

Dose range-finding toxicity study in rats with recombinant human lactoferrin produced in *Komagataella phaffii*

Short title: A dose range-finding study of *K. phaffii*-derived rhLF

Authors: Ross Peterson¹, Robert B. Crawford², Lance K. Blevins², Norbert E. Kaminski², June S. Sass¹, Bryce Ferraro¹, Roma Vishwanath-Deutsch¹, Anthony J. Clark¹, Carrie-Anne Malinczak¹

Affiliations:

¹Helaina Inc., 345 Park Ave, South, New York, NY 10010, USA

²Michigan State University: 1129 Farm Lane, RM 165, Food Safety and Toxicology Building, East Lansing, MI 48824, USA

Ross Peterson, PhD: Director of Regulatory Affairs, Helaina Inc. New York, NY, USA; ross@myhelaina.com; ORCID: 0009-0002-7405-4602

Robert B. Crawford: Institute for Integrative Toxicology, Michigan State University, East Lansing, MI, USA; crawfo28@msu.edu

Lance K. Blevins, PhD: Assistant Professor, Institute for Integrative Toxicology, Michigan State University, East Lansing, MI, USA; blevin24@msu.edu; ORCID: 0000-0002-8911-8843

Norbert E. Kaminski, PhD: Professor, Department of Pharmacology & Toxicology, Director and Food and Consumer Product Ingredient Safety Endowed Chair, Center for Research on Ingredient Safety, Director, Institute of Integrative Toxicology, Michigan State University, East Lansing, MI, USA; kamins11@msu.edu; ORCID: 0000-0002-2144-428X

June S. Sass, BS: Research Associate, Nutritional Biology & Safety, Helaina Inc. New York, NY, USA; june@myhelaina.com; ORCID: 0009-0002-6397-4614

Bryce Ferraro, BS: Lead Engineer, Early-Stage R&D, Helaina Inc. New York, NY, USA;
bryce@myhelaina.com; ORCID: 0009-0003-8259-3478

Roma Vishwanath-Deutsch, MS: Scientist, Nutritional Biology & Safety, Helaina Inc. New York, NY, USA; roma@myhelaina.com; ORCID: 0009-0003-0236-4270

Anthony J. Clark, PhD: Chief Technology Officer, Helaina Inc. New York, NY, USA;
anthony.clark@myhelaina.com; ORCID: 0009-0002-9425-0221

Carrie-Anne Malinczak, PhD: Head, Nutritional Biology & Safety, Helaina Inc. New York, NY, USA; carrie@myhelaina.com; ORCID: 0000-0003-3377-5921

Corresponding author information: Carrie-Anne Malinczak, PhD. Email: carrie@myhelaina.com; Tel: 734-657-2414; Address: 345 Park Ave, South, New York, NY 10010, USA

ABSTRACT

The oral toxicity of recombinant human lactoferrin (rhLF, Helaina rhLF, Effera™) produced in *Komagataella phaffii* was investigated in adult Sprague-Dawley rats by once daily oral gavage for 14 consecutive days. The study used groups of 3-6 rats/sex/dose. The vehicle control group received sodium citrate buffer and the test groups received daily doses of 200, 1000 and 2000 mg of rhLF in sodium citrate buffer per kg body weight. Bovine LF at 2000 mg/kg body weight per day was used as a comparative control. Clinical observations, body weight, hematology, clinical chemistry, iron parameters, immunophenotyping, and gross examination at necropsy were used as criteria for detecting the effects of treatment in all groups and to help select dose levels for future toxicology studies. Quantitative LF levels were also analyzed as an indication of bioavailability. Overall, administration of Helaina rhLF by once daily oral gavage for 14 days was well tolerated

in rats at levels up to 2000 mg/kg/day, or 57x Helaina's intended commercial use in adults, and indicating that a high dose of 2000 mg/kg/day is appropriate for future definitive toxicology studies.

Keywords: *Komagataella phaffii*, lactoferrin, recombinant protein, toxicity, immunophenotyping

Abbreviations:

ALB: Albumin

bLF: Bovine lactoferrin

CH: Mean cellular hemoglobin content

CHCM: Cellular hemoglobin concentration mean

CHOL: Cholesterol

CHr: Reticulocyte hemoglobin content

CL: Chloride

CREAT: Creatinine

FE: Iron

FERR: Ferritin

GRAS: Generally Recognized as Safe

HDW: Hemoglobin distribution width

hLF: Human lactoferrin

hmLF: Human milk lactoferrin

HPLC: High performance liquid chromatography

K. phaffii: *Komagataella phaffii*

LF: Lactoferrin

MCH: Mean corpuscular volume

MCHC: Mean corpuscular hemoglobin concentration (calculated)

MCVr: Mean reticulocyte volume

MPV: Mean platelet volume

NA: Sodium

NOAEL: No observed adverse effect level

PCDW: Platelet component distribution width

PCT: Plateliterit

PDW: Platelet distribution width

PHOS: Phosphorus

RDW: Red blood cell distribution width

rhLF: Recombinant human lactoferrin

SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

TBIL: Total bilirubin

TFRRNSAT: Transferrin saturation

TIBC: Total iron binding capacity

TRIG: Triglycerides

TSAT: Transferrin saturation

UIBC: Unsaturated iron binding capacity

UREAN: Urea nitrogen

INTRODUCTION

Human lactoferrin (hLF), isolated by Bengt Johansson in 1960,¹ is an 80 kD glycoprotein member of the transferrin family of iron binding proteins, made up of a single chain polypeptide of 691 amino acids.² Lactoferrin has received attention due to its bioactive (i.e., functional) properties and beneficial interactions with the immune system.³ It is ubiquitous in the human body, present in human biological fluids and mucous secretions (e.g., human milk, tears, and saliva) as well as in the granules of circulating neutrophils.⁴ The concentration of hLF is highest in human milk (hmLF), from 5–7 mg/mL in colostrum to 1–3 mg/mL in mature milk;⁵ therefore, breastfed babies are exposed to high levels of hmLF in early life.⁶ Additionally, adult humans are also constantly exposed to hLF naturally occurring in tears (7-9 mg/L)⁷, saliva (~0.62 g/L)⁸, and other epithelial cell secretions.⁹ Bovine LF (bLF) is also routinely consumed, at an estimated daily intake of 80 mg/day, through ingestion of cow's milk¹⁰ and was first isolated by Sorensen and Sorensen in 1939.¹¹ Since the discovery of LF, studies have been performed in vitro, in experimental animals, and in humans to analyze the multifunctional roles which include, but are not limited to, iron transport and interaction with the immune system.^{3,12}

Bovine LF isolated from cow's milk is generally recognized as safe (GRAS) for various intended uses in conventional food and infant formula, and it is available as a dietary supplement, taken orally for its nutritional benefits.¹³ Literature has demonstrated improved hematological parameters with daily oral administration of bLF in treating iron deficiency anemia in pregnant and non-pregnant women.^{14–16} However, hmLF has been shown to digest more slowly and has stronger affinity for the human LF receptor in the small intestine, indicating that it may be more bioavailable than bLF.^{17–19} Therefore, human forms of LF have the potential to be more functionally active than bLF as a food ingredient. The use of hmLF isolated from human milk has not been pursued due to economic, ethical, and sustainability reasons. Approximately one liter of human milk is required to yield 100 mg of hmLF²⁰; not only is this practice costly and wasteful, but milk used for this purpose could be unsuitable for feeding infants. The expression of recombinant proteins provides a method for production at commercial scale with prospective sustainability benefits, and at a lower cost and without ethical concerns. Recombinant human LF (rhLF) has been produced by several biological expression systems including rice,²¹ transgenic cows,²² and yeast.^{23–25}

Several general toxicity animal studies have evaluated the safety of previously developed rhLF products following oral ingestion. Cerven et al. used a rat model to demonstrate no toxic effects at 1800 mg/kg body weight per day, for 28 days, of rice-derived apo-rhLF (iron unsaturated form).²⁶ Another study by the same group found the no observed adverse effect level (NOAEL) of holo-rhLF (iron saturated form) to be greater than 1,000 mg/kg body weight per day.²⁷ In a 13-week oral toxicity study in rats, Appel et al. found the NOAEL of rhLF from transgenic cattle to be at least 2,000 mg/kg body weight per day.²⁸ Finally, Zhou et al. found that milk powder with rhLF derived from transgenic cattle (up to ~400 mg/kg) was as safe as conventional milk powder in a

90-day toxicity study in rats.²⁹ These studies provide support for safety regarding the ingestion of rhLF in vivo.

