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The lactoferrin receptor may mediate the reduction of eosinophils in the duodenum of pigs consuming milk containing recombinant human lactoferrin

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Abstract Lactoferrin is part of the immune system and multiple tissues including the gastrointestinal (GI) tract, liver, and lung contain receptors for lactoferrin. Lactoferrin has many functions, including antimicrobial, immunomodulatory, and iron binding. Additionally, lactoferrin inhibits the migration of eosinophils, which are constitutively present in the GI tract, and increase during inflammation. Lactoferrin suppresses eosinophil infiltration into the lungs and eosinophil migration *in -vitro*. Healthy pigs have a large population of eosinophils in their small intestine and like humans, pigs have small intestinal lactoferrin receptors (LFR); thus, pigs were chosen to investigate the effects of consumption of milk containing recombinant human lactoferrin (rhLF-milk) on small intestinal eosinophils and expression of eosinophilic cytokines. In addition, LFR localization was analyzed in duodenum and circulating eosinophils to determine if the LFR could play a role in lactoferrin's ability to inhibit eosinophil migration. In the duodenum there were significantly fewer eosinophils/unit area in pigs fed rhLF-milk compared to pigs fed control milk ($p = 0.025$); this was not seen in the ileum ($p = 0.669$). In the duodenum, no differences were observed in expression of the LFR, or any eosinophil migratory cytokines, and the amount of LFR protein was not different ($p = 0.386$). Immunohistochemistry (IHC) showed that within the duodenum the LFR localized on the brush border of villi, crypts, and within the lamina propria. Circulating eosinophils also contained LFRs, which may be a mechanism allowing lactoferrin to directly inhibit eosinophil migration.

Keywords Eosinophils · Gastrointestinal tract · Human lactoferrin · Inflammation · Lactoferrin receptor · Transgenic

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Introduction

Lactoferrin is an 80 kDa iron binding protein that is secreted in milk and tears, and can also be found in neutrophil granules. Lactoferrin acts as part of the

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immune system and has a variety of functions, acting as an antimicrobial, immunomodulator, and antioxidant (Wakabayashi et al. 2006). Multiple tissue types including the gastrointestinal (GI) tract, liver, and lung contain receptors for lactoferrin (Elfinger et al. 2007; Suzuki et al. 2005). Lactoferrin has targeted control of some cellular processes and can act as a transcription factor, regulating granulopoiesis and DNA synthesis in certain cells types (Kanyshkova et al. 2001). Administration of lactoferrin is an effective treatment for enterogenic endotoxinemia in rats (Nebermann et al. 2001) and suppressed tumor formation in a transgenic mouse lung tumor model (Tung et al. 2013). Lactoferrin can also alter circulating levels of white blood cells (Cooper et al. 2012) and direct differentiation of monocytes (van der Does et al. 2012).

Eosinophils are a class of immune cells often seen during allergic reaction and other bouts of inflammation; however eosinophils are also constitutively present in certain tissues, such as the GI tract. There is growing evidence that eosinophils have a more complex role in immunology than previously thought (Svensson-Frej 2011), including playing a role in tissue remodeling, homeostasis, and the adaptive immune response. In different tissues eosinophils have different phenotypes and life spans (Masterson et al. 2011). Within the GI tract eosinophils comprise a significant subset of the population of resident immune cells seen during steady state conditions, with notably more eosinophils in the duodenum. GI tract eosinophils typically live for at least 7 days, and increased longevity is correlated with changes in cytokine expression (Svensson-Frej 2011).

Changes in eosinophil numbers can occur via changes in eosinophil differentiation in the bone marrow leading to changes in the circulating pool, changes in migration from circulation into a target tissue, and changes in survival longevity within the tissue (Svensson-Frej 2011). The number of intestinal eosinophils is significantly elevated in ulcerative colitis (UC) and Crohns disease, with prior research indicating that they are key to the pathologies of these diseases. This may be caused by increased eosinophil products like major basic protein (MBP) and eosinophil cationic protein (ECP), which are found in UC tissue and correlated with increased disease severity (Maltby et al. 2010).

Lactoferrin can suppress airway inflammation and eosinophil infiltration to the lungs during experimentally induced lung inflammation (Zimecki et al. 2012). Lactoferrin is not toxic to eosinophils and exerts a direct effect on eosinophils by inhibiting their migratory ability in a concentration dependent manner without affecting transcription of chemoattractant molecules (Bournazou et al. 2010; Curran and Bertics 2012). In addition, lactoferrin reduces granulocyte macrophage colony-stimulating factor (GM-CSF) stimulated adhesion of eosinophils, which is possibly one of the mechanisms causing decreased eosinophil migration (Curran and Bertics 2012).

