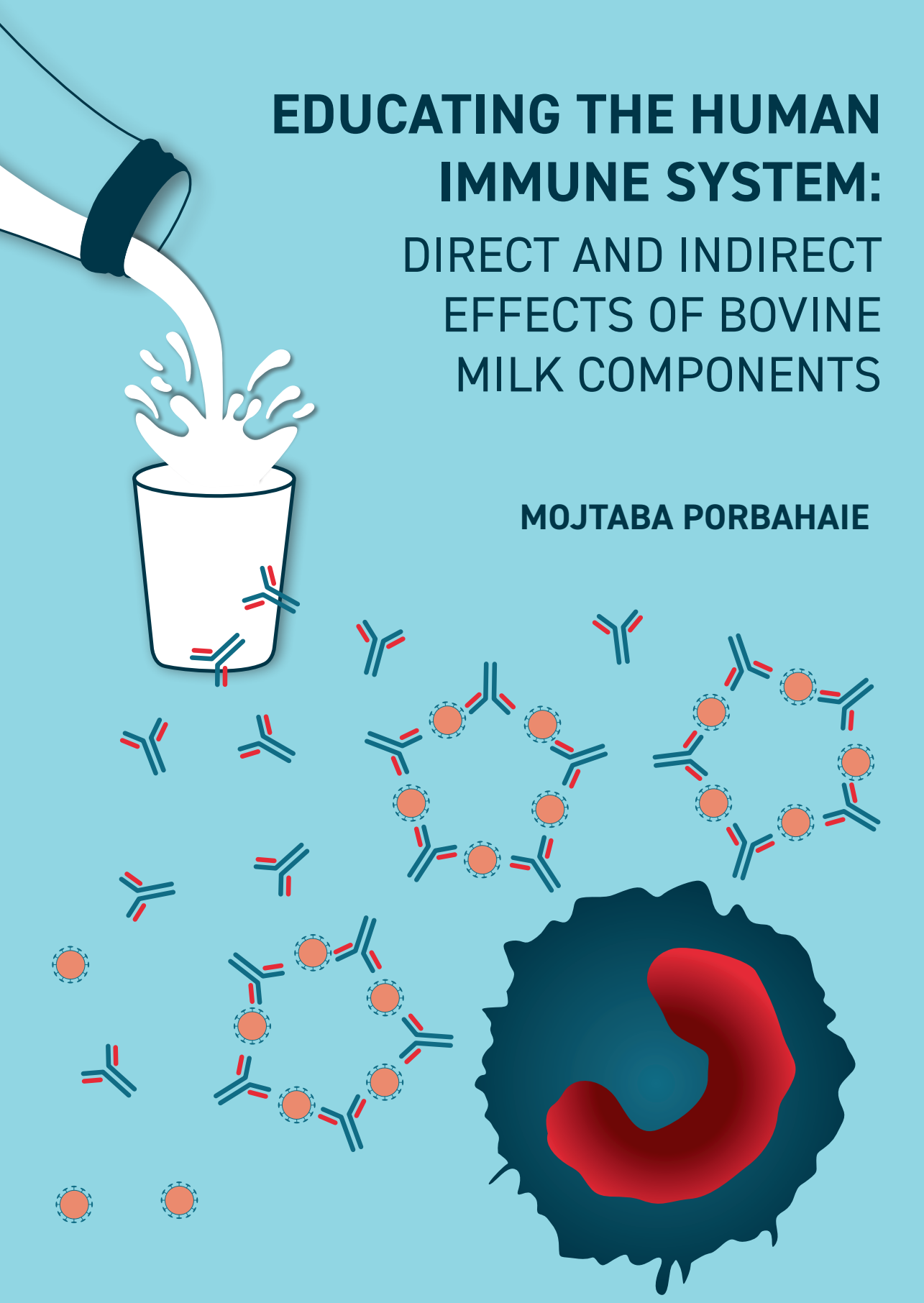
EDUCATING THE HUMAN IMMUNE SYSTEM:

DIRECT AND INDIRECT EFFECTS OF BOVINE MILK COMPONENTS

MOJTABA PORBAHAIE



Propositions

1. The monomeric form of bIgG does not induce innate immune training in human monocytes. (This thesis)

2. Two-tier infection challenge models are essential for studying the effects of (food) components on the adaptive as well as the innate immune system. (This thesis)

3. Epigenetics is the strongest contributor whereby the immune system adapts to environmental stimuli.

4. Academic education does not promote the acquisition of improvising abilities.

5. The combination of a pandemic and generating a PhD thesis is detrimental to one's health.

6. Science and arts mutually improve each other.

Propositions belonging to the thesis, entitled:

Educating the human immune system:
Direct and indirect effects of bovine milk components

Mojtaba Porbahaie
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Educating the human immune system:
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Educating the human immune system:
Direct and indirect effects of bovine milk components

Mojtaba Porbahaie

Thesis

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For Sara and Diba

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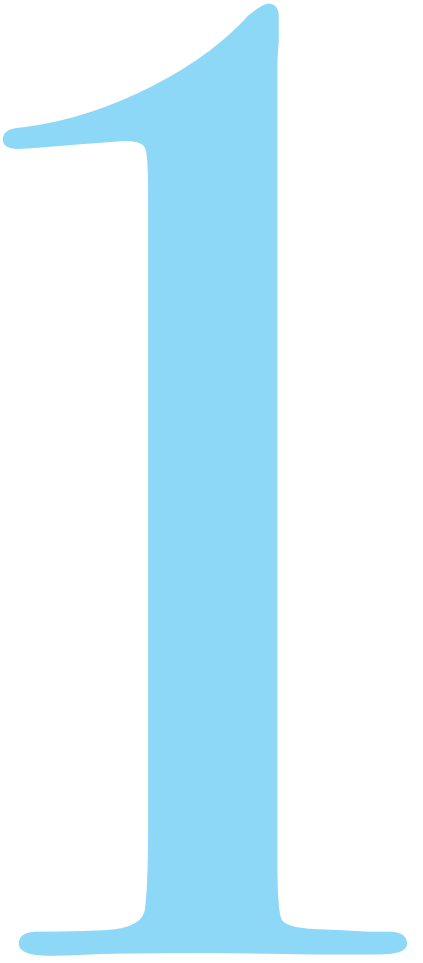
CHAPTER



General introduction

Introduction to the immune system

The key purpose of developing the immune system is to protect oneself from infections and malignancies. In vertebrates, the immune system is a complex multi-organ system composed of several tissues, specialized cells, and molecules that harmonically cooperate to remove intrinsic or extrinsic threats. Conventionally, the immune system is divided into the innate immunity and the acquired (adaptive) immune system. Cells of the innate immune system like neutrophils, monocytes/macrophages, Dendritic Cells (DCs), and Natural Killer (NK) respond rapidly to stimulation [1, 2]. They recognize conserved Microbial-Associated Molecular Patterns (MAMPS) using membrane-bound or cytosolic Pattern Recognition Receptors (PRRs) such as Toll-Like Receptors (TLRs), C-type Lectin Receptors (CLRs), Rig-Like Receptors (RLRs), and Nod-Like Receptors (NLRs) [3, 4]. Neutrophils and mononuclear phagocytes like macrophages phagocytose and kill microbes and secrete cytokines and chemokines to activate and recruit other cells to the site of infection. Whereas NK cells can directly induce apoptosis in (virus) infected cells, DCs have a key role in presenting pathogen-derived peptides to naive T lymphocytes to initiate an adaptive immune response [5]. Adaptive immunity represented by T- and B lymphocytes are involved in cellular and humoral responses, respectively. The generation of antigen-specific T and B cells requires V-D-J recombination at the TCR and BCR gene segments, resulting in high degrees of antigen specificity. Moreover, adaptive immune cells exert a higher magnitude of reaction upon second exposure. This means that T and B cells can develop immunological memory against formerly encountered antigens in contrast to innate immune cells, although this view has been questioned recently. This point will be discussed further in the section on trained immunity.

The mucosal immune system

The epithelial surfaces of the body are constantly exposed to the outside environment, where commensal and potentially pathogenic microbes and allergens are abundant. Epithelial cells, together with the mucus layers that cover the epithelia, form a physical barrier between the environment and the body [6, 7]. Nevertheless, the epithelial layers are generally thin and permeable to very small components due to their additional physiological functions, such as allowing gas exchange (in the lung) and absorption of food (in the gut). The primary role of the mucosal immune system is to effectively prevent the invasion and colonization of the mucosa from pathogenic agents while at the same time developing mucosal tolerance to non-pathogenic molecules and commensal microbiota to avoid excessive inflammatory responses and tissue damage.