However, few previous studies have examined changes in immune function by rhLF that provide insights into its putative immunotoxicologic effects, crucial for ensuring safety. To our knowledge, no published animal study on rhLF has included comprehensive evaluation of its effects on the frequency of major immune cell types by immunophenotyping. Immunophenotyping is an application of flow cytometry to detect proteins of interest on a single-cell level^{30–32} and it is one of the suggested models to identify compounds of potential immunotoxic risk.³³ Given that reductions in the frequency of immune cell types can be an indicator of changes to immune function, immunophenotyping is a valuable technique, in combination with other safety endpoints including hematology and clinical chemistry, to characterize the toxicity profile of novel food ingredients and was addressed in the current work.

Helaina Inc. (New York City, NY, USA) has developed rhLF, derived from a glycoengineered yeast, that is substantively similar to hmLF.³⁴ Specifically, a proprietary technology involving disruption of an endogenous glycosyltransferase gene (OCH1) and a stepwise introduction of heterologous glycosylation enzymes, enables a modified strain of *K. phaffii* to produce rhLF (EfferTM).^{35,36} Despite the structural similarities of rhLF to hmLF, safety of recombinant proteins intended for food use must be demonstrated.

Given that human infants are exposed to naturally occurring hmLF in human milk, and that adults are exposed to hLF in other biological secretions (e.g., saliva), the addition of Helaina rhLF to the diet should be equally as tolerated as other LF forms and not pose a safety risk at appropriately evaluated exposure levels. Tolerability and toxicity of rhLF was evaluated in a 14-day oral gavage dose range-finding study in rats. Endpoints included observable changes in the animals (e.g.,

behavior, body weight), hematology, clinical chemistry, iron parameters, immunophenotyping, and LF detection in the serum. The goal of the current study was to compare ingestion of rhLF to bLF, which has GRAS designation as a food ingredient, as well as to help select dosage levels for future, longer-term definitive toxicology studies.

MATERIALS & METHODS

Regulatory Compliance

This study was not performed under the regulatory guidelines of Good Laboratory Practices (GLP) but all experimental procedures and data collected followed validated methods and standard operating procedures developed by the testing facility (Charles River Laboratories (CRL), Mattawan, MI, USA), Michigan State University (MSU), or at Helaina, Inc. All data was verified through quality control by either the testing facility or independent scientists at MSU and Helaina, Inc.

Test Substances

Recombinant human lactoferrin (rhLF, Helaina Inc.), alpha isoform protein id P02788 of the UniProt database, was produced by fermentation and expressed from a *Komagataella phaffii* recombinant system, purified by microfiltration/diafiltration and cation exchange chromatography, and then spray-dried to a powder. The purity of rhLF was measured by high performance liquid chromatography (HPLC) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and is typically greater than 98%. The iron saturation was determined to be 58% saturated.³⁴ Recombinant human LF was reconstituted and diluted with sodium citrate (50 mM) buffer (pH 5.5) prior to use.

Bovine lactoferrin (bLF) isolated from bovine milk was purchased from Lactoferrin Co, Australia (Product 11683) and reconstituted and diluted with sodium citrate (50 mM) buffer (pH 5.5) prior to use. Purity, provided by the supplier, was greater than 95% and the iron saturation was 9.9%.

Animals and Treatment

Nine-week-old CD® (Sprague Dawley) IGS rats were received from Charles River Laboratories, Kingston, New York. Following a seven-day acclimatization period, 42 healthy male and 42 healthy female adult rats, approximately 10 weeks of age, were randomly selected and assigned to treatment and control groups using a computer randomization process. The body weight range prior to the start of dosing was 250-297 g for males and 206-231 g for females.

The animals were socially housed 2-3/cage in solid bottom cages with nonaromatic bedding and appropriate animal enrichment, adhering to the USDA Animal Welfare Act (9 CFR, Parts 1, 2 and 3)³⁷ and as described in the Guide for the Care and Use of Laboratory Animals (NRC, eighth edition).³⁸ After assignment to study, these animals were implanted with microchips. The individual animal number, implant number, and the Testing Facility (CRL, Mattawan, MI) study number comprised a unique identification for each animal. The animal cage was identified by the study number, animal number, group number, and sex. Block Lab Diet® Certified Rodent Diet #5CR4, PMI Nutrition International, Inc. was provided ad libitum except for the fasting period on the day of designated procedures. Fresh tap water, which was analyzed routinely for impurities, was available ad libitum. During the time frame of this study, there were no impurities found in the food or water which would affect toxicologic evaluation. The animal room had a 12-h

light/dark cycle, only interrupted intermittently for study-related activities. Temperature (68-79°F) and humidity (30-70%) of the room was maintained according to the testing facility SOP.

The oral route is the intended route of administration of this test article in humans. Therefore, recombinant human LF (rhLF, Helaina Inc.), bLF, or control vehicle (50 mM sodium citrate buffer, pH 5.5) was administered orally, once daily by gavage, using a syringe and appropriately sized plastic feeding implement. There were ten dosing groups with 3-6 female and 3-6 male animals in each group (Tables 1 and 2). The total number of animals to be used in this study was considered to be the minimum required to properly characterize the effects of the test article and this study was designed such that it did not require an unnecessary number of animals to accomplish the objectives. All doses were given at a volume of 10 mL/kg. Three groups of animals (3/sex) received rhLF in sodium citrate at levels of 200, 1000, or 2000 mg/kg bw/day; one group of animals (3/sex) received bLF at 2000 mg/kg bw/day, and one group of animals (3/sex) received sodium citrate vehicle only for the toxicological (main) phase of the study. Three additional satellite groups of animals (6/sex) received rhLF in sodium citrate at levels of 200, 1000, or 2000 mg/kg bw/day, one additional group of animals (6/sex) received bLF at 2000 mg/kg bw/day, and one additional satellite group of animals (3/sex) received sodium citrate vehicle only for the LF systemic exposure phase of the study. The dose levels were selected based on the suspected lack of toxicity as determined in published literature for a similar recombinant human protein delivered to rats via the oral gavage route.²⁶⁻²⁸ The start of dose administration for the high dose level was staggered by at least 4 days to confirm initial tolerability at low and mid-dose levels. The test articles were stored in powder form at room temperature and have at least 6 months of stability under these conditions. The test protein powders were mixed into a fresh suspension daily with

sodium citrate (50 mM) buffer, pH 5.5 to the proper concentration, and continuously mixed at room temperature during the dosing procedure. The prepared test articles in solution were stable for at least 10 days when stored at 4°C or at room temperature. Individual dose volumes were adjusted twice weekly based on body weight.