Pigs, like humans, have lactoferrin receptors throughout their small intestine (Liao et al. 2007). Due to the high number of lactoferrin receptors located in the small intestine, it is a logical target for lactoferrin mediated inhibition of eosinophil migration. To determine the effects of consumption of human lactoferrin on the population of eosinophils in the small intestine, young pigs were fed transgenic cows' milk containing recombinant human lactoferrin (rhLF-milk) or control cows' milk. Pigs are an ideal model because, in addition to having small intestinal lactoferrin receptors, healthy pigs are known to have high numbers of eosinophils in their GI tracts (Tsukahara et al. 2007). The effects of feeding milk containing recombinant human lactoferrin (rhLF-milk) on expression of key cytokines in the small intestine related to eosinophil migration were also investigated. To help elucidate if the lactoferrin receptor could play a role in inhibiting eosinophil migration the presence and localization of the lactoferrin receptor was also analyzed in circulating eosinophils and the small intestine which is a key sites that eosinophils migrate to.

Materials and methods

Milk collection and pasteurization

Transgenic cow's milk containing recombinant human lactoferrin (rhLF-milk) was provided by Pharming Group NV from a second parity Holstein from their herd in Wisconsin (USA). Milk was collected, pooled, frozen and then sent to the University of California Davis. A non-transgenic Holstein matched for parity and stage of lactation (mid-lactation) from the UC Davis dairy herd was selected and control milk was collected and frozen. Both control and rhLF containing milk were pasteurized at 73.8 °C for ten seconds

then immediately cooled and stored at 4 °C until consumption by the pigs. Pre and post-pasteurisation samples were collected and tested for concentrations of rhLF and bLF and for lactoferrin activity through a bacterial lysis assay. The rhLFmilk contained 1.2 g/L of rhLF and 0.13 g/L bLF and retained 85 % of its activity after pasteurisation.

Animals, Necropsy, and Sample Collection

Male Hampshire Yorkshire crossbred pigs were obtained from the University of California specific pathogen free (SPF) swine facility. Pigs were weaned at 3 weeks of age and then moved to a containment facility at 6 weeks of age. Pigs were weighed upon arrival and kept in a temperature-controlled room between 25 and 27 °C with ad libitum access to food and water for the duration of the trials. The pigs were monitored twice daily for physical and general well-being. The specific diet and rearing of the pigs used for this study was previously described (Brundige et al. 2008). Pigs were singly housed and randomly assigned to groups and twice daily for one week fed 250 mL of either pasteurized rhLF-milk from one transgenic cow (n = 8) or pasteurized milk from a non-transgenic control cow (n = 8). Groups were balanced to have equal numbers of pigs from each litter. The amount of milk was increased to 350 mL twice daily for the second week. At 8 weeks of age the pigs were weighed and euthanized using pentobarbital sodium (Fatal-Plus®, Vortech Pharmaceuticals, Ltd.) and tissue samples were collected. Duodenum samples were taken 20 cm below the pyloric sphincter and ileum samples were taken 20 cm above the ileocecal junction. Tissue samples for qRT-PCR analysis were snap frozen in liquid nitrogen before being stored at −70 °C until RNA extraction. Samples for histology were washed in PBS then placed in formalin. The use and care of all animals in this study was approved by the UC Davis Institutional Animal Care and Use Committee, under Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) approved conditions.

Blood collection and eosinophil purification

Blood was collected from two healthy 6-week-old male Hampshire Yorkshire pigs housed at the University of California swine facility. Pigs were placed in a recumbent position on a V shaped table to restrict their movement and blood was collected from the cranial vena cava. Samples were collected into 10 mL tubes containing EDTA (Becton–Dickinson Company, Franklin Lakes, NJ). Blood samples were pooled and whole blood smears were made on lysine coated adhesive slides and fixed in either paraformaldehyde or 4 °C methanol for 10 min. Eosinophils were isolated from the remaining whole blood according to Samoszuk (2006). Briefly, 2 mL of whole blood was added to 40 mL of sterile distilled water in a 50 mL conical tube and slowly inverted for 1 min, then 4 mL of sterile 10X PBS was added. The tubes were centrifuged at $1{,}200\times g$ for 5 min and the supernatant was aspirated, leaving a light tan pellet of eosinophils. The pellet was resuspended in 1 mL of distilled water and transferred to a 2 mL centrifuge tube and centrifuged again at $1{,}200\times g$ for 5 min. Supernatant was again aspirated and the pellet resuspended in 0.2 mL of distilled water and 50 µL of the resuspended eosinophil pellet was applied to lysine coated adhesive slides and fixed in either paraformaldehyde or 4 °C methanol for 10 min. Slides were examined under a confocal microscope to assess the purity of the isolated eosinophil samples.