Up to 70% of all immune cells in the mammalian body are found in the mucosal immune system, also known as Mucosa-Associated Lymphoid Tissues (MALT) [7, 8]. It consists

of specialized tissues and cells that are present on the linings of the gastrointestinal, respiratory, and urogenital tract, as well as eye conjunctiva, inner ear, and ducts of all exocrine glands [7]. Each anatomically distinctive mucosal tissue has its distinguishing lymphoid tissue; Gut-Associated Lymphoid Tissue (GALT), Bronchus-Associated Lymphoid Tissue (BALT) in the lower respiratory tract, and Nasal-Associated Lymphoid Tissue (NALT). GALT in the gastrointestinal (GI) tract mainly consists of the Lamina Propria (LP), lymphoid follicles such as Peyer's Patches (PPs), and Mesenteric Lymph Nodes (MLNs) (Fig 1.). Microfold cells (M cells) laying in between intestinal epithelial cells are highly specialized in phagocytosis and transcytosis of large antigens from the lumen and transporting them into the subepithelial PPs where macrophages, DCs, and lymphocytes are present [9]. NALT in the nasopharynx mainly consists of the tonsils and adenoids that make up Waldeyer's ring. Waldeyer's ring serves as the first interaction point of immune cells with swallowed pathogens and structurally intact (undigested) food components prior to their transit to the lower regions of the digestive tract for hydrolysis [10]. Waldeyer's ring comprises lymphoid tissue located in the nasopharynx, palatine tonsil, and lingual tonsil [11, 12]. The crypt of the palatine tonsil has a massive surface area of 300 cm^2, which is suitable for immune cells to encounter and monitor ingested and inhaled antigens and have prolonged interaction time. The epithelial cells in the tonsils and adenoids express polymeric immunoglobulin receptors (pIgR) required for IgA transport to the nasopharynx area [11, 12]. Similar to M cells found in the intestine's Peyer's patches, M-like cells in the crypt epithelium facilitate the interaction of the immune cell with antigens. Antigens are taken up by epithelial cells, tissue-resident macrophages, and specialized mucosal DCs that sample the luminal contents. In some cases, the uptake process is facilitated by the neonatal Fcγ receptor (FcRn) when the antigen is coated with IgG in the form of an immune complex (IC). The role of ICs and their effector functions will be discussed in this thesis.

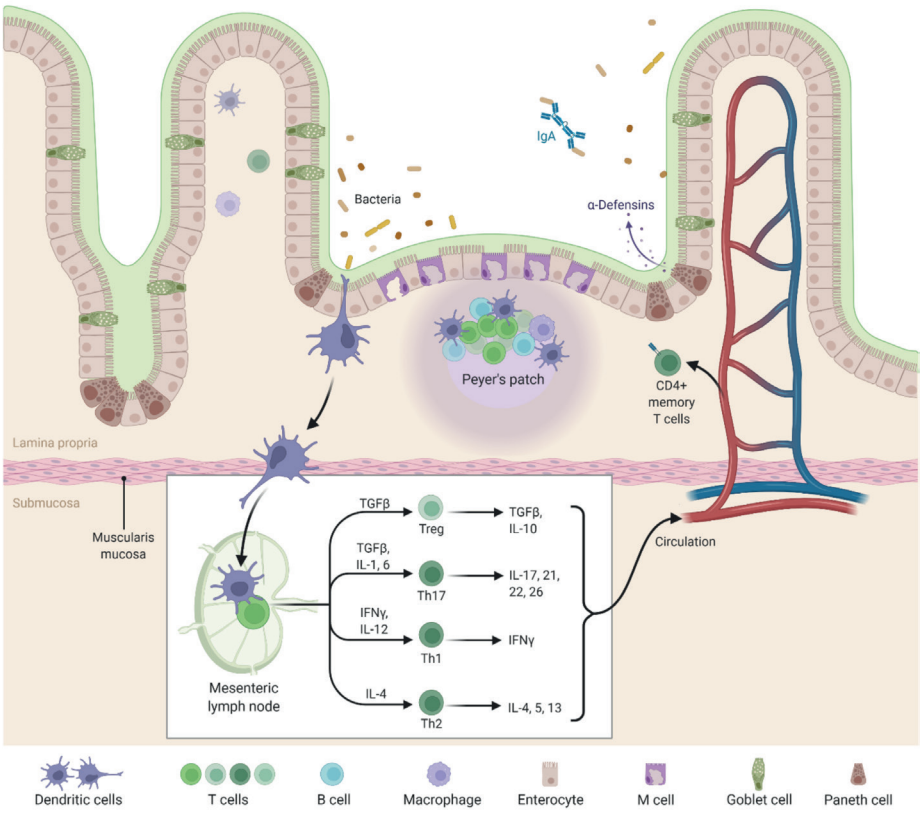


Figure 1. Structure of Gut-Associated Lymphoid tissue (GALT). Mucosal DCs sample luminal content and migrate to the draining mesenteric lymph node to activate the T cells. Depending on the type of antigen, DCs secrete various cytokines that drive T cells differentiation towards Th1, Th2, Th17, or Tregs. M cells transport luminal antigens and bacteria toward the underlying immune cells in Peyer's Patches (PP) in the lamina propria (LP). The antigens can activate or inhibit the immune response leading to either systemic immune cell response or tolerance. Secretory IgA (sIgA) is present in the mucosal surface, which neutralizes pathogens and prevents contact with intestinal epithelial cells. Goblet cells are responsible for producing mucus, and the Paneth cells secrete antimicrobial peptides such as α-defensins. The figure was created with BioRender.com.

Upon pathogen recognition by PRRs, tissue macrophages and mucosal DCs become activated. DCs migrate to the draining lymph nodes to activate T cells by presenting the antigen and producing cytokines [13, 14]. The mucosal immune system contains a distinctive repertoire of lymphocytes in the submucosal tissues and the regional lymph nodes. Apart from T cells, B lymphocytes in the submucosal tissues get activated to produce IgA [15]. Two IgA molecules and the joining chain form dimeric IgA (dIgA), which binds to pIgR and after transcytosis is released as secretory IgA (sIgA) into the mucosal tissues. sIgA plays an essential role in defending the mucosal surfaces by neutralizing any pathogen or toxin trying to make contact with epithelial cells [16, 17].

Another factor that impacts the immune system at the mucosal barriers is the microbiota. Mucosal surfaces, particularly those of the large intestine, are colonized by a diverse and dynamic population of microorganisms such as bacteria, archaea, and viruses. Microbial colonization begins at birth and is shaped by factors such as mode of birth, breast milk, nutrition, and environment. Human milk oligosaccharides (HMOs) provide a substrate for the gut microbiota, resulting in the proliferation of Bifidobacteria and other beneficial bacteria [18]. The microbiota composition and metabolic activity contribute to gut immune homeostasis by enhancing epithelial cell integrity, preventing harmful bacteria from spreading, and promoting the development of the immune system [19-21]. Bacterial metabolites produced after fermentation of complex sugars and fibers are Short-Chain Fatty Acids (SCFAs), serving as key mediators of the microbiota to interact with the host. SCFAs, particularly butyrate, have been shown to have anti-inflammatory activities locally and systemically *in vivo* as well as in animal models [22-24]. SCFAs have the ability to modify the immune cells epigenetically. Epigenetic modifications include DNA methylation, histone modifications (e.g., acetylation, phosphorylation, and methylation), as well as interference with RNA by microRNA (miRNA) [25]. These modifications can influence the cellular response by promoting the expression of specific genes or silencing others.

As mentioned earlier, one critical function of the mucosal immune system is to develop immunological tolerance to innocuous commensal microorganisms and dietary antigens. Mucosal DCs are present in Peyer's patches, mesenteric lymph nodes, and the Lamina Propria, where they constantly sample the intestinal contents. Upon encountering food antigens, mucosal $CD103^+$ DCs modulate the activity of $FoxP3^+$ regulatory T cells (Tregs) to produce immunosuppressive cytokines like IL-10 and TGF-β [13, 26]. Together with commensal microbial metabolites such as SCFAs, these cytokines regulate intestinal (oral) tolerance and help maintain gut homeostasis [27]. However, these intestinal DCs can initiate a robust inflammatory response towards pathogenic agents by migrating to the local lymph nodes and priming T cell activity [13].

Trained immunity

In classical immunology, the cells of the innate arm of the immune system are considered to be rapid-acting and robust, lacking highly specific antigen receptors. Most importantly, they were assumed not to contribute to immunological memory to previously encountered pathogens. This general perception was challenged a few years ago by the group of Netea that introduced the concept known as trained immunity or innate immune training [28, 29]. It was observed that vaccination with Bacillus Calmette–Guérin (BCG) vaccine protected the vaccinated individuals against infections with pathogens other than *Mycobacterium tuberculosis* [30-33]. The Peripheral Blood Mononuclear Cells (PBMCs) of the BCG-vaccinated subjects showed an increased production of IFN-γ, TNF-α, and

IL-1β upon *ex vivo* stimulation with unrelated bacterial and fungal pathogens [34]. The increased cytokine response was shown to be T- and B lymphocyte-independent, implying that it was associated with enhanced innate immune cell function [34]. Monocytes with higher expression levels of CD11b and TLR4 were detected at least three months after vaccination [34], longer than the lifespan of these cells, and "trained" natural killer (NK) cells expanded rapidly and released cytokines several months after the primary exposure to the training components [35-37]. *In vitro* and *ex vivo* research confirmed the trainability of monocytes/macrophages [34, 38, 39], NK cells [40, 41], and another myeloid cell, DCs [42]. Besides, a recent paper showed the inheritability of the effects, which is thought to be associated with epigenetic changes in the bone marrow-resident myeloid effector and progenitor cell compartment [43]. Since the concept's inception, training effects have been documented for a variety of microbial substances, including b-glucan from *Candida albicans* [38] and chitin from *Saccharomyces cerevisiae* [44]. Likewise, dietary components such as oxidized Low-Density Lipoprotein (oxLDL) [45] and bovine milk immunoglobulin G (bIgG) [46, 47] have been shown to induce innate immune training.