Table 1. Experimental Design (Main Study)

Group No.	Test Material	Dose Level (mg/kg/day)	Dose Volume ^a (mL/kg)	Dose Concentration (mg/mL)	Main Study	
					No. of Males	No. of Females
1	Vehicle Control	0	10	0	3	3
2	HE-001	200	10	20	3	3
3	HE-001	1000	10	100	3	3
4	HE-001	2000	10	200	3	3
5	Bovine Lactoferrin	2000	10	200	3	3

No. = Number

^a Based on the most recent body weight measurement.

Table 2. Experimental Design (Satellite)

Group	Test Material	Dose Level (mg/kg/day)	Dose Volume ^a (mL/kg)	Dose Concentration (mg/mL)	Satellite Phase ^b	
					No. of Males	No. of Females
6	Vehicle Control	0	10	0	3	3
7	HE-001	200	10	20	6	6
8	HE-001	1000	10	100	6	6
9	HE-001	2000	10	200	6	6
10	Bovine Lactoferrin	2000	10	200	6	6

No. = Number

^a Based on the most recent body weight measurement

^b Animals will be treated, and samples collected for exposure assessment. Additionally, following final blood collection, the animals will be euthanized and spleen samples will be collected for immunophenotyping analysis

Animal Observations

All animals were observed once daily for cage-side observations and twice daily for morbidity and mortality. Detailed clinical observations were performed twice weekly. Body weights were recorded pretest, twice weekly, and on the day prior to study termination.

Terminal Procedures

Animals were euthanized by carbon dioxide inhalation followed by a Testing Facility SOP approved method to ensure death, (e.g., exsanguination). All animals that underwent scheduled terminal procedures were subjected to a complete necropsy examination that included evaluation of the carcass and musculoskeletal system, all external surfaces and orifices, cranial cavity and external surfaces of the brain, and thoracic, abdominal, and pelvic cavities with their associated organs and tissues. Organs examined and associated weights collected included brain, epididymis, adrenals, prostate, heart, kidney, liver, spleen, testis, thymus, ovaries, and uterus/cervix. Following collection and weight measurement, the spleen was cut in half from the main study animals and three satellite animals/sex/group, and one half was placed into a tube containing media, blind coded, and stored refrigerated (2-8°C) prior to shipment to the immunophenotyping testing facility (MSU) the next day. A veterinary pathologist, or other suitably qualified personnel, was available for consultation during scheduled necropsy. All animals were examined carefully for external abnormalities including palpable masses.

Clinical Pathology

Blood samples were obtained from each study animal immediately after euthanasia via vena cava venipunctures prior to terminal necropsy. Clinical chemistry and hematologic parameters are summarized in Supplemental Table 1.

Lactoferrin Absorption into Serum

Blood (0.3 mL) was collected from two cohorts of satellite animals 3/sex/group (Groups 6-10) at alternating time points via sublingual or other suitable vein at the following time points on Days 1 and 14: 0.5, 1, 3, 6, 12, and 24 hours from rhLF (low, mid, high) and bLF test groups 7-10, respectively, and only at hour 1 for Group 6 (vehicle control). Blood was allowed to clot prior to centrifugation to separate serum from the cellular clot. Serum was carefully removed and transferred to a collection tube and stored at $\leq -20^{\circ}\text{C}$ until ELISA analysis for LF quantification.

Human rhLF ELISA

A direct sandwich ELISA method, conducted at Helaina Inc., was used to determine the concentration of rhLF in the rat serum. 96-well plates were coated overnight with capture antibody (100 $\mu\text{g/mL}$; lactoferrin goat anti-human polyclonal, Bethyl Labs, Cat: A80-143A). The wells were washed and blocked using fish gelatin and incubated for 2 hours. Standards (3.6 to 210 ng/mL) were prepared in 1:10 rat sera. Plates were washed and standards, positive control (known concentration of rhLF spiked into 1:10 rat sera), samples (diluted 1:10), and blanks (1:10 rat sera) were added to appropriate wells, and plates were incubated for 1.5 hours at room temperature. Plates were washed, a 1:5,000 dilution of detection antibody (Rabbit anti-Lactoferrin (006) [HRP] (Novus; Cat: NBP2-89794H)) was added and incubated at room temperature for 1 hour. Plates were washed and then developed by adding Slow Step ELISA TMB substrate (Thermofisher, Cat: 34024) to each well. Plates were incubated for 12 minutes, and 2.0M aqueous sulfuric acid was added to quench the reaction. Absorbance was measured using a plate reader; at 450 nanometers and 652 nanometers (Spectramax M2). Lactoferrin concentration was interpolated from the

standard curve; the lower and upper limits of quantitation were 36 and 2100 ng/mL, respectively due to the minimum required dilution of 1:10 for the study samples. An R² of at least 0.990 with a sigmoidal curve fit was required with a blank (1:10 rat serum) absorbance of <0.050 for the assay to be considered valid.

Bovine Lactoferrin ELISA

A Sandwich ELISA (Bovine Lactoferrin ELISA kit, NBP3-12185, Novus Biologicals) was used and adopted to determine bovine lactoferrin concentrations in rat serum. This assay was modified to detect bovine lactoferrin ranging from 6.9 to 5000 ng/mL. The assay was performed per the manufacturer's instructions with the following modifications: standards were prepared using bovine LF (Lactoferrin Company; Product 11683) that was used in the oral administration to rats, diluted in 1:10 rat serum for final concentration on the plate ranging from 6.9 to 5000 ng/mL, positive controls were prepared by spiking known concentrations of bovine LF into 1:10 rat serum, and the study samples were diluted 1:10 prior to testing. Lactoferrin concentration was interpolated from the standard curve; the lower and upper limits of quantitation were 69 and 50,000 ng/mL, respectively due to the minimum required dilution of 1:10 for the study samples. An R² of 0.970 with a sigmoidal curve fit was required with a blank (1:10 rat serum) absorbance of <0.05 for the assay to be considered valid.

Immunophenotyping

After terminal necropsy, spleen samples were collected from the main study animals and three satellite animals/sex/group on Day 15, blind coded, stored refrigerated (2-8°C) and shipped to the test site at Michigan State University (East Lansing, MI) for exploratory spleen

immunophenotyping analysis to determine whether oral administration of rhLF protein to rats produces changes in the frequency of major immune cell types in the spleen as evaluated by flow cytometry. Immunophenotyping was conducted blinded to dose level.

Tissue processing

Splenocytes were isolated by mechanical disruption of spleen samples and made into single cell suspensions in RPMI 1640 medium supplemented with 5% FCS and penicillin/streptomycin. Red blood cells were lysed using Zap-oglobin-II Lytic Reagent (Beckman Coulter) prior to counting splenocytes on a Z1 Beckman Coulter Counter.

Flow Cytometry

A total of 2×10^6 splenocytes were placed in a 96-well round bottom culture plate for surface and intracellular antibody staining. Splenocytes were washed using Hank's Balanced Salt Solution (HBSS, (pH 7.4)), (Invitrogen) and stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain (Gibco Invitrogen) to assess cell viability. Splenocytes were washed with FACS buffer ($1 \times$ HBSS (pH 7.5) containing 1% BSA and 0.1% sodium azide) and Cell FcRs were blocked with purified mouse anti-rat CD32 (BD Biosciences). After a 15-minute incubation at 4°C, splenocytes were stained for surface proteins.

