activation in the presence or absence of iron and BLG and Cyp1a1 expression were tested by one-way ANOVA following Tukey's multiple comparisons test, Mean ± STD; ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

Fig. 5 HoloBLG increases intracellular iron in monocytic cells but not T cells. Peripheral blood mononuclear cells isolated by density gradient (n=16) were incubated overnight in iron-free media and in the presence or absence of apoBLG (blue curve) or holoBLG (orange curve), before analysis for increase in intracellular iron-levels by means of quenching of the calcein-signal. Summary of the calcein-signal in the **A**, monocytic gate and **B**, CD3⁺ cells. **C**, HLADR+CD86⁺ expression of CD14⁺ cells. **D**, IL10 **E**, IL13 **F**, IFN γ levels after 18h incubation in iron-free media. Data from five independently performed experiments with a total of n=16 subjects are shown. Groups were compared with Wilcoxon matched-pairs signed test, Mean $\pm$ SEM; **P* < 0.05, ***P* < 0.01

Fig. 6 Ligand binding masks B cell and T cell epitopes of BLG affecting IgE binding and antigen-independent mast cell degranulation. Localization in the structure (cartoons and sticks in upper row) and in the surface (lower row) of **A**, two IgE-binding epitopes in BLG: epitope 1 (residues 75-85) colored magenta and epitope 2 (residues 127-144) colored cyan. Labels in red indicate residues within 4 Å distance of the Fe(queracetin)₂ ligand, shown as sticks with carbons in deep blue in the three cases. In the right column ligands are depicted as partially transparent spheres and sticks underneath. **B**, IgE binding to BLG alone or in combination with deferoxamine and iron-queracetin in milk allergic (n=20) and milk tolerant children (n=20) respectively. **C**, The dominant T-cell epitope defined by residues Y102-E112 marked in orange in two views of the BLG-Fe(queracetin)₂ FeQ2 complex. Surface images correspond to the orientation shown in the cartoon images. **D**, Antigen-independent impact of BLG in combination with the ligands on human mast cell degranulation. Groups were compared for B, by RM-one-way ANOVA following Tukey's multiple comparisons test and for D, by one-way ANOVA following Tukey's multiple comparisons test. Mean $\pm$ SEM; **P* < 0.05, *****P* < 0.0001

Table S1. Characteristics of milk allergic and milk tolerant children

Characteristics of patients	milk-allergic	milk tolerant	p-Value
n	20	20	ns.
Age in months; Mean $\pm$ SD	71 $\pm$ 57	52 $\pm$ 30	0,197
gender; f/m	7/13	5/15	
IgE total kU/L ; Mean $\pm$ SD	867 $\pm$ 2718	235 $\pm$ 283	0,334
IgE milk kU/L ; Mean $\pm$ SD	29 $\pm$ 25	2.1 $\pm$ 6.9	0,001

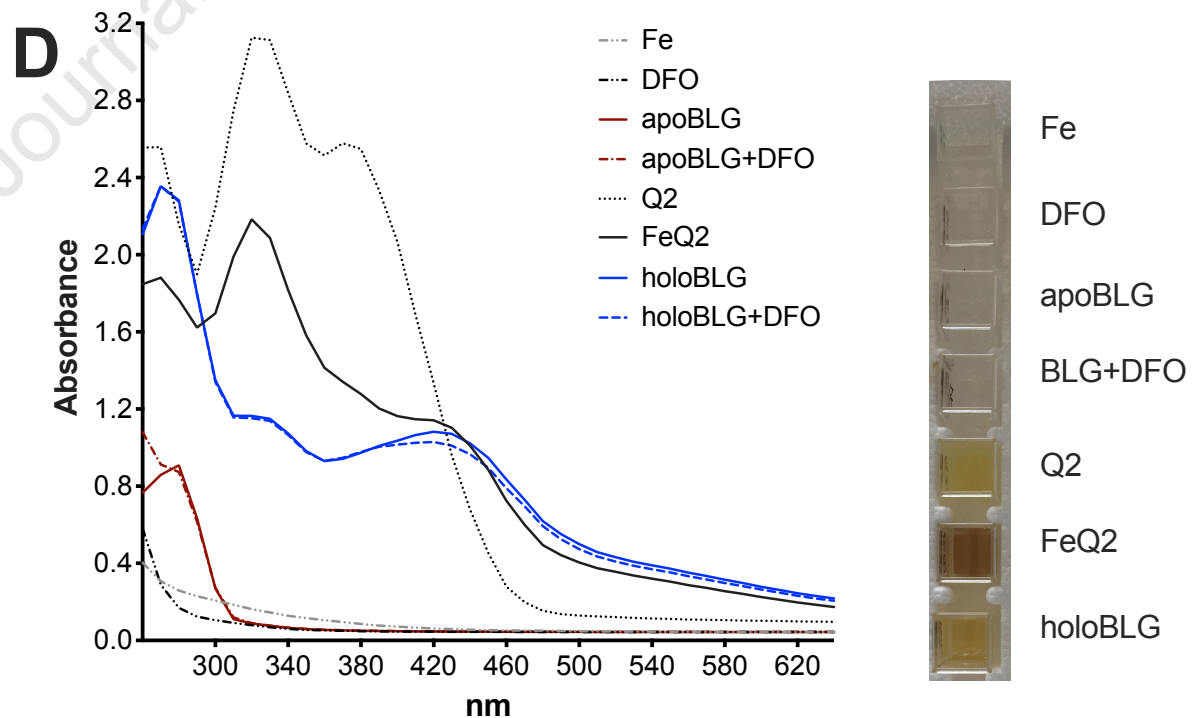
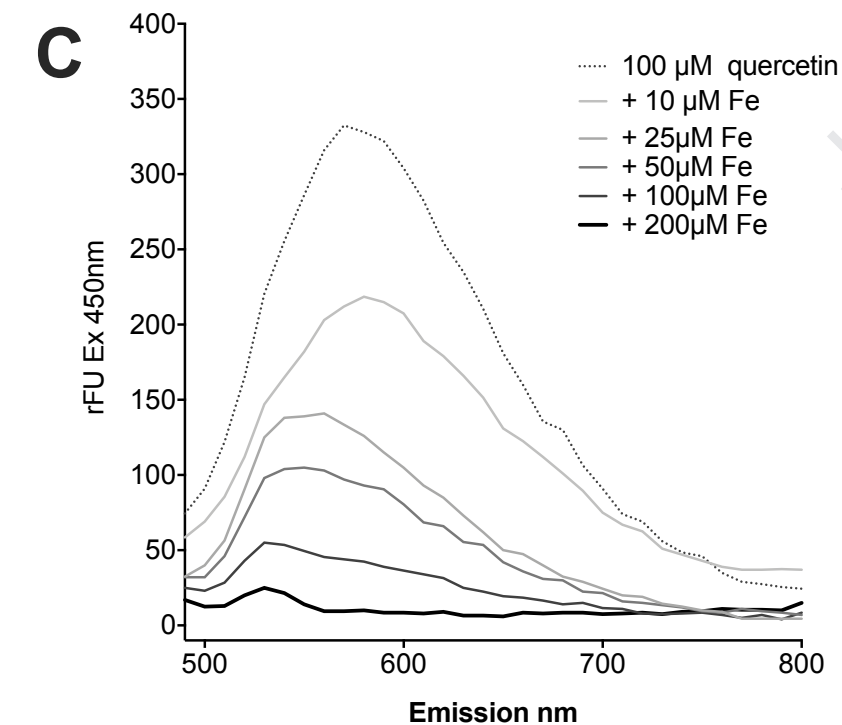