Histology

Sections from the duodenum and ileum were placed in formalin for 48 h, and then progressively dehydrated in ethanol. Sections were embedded in paraffin and then cut and mounted on slides. Slides from the duodenum and ileum were stained with hematoxylin and eosin and were photographed. Analysis was done by measuring the villi height, width, lamina propria thickness, and crypt depth at 10x magnification, using Spot Advanced Software (v3.4, Diagnostic Instruments, Sterling Heights, MI). In addition, the number of eosinophils within the villi, lamina propria, and migrated into the epithelial layer were counted at 40x magnification and analyzed as cells per millimeter of area. At least five villi were measured per intestinal section for each pig.

Immunohistochemistry (IHC) and immunocytochemistry (ICC)

Slides with two tissue sections each from the duodenum were dehydrated in citrosolve (Fisher Scientific,

Pittsburgh, PA), then dehydrated in graded ethanol (95, 90, and 66.5 %), with a final wash in distilled water for 5 min. Paraformaldehyde and methanol fixed whole blood smear slides and isolated eosinophil slides were rehydrated in distilled water and then in PBS. A PAP pen was used to circle a control and experimental side on each slide. Peroxide block solution (3 % H_20_2 in methanol) was applied to the lung and duodenum slides for 10 min, and then the slides were washed in PBS for 5 min. Serum blocking solution (1:200 dilution of goat serum (Vector Labs, Vectastain kit 6101) in PBS) was applied to all the slides and they were incubated at room temperature for 20 min. Serum blocking solution was removed and the primary antibody was applied. For control tissue a 1:20,000 dilution for rabbit serum (Vector Labs, Vectastain kit 6101) in PBS was used, and for experimental tissue a 1:200 dilution of ITLN1 (Intelectin-1, Abcam, ab118232) in PBS was used. Samples were incubated with primary antibody at room temperature for 2 to 6 h. After incubation the slides were washed in TBST and TBS. Secondary antibody (3:200 dilution of goat anti-rabbit antibody (Vector Labs, Vectastain kit 6101) in PBS) was applied to the slides and incubated at room temperature for 30 min. After incubation the slides were washed in Tris-Buffered Saline-Tween (TBS-T) and TBS. After incubation with the secondary antibody an abotin and biotin solution (1:1:50 dilution of abotin:biotin:PBS, (Vectastain Elite ABC Kit))was applied to the slides and incubated at room temperature for 30 min. After incubation the slides were washed in TBS-T and TBS. After this Vector NovaRED (Vector Labs) solution was prepared according to the manufactures protocol and applied to slides for 5 min, and then slides were washed in distilled water for 5 min. A hematoxylin counter stain was performed and coverslips were applied to the slides.

RNA preparation, cDNA synthesis, and qPCR

Samples from the duodenum were used for cytokine expression analysis. The isolation of and preparation of RNA, cDNA synthesis, and qPCR conditions have been previously described (Cooper et al. 2011). The transcription levels of lactoferrin receptor, IL-4, IL-5, IL-6, and CCL-11 (eotaxin) (Table 1) were determined using the Pfaffl method with REST-MCS software (Pfaffl et al. 2002). Briefly, the efficiency of each porcine specific and validated primer pair was calculated from standard curve data. Each target gene was normalized to the housekeeping gene β-actin to determine pair-wise fold differences in expression.