For the Granulocyte/Macrophage/DC and B cell panel: CD45 (OX-1) (Biolegend), CD172a (clone OX-41) (BD Bioscience), CD11b (clone WT.5) (Biolegend), CD25 (clone OX-39) (Biolegend), and AffiniPure Anti-Rat IgG + IgM (Jackson ImmunoResearch). For the T cell panel: CD45 (OX-

1) (Biolegend), CD3 (clone 1F4) (Biolegend), CD4 (clone W3/25) (Biolegend), CD8 (clone OX-8) (Biolegend) and CD25 (clone OX-39) (Biolegend).

For FoxP3+ T cells: Splenocytes were incubated at 4°C and washed three times with FACS buffer and fixed with FoxP3/Transcription Factor Staining Buffer Set (Invitrogen) fixative and stored at 4°C until intracellular staining. To measure intracellular FoxP3, splenocytes were washed and incubated with 1x Permeabilization buffer solution (Invitrogen) for 15 minutes, then incubated with anti-rat FoxP3 (Invitrogen) for 30 minutes. Splenocytes were washed 3X with the permeabilization buffer and then washed one more time with FACS buffer and resuspended in FACS buffer.

Flow cytometric analysis was performed on a Cytex Northern Lights full spectral analyzer (Cytex Biosciences) and analyzed using FlowJo v10.9.0 (Tree Star, Ashland, OR) software.

Statistical analysis

In-life, clinical pathology, and postmortem measures

Statistical analyses for in-life, clinical pathology, and postmortem measures were conducted with Provantis version 9. Raw data was tabulated within each time interval, and the mean and standard deviation and/or incidence counts (categorical variables) were calculated for each endpoint by sex and group. For each endpoint, treatment groups were compared to the control group. The groups were compared using an overall one-way ANOVA F-test if Levene's test was not significant, or the Kruskal-Wallis test if it was significant. If the overall F-test or Kruskal-Wallis test was found to be significant, then pairwise comparisons were conducted using Dunnett's or Dunn's test,

respectively. Results of all pairwise comparisons were reported at the 0.05 and 0.01 significance levels and all endpoints were analyzed using two-tailed tests unless indicated otherwise.

Immunophenotyping

Statistical analyses were performed using GraphPad Prism version 10.0.02 (GraphPad Software, La Jolla, CA). To determine statistically significant changes between the treatment groups and the control (0 mg/kg/day) within male and female rats, a one-way ANOVA with a Dunnett's multiple comparisons posttest was used. The mean $\pm$ SD is displayed in all bar graphs (* $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$).

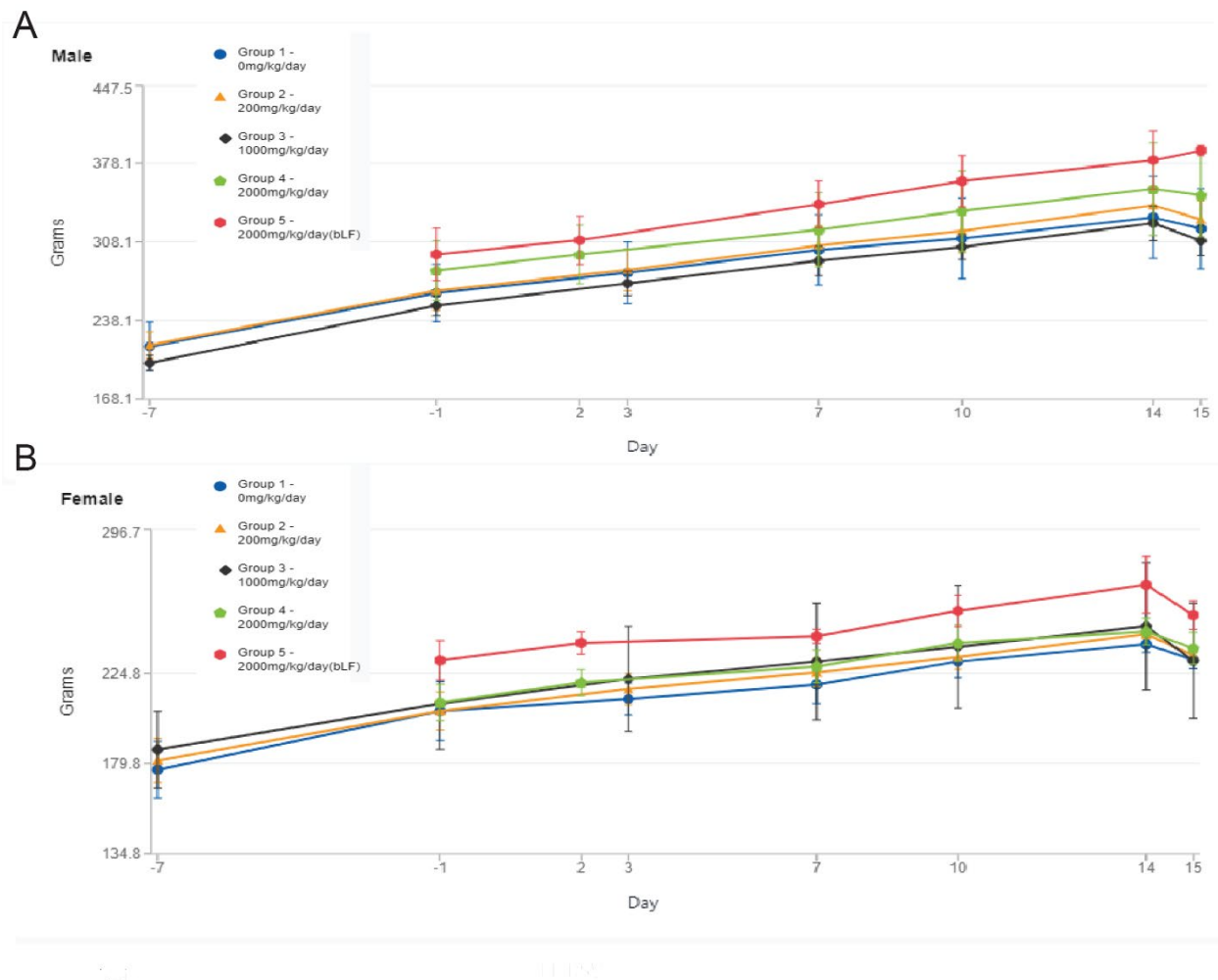
RESULTS

Ingestion of Helaina rhLF was well tolerated up to 2000 mg/kg/day

All animals survived the 14-day oral dosing. There was no morbidity or mortality associated with administration of rhLF or bLF and in-life observations showed minimal instances of abnormal physical signs, none that were attributed to treatment with rhLF or bLF. Changes in mean body weight per group during the study are shown in Figure 1 and Supplemental Figure 1. No significant body weight changes were observed during the course of treatment for any dose level. The drop in body weights for some animals on Day 15 is expected as the animals were fasted prior to the necropsy procedures. At necropsy, there were no changes attributable to the administration of test articles in males or females, and there were no significant differences in absolute organ weights or the relative organ weights between male or female control and dosed groups. Any observed differences were not statistically significant, were not clearly dose-responsive in nature, and/or were generally due primarily to differences for an individual animal. No macroscopic observations

were noted at any dose level evaluated, including controls. Collectively, these data show that ingestion of rhLF and bLF is well tolerated up to at least 2000 mg/kg/day.