Innate immune cells exhibit a memory-like phenotype defined by an increased response upon secondary exposure to the same and unrelated antigen, different from immunological memory in adaptive immune cells. Trained immunity has been reported to be due to epigenetic modification and metabolic reprogramming [29]. Activation of the Dectin-1 receptor by β-1, 3-(D)-glucan derived from *Candida albicans* followed by Raf-1 signaling was shown to induce histone methylation and acetylation of H3K4me1 or H3K27Ac [38, 48]. Besides, the shift of central metabolism from oxidative phosphorylation (OXPHOS) to glycolysis was reported in the trained cells [49-51]. These epigenetic modifications increase the activity of the enhancers of proinflammatory genes and the accessibility of DNA for gene transcription. Thereafter, upon secondary stimulation with TLR ligands in general as well as with pathogenic molecules, a rise in the production of inflammatory cytokines such as IL-6 and TNF-a occurs [29].

Immune tolerance is another side of the trained immunity coin that may be equally important (Fig 2.). Exposure to LPS induced innate immune tolerance in murine macrophages characterized the cells' decreased reactivity to LPS and a higher activation threshold [52]. To induce tolerogenic phenotypes in innate immune cells, the epigenetic and metabolic program of the cells must be rewired [52]. In fact, the tolerance induction mechanism is thought to be similar to the trained immunity but in the opposite direction [53].

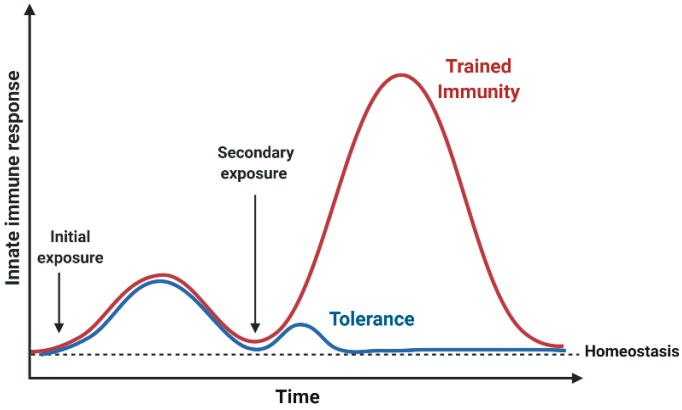


Figure 2. Schematic presentation of innate immune responses during trained immunity and tolerance. Following the training, the cells respond more vigorously to the second stimulation to the same and homologous stimuli, while after induction of tolerance, the response to the second stimulation is reduced. The figure was generated with Biorender.com.

Immunomodulation by nutrition

Immunomodulation is the regulatory adjustment of the immune system [54]. It may relate to immune system homeostasis, in which the system self-regulates to maintain appropriate levels of immunological responses via regulatory T cells and cytokines such as IL-10. Alternatively, immunomodulation can be used in conjunction with immunotherapy, which involves inducing or altering the immune responses to achieve therapeutic aims.

Dietary components have been shown to affect immune health and possess immunomodulatory properties [55-57]. Different diets have been linked to the development, management, and treatment of non-communicable diseases such as allergies, diabetes, cancer, and cardiovascular diseases [58]. While the presence of micronutrients such as zinc, iron, selenium, vitamin A, C, and D are needed for the normal function of the immune system [59], supplementation with other dietary components like n-3 Polyunsaturated Fatty Acids (PUFA) has been demonstrated to be preventive in the development of inflammatory diseases [60, 61]. Besides, nutritional interventions were shown to reduce the prevalence or severity of infections by improving the host's immunocompetence [62-64]. The prophylactic aspect of nutritional intervention is especially appealing for preventing and alleviating the infection symptoms and, at the same time, lowering the risk of antibiotic-resistant microbes expansion.

While everyone can benefit from a balanced healthy diet, the positive effects of nutritional interventions might have a more profound impact on newborns, the elderly, and immunocompromised individuals. Newborns are particularly susceptible to respiratory and gastrointestinal tract infections since their immune system is not yet fully mature. At birth, innate immune cells are substantially less responsive to TLR stimulation [65]. Monocytes and macrophages are still immature with reduced TLR4 expression [66], and pDCs in newborns produce a limited amount of interferon

α/β upon exposure to different viruses [67]. Passive immunity conferred to breastfed infants by maternal IgA and IgG partially compensates for the immune system's immaturity and contributes to the protection against pathogens while giving the neonate the time to develop their immune system [68, 69]. However, malnutrition and deprivation from breastfeeding impair immune function and development, putting children at a higher risk of infectious diseases [70]. Similarly, the competence of the immune system declines with advancing in age. The number of proliferating T and B cells decline by aging [71], and the sensitivity of the innate immune cells to antigenic stimulations via, e.g. TLRs decreases [72, 73]. Since the immune system is less efficient, the elderly are more vulnerable to prevalent infections such as influenza [74], and their response to vaccination is not as strong as healthy young adults [75]. Dietary supplementation with immunomodulatory components can somewhat improve the immune competence of vulnerable individuals. Infant formulas enriched with dietary fibers and bioactive proteins are currently being used to improve immune function in young children that cannot be breastfed. Also, dietary products containing, e.g. pre- and probiotics, have been studied and shown to help support the immune system in preventing and managing infections in the elderly and immunocompromised people.

Several mechanisms exist for dietary components to exert beneficial effects on the immune system. Direct neutralization of microbes or toxins and regulation of enteric inflammation [76], acting as bactericidal agents [77-79], and preventing harmful bacteria from colonizing the gut [80, 81] are among these mechanisms. Other ways that dietary compounds contribute to well-being are by, e.g. modulating pro- or anti-inflammatory responses, modifying cell-mediated immunity, and altering antigen-presenting cell functions [82]. Dietary compounds can directly influence the gene expression of the exposed cells [82, 83]. However, certain compounds like β-glucan have been shown to induce epigenetic changes at the levels of immune cell progenitors that result in inheritable changes, as discussed earlier in the section on trained immunity.

In vitro assays are being used to select and screen for potential food components and validate their immunomodulatory properties. Subsequently, to substantiate the contribution of dietary components in supporting the immune system against infections, these ingredients need to be tested *in vivo*. Randomized double-blind intervention trials are ideally performed to study the effects of nutritional interventions. However, those trials are costly and require a considerable amount of time. Alternatively, controlled human challenge models with attenuated pathogens are nowadays used to study the effects of (dietary) components on symptoms of infection *in vivo* [84, 85]. These models can replace large-scale nutritional intervention studies that look into the impact of dietary components in preventing infections that occur at low frequency and, therefore, require large sample sizes and extended study durations. Challenge models are quicker, better defined, and useful in studying immunological biomarkers and mechanisms and thereby reducing the need for animal testing.

Immunomodulatory properties of Milk

Breast milk is the primary source of nutrients and immune-supporting components for the neonate, and WHO recommends breastfeeding for at least the first six months of life [86]. The composition of breast milk and the IgA content vary over time according to the baby's needs (IgA content: 2.0 g/L in colostrum vs. 0.5 g/L in mature milk) [87, 88]. As discussed earlier, maternal IgA transferred via colostrum and milk helps to protect the newborn against early-life infections. The HMOs present in the milk shape the child's microbiota composition and promotes the growth of beneficial bacteria like Bifidobacteria [89]. Other bioactive milk proteins, including lactoferrin, osteopontin, as well as the Milk Fat Globule Membranes (MFGM) and the proteins therein, have been demonstrated to have immunomodulatory properties [90]. The presence of anti-inflammatory cytokines like TGF-β in breast milk supports the neonatal immune system by enhancing the intestinal barrier function [78, 79] and has been correlated with preventing allergies [91].

Human breast milk and cow's milk are quite comparable in terms of the presence of various protein and fat components, albeit some components have different concentrations [92]. The abundance and composition of milk oligosaccharides and the presence of different antibody isotypes are two major variances. Breast milk, in general, contains considerably larger amounts (100x higher) of oligosaccharides containing fucose and sialic acid [93, 94], whereas in bovine milk, much fewer oligosaccharides and only sialylated ones are available [95]. In contrast to bovine milk, where IgG predominates with smaller quantities of IgA and IgM, IgA is the most prevalent antibody isotype in breast milk [92]. Because cows do not transfer IgG across the placenta, the antibodies must be supplied by milk/colostrum to the offspring and are taken up into the circulation just after birth. Besides that, human milk contains a larger quantity of lactoferrin and lysozyme, but bovine milk contains a higher concentration of lactoperoxidase, TNF-a, TGF-β1 and 2 [92].