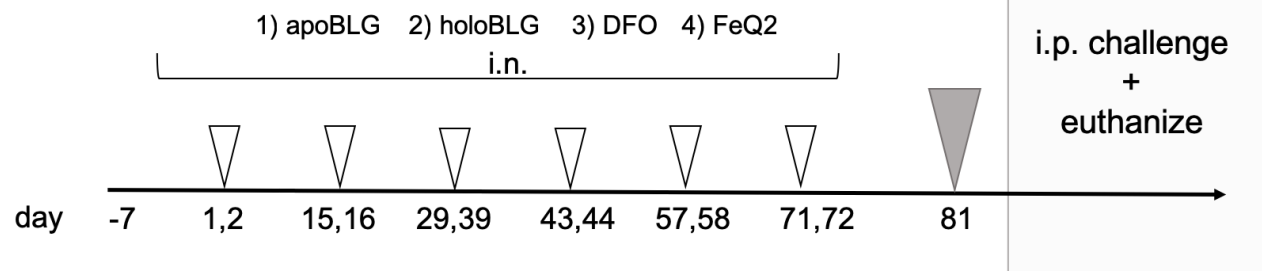
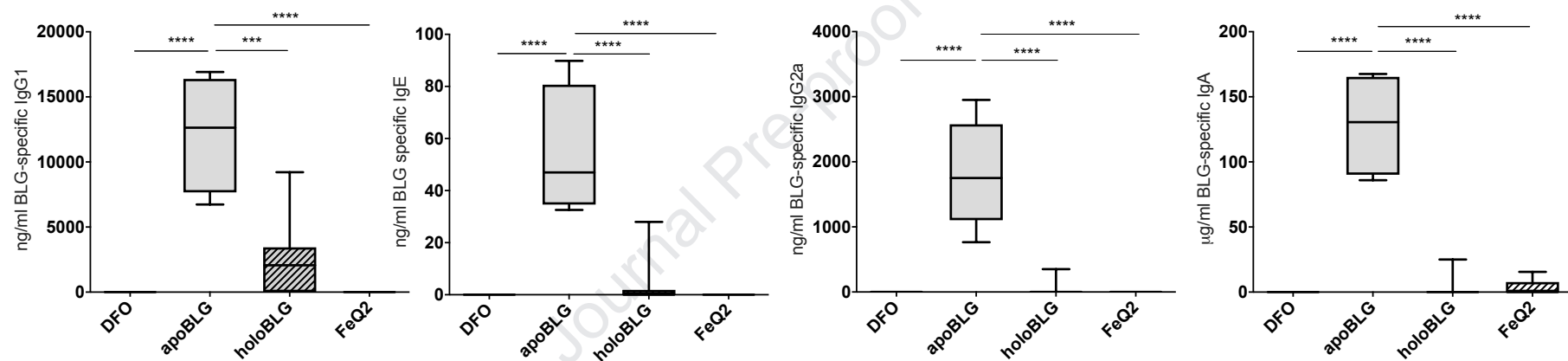
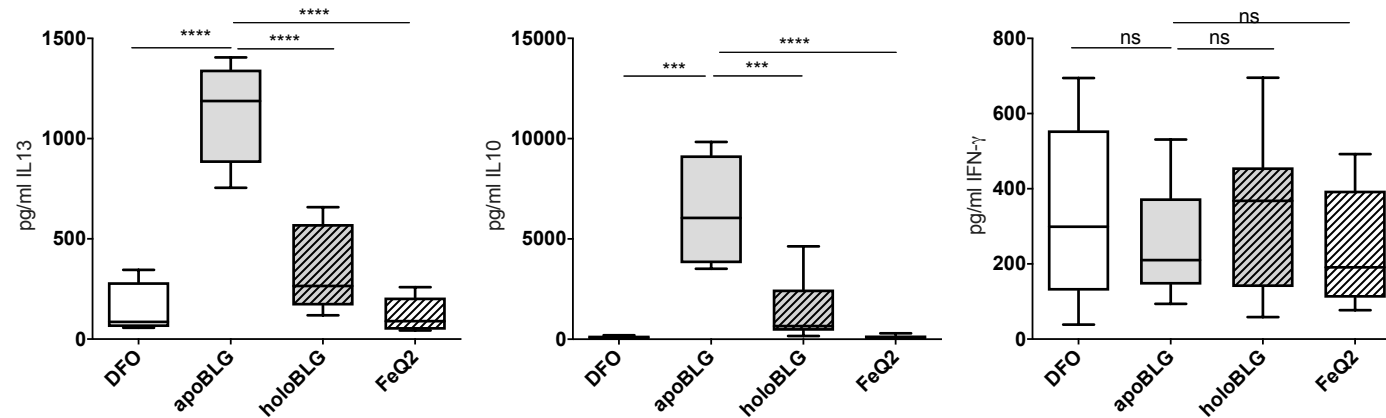
B