Figure 1: Body Weight Changes by Sex



Minimal to moderate changes in hematology and chemistry parameters were observed at ≥ 1000 mg/kg/day

Recombinant hLF and bLF-related alterations in measured hematology parameters were observed in both sexes at 2000 mg/kg/day and are displayed in Table 3. At termination on Day 15, in females at 2000 mg/kg/day rhLF and 2000 mg/kg/day bLF, there were test article-related, minimally higher

hemoglobin distribution width (HDW), reflecting greater variation in erythrocyte hemoglobin content. In both sexes at 2000 mg/kg/day rhLF and 2000 mg/kg/day bLF, there were test article-related, minimally to mildly higher mean platelet volume (MPV), reflecting larger mean platelet size. Additionally, females at 2000 mg/kg/day rhLF and 2000 mg/kg/day bLF exhibited test article-related, minimally lower platelet distribution width (PDW), reflecting less variation in platelet size. In females at 2000 mg/kg/day rhLF and 2000 mg/kg/day bLF, there were test article-related, minimally lower mean platelet component (MPC) values, reflecting lower platelet refractive index, or lower platelet density / granularity; additionally, both sexes at 2000 mg/kg/day rhLF and 2000 mg/kg/day bLF exhibited test article-related, minimally to mildly lower platelet component distribution width (PCDW), reflecting less variation in platelet density / granularity.

Helaina rhLF-related alterations in measured clinical chemistry parameters were observed in males at 2000 mg/kg/day and females at ≥ 1000 mg/kg/day, and bLF-related alterations in measured clinical chemistry parameters were observed in both sexes at 2000 mg/kg/day, as shown in Table 4. These included test article-related changes: minimally higher total bilirubin (TBIL) concentrations, minimally higher urea nitrogen (UREAN) concentrations, minimally lower mean creatinine (CREAT) concentrations, minimally to mildly higher cholesterol (CHOL) concentrations, mildly higher triglyceride (TRIG) concentrations, minimally to mildly higher albumin (ALB) concentrations with resultant higher A:G ratios, minimally to mildly lower mean phosphorus (PHOS) concentrations, minimally lower sodium (NA) and/or chloride (CL) concentrations. In addition, two individual females at 2000 mg/kg/day rhLF and one male and two females at 2000 mg/kg/day bLF also exhibited minimally to mildly higher CHOL concentrations which were potentially test article-related, though the small magnitude of difference and inconsistent effect among cohort group members precluded definitive interpretation. One male at

2000 mg/kg/day rhLF and one male at 2000 mg/kg/day bLF also exhibited mildly higher TRIG concentrations which were potentially test article-related, though the small magnitude of difference, inconsistent effect among cohort group members, and high variability inherent to TRIG concentration precluded definitive interpretation.

In terms of iron-related parameters (Table 5), on Day 15, in males at 2000 mg/kg/day rhLF and 2000 mg/kg/day bLF, there were mildly to moderately lower ferritin (FERR) concentrations, suggesting lower quantities of stored iron; conversely, there were concurrent minimally to mildly higher serum iron (FE) and total iron binding capacity (TIBC) concentrations and transferrin saturation (TFRNSAT) values. These parameters tended to be highly variable among females and lacked a clear test article-related pattern of results.

Remaining fluctuations among individual and mean values, regardless of statistical significance, were considered sporadic, consistent with biologic variation, and/or negligible in magnitude, and not related to test article administration. Overall, the clinical pathology changes that were observed in rhLF were also observed in bLF, suggesting a similar toxicological profile between the two proteins. Additionally, changes in hematology parameters may be related to the iron binding and modulating characteristics of these proteins but did not affect the overall health of the animals.

Ingestion of Helaina rhLF or bLF leads to minimal alterations in immune cell phenotypes within the spleen

Ex vivo immunophenotyping techniques using immune organs have been used as a measure of immunotoxicity related to test article administration.³³ To determine whether ingestion of rhLF has an impact on immune cell types within the spleen, immunophenotyping via flow cytometry was performed after 14 days of continuous exposure and the gating strategy is shown in Supplemental Figure 2A. The spleens were collected following euthanasia and dispersed into

single cell suspension for flow cytometry staining and first analyzed for cell viability. It was determined that oral gavage with Helaina rhLF or with bLF did not have any significant effects on cell viability in either female or male rats in any of the treatment groups (Table 6).

Next, spleen resident immune cells were assessed for the myeloid marker, CD172a⁺, expression, and there was a significant, and similar, increase in the frequency of immune cells expressing CD172a⁺ in both male and female rats administered the highest dose (2,000 mg/kg/day) of either rhLF or bLF (Table 6). While there was a significant increase in the percentage of CD172a⁺ spleen cells in response to high dose LF (i.e., rhLF and bLF), there was no change in the percentage of cells expressing CD11b, suggesting that the change in CD172a⁺ cells was not due to an increase in monocyte or macrophage cells. While treatment with high doses of LF did not affect the frequency of monocytes/macrophages, when the percentage of CD172a⁺ cells expressing CD11b and CD25, a marker of dendritic cells (DC), was assessed, a significant increase was found at the 2,000 mg/kg/day dose group (Table 6). This would suggest that most of the changes in the CD172a⁺ expressing cell population are due to the observed increase in DC. The final immune cell type evaluated, via use of antibody panel 1, (Supplemental Figure 2A) were B cells as identified by the presence of B cell receptors on the cell surface plasma membrane (IgM⁺ or IgG⁺). When the frequency of B cells in the spleen were quantified, no treatment-related changes were observed in either female or male rats following oral gavage with LF protein (Table 6).

Antibody panel 2 (Supplemental Figure 2A) was used to quantify the frequency of T lymphocyte populations in the spleen of rats gavaged with either rhLF or bLF. There were no treatment-mediated changes in the frequency of immune cells in the spleen expressing CD3, which is indicative of total splenic T cells (Table 6). Despite observing no effect on the total frequency of CD3⁺ cells in the spleen in any of the treatment groups, when the populations of T cells were