Despite these differences, bovine milk components have been shown to be able to support the human immune system. Early epidemiological investigations showed that bovine colostrum from vaccinated cows was protective against human rotavirus [96], and raw farm milk consumption was associated with reduced common respiratory tract infections in infants [97]. Consumption of unprocessed cow's milk is also associated with protection against the development of asthma and allergies [92, 98-101]. Several milk components, including bovine milk IgG (bIgG), have been shown to have immunomodulatory effects on humans' immune systems. bIgG can bind to various human pathogenic bacteria and viruses [102-104], as well as allergens [105]. Accordingly, bIgG has been administered to reduce gastrointestinal and respiratory tract infections (reviewed in [106]). Several mechanisms of action for bIgG have been described. bIgG-rich preparations prevented the adhesion of pathogens to intestinal epithelial cells [107-109] and inhibited the NF-κB

mediated proinflammatory cytokine expression, thus showing direct anti-inflammatory effects on the intestinal epithelium [110]. The direct effector function of bIgG on the cells is mediated by the interaction of their Fc portion with cellular Fcγ receptors (FcγRs), which are predominantly expressed on the immune cells [102, 106]. bIgG-coated antigen, especially in the form of large immune complexes, can bind to FcγRII on human monocytes, DCs, and B cells [102, 111], and this topic will be discussed in this thesis.

Aims and outline of the thesis

Dietary intervention can possibly contribute to preventing and managing human infections and allergies by supporting the immune system. The impact of nutritional interventions is more relevant in people whose immune system is less robust, especially during infancy and in the elderly. Elucidating the mechanisms by which selected dietary compounds support the immune system may generate scientific basics for new applications. Such studies can provide evidence for the immune-supporting effects of food ingredients and may result in the development of new or improved food products. The aim of this thesis was to investigate the immunomodulatory properties of bovine milk components and metabolites induced by milk oligosaccharides by applying *in vitro* assays and also evaluating the *in vivo* effects of dietary (dairy) interventions using human infection challenge models.

Maternal diet during pregnancy, early-life nutrition, microbiota, and exposure to various antigens/allergens throughout infancy imprint long-term impacts on the immune health of the newborn. Many environmental and nutritional factors (including milk) may exert health-promoting effects by altering the immune cells' epigenomes. While breast milk is the ideal diet for neonates, cow's milk-based formula is used as an alternative that confers part of the functional effect of breastmilk. In **Chapter 2** of this thesis, we summarized the current knowledge on human and bovine milk bioactive components with the potential to support immune function through epigenetics. We discuss the potential effects of milk components on rewiring the function of the immune cells, emphasizing allergies and asthma.

Milk oligosaccharides are important components of milk that provide a substrate for the gut microbiota to produce SCFAs. In **Chapter 3**, we study the direct and indirect effects of the individual SCFAs acetate, butyrate, and propionate on inflammatory responses in innate and adaptive cells. PBMCs were incubated with SCFAs, and the production of cytokines in various innate (monocytes, mDC and pDC) and adaptive (T- and B cells) immune cells were studied. The differential effects on the cells are explained in light of the expression of SCFA receptors and transporters. Since SCFAs are known to have the ability to inhibit HDACs and influence gene expression, we investigated their potential to induce innate immune training *in vitro*.

The process of antigen uptake by APCs and subsequent presentation to T cells can be facilitated when antigen: antibody immune complexes (ICs) are formed. Only antibodies in large ICs can induce maximal effector signaling through interacting with low-affinity receptors like CD32. The ability of bIgG to bind to human pathogens such as Respiratory Syncytial Virus (RSV) and enhance the activation of T cells was shown previously. However, the intermediate step, which is the interaction of bIgG with APCs, has not been addressed. In **Chapter 4**, we examined the binding of bovine IgG alone or in immune complexes to monocytes. Besides, although bIgG was shown to train monocytes *in vitro*, it is not clear whether the effect is induced by bIgG monomers or the ICs containing bIgG. We evaluated the ability of the ICs composed of bovine IgG and human RSV preF protein to induce innate immune training.

In **Chapter 5**, we investigated whether a two-week consumption of a dairy whey product rich in bovine IgG can modify the gene expression pattern of PBMCs from healthy volunteers or change the responsiveness of monocytes and DCs to TLR ligands (LPS and R848) stimulation *ex vivo*. While this double-blind placebo-controlled single-tier infection model focused on preventing the primary infection, in **Chapter 6**, we performed a 2-tier human challenge pilot study. In this study, we optimized the model by adjusting the dose of primary infection, looking for a dose that would still lead to symptoms after secondary infection. These optimized models enable studying the protective effects of dietary components against reinfections. Besides, we investigated the correlates of protection against a diarrheagenic *E. coli* reinfection. Healthy volunteers in this double-blind study were challenged with increasing concentrations of attenuated *E. coli,* and three weeks later, they received a high dose of the same pathogen that is known to cause clinical symptoms in healthy individuals. Several local and immunological parameters were monitored prior to and after each bacteria inoculation, the severity of clinical diarrhea caused by the bacteria was assessed. Also, the responsiveness of monocytes, mDCs, and pDCs to TLR ligand stimulation as well as induction of *E.coli*-specific antibodies was compared prior to and after the primary infection.

The findings from earlier chapters are discussed in light of recent literature and summarized in the discussion of this thesis (**Chapter 7**). We tried to highlight the immunomodulatory properties of milk components, emphasizing their potential to epigenetically reprogram myeloid innate immune cells toward trained immunity. Additionally, we attempted to outline some potential applications of the findings for protecting vulnerable populations against infections and made new suggestions for future research.

References

1. Medzhitov, R. and C. Janeway, *Innate Immunity.* New England Journal of Medicine, 2000. **343**(5): p. 338-344.
2. Turvey, S.E. and D.H. Broide, *Innate immunity.* The Journal of allergy and clinical immunology, 2010. **125**(2 Suppl 2): p. S24-S32.
3. Fu, Y.L. and R.E. Harrison, *Microbial Phagocytic Receptors and Their Potential Involvement in Cytokine Induction in Macrophages.* Frontiers in immunology, 2021. **12**: p. 662063-662063.
4. Amarante-Mendes, G.P., et al., *Pattern Recognition Receptors and the Host Cell Death Molecular Machinery.* Frontiers in Immunology, 2018. **9**.
5. Collin, M., N. McGovern, and M. Haniffa, *Human dendritic cell subsets.* Immunology, 2013. **140**(1): p. 22-30.
6. Strober, W., *Chapter 47 - Overview: Mucosal Immunity and Infections*, in *Mucosal Immunology (Fourth Edition)*, J. Mestecky, et al., Editors. 2015, Academic Press: Boston. p. 945-948.
7. Holmgren, J. and C. Czerkinsky, *Mucosal immunity and vaccines.* Nature Medicine, 2005. **11**(4): p. S45-S53.
8. Brandtzaeg, P., *Mucosal Immunity: Induction, Dissemination, and Effector Functions.* Scandinavian Journal of Immunology, 2009. **70**(6): p. 505-515.
9. Williams, I.R. and R.L. Owen, *Chapter 13 - M Cells: Specialized Antigen Sampling Cells in the Follicle-Associated Epithelium*, in *Mucosal Immunology (Fourth Edition)*, J. Mestecky, et al., Editors. 2015, Academic Press: Boston. p. 211-229.
10. Govers, C., et al., *Ingestion, Immunity, and Infection: Nutrition and Viral Respiratory Tract Infections.* Frontiers in Immunology, 2022. **13**.
11. Hellings, P., M. Jorissen, and J.L. Ceuppens, *The Waldeyer's ring.* Acta Otorhinolaryngol Belg, 2000. **54**(3): p. 237-41.
12. Brandtzaeg, P., *Immunology of tonsils and adenoids: everything the ENT surgeon needs to know.* International Journal of Pediatric Otorhinolaryngology, 2003. **67**: p. S69-S76.
13. Savelkoul, H.F., et al., *Choice and Design of Adjuvants for Parenteral and Mucosal Vaccines.* Vaccines (Basel), 2015. **3**(1): p. 148-71.
14. Schulz, O., et al., *Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions.* J Exp Med, 2009. **206**(13): p. 3101-14.
15. Tezuka, H. and T. Ohteki, *Regulation of IgA Production by Intestinal Dendritic Cells and Related Cells.* Frontiers in Immunology, 2019. **10**.
16. Brandtzaeg, P., *Role of secretory antibodies in the defence against infections.* International Journal of Medical Microbiology, 2003. **293**(1): p. 3-15.
17. Brandtzaeg, P., *Induction of secretory immunity and memory at mucosal surfaces.* Vaccine, 2007. **25**(30): p. 5467-5484.
18. Bode, L., *The functional biology of human milk oligosaccharides.* Early Human Development, 2015. **91**(11): p. 619-622.
19. Maslowski, K.M. and C.R. Mackay, *Diet, gut microbiota and immune responses.* Nature Immunology,

2011. **12**(1): p. 5-9.

