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The farm effect revisited: from β-lactoglobulin with zinc in cowshed dust to its application

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Summary
Background Numerous factors such as microbiota and their products are discussed in the context of the hygiene hypothesis and the associated allergy-preventive farm effect. Besides inhalation of dust from farms, consumption of raw milk also counteracts the development of asthma and allergies. Since cattle barns and cow's milk in particular have been described as effective, the involvement of a bovine protein seems likely. β-Lactoglobulin (BLG) is a major protein in milk and, as a member of the lipocalin family, has an intramolecular pocket that allows binding to hydrophobic ligands.
Results Our *in vitro* and *in vivo* studies show that unloaded BLG promotes the development of allergy, while loaded, so-called holo-BLG, prevents allergies. BLG associated with zinc could also be detected in stable dust and ambient air of cattle farms.
Conclusion It seems obvious that in addition to microbes and their products, holo-BLG also plays an important role in the protective farm effect. Therefore, in a newly developed lozenge for dietary management of allergies, based on the farm effect, zinc attached to holo-BLG is one of the key ingredients.

Keywords Beta-lactoglobulin · Farm effect · Lipocalins · Allergy prevention

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Abbreviations

apo-BLG	Unloaded β-lactoglobulin
BLG	β-Lactoglobulin
BSA	Bovine serum albumin
CD4	Surface protein on cells of the immune system; CD, cluster of differentiation
CD14	Surface protein on cells of the immune system; CD, cluster of differentiation
holo-BLG	Loaded β-lactoglobulin
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL-13	Interleukin-13
LCN-2	Lipocalin-2
LIMR	Lipocalin-interacting membrane receptor
LPS	Lipopoly-saccharides
miRNA	MicroRNA
Neu5Gc	N-glycolylneuraminic acid
NGAL	Neutrophil gelatinase-associated lipocalin
PAULA	Perinatal Asthma Environment Long-Term Allergy Study
PBMC	Peripheral blood mononuclear cells
PRR	Pattern-recognition receptor
STATs	Signal transducers and activators of transcription
Th1	T helper cells 1
Th2	T helper cells 2
TLR	Toll-like receptor
TNFAIP3	Tumor necrosis factor α-induced protein 3
Treg	Regulatory T cells
UHT	Ultra-high temperature

Allergies and the role of hygiene

The hygiene hypothesis emerged from an attempt to establish a link between infectious diseases and the development of allergies [1]. Contrary to the prevailing view at that time that viruses caused allergies, David Strachan claimed in 1989 that viral infections transmitted through "unhygienic contact" in early childhood could prevent the development of allergies [2]. This claim has been substantiated by several studies demonstrating an indirect-proportional relationship between allergies and endemic infections (e.g. hepatitis A virus, *Helicobacter pylori, Toxoplasma gondii*) [3]. However, the term "hygiene" has often been misinterpreted in terms of personal hygiene or cleanliness in the household. The Perinatal Asthma Environment Long-Term Allergy Study (PAULA) followed up exactly on this misinterpretation and described that excessive household hygiene does not influence the development or prevention of allergies. Thus, increased household cleanliness and personal hygiene cannot prevent the development of allergy [4]. However, an inverse relationship between cleanliness and certain dust components (endotoxins, muramic acid) was found in this study. The presence of these dust components correlated with a reduced incidence of allergy and asthma. Nevertheless, the direct correlation between cleanliness and the incidence of allergies could not be demonstrated. The authors therefore summarize that the validity of the hygiene hypothesis is independent of cleanliness. Whether specific microbes, their relationship to each other, or microbial diversity play a role remains to be elucidated.

The farm effect: all about microbes?

Numerous studies show that the risk of developing asthma or allergies is reduced by up to 50% in children who grow up on farms [1]. This so-called farm effect is mediated among other things by a bacteria-rich environment, and bacterial products and components, for example endotoxins (LPS, lipopoly-saccharides) and N-glycolylneuraminic acid (Neu5Gc) [5].