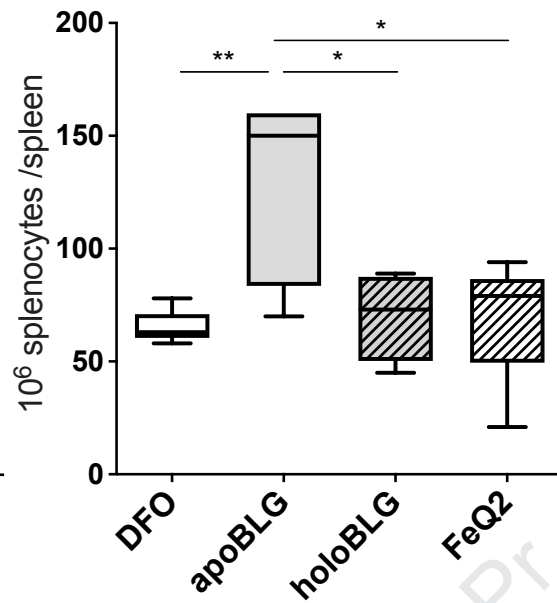
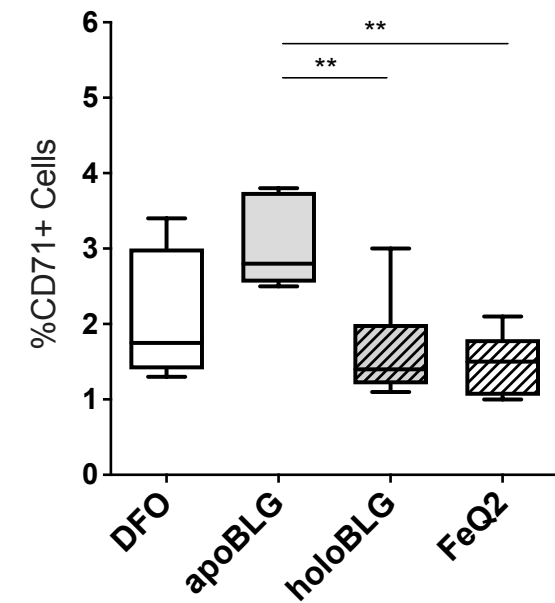
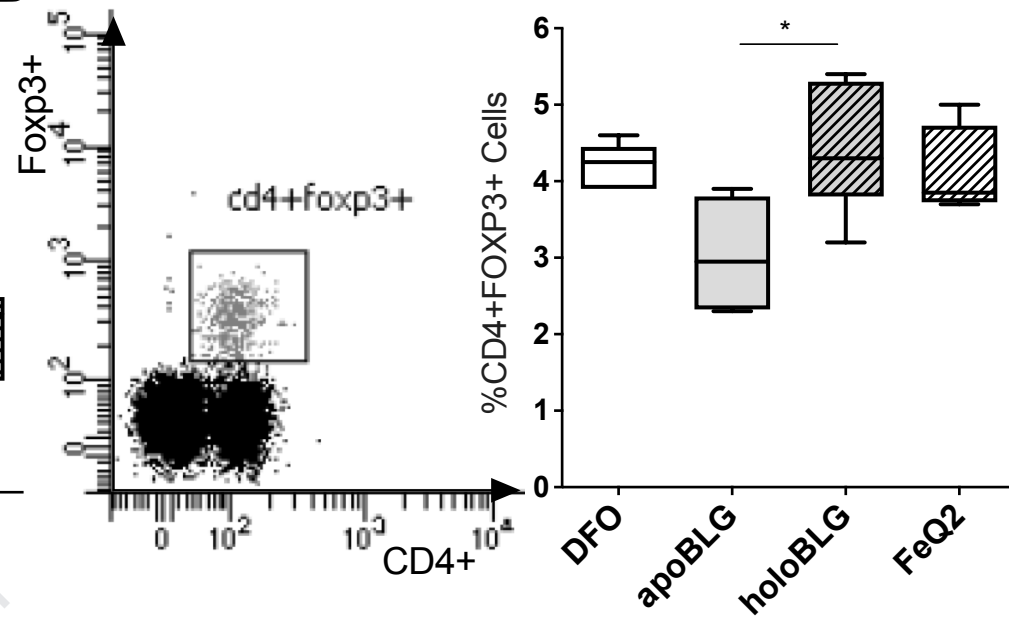
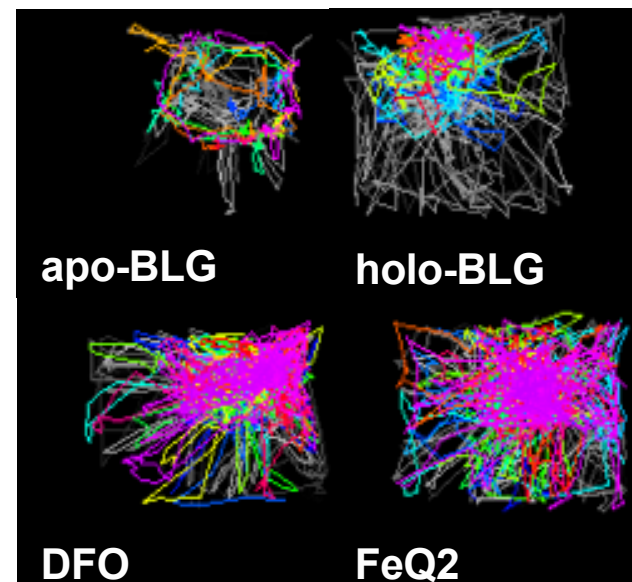
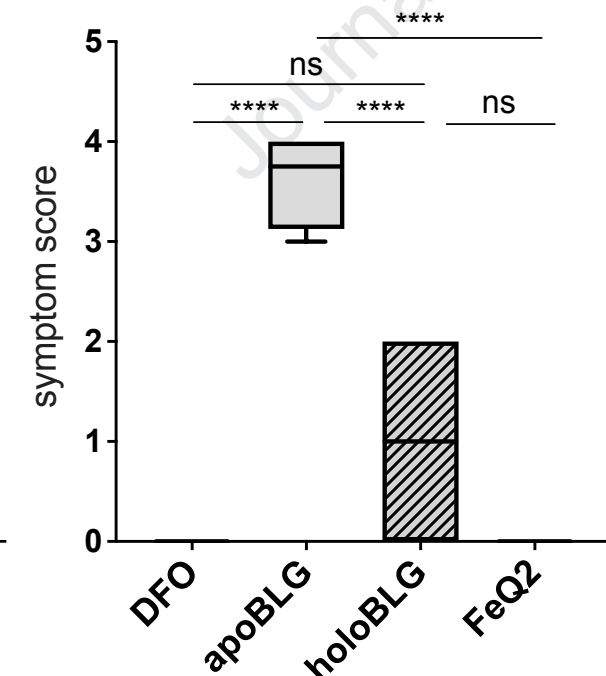
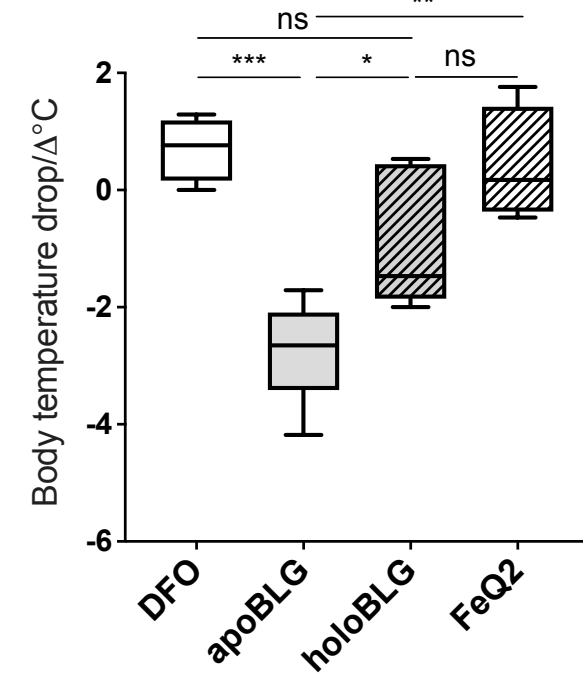
BLG (1GX9)		
Ligand	Energy	Kd (μ M)
Quercetin	-7.9	1.42
Fe(quercetin)1	-8.5	0.51
Fe(quercetin)2	-9.4	0.11
Desferrioxamine, DFO	-5.9	47
Ferrioxamine	-6.3	24