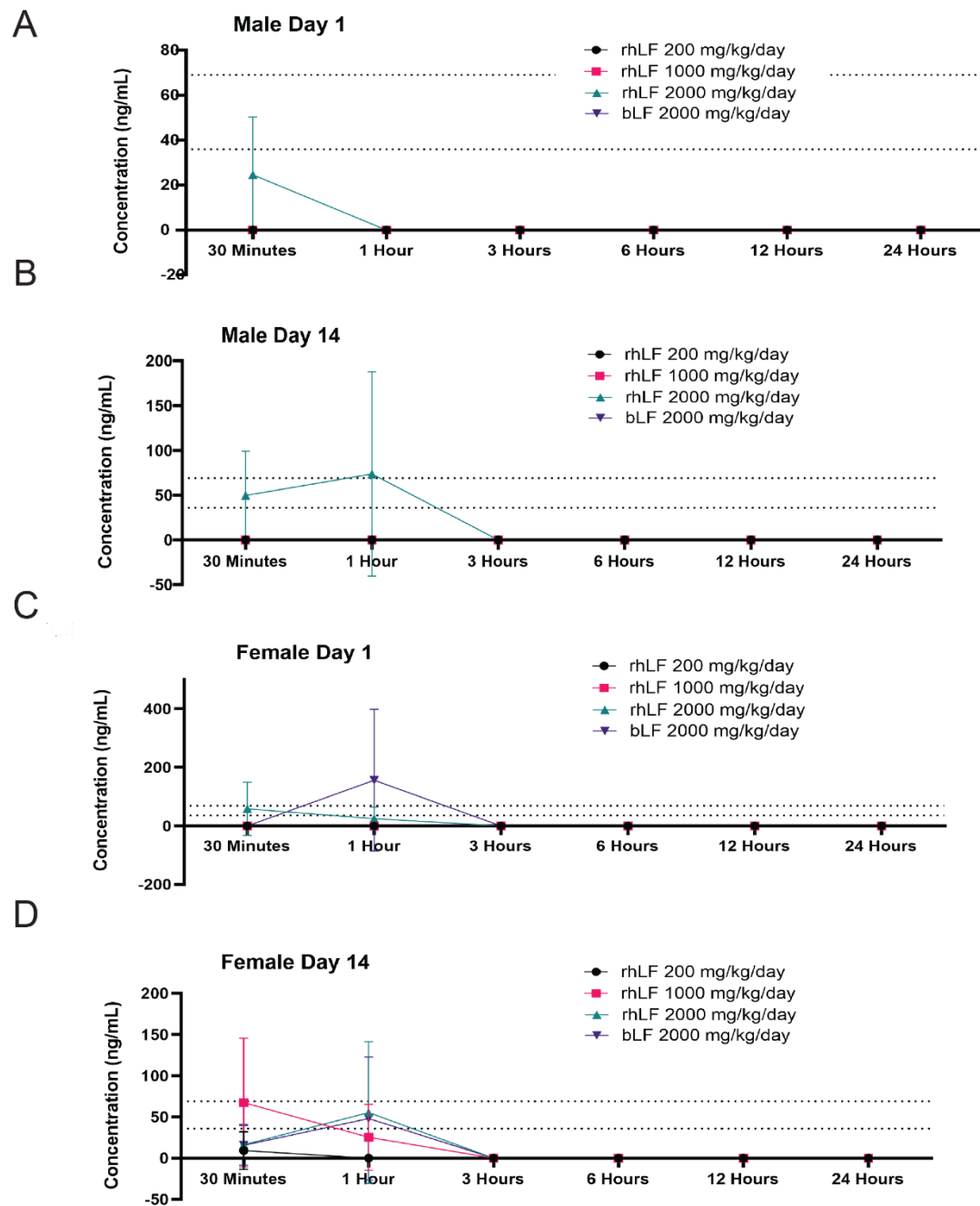
evaluated, there were treatment-related effects at the population level. For example, when the frequencies of CD8⁺ T cells within the spleen were assessed, we found that the highest dose treatment with rhLF resulted in statistically significant increases in the percentage of CD8⁺ T cells in both female and male rats, and an increase in male rats given bLF (Table 6). This increase was not observed in the frequencies of spleen resident helper CD4⁺ T cells as there was no change in any of the treatment groups in either male or female rats. We next assessed a minor population of T cells with regulatory function (Treg), which are typically induced in response to inflammation and T cell activation. These cells are identified within the CD4⁺ T cell compartment by their expression of CD25 and the intracellular protein FoxP3. No significant changes in the frequency of Treg cells was observed in any of the treatment groups (Table 6). However, when the percentage of activated T cells was assessed by measuring the percentage of CD25⁺ CD3⁺ T cells, there was a marked decrease in the frequency of these cells within the spleen at the highest dose (2,000 mg/kg/day) of either rhLF or bLF (Table 6).

Finally, we examined the effects of oral administration of LF protein on the frequencies of natural killer (NK) cells as evidenced by changes in the expression of CD161⁺ (Supplemental Figure 2A). Recombinant hLF administration did not produce any changes in the frequency of CD161⁺ cells. By contrast, administration of bLF (2000 mg/kg/day) produced a modest reduction in the frequency of CD161⁺ cells in female, but not male rats (Table 6). We then examined a subpopulation of NK cells, NK T cells, which are marked by the expression of CD3. There were no treatment-related changes in either male or female rats in the frequencies of spleen resident NK T cells (Table 6).

Postprandial levels of LF detected in the serum

In order to assess relative bioavailability of rhLF and bLF in circulation, serum was collected at multiple time points (0.5, 1, 3, 6, 12, 24 hours) following LF administration on Days 1 and 14 and tested for the presence of LF using ELISA. Results indicate that rhLF is quickly absorbed and detected in serum within 30 minutes of administration for males dosed at 2000 mg/kg/day on both Day 1 and Day 14 (Figure 2A, B), and very quickly cleared from circulation with no detection observed by 3 hours post-administration (Figure 2A,B). A similar trend was observed in female rats administered 2000 mg/kg/day rhLF on Day 1 (Figure 2C). However, on Day 14, female rats administered ≥ 1000 mg/kg/day had detectable levels of rhLF that cleared circulation by 3 hours post-administration (Figure 2D). No bLF was detected in the serum from male rats administered 2000 mg/kg/day (Figure 2A,B), while bLF was detected in female rats (Figure 2C,D). These data suggest that Helaina rhLF may be more bioavailable than bLF with a stronger response in females; however, the changes observed in clinical pathology suggest similar bioavailability of both rhLF and bLF. Overall, a lack of detectable LF in most samples tested and high variability amongst responders make it difficult to fully assess. Furthermore, due to a lack of responders overall, standard toxicokinetic parameters (e.g. T_{max}, C_{max}, AUC) could not be calculated.

Figure 2. Postprandial levels of LF detected in the serum



DISCUSSION

To date, no published study has evaluated the safety and oral toxicity of the novel protein, Helaina rhLF, produced in *K. phaffii*. Currently, the only LF commonly used for commercial food

applications (e.g., added to yogurt and consumed as a dietary supplement) is bLF. The present study aimed to assess the oral toxicity of Helaina rhLF compared to bLF administration in adult Sprague Dawley rats by once daily oral gavage for 14 days and was designed as a dose range-finding study to help select dosages for future definitive studies. Results showed that Helaina rhLF was well tolerated in rats up to 2000 mg/kg/day, or 57x Helaina's commercial use intended only for use in non-infant/non-toddler populations (e.g. 0.34 g/day in an average 60 kg adult). All animals survived to terminal necropsy. There were no test article-related effects on clinical observations, body weights, organ weights or macroscopic observations.

Changes in hematology parameters (increased HDW, higher MPV, lower PDW, lower MPC, and/or lower PCDW) were limited to the 2000 mg/kg groups and were similar in both sexes. Minimal to moderate rhLF-related alterations in measured clinical chemistry parameters (higher TBIL, UREAN, CHOL or TRIG, ALB, A:G ratios, and lower CREAT, PHOS, NA and/or CL concentrations) were observed in males at 2000 mg/kg/day and females at ≥ 1000 mg/kg/day, and bLF-related alterations in measured clinical chemistry parameters were observed in both sexes at 2000 mg/kg/day. While these changes were considered statistically significant and potentially biologically relevant, the consequences of these changes are unknown but did not outwardly affect the health and well-being of the animals and justifies the use of 2000 mg/kg/day as a high dose for future toxicological studies:

Lactoferrin is an iron-binding protein³⁹; therefore, this study also evaluated parameters essential for iron homeostasis. Changes in these parameters were observed in males at 2000 mg/kg/day and females at ≥ 1000 mg/kg/day, as well as bLF-treated groups in both sexes at 2000 mg/kg/day. Results included lower ferritin concentrations, higher serum iron concentrations, higher total iron binding capacity, and/or higher transferrin saturation. These changes could indicate that iron is

being transported out of the cells and into circulation, and correlates with clinical studies using bLF to treat inflammatory-driven anemia in pregnant and non-pregnant women.^{15,16} It is important to note that while Helaina rhLF is greater than 50% saturated with iron, the bovine LF used in these studies was less than 10% iron saturated, indicating that inherent iron saturation status is not a pivotal indicator for iron homeostasis properties of LF; however, the level of iron absorbed could contribute to these changes.