Important interacting molecules on cells are Toll-like receptors 2 and 4, which are pattern-recognition receptors (PRRs) of the innate immune system.

TLR2 is expressed primarily on monocytes, macrophages, dendritic cells, B cells and T cells, including Treg (regulatory T cells) and is responsible for recognizing and binding cell wall components of Gram-positive bacteria.

The major receptor for endotoxin (LPS) is TLR4, which is expressed on monocytes/macrophages, neutrophils, dendritic cells, mast cells, B lymphocytes and intestinal epithelial cells.

The action of endotoxin is subsequently thought to be mediated via the ubiquitin-modifying enzyme A20 (also known as "tumor necrosis factor α-induced protein 3" or TNFAIP3), an endogenous regulator of inflammation [6].

However, allergies and asthma have a rather contradictory relationship with endotoxins: While LPS protect against allergies, they promote the development of non-atopic asthma [7]. These opposite effects indicate that the allergy- and asthma-preventive effects of microbes depend on other factors such as environmental influences, genetics, diet or possibly endotoxin concentration and microbial diversity.

Children who grow up in a farm environment are exposed to a greater diversity of bacteria and fungi than children who do not grow up on a farm [8]. This diversity and the production of butyrate, which is dependent on specific taxa, also appears to contribute to the maturation of the gut microbiota in children and to the subsequent reduction of asthma risk [9].

Pets also contribute to allergy prevention in terms of a favorable household microbiome, with studies showing that dog ownership is more favorable than cat ownership, and may even reduce the risk of food allergies [10]. One explanation may be the more evenly distributed, diverse microbiome of bacteria and molds in the household when dogs *versus* cats are kept [11].

In addition to the broader spectrum of microbes on farms, the specific taxa probably also play an important role. The comparison between farm and urban households showed that the risk of developing asthma decreases in children as the microbiome of urban households becomes more similar to that of farm households. Farm households primarily contain bacteria of the taxa Bacteroidales, Clostridiales, and Lactobacillales which are primarily associated with cattle [12].

Consistently with these findings, not all farms show the same protective effect: cattle and pig farms in particular provide a beneficial environment, demonstrating the allergy-preventive effect within a radius of up to 327 meters around the farm (up to 100 meters in the GABRIELA studies) [13–15].

Another important factor in the context of the hygiene hypothesis seems to be genetics. Genetic predisposition significantly influences the response to microbial components. Thus, primarily individuals with a specific polymorphism in the Toll-like receptor 2 (TLR2) gene appear to benefit from the protective effect of farms [16].

However, not only microbial substances on farms are likely to contribute to the protection against allergy. Studies on the environment of alpine farms found a high plant content in dust. The most important components are the so-called arabinogalactans, a group of branched polysaccharides from plant material (e.g. larch) for which an anti-Th2 effect and thus prevention of atopic asthma has already been shown in animal models [17].

Raw milk as another key to success?

In addition to endotoxins, microbial diversity and genetics, the consumption of raw milk contributes to the protective farm effect [18]. The GABRIELA study was the first to investigate the effect of raw milk on asthma, atopy and hay fever in a large cohort of school children and identified the whey content of raw milk as the important component that protects against these diseases. In particular, higher levels of the whey proteins bovine serum albumin (BSA), α-lactalbumin, and β-lactoglobulin (BLG) correlated with reduced asthma incidence. Moreover, it was shown that consumption of cooked milk, in which the proportion of whey proteins is significantly lower, no longer contributes to the protective farm effect [19].

Other molecules appear to contribute to allergy and asthma prevention in raw milk as well (review article Van Esch et al. 2020) [20]. For example, the effectiveness of "small non-coding microRNA" (miRNA) of cow's milk has also been discussed. Cow's milk comprises a high miRNA content including such with similarity to miRNA in human milk. This miRNA may also affect the gene expression of immune system-associated products such as STATs, interleukins and prostaglandins via mRNA modulation. The exact effect on the immune system remains to be determined; however, it is thought to be associated with reduced asthma and allergy incidence. Consistently, a much lower level of miRNA has been demonstrated in heat-treated milk (cooked, UHT) than in raw milk [21]. Thus, current studies are investigating the effect of minimally processed milk on asthma prevalence in young children [22].