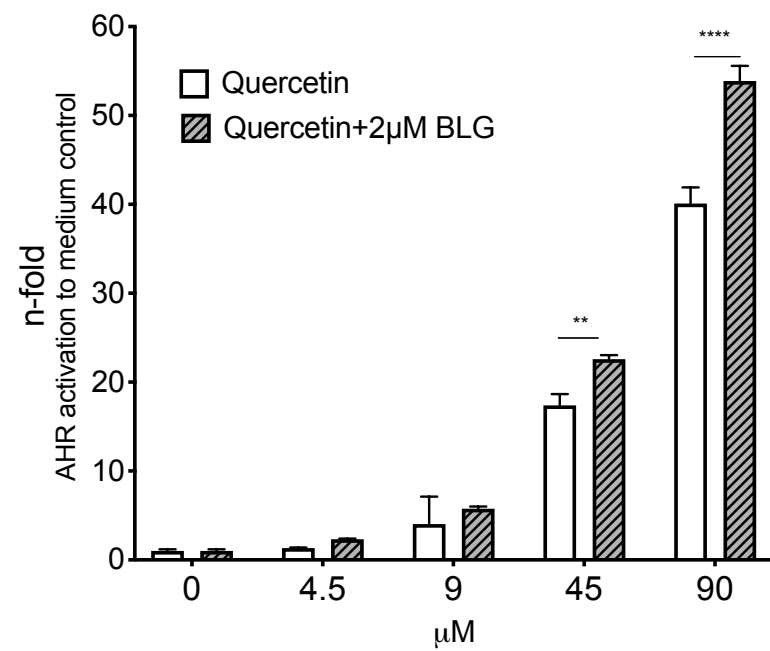
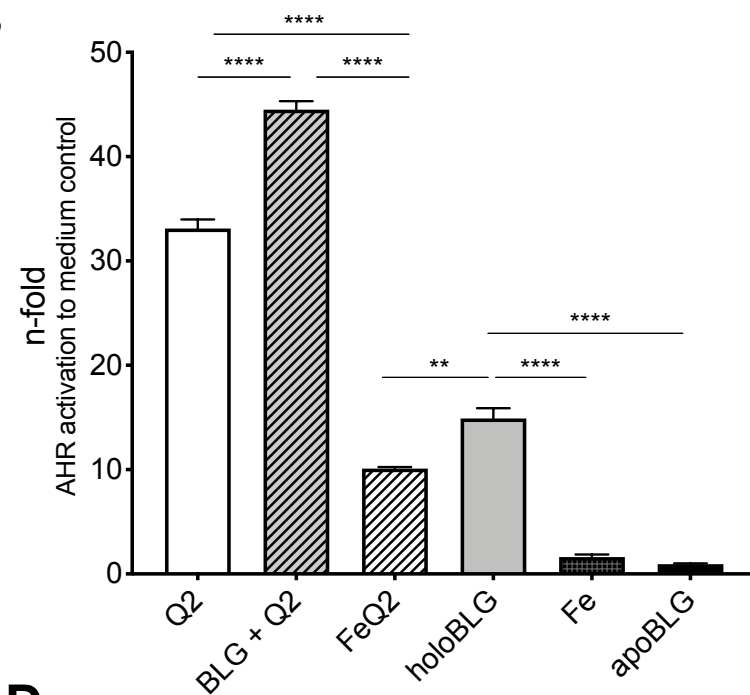
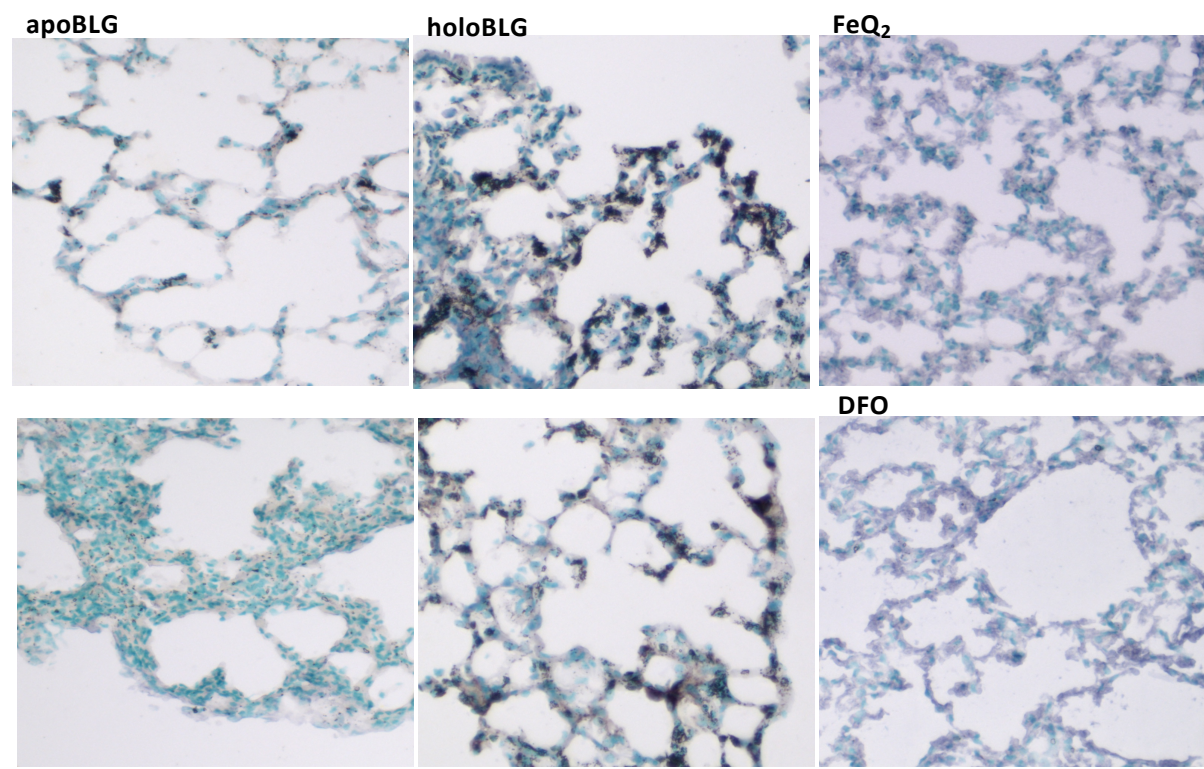
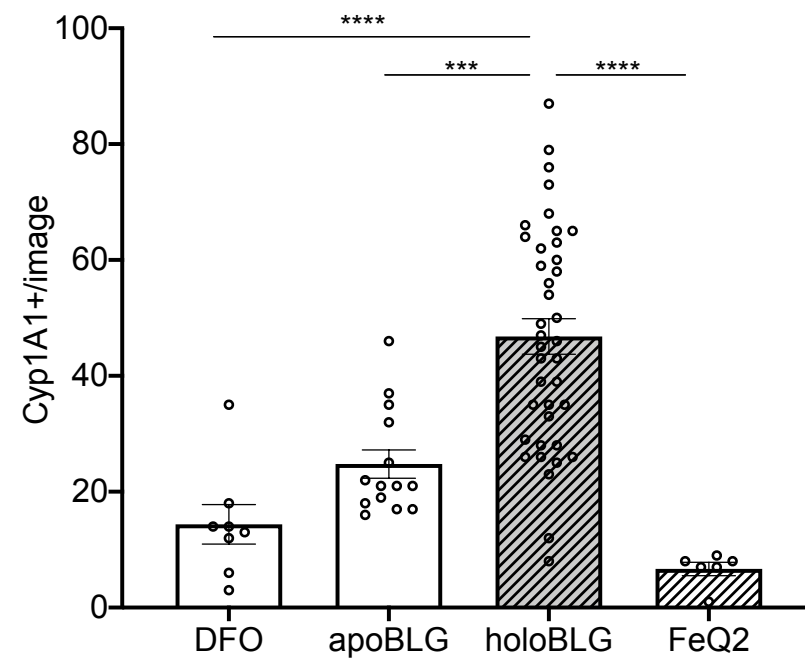
Fe(quercetin)2