20. Thursby, E. and N. Juge, *Introduction to the human gut microbiota.* The Biochemical journal, 2017. **474**(11): p. 1823-1836.
21. Krautkramer, K.A., et al., *Diet-Microbiota Interactions Mediate Global Epigenetic Programming in Multiple Host Tissues.* Molecular Cell, 2016. **64**(5): p. 982-992.
22. Tan, J., et al., *Chapter Three - The Role of Short-Chain Fatty Acids in Health and Disease*, in *Advances in Immunology*, F.W. Alt, Editor. 2014, Academic Press. p. 91-119.
23. Maslowski, K.M., et al., *Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43.* Nature, 2009. **461**(7268): p. 1282-1286.
24. Blaak, E.E., et al., *Short chain fatty acids in human gut and metabolic health.* Benef Microbes, 2020. **11**(5): p. 411-455.
25. Portela, A. and M. Esteller, *Epigenetic modifications and human disease.* Nature Biotechnology, 2010. **28**(10): p. 1057-1068.
26. Mörbe, U.M., et al., *Human gut-associated lymphoid tissues (GALT); diversity, structure, and function.* Mucosal Immunology, 2021. **14**(4): p. 793-802.
27. van der Hee, B. and J.M. Wells, *Microbial Regulation of Host Physiology by Short-chain Fatty Acids.* Trends in Microbiology, 2021.
28. Netea, Mihai G., J. Quintin, and Jos W.M. van der Meer, *Trained Immunity: A Memory for Innate Host Defense.* Cell Host & Microbe, 2011. **9**(5): p. 355-361.
29. Netea, M.G., et al., *Defining trained immunity and its role in health and disease.* Nature Reviews Immunology, 2020.
30. Velema, J.P., et al., *Childhood mortality among users and non-users of primary health care in a rural west African community.* Int J Epidemiol, 1991. **20**(2): p. 474-9.
31. Niobey, F., et al., *Risk factors for death caused by pneumonia in children younger than 1 year old in a metropolitan region of southeastern Brazil. A case-control study.* Revista de saude publica, 1992. **26**(4): p. 229-238.
32. Garly, M.-L., et al., *BCG scar and positive tuberculin reaction associated with reduced child mortality in West Africa: a non-specific beneficial effect of BCG?* Vaccine, 2003. **21**(21-22): p. 2782-2790.
33. Vaugelade, J., et al., *Non-specific effects of vaccination on child survival: prospective cohort study in Burkina Faso.* bmj, 2004. **329**(7478): p. 1309.
34. Kleinnijenhuis, J., et al., *Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes.* Proc Natl Acad Sci U S A, 2012. **109**(43): p. 17537-42.
35. Sun, J.C., *Re-educating natural killer cells.* The Journal of experimental medicine, 2010. **207**(10): p. 2049-2052.
36. Sun, J.C., J.N. Beilke, and L.L. Lanier, *Immune memory redefined: characterizing the longevity of natural killer cells.* Immunological reviews, 2010. **236**: p. 83-94.
37. Sun, J.C., J.N. Beilke, and L.L. Lanier, *Adaptive immune features of natural killer cells.* Nature, 2009. **457**(7229): p. 557-561.
38. Quintin, J., et al., *Candida albicans infection affords protection against reinfection via functional*

reprogramming of monocytes. Cell host & microbe, 2012. **12**(2): p. 223-232.
39. Saeed, S., et al., *Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity.* Science (New York, N.Y.), 2014. **345**(6204): p. 1251086-1251086.
40. Dou, Y., et al., *Influenza vaccine induces intracellular immune memory of human NK cells.* PLoS One, 2015. **10**(3): p. e0121258.
41. Sun, J.C., J.N. Beilke, and L.L. Lanier, *Adaptive immune features of natural killer cells.* Nature, 2009. **457**(7229): p. 557-61.
42. Hole, C.R., et al., *Induction of memory-like dendritic cell responses in vivo.* Nature communications, 2019. **10**(1): p. 2955-2955.
43. Katzmarski, N., et al., *Transmission of trained immunity and heterologous resistance to infections across generations.* Nature Immunology, 2021.
44. Rizzetto, L., et al., *Fungal Chitin Induces Trained Immunity in Human Monocytes during Cross-talk of the Host with Saccharomyces cerevisiae*.* Journal of Biological Chemistry, 2016. **291**(15): p. 7961-7972.
45. Bekkering, S., et al., *Oxidized Low-Density Lipoprotein Induces Long-Term Proinflammatory Cytokine Production and Foam Cell Formation via Epigenetic Reprogramming of Monocytes.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2014. **34**(8): p. 1731-1738.
46. van Splunter, M., et al., *Induction of Trained Innate Immunity in Human Monocytes by Bovine Milk and Milk-Derived Immunoglobulin G.* Nutrients, 2018. **10**(10): p. 1378.
47. Hellinga, A.H., et al., *In Vitro Induction of Trained Innate Immunity by bIgG and Whey Protein Extracts.* International Journal of Molecular Sciences, 2020. **21**(23): p. 9077.
48. Ifrim, D.C., et al., *Trained immunity or tolerance: opposing functional programs induced in human monocytes after engagement of various pattern recognition receptors.* Clinical and vaccine immunology : CVI, 2014. **21**(4): p. 534-545.
49. Cheng, S.-C., et al., *mTOR- and HIF-1α-mediated aerobic glycolysis as metabolic basis for trained immunity.* Science (New York, N.Y.), 2014. **345**(6204): p. 1250684-1250684.
50. Arts, R.J.W., et al., *Immunometabolic Pathways in BCG-Induced Trained Immunity.* Cell reports, 2016. **17**(10): p. 2562-2571.
51. Domínguez-Andrés, J., L.A.B. Joosten, and M.G. Netea, *Induction of innate immune memory: the role of cellular metabolism.* Current Opinion in Immunology, 2019. **56**: p. 10-16.
52. Foster, S.L., D.C. Hargreaves, and R. Medzhitov, *Gene-specific control of inflammation by TLR-induced chromatin modifications.* Nature, 2007. **447**(7147): p. 972-978.
53. Netea, M.G., et al., *Trained immunity: A program of innate immune memory in health and disease.* Science, 2016. **352**(6284).
54. Saroj, P., et al., *An overview on immunomodulation.* J Adv Sci Res, 2012. **3**(1): p. 7-12.
55. De Rosa, V., et al., *Nutritional control of immunity: Balancing the metabolic requirements with an appropriate immune function.* Seminars in Immunology, 2015. **27**(5): p. 300-309.
56. Prescott, S.L., *Early Nutrition as a Major Determinant of 'Immune Health': Implications for Allergy, Obesity and Other Noncommunicable Diseases.* Nestle Nutr Inst Workshop Ser, 2016. **85**: p. 1-17.
57. Childs, C.E., P.C. Calder, and E.A. Miles, *Diet and Immune Function.* Nutrients, 2019. **11**(8): p.

1933.

58. Venter, C., et al., *Nutrition and the Immune System: A Complicated Tango.* Nutrients, 2020. **12**(3): p. 818.
59. (EFSA), E.F.S.A., *Nutrition and Health Claims (Article 13 claims).* 2020.
60. Calder, P.C., *The role of marine omega-3 (n-3) fatty acids in inflammatory processes, atherosclerosis and plaque stability.* Mol Nutr Food Res, 2012. **56**(7): p. 1073-80.
61. Calder, P.C., *n–3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases.* The American Journal of Clinical Nutrition, 2006. **83**(6): p. 1505S-1519S.
62. Purssell, E., *Prevention and management of gastrointestinal infections in infants from a nutritional perspective.* J Fam Health Care, 2009. **19**(6): p. 200-3.
63. Bührer, C., H.S. Fischer, and S. Wellmann, *Nutritional interventions to reduce rates of infection, necrotizing enterocolitis and mortality in very preterm infants.* Pediatr Res, 2020. **87**(2): p. 371-377.
64. Calder, P.C. and S. Kew, *The immune system: a target for functional foods?* British Journal of Nutrition, 2002. **88**(S2): p. S165-S176.
65. Kollmann, T.R., et al., *Innate immune function by Toll-like receptors: distinct responses in newborns and the elderly.* Immunity, 2012. **37**(5): p. 771-783.
66. Förster-Waldl, E., et al., *Monocyte Toll-Like Receptor 4 Expression and LPS-Induced Cytokine Production Increase during Gestational Aging.* Pediatric Research, 2005. **58**(1): p. 121-124.
67. Schüller, S.S., et al., *Preterm neonates display altered plasmacytoid dendritic cell function and morphology.* Journal of Leukocyte Biology, 2013. **93**(5): p. 781-788.
68. Macpherson, A.J., M.G. de Agüero, and S.C. Ganal-Vonarburg, *How nutrition and the maternal microbiota shape the neonatal immune system.* Nature Reviews Immunology, 2017. **17**: p. 508.
69. Hurley, W.L. and P.K. Theil, *Perspectives on immunoglobulins in colostrum and milk.* Nutrients, 2011. **3**(4): p. 442-474.
70. Duijts, L., M.K. Ramadhani, and H.A. Moll, *Breastfeeding protects against infectious diseases during infancy in industrialized countries. A systematic review.* Maternal & child nutrition, 2009. **5**(3): p. 199-210.
71. Agarwal, S. and P.J. Busse, *Innate and adaptive immunosenescence.* Annals of Allergy, Asthma & Immunology, 2010. **104**(3): p. 183-190.
72. Shaw, A.C., et al., *Dysregulation of human Toll-like receptor function in aging.* Ageing research reviews, 2011. **10**(3): p. 346-353.
73. Pawelec, G., *Age and immunity: What is "immunosenescence"?* Experimental Gerontology, 2018. **105**: p. 4-9.
74. Schanzer, D., J. Vachon, and L. Pelletier, *Age-specific Differences in Influenza A Epidemic Curves: Do Children Drive the Spread of Influenza Epidemics?* American Journal of Epidemiology, 2011. **174**(1): p. 109-117.
75. Pera, A., et al., *Immunosenescence: Implications for response to infection and vaccination in older people.* Maturitas, 2015. **82**(1): p. 50-55.
76. Andersen-Civil, A.I.S., P. Arora, and A.R. Williams, *Regulation of Enteric Infection and Immunity by Dietary Proanthocyanidins.* Frontiers in Immunology, 2021. **12**.