The present work also determined whether oral administration of rhLF produces changes in the frequency of major immune cell types in the spleen as evaluated by flow cytometry as a measure of potential immunotoxicity.³¹⁻³³ Collectively, this study shows an absence of any changes in the frequency of major immunocompetent cell populations in the spleen, comprising both myeloid and lymphoid lineages, after administration of rhLF at doses below 2000 mg/kg/day. Modest changes in the frequency of several spleen cell populations, including increases in CD172a⁺ DC, and CD8⁺ T cells, and decreases in activated CD25⁺ T cells, were observed in both rhLF and bLF treatment groups, which was restricted to the highest dose group (2000 mg/kg/day). Predominantly, these changes in cell population frequency occurred in both male and female rats, suggesting no sex-specific alterations. Further, these results suggest that there are no differences between rhLF and bLF in inducing immunological changes in the Sprague Dawley rat. These changes observed in spleen cell type frequencies in both rhLF and bLF at 2,000 mg/kg/day treatment groups can neither be deemed detrimental nor beneficial, as no other measurements of the immune system were conducted in this analysis and should be considered in future safety studies. Furthermore, due to differences between the rat and human immune system, it is difficult to directly translate these results into clinical relevance.

Assessment of LF absorption was performed at multiple time points ranging from 30 minutes to 24 hours and indicated very low levels of LF absorption overall. Males were observed to have lower levels of both rhLF and bLF than females, indicating a potential sex difference. Importantly, none of the male rats had detectable levels of bLF in the serum, and detectable levels of bLF were only observed in three female samples, suggesting better bioavailability of intact rhLF compared to bLF. However, similar changes in clinical pathology (e.g. clinical chemistry and hematology) for both rhLF and bLF suggest similar bioavailability. These discrepancies could potentially be explained by the ELISA methods used to detect rhLF vs bLF; the sensitivity of the assay for rhLF was greater (36 ng/mL) compared to that of the bovine assay (69 ng/mL). Furthermore, it has been documented that LF peptides following digestion are responsible for many of the bioactivity properties of LF¹⁹ and bLF has been shown to digest more quickly than hLF¹⁷⁻¹⁹ and peptide specific quantification in the serum was not performed in this study. It was observed that LF was quickly absorbed into the serum (average 30 minutes to one hour) and quickly cleared from circulation (no detectable levels after ~3 hours), with no signs of accumulation. These findings are correlated with the literature that identified LF in circulation within 10-20 minutes after ingestion in a mouse model⁴⁰ and within ~30 minutes in humans.⁴¹ Finally, studies that injected LF directly into the blood via intravenous injection showed the half-life of LF was 12.6 minutes in adult rats⁴² supporting these findings.

In conclusion, this study showed that administration of EfferTM (Helaina rhLF) or bLF by once daily oral gavage for 14 days was well tolerated in rats and justifies the use of doses up to 2000 mg/kg/day for future toxicological studies.

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FOOTNOTES

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Declaration of Conflicting Interest: Authors RP, JSS, BF, RV-D, AJC, and C-AM are all employees of Helaina, Inc.

Figure legends:

Figure 1: Body Weight Changes by Sex. Body weight was measured bi-weekly for each animal during the treatment period for both main (Groups 1-5) and the mean results shown over time. Males are shown on the top figure and females on the bottom figure.

Figure 2. Postprandial levels of LF detected in the serum. Serum was collected over multiple time points after administration on Day 1 and Day 14 of dosing. ELISA was performed to detect LF (rhLF or bLF) in the serum. **A.** Day 1 LF results in male rats **B.** Day 14 LF results in male rats. **C.** Day 1 LF results in female rats. **D.** Day 14 results in female rats. N = 3 animals per time point. No statistical analysis was performed due to insufficient data/lack of responses above the limit of detection of the assays.

Supplemental Figure 1. Body Weight Changes by Sex. Body weight was measured bi-weekly for each animal during the treatment period for the LF absorption animals (Group 6-10) and the

mean results shown over time. Males are shown on the top figure and females on the bottom figure.

Supplemental Figure 2. Spleens were collected after the 14-day exposure period and dispersed into single-cell suspension for immunophenotyping analysis via flow cytometry. Overall gating strategy. Left side: Panel 1; Right side: Panel 2

Data Availability: Not applicable

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Tables

Table 3: rhLF- and bLF-Related Hematology Changes – Day 15

Group	2		3		4		5	
Test Article	rhLF		rhLF		rhLF		bLF	
Dose (mg/kg/day)	200		100 0		200 0		2000	
Sex	M	F	M	F	M	F	M	F
HDW	—	—	—	—	—	1.07 x	—	1.08 x
MPV	—	—	—	—	1.08 x	1.07 x	1.10 x	1.18 x
PDW	—	—	—	—	—	0.93 x	—	0.91 x
MPC	—	—	—	—	—	0.96 x	—	0.91 x
PCDW	—	—	—	—	0.91 x	0.92 x	0.93 x	0.87 x

M = Males; F = Females.

A dash (—) indicates absence of a test article-related change. Numerical values indicate fold change of the treated group mean value relative to the control group mean value. N = 3/sex/group

Table 4: rhLF- and bLF-Related Clinical Chemistry Changes – Day 15

Group	2		3		4		5	
Test Article	rhLF		rhLF		rhLF		bLF	
Dose (mg/kg/day)	200		100 0		200 0		2000	
Sex	M	F	M	F	M	F	M	F
TBIL	—	—	—	—	1.39 x	1.20 x	1.32 x	—
UREAN	—	—	—	—	1.33 x	—	—	1.35 x
CREAT	—	—	—	—	0.75 x	0.78 x	—	—
CHOL	—	—	—	—	1.35 x	—	—	—
TRIG	—	—	—	—	—	1.54x	—	1.58x
ALB	—	—	—	—	1.14 x	1.09x	1.13x	1.11x
A:G	—	—	—	—	1.14 x	1.13x	1.17x	1.11x
PHOS	—	—	—	0.89x	0.89 x	0.89x	0.87x	0.81x
NA	—	—	—	—	0.97 x	0.97x	—	0.97x
CL	—	—	—	—	0.95 x	0.97x	—	—

M = Males; F = Females.

A dash (—) indicates absence of a test article-related change. Numerical values indicate fold change of the treated group mean value relative to the control group mean value. N = 3/sex/group

Table 5: rhLF- and bLF-Related Clinical Chemistry Changes – Iron-Related Parameters-Day 15

Group	2		3		4		5	
Test Article	rhLF		rhLF		rhLF		bLF	
Dose (mg/kg/day)	200		100 0		200 0		2000	
Sex	M	F	M	F	M	F	M	F
FERR	—	—	—	—	0.70 x	—	0.46 x	—
FE	—	—	—	—	1.47 x	—	1.24 x	—
TIBC	—	—	—	—	1.08 x	—	1.07 x	—
TFRNSAT	—	—	—	—	1.36 x	—	1.16 x	—

M = Males; F = Females.