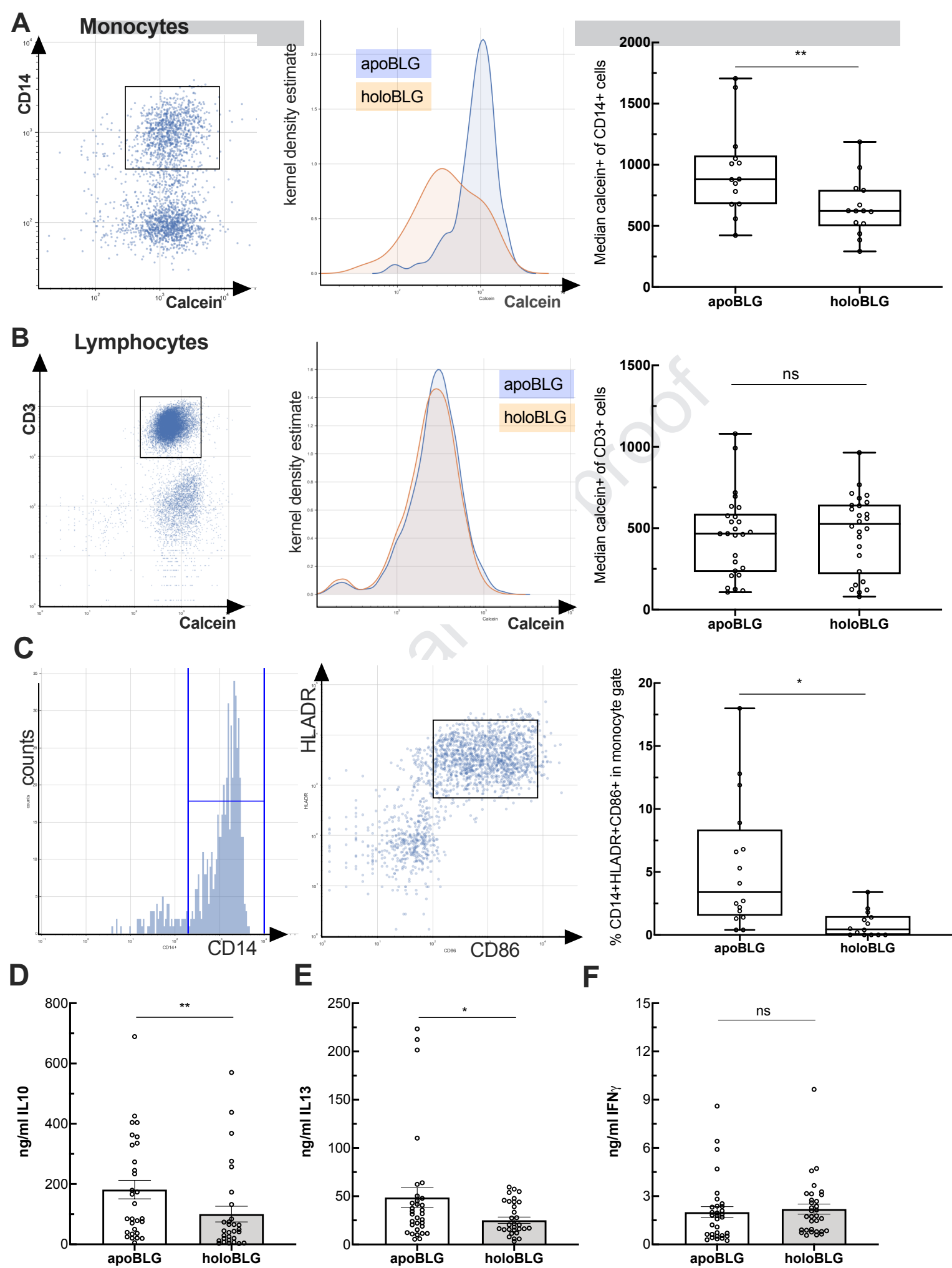
Ferrioxamine



A**Sensitization model****B****C**

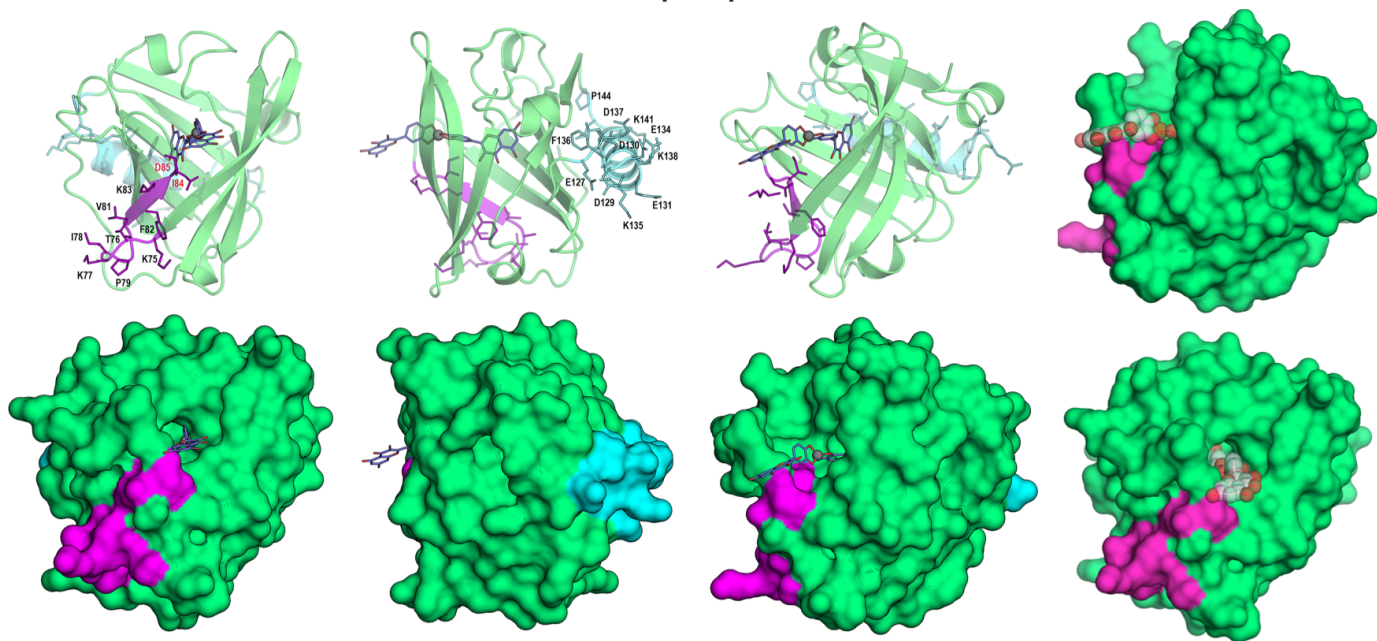
A**B****C**

A**B****C****D**

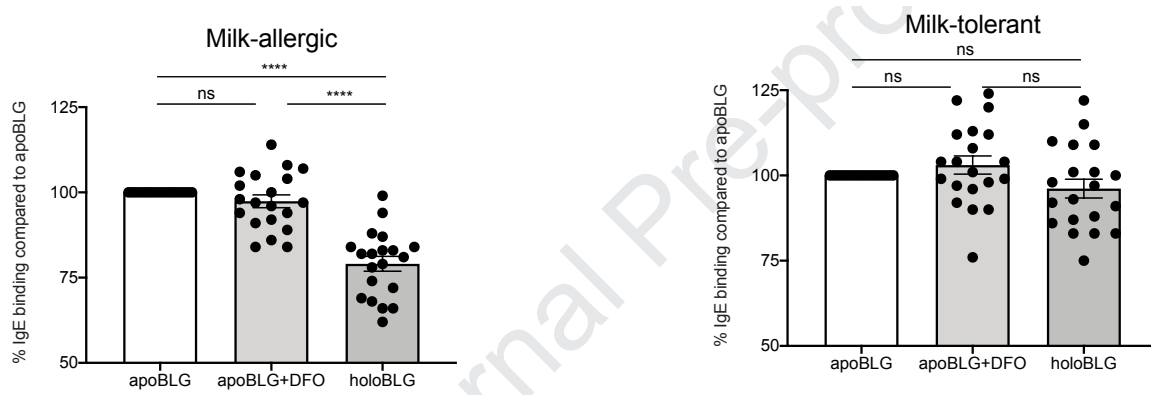


B cell epitopes

a