Protein isolation and western blot analysis

Duodenum portions were homogenized in RIPA buffer (20 mM Tris pH 7.4, 150 mM NaCl, 0.5 % Triton X-100, 10 mM EDTA) including protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and centrifuged at $12{,}000\times g$ for 30 min. The protein concentration of the resulting aqueous phase was determined by the bicinchoninic acid (BCA) assay (Pierce, Thermo Scientific, Rockford, IL). Duodenal LFR abundance was assessed by SDS-PAGE (reducing conditions). Briefly, 50 µg protein sample was added to each gel lane, electrophoresed, and transferred to nitrocellulose membranes (Amersham Hybond-ECL: GE Healthcare, Pittsburgh, PA). Membranes were blocked in PBS-Tween containing 5 % [w/v] non-fat milk prior to probing with rabbit-polyclonal anti-ITLN-1 primary antibody (Abcam, Cambridge, MA) diluted 1:2,000 in PBS-T containing 5 % [w/v] BSA (Sigma-Aldrich). Following overnight incubation at 4 °C blots were incubated in 1:8,000 donkey-anti-rabbit IgG HRP secondary antibody (GE Healthcare), rinsed, and exposed to chemiluminescence reagent (Pierce ECL Western Blotting Substrate: Peirce, Thermo Scientific) for analysis. Blots were re-probed with a 1:5,000 dilution of mouse-monoclonal anti-β-actin primary antibody (Santa Cruz Biotechnology, Dallas, TX) and 1:8,000 rabbit–anti-mouse IgG HRP secondary antibody (DakoCytomation, Carpinteria, CA) to confirm equivalent protein loading. Membranes were exposed on Blue Ultra Autorad Film (GeneMate, BioExpress, Kaysville, UT) and resulting blots were quantified using Image Studio Lite software (LI-COR Biosciences, Lincoln, NE).

Statistical analysis

Statistical analysis of histological and western blot data was performed using SAS statistical software (SAS, Cary, NC, USA). Tukey's test was used to determine p-values and standard errors. Western blot data were normalized according to β-actin expression. Statistical analysis for fold expression differences

Table 1 Gene expression in the duodenum of hLF-milk fed pigs compared to control milk fed pigs

Gene name	Forward primer (3′–5′)	Reverse primer (3′–5′)	Fold change	*p* value
LFR	TGGAATGGTTCCCGGAGAA	GCAACTCCGAGGCAGGAAA	1.198	0.701
IL-4	GTCTGCTTACTGGCATGTACCA	GCTCCATGCACGAGTTCTTTCT	0.911	0.858
IL-5	GACTGGTGGCAGAGACCTTGAC	CTTCAATGCATAGTTGGTGATTTGT	0.776	0.495
IL-6	CTGGCAGAAAACAACCTGAACC	TGATTCTCATCAAGCAGGTCTCC	0.967	0.94
CCL-11 (eotaxin)	AGCTTCTGTCGCCACCATCT	TTCTCTTGGGCATCAGCACAG	1.059	0.888

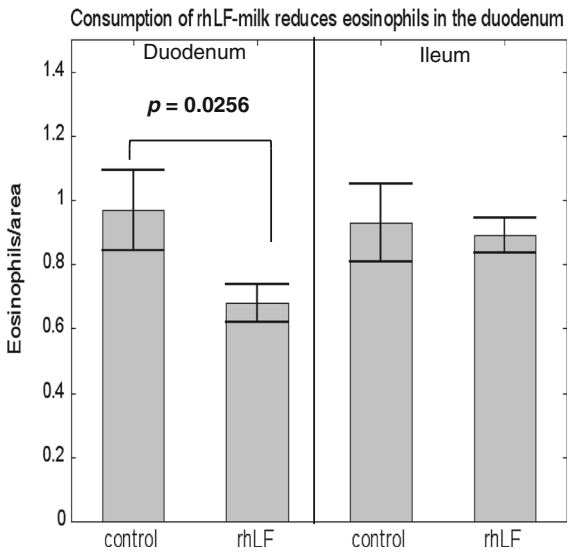

Fig. 1 Consumption of rhLF-milk reduces duodenal eosinophils. Average eosinophils/area counts from the duodenum and ileum of pigs drinking rhLF-milk and control milk. Eosinophils were significantly reduced ($p = 0.0256$) in the duodenum of pigs consuming rhLF-milk, however no differences were observed in the ileum

from the qPCR assay was performed using REST-MCS software using β-actin expression to normalize the data. For all analyses a p value of ≤ 0.05 was considered statistically significant.

Results

Histology

There was a significant reduction in the total number of eosinophils in the duodenum of rhLF-milk fed pigs in comparison to control milk fed pigs ($p = 0.0256$). There was no difference between milk feeding groups in the total number of eosinophils in the ileum ($p = 0.6714$) (Fig. 1). The subset of total intestinal eosinophils that had migrated into the single cell epithelial layer was also analyzed and no differences were observed in the duodenum ($p = 0.5996$) or the ileum ($p = 0.6697$) between the two groups.

qRT-PCR

In the duodenum there were no differences in the expression of lactoferrin receptor, IL-4, IL-5, IL-6, or eotaxin between the two milk feeding groups (Table 1)

Immunohistochemistry

Immunohistochemistry showed that within the porcine small intestine the lactoferrin receptor is present in the apical membrane of enterocytes as well as the cells residing in the crypts. There were also cells within the lacteal of the villi that contained the lactoferrin receptor (Fig. 2a, b).