77. Graham, D.Y., M.K. Estes, and L.O. Gentry, *Double-blind comparison of bismuth subsalicylate and placebo in the prevention and treatment of enterotoxigenic Escherichia coli-induced diarrhea in volunteers.* Gastroenterology, 1983. **85**(5): p. 1017-22.
78. Nabavi, S.F., et al., *Antibacterial Effects of Cinnamon: From Farm to Food, Cosmetic and Pharmaceutical Industries.* Nutrients, 2015. **7**(9): p. 7729-7748.
79. Ozogul, Y., et al., *Antimicrobial Impacts of Essential Oils on Food Borne-Pathogens.* Recent Patents on Food, Nutrition & Agriculture, 2015. **7**(1): p. 53-61.
80. Bovee-Oudenhoven, I.M.J., et al., *Diarrhea caused by enterotoxigenic Escherichia coli infection of humans is inhibited by dietary calcium.* Gastroenterology, 2003. **125**(2): p. 469-476.
81. Top, J., et al., *Low-calcium diet in mice leads to reduced gut colonization by Enterococcus faecium.* MicrobiologyOpen, 2019. **8**(12): p. e936-e936.
82. Wu, D., et al., *Nutritional Modulation of Immune Function: Analysis of Evidence, Mechanisms, and Clinical Relevance.* Frontiers in Immunology, 2019. **9**.
83. Cousins, R.J., *Nutritional regulation of gene expression.* The American Journal of Medicine, 1999. **106**(1, Supplement 1): p. 20-23.
84. Hanevik, K., et al., *The way forward for ETEC controlled human infection models (CHIMs).* Vaccine, 2019. **37**(34): p. 4794-4799.
85. Bekeredjian-Ding, I., et al., *Controlled Human Infection Studies: Proposals for guidance on how to design, develop and produce a challenge strain.* Biologicals, 2021. **74**: p. 16-23.
86. World Health organization, *Infant and Young Child Nutrition. Global Strategy on Infant and Young Child Feeding.* 2002.
87. Oddy, W.H., *The impact of breastmilk on infant and child health.* Breastfeed Rev, 2002. **10**(3): p. 5-18.
88. Haschke, F., N. Haiden, and S.K. Thakkar, *Nutritive and Bioactive Proteins in Breastmilk.* Ann Nutr Metab, 2016. **69 Suppl 2**: p. 17-26.
89. Bäckhed, F., et al., *Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life.* Cell Host Microbe, 2015. **17**(5): p. 690-703.
90. Demmelmair, H., et al., *Benefits of Lactoferrin, Osteopontin and Milk Fat Globule Membranes for Infants.* Nutrients, 2017. **9**(8).
91. Oddy, W.H. and F. Rosales, *A systematic review of the importance of milk TGF-β on immunological outcomes in the infant and young child.* Pediatric Allergy and Immunology, 2010. **21**(1-Part-I): p. 47-59.
92. van Neerven, R.J.J., et al., *Which factors in raw cow's milk contribute to protection against allergies?* Journal of Allergy and Clinical Immunology, 2012. **130**(4): p. 853-858.
93. Wu, S., et al., *Development of an annotated library of neutral human milk oligosaccharides.* J Proteome Res, 2010. **9**(8): p. 4138-51.
94. Wu, S., et al., *Annotation and structural analysis of sialylated human milk oligosaccharides.* Journal of proteome research, 2011. **10**(2): p. 856-868.
95. Tao, N., et al., *Bovine milk glycome.* J Dairy Sci, 2008. **91**(10): p. 3768-78.
96. Ebina, T., et al., *PREVENTION OF ROTAVIRUS INFECTION BY COW COLOSTRUM CONTAINING ANTIBODY AGAINST HUMAN ROTAVIRUS.* The Lancet, 1983. **322**(8357): p.

1029-1030.

97. Loss, G., et al., *Consumption of unprocessed cow's milk protects infants from common respiratory infections.* Journal of Allergy and Clinical Immunology, 2015. **135**(1): p. 56-62.e2.
98. C., B.F. and V.M. E., *Can farm milk consumption prevent allergic diseases?* Clinical & Experimental Allergy, 2011. **41**(1): p. 29-35.
99. Loss, G., et al., *The protective effect of farm milk consumption on childhood asthma and atopy: The GABRIELA study.* Journal of Allergy and Clinical Immunology, 2011. **128**(4): p. 766-773.e4.
100. van Neerven, J., *The effects of milk and colostrum on allergy and infection: Mechanisms and implications*. Vol. 4. 2014. 16-22.
101. Brick, T., et al., *The Beneficial Effect of Farm Milk Consumption on Asthma, Allergies, and Infections: From Meta-Analysis of Evidence to Clinical Trial.* The Journal of Allergy and Clinical Immunology: In Practice, 2020. **8**(3): p. 878-889.e3.
102. den Hartog, G., et al., *Specificity and Effector Functions of Human RSV-Specific IgG from Bovine Milk.* PLOS ONE, 2014. **9**(11): p. e112047.
103. Rump, J.A., et al., *Treatment of diarrhoea in human immunodeficiency virus-infected patients with immunoglobulins from bovine colostrum.* The clinical investigator, 1992. **70**(7): p. 588-594.
104. Lissner, R., H. Schmidit, and H. Karch, *A standard immunoglobulin preparation produced from bovine colostra shows antibody reactivity and neutralization activity against Shiga-like toxins and EHEC-hemolysin of Escherichia coli O157:H7.* Infection, 1996. **24**(5): p. 378-83.
105. Collins, A.M., et al., *Bovine Milk, including Pasteurised Milk, Contains Antibodies Directed against Allergens of Clinical Importance to Man.* International Archives of Allergy and Immunology, 1991. **96**(4): p. 362-367.
106. Ulfman, L.H., et al., *Effects of Bovine Immunoglobulins on Immune Function, Allergy, and Infection.* Frontiers in Nutrition, 2018. **5**(52).
107. Brooks, H.J., et al., *Potential prophylactic value of bovine colostrum in necrotizing enterocolitis in neonates: an in vitro study on bacterial attachment, antibody levels and cytokine production.* FEMS Immunology & Medical Microbiology, 2006. **48**(3): p. 347-354.
108. Casswall, T., et al., *Bovine anti-Helicobacter pylori antibodies for oral immunotherapy.* Scandinavian journal of gastroenterology, 2002. **37**(12): p. 1380-1385.
109. Naaber, P., et al., *Inhibition of adhesion of Clostridium difficile to Caco-2 cells.* FEMS Immunology & Medical Microbiology, 1996. **14**(4): p. 205-209.
110. An, M.J., et al., *Bovine colostrum inhibits nuclear factor κB–mediated proinflammatory cytokine expression in intestinal epithelial cells.* Nutrition research, 2009. **29**(4): p. 275-280.
111. Nederend, M., et al., *Bovine IgG Prevents Experimental Infection With RSV and Facilitates Human T Cell Responses to RSV.* Frontiers in immunology, 2020. **11**: p. 1701-1701.

1

CHAPTER

The impact of milk and its components on epigenetic programming of immune function in early life and beyond: implications for allergy and asthma

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Abstract

Specific and adequate nutrition during pregnancy and early life is an important factor in avoiding non-communicable diseases such as obesity, type 2 diabetes, cardiovascular disease, cancers, and chronic allergic diseases. Although epidemiologic and experimental studies have shown that nutrition is important at all stages of life, it is especially important in prenatal and the first few years of life. During the last decade, there has been a growing interest in the potential role of epigenetic mechanisms in the increasing health problems associated with allergic disease. Epigenetics involves several mechanisms including DNA methylation, histone modifications, and microRNAs which can modify the expression of genes. In this study, we focus on the effects of maternal nutrition during pregnancy, the effects of the bioactive components in human and bovine milk, and the environmental factors that can affect early life (i.e., farming, milk processing, and bacterial exposure), and which contribute to the epigenetic mechanisms underlying the persistent programming of immune functions and allergic diseases. This knowledge will help to improve approaches to nutrition in early life and help prevent allergies in the future.