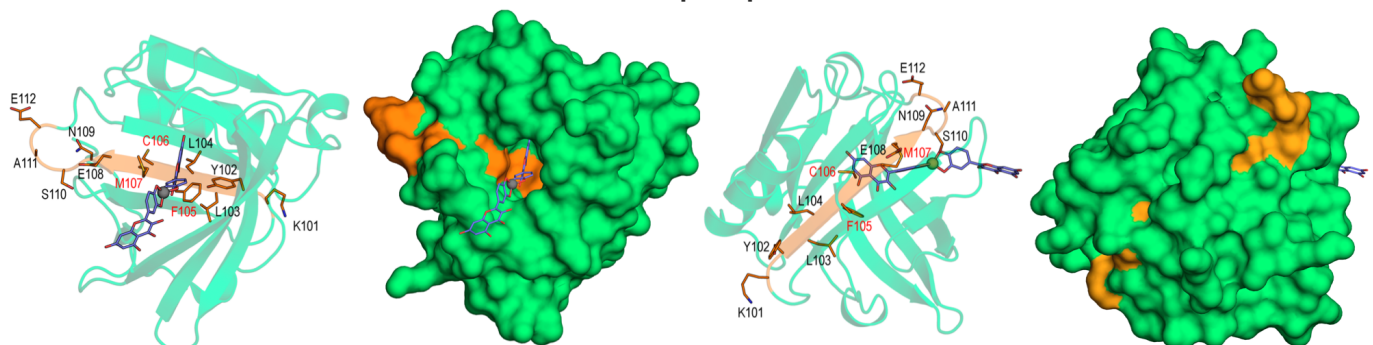


b

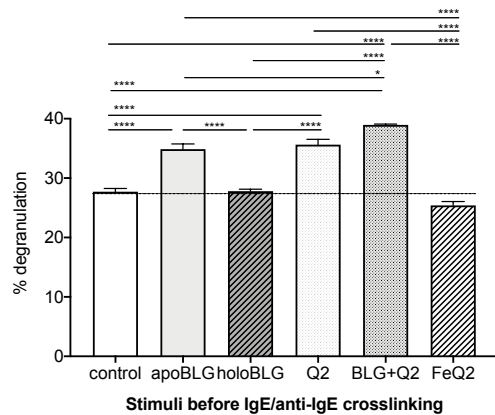


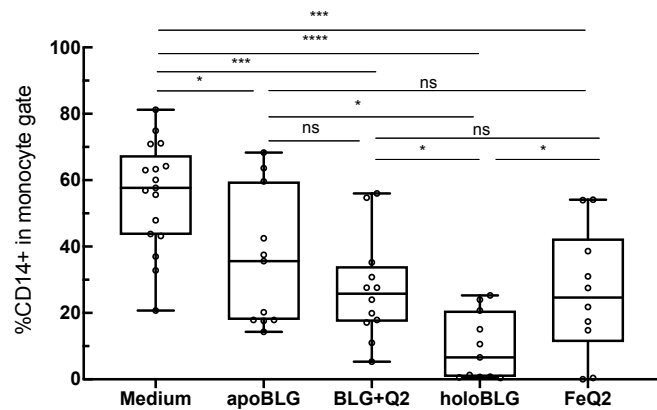
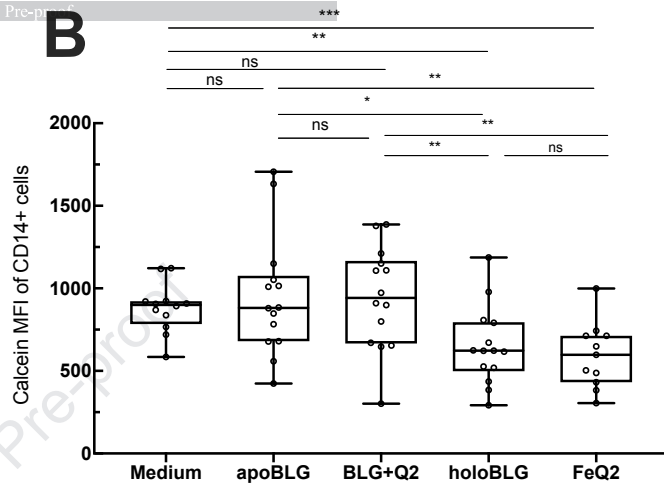
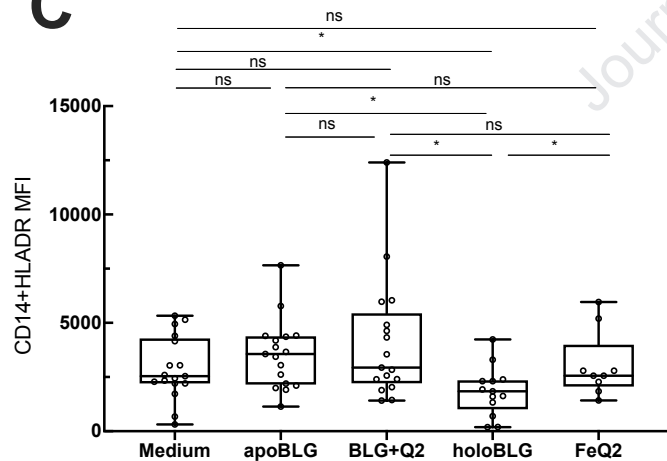
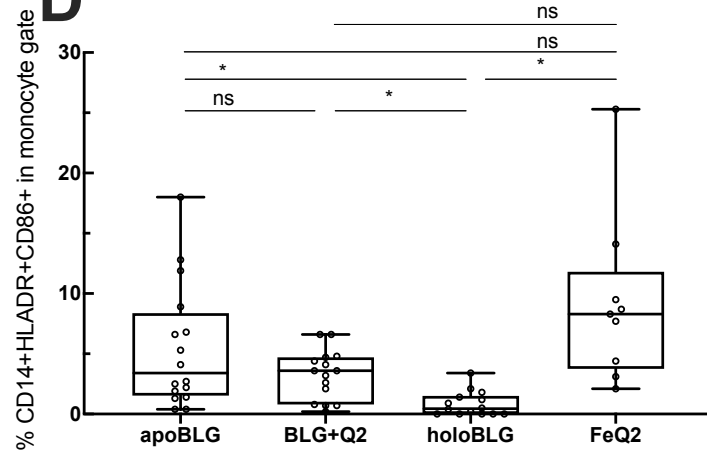
c