Another milk component that may have a lasting immunologic effect on allergen development is bovine IgG (immunoglobulin G). Cow's milk contains large amounts of bovine IgG, which could bind and neutralize aeroallergens by forming immune complexes, and furthermore appears to be responsible for a Th1 response [23].

Oligosaccharides in cow's milk are thought to have a prebiotic function by promoting the growth of beneficial, immunomodulatory bacteria in the gut, which may protect against asthma and allergies [24].

Last but not least, the content of omega-3 fatty acids in milk seems to be crucial for allergy protection, again more of them being present in raw milk than in processed milk [25].

As observed for endotoxin, the effectiveness of milk in allergy-protection depends also on genetic factors: children homozygous for the G-allele in CD14-positive leukocytes are less likely to benefit from the effects of raw milk [26].

Raw milk as a tool for allergy prevention seems to be an effective natural remedy, but regular consumption is not recommended due to a potentially high pathogen load [27]. A promising alternative seems to be offered by minimally processed milk [22].

The ambivalence of BLG

In addition to its function as a whey protein, BLG (also known as Bos d 5 as an allergen) is considered one of the major allergens in cow's milk. BLG accounts for up to 50% of the whey fraction and about 12% of the total protein in cow's milk [28]. BLG belongs to the protein family of lipocalins, which possess an intramolecular pocket that allows them to bind small, hydrophobic ligands. Vitamin metabolites (retinoic acid, vitamin D3), hormones (epinephrine) and iron-binding siderophores (catecholates) can act as potential ligands [29]. The loading of lipocalin proteins with their ligands seems to play an important role in allergy development: While unloaded BLG (apo-BLG) promotes an increase in CD4-positive T helper cells as well as IL-13 (Th2 cytokine) and IFN (interferon) and thus inflammation, loaded holo-BLG suppresses CD4-positive T cells and therefore has immunosuppressive properties *in vitro* and *in vivo* [30–32]. These lipocalin-like properties have also been observed for the birch pollen allergen Bet v 1 [33, 34].

BLG in stable dust

To prove the actual importance of BLG in connection with the farm effect, its occurrence on cattle farms was examined. There, holo-BLG could be identified as a major protein, associated with zinc, in air as well as in barn dust [Pali-Schöll et al., manuscript in review]. Closer examination showed that bovine urine from both female and male animals was the primary source of BLG in barn dust. BLG accumulates in stable dust and reaches the air as aerosol. It was also detectable in aerosols surrounding cattle barns in decreasing concentration with increasing distance up to 300 meters around the barn—another fact confirming the involvement of holo-BLG in the described allergy-protective environment around cow farms [14]. Female and male animals produce and secrete this protein; however, its significance for the animal itself is unclear to date. Our data indicate an important function within the innate immune system. Accordingly, the human body also produces lipocalin proteins, some of which also exhibit immunomodulatory properties, for example, lipocalin-2 (LCN-2), also known as neutrophil gelatinase-associated lipocalin (NGAL). This protein is predominantly expressed in tissues exposed to environmental agents (lung, intestine) and its immunoregulatory functions depend on iron-siderophore loading [35, 36]. In fact, LCN-2 is also excreted in the urine of humans during acute kidney disease [37] and is considered a biomarker in this case as well as in tumor diseases [38]. However, it is significantly decreased in allergic patients [39].

BLG could be detected not only in stable dust and bovine urine but also in households of farms [Pali-Schöll et al., manuscript in review]; thus, people who live on a farm but do not spend time directly or regularly in the barn could also benefit from it.