A dash (—) indicates absence of a test article-related change. Numerical values indicate fold change of the treated group mean value relative to the control group mean value. N = 3/sex/group

Table 6: Ingestion of Helaina rhLF or bLF Leads to Minimal Alterations in Immune Cell Phenotypes within the Spleen

	Male Mean (SD)					Female Mean (SD)				
	0	200 rhLF	1000 rhLF	2000 rhLF	2000 bLF	0	200 rhLF	1000 rhLF	2000 rhLF	2000 bLF
Viable Splenocytes	98.27 (0.30)	98.12 (0.46)	98.10 (0.42)	97.40 (0.71)	96.65 (2.82)	98.10 (0.58)	98.25 (0.37)	98.28 (0.26)	97.98 (0.75)	97.98 (0.44)
Gran/Mono/Mac/DC (CD172a+)	14.88 (1.16)	15.93 (3.72)	16.55 (1.35)	21.58*** (2.54)	23.95**** (3.54)	14.23 (1.90)	12.85 (3.13)	13.03 (1.65)	22.05*** (3.87)	21.13*** (2.88)
Mono/Mac	26.75 (3.78)	26.35 (4.29)	28.10 (3.35)	28.00 (4.52)	26.08 (4.33)	20.53 (4.73)	21.37 (3.74)	21.92 (7.58)	20.10 (3.73)	21.05 (5.14)
Dendritic Cells	9.11 (1.27)	10.80 (2.94)	8.35 (2.04)	16.75*** (1.74)	14.83** (5.12)	9.43 (3.24)	10.75 (2.25)	11.02 (4.28)	21.83*** (4.20)	27.53**** (6.65)
B Cells	49.18 (6.41)	46.05 (5.07)	44.93 (3.00)	43.70 (7.09)	41.83 (3.68)	47.97 (3.70)	49.57 (2.18)	45.40 (3.72)	41.70 (6.63)	46.72 (5.15)
T Cells	28.47 (5.68)	34.57 (2.65)	31.50 (1.13)	29.52 (3.12)	32.60 (7.09)	33.22 (5.16)	29.38 (3.30)	32.25 (3.67)	36.97 (1.73)	32.17 (5.42)
CD8⁺ T Cells	22.17 (6.48)	26.57 (2.36)	26.57 (2.37)	31.13** (4.44)	32.67** (5.79)	25.72 (3.25)	27.05 (3.82)	27.47 (3.23)	33.00* (5.53)	27.45 (3.15)
Helper T Cells (CD4+)	53.33 (3.95)	51.32 (3.27)	52.68 (2.41)	48.97 (2.37)	49.50 (4.80)	49.87 (2.35)	47.48 (3.36)	52.50 (2.87)	47.45 (6.10)	51.90 (4.00)

Treg Cells	3.03 (1.45)	1.72 (1.22)	1.84 (1.56)	3.75 (2.54)	2.52 (2.01)	1.85 (1.37)	1.55 (0.57)	1.06 (0.34)	2.91 (1.88)	3.42 (1.23)
Activated T Cells	19.97 (3.23)	17.30 (1.04)	18.45 (1.19)	5.23**** (1.08)	5.20**** (1.49)	17.75 (2.14)	16.70 (0.70)	15.30 (4.40)	6.08**** (1.45)	4.40**** (0.91)
Natural Killer Cells	10.37 (2.86)	11.28 (1.25)	11.41 (1.37)	8.26 (1.56)	8.72 (1.06)	9.88 (1.91)	9.37 (1.80)	8.86 (1.05)	8.63 (1.82)	6.50** (1.12)
NKT Cells	17.65 (3.92)	18.03 (1.48)	18.67 (2.29)	17.67 (2.77)	15.87 (2.86)	16.80 (2.72)	16.88 (2.50)	14.48 (1.72)	16.40 (3.45)	13.75 (2.46)

DC = dendritic cells, Gran = granulocytes, Mac = macrophages, Mono = monocytes, Treg = regulatory T cells

N = 6 rats per group. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

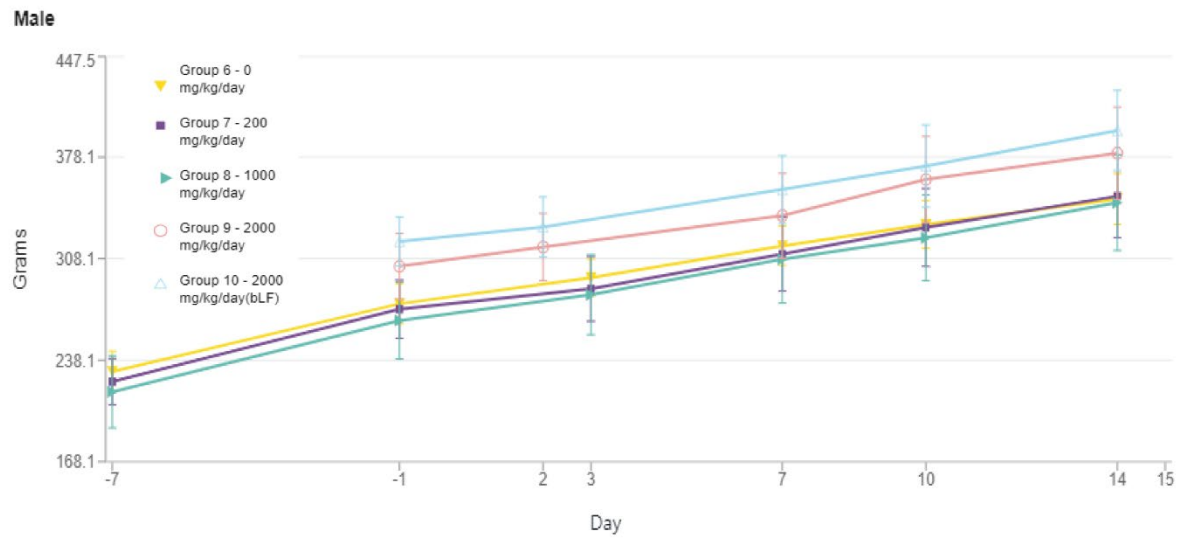
Supplemental Table 1: Hematologic and Clinical Chemistry Parameters

Hematology	Clinical Chemistry (Without Iron)	Clinical Chemistry (Iron Parameters)
• Leukocyte count (total and differential) • Erythrocyte count • Hemoglobin • Hematocrit • Absolute reticulocytes • Mean platelet volume (MPV) • Cellular hemoglobin concentration mean (CHCM) • % reticulocytes • Platelet distribution width (PDW) • Plateletcrit (PCT) • Mean cellular hemoglobin content (CH) • Mean corpuscular hemoglobin (MCH) • Mean corpuscular volume (MCV) • Mean corpuscular hemoglobin concentration (calculated) (MCHC)) • Red blood cell distribution width (RDW) • Platelet count	• Alkaline phosphatase • Total bilirubin^a • Aspartate aminotransferase • Alanine aminotransferase • Urea nitrogen • Creatinine • Total protein • Albumin • Globulin • Albumin/globulin (A/G) ratio (calculated) • Glucose • Total cholesterol • Triglycerides • Electrolytes (sodium, potassium, chloride) • Calcium • Phosphorus • Sample quality	• Unsaturated iron binding capacity (UIBC) • Total iron binding capacity (TIBC) calculated • Iron • Ferritin • Transferrin saturation (TSAT)

• Blood smear (preserve and stain) • Hemoglobin distribution width (HDW) • Platelet component distribution width (PCDW) • Reticulocyte hemoglobin content (CHr) • Mean reticulocyte volume (MCVr)		
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*When total bilirubin was >0.5 mg/dL, direct bilirubin was also measured, and indirect bilirubin was calculated.

A



B