T cell epitope



d



A**B****C****D**

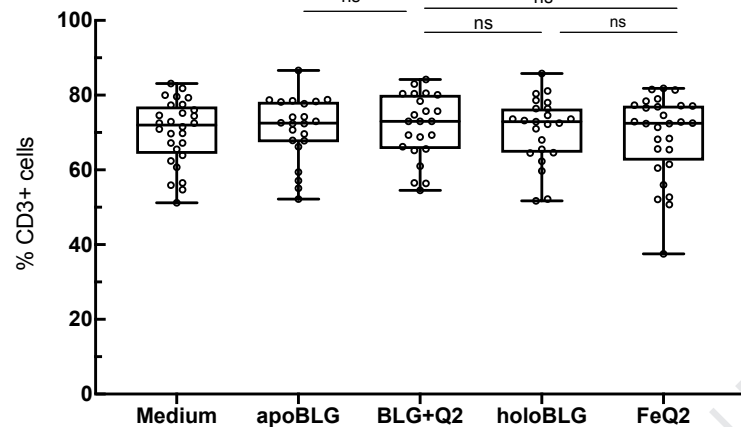
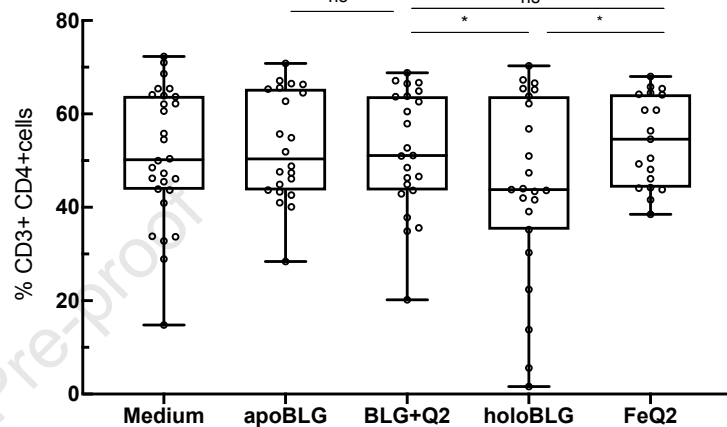
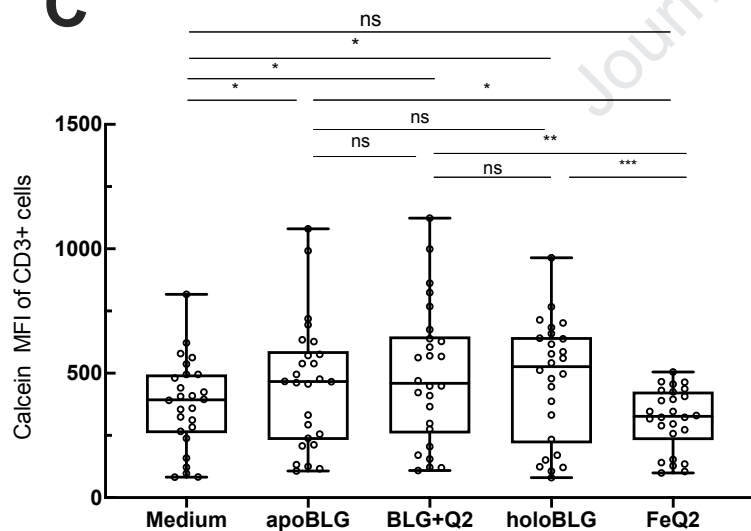
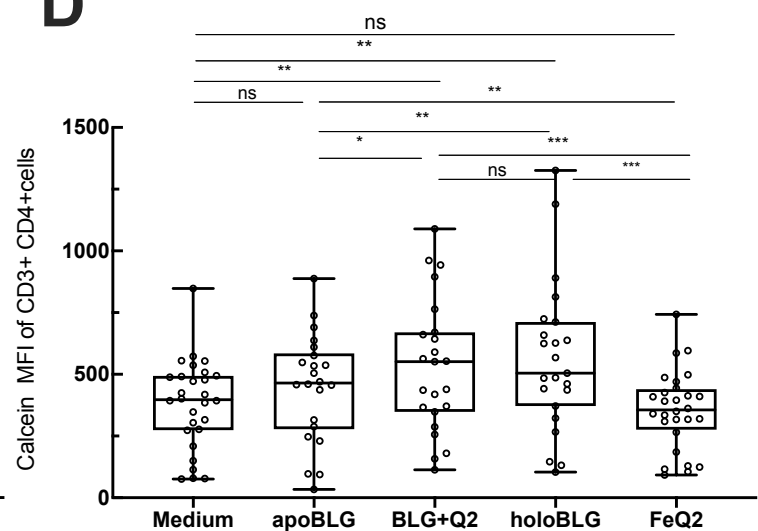
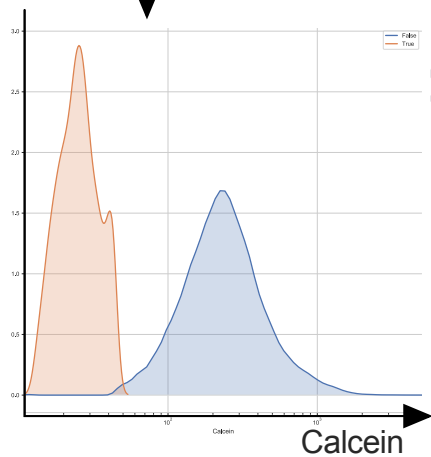
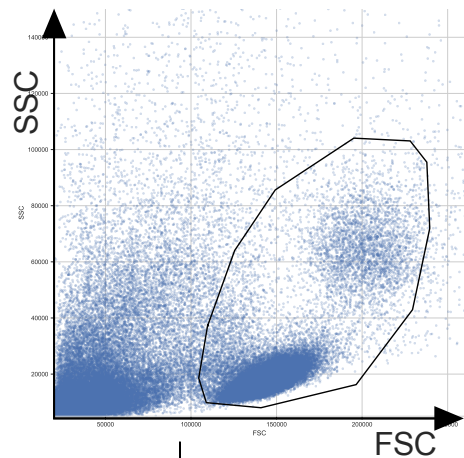
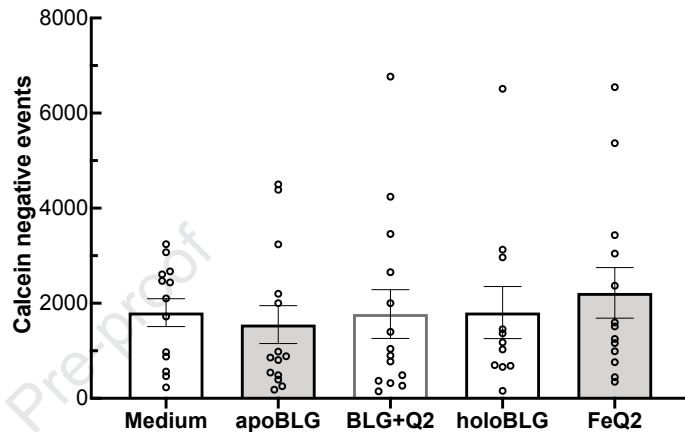
A**B****C****D**

Figure legends

Fig S1. HoloBLG treatment increased intracellular iron in CD14+ monocytic cells, while decreasing HLADR expression and the relative CD14+ population. Impact of stimulants on intracellular iron and cell populations. Peripheral blood mononuclear cells were incubated overnight in iron-free media alone or with quercetin, iron-quercetin in the presence or absence of BLG, before analysis for CD14+, HLADR and intracellular iron-levels by calcein. (A) Relative numbers and (B) intracellular iron-levels of CD14+cells, MFI of (C) HLADR and (D) CD86 of CD14+ cells; Groups were compared by RM-one-way ANOVA following Tukey's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig S2. No significant differences in cell death in the gated cell population. Peripheral blood mononuclear cells were incubated overnight in iron-free media alone or with quercetin, iron-quercetin in the presence or absence of BLG. Cells were stained with calcein and gated to the lymphocytic and monocytic compartment. Calcein negative cells within the gate were considered dead. (A) Gating strategy, (B) absolute and (C) relative numbers of calcein-negative cells within the gate. Groups were compared by RM-one-way ANOVA following Tukey's multiple comparisons test, Mean $\pm$ SEM.

Fig S3. HoloBLG treatment reduced intracellular iron and relative numbers of CD3+CD4+ cells. Impact of stimulants on intracellular iron and cell populations. Peripheral blood mononuclear cells were incubated overnight in iron-free media alone or with quercetin, iron-quercetin in the presence or absence of BLG, before analysis for CD3+CD4+ cells and intracellular iron-levels by means of quenching the calcein-signal. (A) Relative numbers of CD3+cells and (B) CD3+CD4+ cells; intracellular iron-levels in (C) CD3+ and (D) CD3+CD4+ cells; Groups were compared by RM-one-way ANOVA following Tukey's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

**B****C**