The association of BLG with zinc

As our recent studies showed that the BLG loading status plays an important role and BLG was found associated with zinc in stable dust, BLG with and without

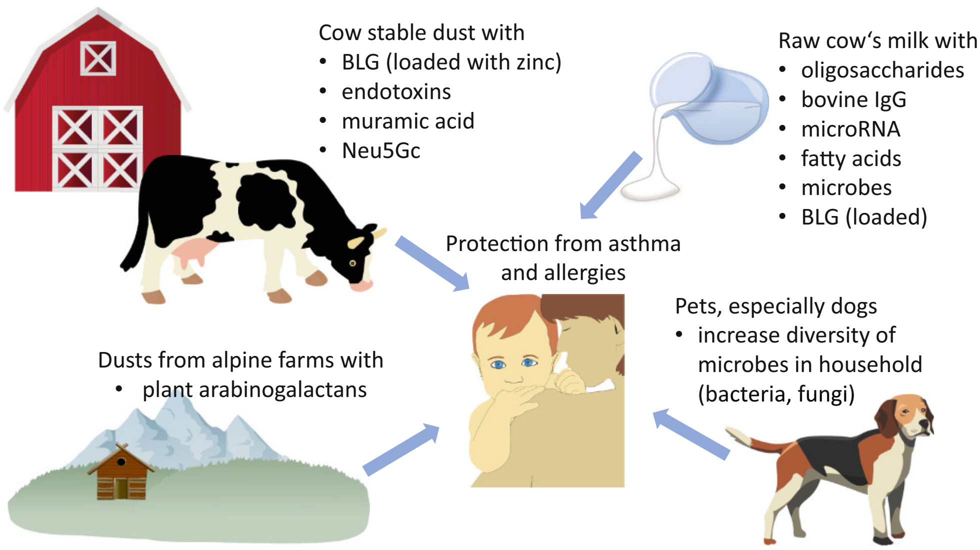

Fig. 1 The hygiene hypothesis: different factors influence the development of allergy and asthma, many of which are discussed as protective, especially if they act during pregnancy and childhood. *BLG* β-lactoglobulin, *Neu5Gc* N-glycolylneuraminic acid, *IgG* immunoglobulin G. *Image sources:* Stable, hut: Clker-Free-Vector-Images@Pixabay; cow: grafikacesky@Pixabay; mountain pasture, mother–child, milk: OpenClipart-Vectors@Pixabay; dog: Jose R. Cabello@Pixabay

zinc was studied in the cell system on PBMCs (peripheral blood mononuclear cells) of healthy donors. Zinc-loaded BLG induced lower proliferation of immune cells and favored the release of Th1-associated cytokines which counteract allergy induction [Pali-Schöll et al., manuscript in review].

The receptor responsible for the uptake or binding of BLG on immune cells is currently not completely clarified, but the lipocalin-interacting membrane receptor (LIMR) seems to be a promising candidate [Pali-Schöll et al., manuscript in review]. Among others, the uptake of BLG by LIMR was observed in different cell lines [40], which further supports the significance of BLG in the innate immune system.

BLG in practical application

Numerous studies prove the allergy-preventive as well as allergy-reducing properties of loaded holo-BLG. Following the convincing preclinical data, its efficacy could be confirmed in double-blind, placebo-controlled, randomized studies in pollen allergic patients (Bartosik et al., in preparation) ingesting holo-BLG in the form of a lozenge as well as in an application study on house dust mite allergic patients in the Berlin Provocation Chamber [41]. Zinc is an important immunomodulatory component in this lozenge. Ongoing studies will clarify further details of the mechanism of action with emphasis on the cellular uptake kinetics of holo-BLG and the influence of associated ligands such as zinc.

Conclusion

Several different mechanisms of action explaining the protective farm effect in the context of allergy prevention have been identified to date (Fig. 1); however, none of them offered an explanation for the efficacy of specifically bovine stables and bovine raw milk. According to our current studies, the presence of lipocalins such as BLG with its ligands in the environment and in milk seems to be crucial in the protection against allergy.

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