Immunocytochemistry

Immunocytochemistry analysis showed that the lactoferrin receptor is present on circulating porcine eosinophils (Fig. 2c, d).

Western blot analysis

Western blot analysis showed no differences in the amount of lactoferrin receptor protein in the duodenum of pigs fed rhLF-milk compared to pigs fed control milk ($p = 0.3859$) (Fig. 3).

Discussion

Eosinophils are a class of granulocytes which complete their differentiation in the bone marrow, upon

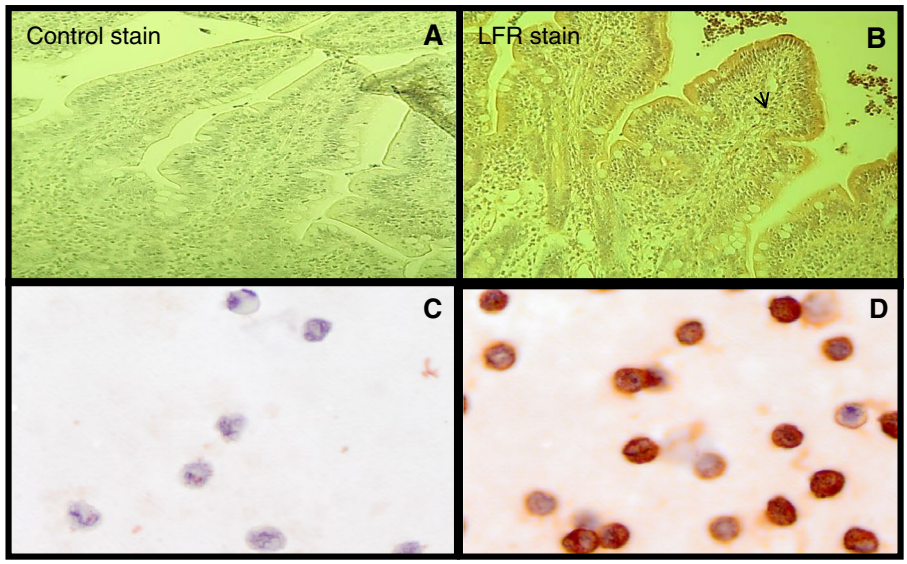

Fig. 2 Lactoferrin receptor localization in the duodenum and eosinophils. Immunohistochemistry and immunocytochemistry slides with control serum and lactoferrin receptor (LFR)antibody staining demonstrating that in the duodenum (**a**, **b**) the LFR is localized to the apical membrane of enterocytes and cells residing within the crypts and some cells within the lacteal of the villi also stained positive for the lactoferrin receptor. Circulating eosinophils also express the lactoferrin receptor on their membrane (**c**, **d**)

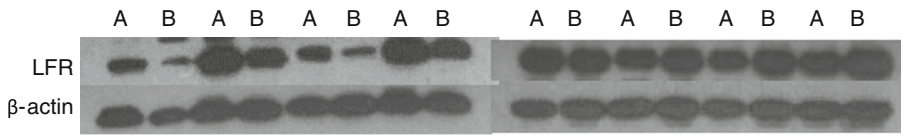

Fig. 3 Consumption of rhLF-milk does not affect lactoferrin receptor density in the duodenum. Western blot analysis of lactoferrin receptor and β-actin protein expression in the duodenum of pigs fed rhLF-milk (A group) and pigs fed control milk (B group)

which they enter into circulation and then migrate into different tissues throughout the body. Increased infiltration of eosinophils is seen in multiple tissues during periods of increased inflammation, including the lung during asthma and allergic reactions (Winther et al. 1999), and the GI tract during irritable bowel disease (IBD) and Crohn's disease (Katsanos et al. 2011; Wedemeyer and Vosskuhl 2008). Eotaxin is one of the chemokines responsible for eosinophil migration into tissue and in a chemically induced model of colitis mice lacking eotaxin had impaired eosinophil migration and ameliorated cases of colitis (Maltby et al. 2010). This supports the concept that inhibiting eosinophil migration into tissues during inflammation could help reduce tissue damage.