There is increasing evidence to suggest that maternal diet during pregnancy, breastfeeding, early life nutrition, and early life malnutrition can have sustained effects on immunological outcomes, such as respiratory allergies, and metabolic outcomes such as type 2 diabetes and obesity. Nutritional programming during gestation might permanently affect the immunological competence and nutritional status in early life Figure 1. This is exemplified by the thrifty phenotype, where the metabolic response to undernutrition during the fetal period is inappropriate during overnutrition later in life, leading to disease manifestations (1). Several studies have since shown that prenatal exposure to famine is associated with the development of type 2 diabetes later in life (2–4), and an epigenetic link was demonstrated in relation to the Dutch hunger winter where epigenetic modification of the IGF2 gene was shown to be linked to famine during prenatal development (5).

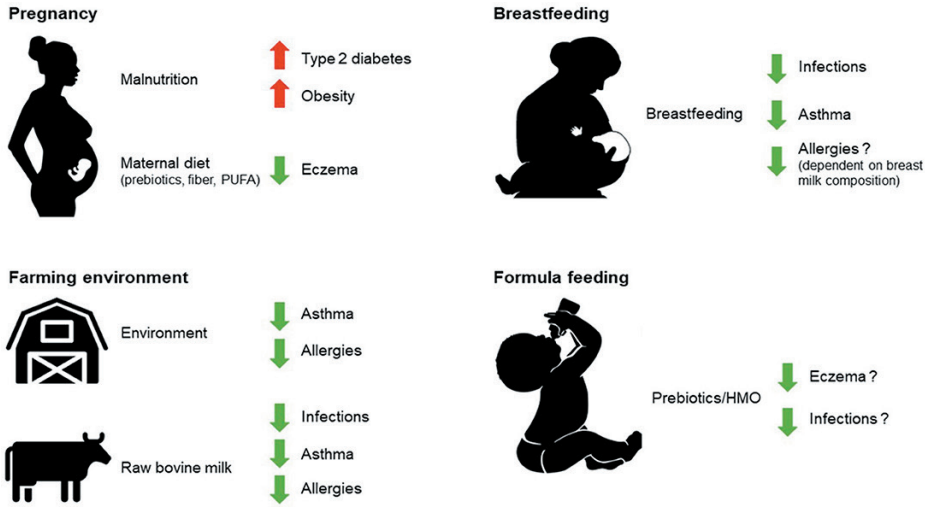
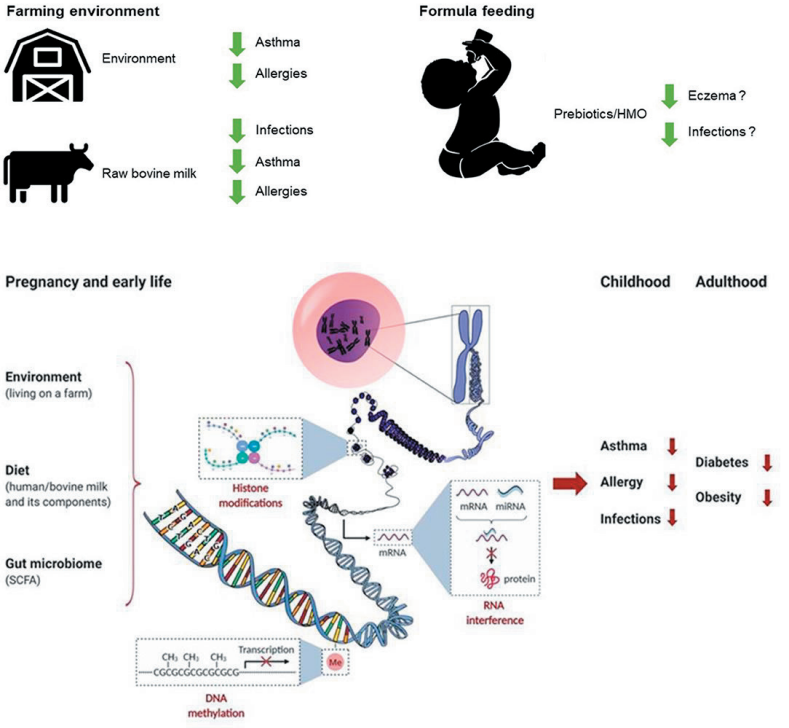


Figure 3. As described in this review, early life nutrition (breastfeeding, raw milk consumption, and some infant formula components), early life environmental exposures (such as farming environment), as well as prenatal development under the influence of maternal diet can all have sustained effects on health outcomes later in life. PUFA, polyunsaturated fatty acids; HMO, human milk oligosaccharides.

Epigenetic mechanisms may play an important role in these effects. It has even been suggested that early life nutrition forms the basis for susceptibility to a plethora of chronic age-related non-communicable diseases (NCD), like respiratory allergies (6–9). Thus, specific and adequate nutrition during pregnancy and early life are considered important factors that could reduce instances of allergic diseases. Epidemiologic and experimental studies show that nutrition is important for (immunological) health, especially when we are very young and during prenatal development, which may influence health and disease throughout our lives (6, 10). The structures of the mucosal immune system in the gastrointestinal (GI) tract are fully developed in utero by gestational week 28 (11). Increasing evidence suggests

that maternal diet and other prenatal exposures can influence this development by crossing the placenta (12–14). In the first year of life, the mucosal immune system is further shaped by microbial colonization and oral feeding (15). Breastfeeding is the normal way of providing newborns with nutrients for healthy growth and development and a diet exclusively comprised of breastfeeding has various beneficial outcomes, such as reducing the risk of GI diseases, allergies, colitis, and respiratory infections (16). Besides conferring protection against these short-term outcomes, breastfeeding also reduces the long-term risks of developing diseases like type 2 diabetes and obesity (17). In analogy to breast milk, raw, unprocessed, bovine milk is a rich source of immunomodulatory components (18–20). Studies have indicated that it may protect against common respiratory infections in infants that consume unprocessed bovine milk (21). In addition, epidemiological evidence shows a clear association between the consumption of raw cow's milk and the prevention of allergy development (22–29). Epigenetic mechanisms that are regulated by many immune processes can thereby influence the course of allergic diseases.

Epigenetic mechanisms (Box 1) and transcription regulatory factors allow a flexible adaptation in the fetus. They neonate to a fluctuating external environment whereby heritable, non-DNA encoded, alterations in gene expression patterns occur. Especially relevant in early life, several factors drive the epigenetic changes that occur throughout life: environment (e.g., exposure to microbial components in inhaled dust), diet (e.g., components present in breast milk and bovine milk), and the GI microbiota and its metabolites (e.g., through the production of short-chain fatty acids [SCFA] after fermentation of dietary non-digestible oligosaccharides). Thus, environmental, dietary, and microbiota-derived epigenetic modifications during gestation and early life can shape future immunity to the development of diseases like obesity, type 2 diabetes, allergy, asthma, and infections. Most of our current knowledge on the environmental and dietary effects on epigenetics and early life immune function comes from epidemiological findings which indicate that children growing up on farms have a decreased risk of developing allergies, especially asthma. For this reason, we will focus this review on the effects of maternal nutrition during pregnancy, the effects of bioactive components in human and bovine milk, and the environmental factors in early life that can contribute to the epigenetic mechanisms involved in the course of allergic diseases.



Box 1. Epigenetics refers to systems that control gene expression in a heritable fashion without changing the genomic sequences. The epigenome is much more flexible than the genome and shows different phenotype variations that are influenced by environmental factors and dietary habits. Epigenetic mechanisms include DNA methylation, histone modifications, and RNA interference by microRNAs (miRNAs) (See in this Box figure). Epigenetic mechanisms thus contribute to the regulation of gene expression at the level of transcription by DNA methylation and by modifying chromatin accessibility through posttranslational modifications of histones, and after transcription by mRNA silencing. These epigenetic mechanisms can regulate gene expression by modifying the accessibility of the DNA to transcription enzymes without altering the DNA nucleotide sequence, influencing stability of mRNA or translation efficiency, and others (30–32). The transfer of a methyl group onto DNA, performed by DNA methyltransferases (DNMTs), can directly regulate the rate of gene transcription. DNA demethylation is catalyzed by several enzymes serving as controllers for the equilibrium of DNA methylation (33). For example, methylation of DNA in the promoter regions of cytokines can influence immune responsiveness by steering Th cell differentiation into Th1, Th2, Th17, or Treg (34, 35). For more details see Box 2. In addition, histone modifications like acetylation, methylation, phosphorylation and others can also modulate the development and activity of immune cells. Histone acetylation is an important remodeling activity that is catalyzed by a series of enzymes called histone acetyltransferases (HATs). Acetylation is generally considered as a permissive activity that facilitates gene transcription. On the contrary, histone deacetylases (HDACs) reverse HAT activity and tighten up the folding of DNA around the histones and make them less accessible for transcription factors (31, 36). The interplay between HATs and HDACs determines the histone acetylation balance and regulates the gene expression (37, 38) and production of pro-inflammatory (IL-1β, IL-5, IL-6, IL-8, IL-12, and TNFα) and anti-inflammatory mediators (IL-10). Histone methyltransferases (HMTs) and demethylases (HDMs) serve as controller enzymes for the equilibrium of histone methylation (31). Finally, RNA interference can occur by small noncoding RNAs, most notably miRNAs that are found in biological fluids as well as in extracellular vesicles (e.g., in milk). MiRNAs represent short noncoding RNA molecules of 18 to 23 nucleotides that control gene expression by inducing mRNA degradation and/or inhibit post-transcriptional translation. As a result, specific miRNA can silence selective gene expression (32). For example, milk contains extracellular vesicles or exosomes that contain a wide range of microRNAs, including miR-21, miR-29b, miR-148a, and miR-155 that is known to influence Foxp3 expression and Treg development (39).