Lactoferrin has been shown to inhibit eosinophil migration in in-vitro-models (Bournazou et al. 2010) as well as in an induced model of asthma (Zimecki et al. 2012). Our study shows that consumption of recombinant human lactoferrin in milk (rhLF-milk) by healthy young pigs can reduce eosinophil infiltration in the duodenum of the small intestine. Lactoferrin in-vitro- directly inhibits eosinophil migration without changing transcription of eosinophil chemoattractant molecules such as IL-6 or eotaxin (Bournazou et al. 2010). Our in-vivo results from the duodenum support this finding, showing that the reduction in eosinophils in the duodenum was not related to changes in expression of IL-4, IL-5, IL-6, or CCL-11 (eotaxin).

The actual mechanism that lactoferrin utilizes to inhibit eosinophil migration is still unknown, although there is evidence that lactoferrin reduces integrin dependent adherence of eosinophils (Curran and Bertics 2012). Adherence is a process that is necessary

for eosinophils to be able to migrate from circulation into a specific tissue. Lactoferrin also inhibits expression of vascular endothelial adhesion molecules, E-selectin and ICAM-1 (Baveye et al. 2000). It is unclear exactly how lactoferrin is able to alter eosinophilic and endothelial adherence molecules; however, based on our data demonstrating that eosinophils have lactoferrin receptors the lactoferrin receptor could play a role in this phenomenon.

Pigs and humans both express lactoferrin receptors (LFRs) throughout their small intestines, with the highest density in the duodenum (Liao et al. 2007; Suzuki et al. 2005). Pigs fed lactoferrin have been shown to transport the consumed lactoferrin across the apical membrane of intestinal enterocytes, where it can then bind to cells within the intestine or travel into circulation and eventually enter other tissues including the liver (Harada et al. 1999). Due to the high level of LFRs in the small intestine, the internalization of consumed lactoferrin, and the presence of eosinophils in the small intestine even in healthy animals, the small intestine is a logical target tissue for eosinophil reduction due to lactoferrin consumption. We found that in the duodenum the LFR was localized along the apical membrane of the cells lining the intestinal crypts and villi, and that certain cells within the villi lacteal also stained positive for LFRs (Fig. 2b). Consumption of rhLF-milk did not affect the transcription of the LFR or the amount of LFR protein in the duodenum, indicating that consumption of lactoferrin does not directly up- or down-regulate LFR expression.

Along with allowing for the internalization and transport of lactoferrin we wanted to determine if the LFR could play a role in facilitating the direct effect that lactoferrin has on eosinophils. Lactoferrin is known to have multiple immune modulating properties including being able to bind and direct immune responses in certain subsets of leukocytes including monocytes (Crouch et al. 1992; van der Does et al. 2012) and lymphocytes (Dhennin-Duthille et al. 2000), which are known to contain the LFR (Bi et al. 1994). Through ICC we were able to confirm that circulating porcine eosinophils also contain the lactoferrin receptor (Fig. 2d). It is still unknown if the LFR plays a role in transducing the effects of lactoferrin on eosinophils; however, it is plausible given that lactoferrin exerts its effects on other leukocytes through LFRs.

This study found that consumption of milk containing recombinant human lactoferrin by pigs resulted in a significant reduction in the number of eosinophils in the duodenum, which supports other findings in the lung (Zimecki et al. 2012), and in in-vitro models (Bournazou et al. 2010). Further, we showed that in pigs LFRs are expressed on the apical membranes of the enterocytes and crypt cells of the intestinal villi in the duodenum. The current study and previous research show that both the lung and intestines are targets for the effects of lactoferrin, and it is possible that the LFR plays a role in mediating these effects by localizing lactoferrin to those tissues. This study supports previous research that shows that lactoferrin directly inhibits eosinophil migration independent of cytokine production (Bournazou et al. 2010) as we observed no changes in expression of key eosinophilic cytokines in the duodenum of the rhLF-fed pigs. The data also shows that porcine eosinophils contain the LFR, revealing a possible mechanism for the direct effects of lactoferrin on eosinophils seen in previous research; however additional studies are needed to determine what if any role the LFR plays in inhibiting eosinophils migration. Chiefly, further research must be done to confirm that lactoferrin binds to eosinophilic LFRs, and to determine the specific cellular changes that this binding causes. Due to the role that eosinophils play in allergic reactions and inflammatory disorders this research supports the possible use of recombinant human lactoferrin as a therapeutic treatment for the eosinophilia that occurs during inflammatory conditions in the GI tract and suggests that additional research in needed on eosinophilic lactoferrin receptors.

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Conflict of Interest The authors declare no conflict of interest.

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