Epigenetic regulation of Th2 development in allergic disease

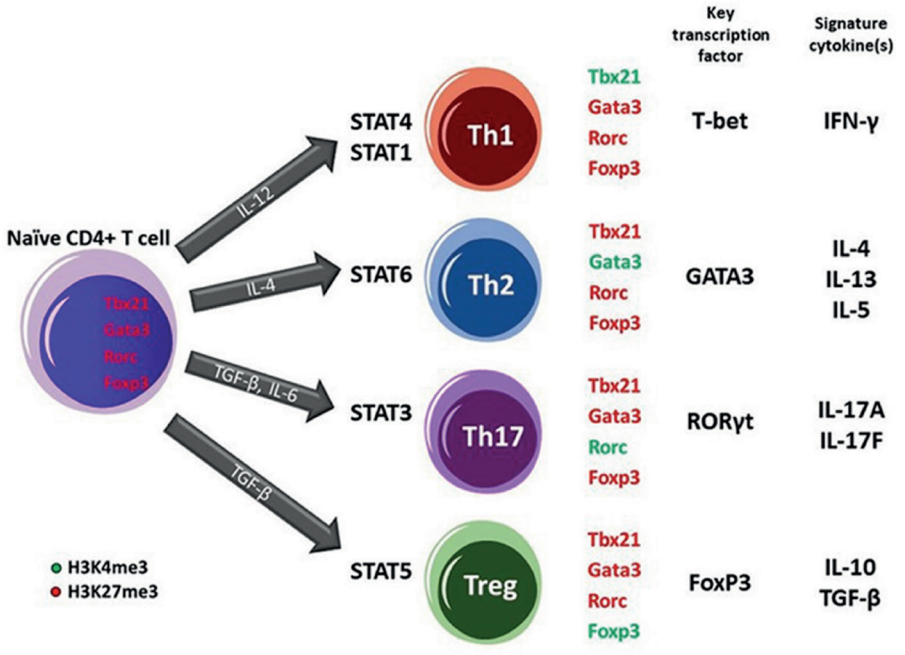
Epigenetic changes have been strongly associated with allergies and asthma and might thereby serve as biomarkers. The role of epigenetic mechanisms, particularly DNA methylation, in allergic diseases is at the interface of gene regulation, environmental stimuli, and developmental processes, thereby determining the pathogenesis of asthma and allergy. Alterations of the DNA methylation status in the genes specific for a different subset of T helper (Th) cells that are considered to be a good example of how epigenetic modulation can influence the development of asthma and other allergic diseases.

The differentiation of naïve CD4+ T cells into Th subpopulations is strictly regulated, with changes in epigenetic marks at main lineage-determining loci encoding transcription factors like GATA3, RORγt, TBX21, and Foxp3 playing a pivotal role. These changes affect the differentiation into mature Th subpopulations, such as Th1, Th2 (and Th9), regulatory T cells (Treg cells), and Th17 (30, 35, 47, 48). In naïve CD4+ T cells, which express a moderate level of GATA3 mRNA after receiving signals via the T cell receptors (TCRs) in the presence of IL-4, activated STAT6 proteins bind to the GATA3 gene locus, driving Th2 differentiation, which is a characteristic in the development of allergy. Differentiation of human CD4+ cells into the Th2 subtype is accompanied by the induction of DNase I hypersensitive (DHS) sites and CpG demethylation around these (DHS) regions within the IL-4 and IL-13 promoters. Extensive studies of the Th2 cytokine locus control region have shown that specific sites undergo rapid demethylation during Th2 differentiation (49).

In addition to DNA methylation, histone modifications are also important in guiding T-cell differentiation. T-bet and GATA3 transcription factors control lineage-specific histone acetylation of IFN-γ and IL-4 loci during Th1/Th2 differentiation. Rapid methylation of H3K9 and H3K27 residues (repressive marks) at the IFN-γ locus was associated with differentiating toward Th1 cells, while demethylation of H3K9 and methylation of H3K27 was associated with Th2 differentiation (49). Epithelial alarmins (IL-25, IL-33, thymic stromal lymphopoietin [TSLP]) induce an inflammatory response in the respiratory mucosal membrane. IL-33 binds to its receptor ST2 on memory Th2 cells and induces epigenetic changes of the IL-5 gene, resulting in the generation of IL-5-producing Th2 cells (47). Thus, Th2 differentiation, which is characteristic of allergy, is triggered by phosphorylation of STAT6 signal transducers and expression of GATA3 and Th2 cytokines, including IL-4 (47).

Demethylation of the IL-4 promoter leads to allergic sensitization (48). Th1 differentiation is in turn triggered by phosphorylation of STAT4 signaling, and expression of the transcription factor T-bet and cytokine. For a more detailed description of epigenetics and

T cell development, see Box 2. Asthmatic individuals show a lower histone deacetylase (HDAC): histone acetylase (HAT) ratio, i.e., a relative decrease of HDAC enzymes, which is corrected by proper anti-asthma treatment (50). The DNA methylation status of Foxp3 is regulated within a highly conserved region within the CpG-rich Treg-specific demethylated region with a differential Foxp3 demethylation status in children with an active cow's milk allergy (CMA) and acquisition of immune tolerance (51).



Box 2. The differentiation of naïve CD4+ T cells upon antigen exposure into effector T helper (Th) subsets (Th1, Th2, and Th17) or induced regulatory T (iTreg) cells relies on epigenetic regulation and the establishment of cell-fate programs (40, 41). DNA methylation and chromatin modifications at pivotal loci in Th cells such as IFN-γ, IL-4 and, Foxp3 contribute to the formation of stable, heritable gene expression patterns. Methylation of CpG dinucleotides specially at promoter or other regulatory regions of genes is generally considered a repressive feature causing silenced genes what mostly seen in (embryonic) stem cells. Targeted loci DNA demethylation is required during early or late hematopoietic cell differentiation (41, 42). For instance, DNA demethylation plays a role in the expression of Th2 cell-related cytokine, IL-4 (43) and, Treg cell-related regulators (44, 45). Besides DNA methylation, histone modifications including acetylation and methylation have a role in the development of Th cell lineage. Histone acetylation, associated with the control of gene expression by condensing or relaxing the chromatin structure to repress or activate transcription, respectively, regulates the expression of several inflammatory mediators of the immune system. In this regard, modifications of histones occur in the enhancer and promoter regions of the STAT4 and STAT1 transcription factor binding sites upstream of the IFN-γ and TBX21 (T-bet) gene to direct Th1 differentiation. In contrast, activation of STAT6 in response to IL-4 occurs leading to the expression of IL-4 and GATA3 transcription factor genes in Th2 differentiating cells. Driving naïve CD4+ T cells toward Th17 phenotype requires STAT3 activation followed by expression of RORC gene encoding RORγt transcription factor and subsequently the production of IL-17 cytokines. Alternatively, upon naïve CD4+ T cells exposure to TGF-β, STAT5 transcription factor engages leading to changes in Foxp3 gene promoter site and commitment of cells into Treg fate. These specific histone modifications lead to engagement of lineage-specific key transcription factors which ensures Th phenotype stabilization and prevents the cells from skewing toward alternative commitments (35, 42, 46).

Effects of early life nutrition on allergic disease

The WHO recommends exclusive breastfeeding for infants during the first 6 months of life, and that it should be given alongside complementary feeding up until children are 2 years old (52). If mothers are unable to breastfeed, many children receive early life nutrition alternatives that are based on bovine milk. Therefore, this section of the study is focused on breast milk, bovine milk, and their components.

Effects of maternal diet in pregnancy & breastfeeding on allergic disease

There is increasing evidence to suggest that the maternal diet during pregnancy and breastfeeding can have sustained effects on immunological outcomes in the infant and even have ramifications for their health later in life. The maternal diet can modify some immune supporting micronutrients in breast milk, such as the fat-soluble vitamins A and D, as well as the water-soluble B vitamins, and polyunsaturated fatty acids (PUFA), but maternal diet does not influence other components such as iron and zinc (53). Although there is some conflicting data, supplementation of maternal diet with vitamins and micronutrients during pregnancy and breastfeeding does not seem to prevent infections and allergies in offspring (54, 55).

Supplementation of maternal diet with PUFA

Long-chain PUFA (LCPUFA) induce inflammation by modulating inflammatory mediators like prostaglandins and immunomodulatory factors like IL-10 and TSLP (56). Consumption of omega-3 PUFA correlates with the inhibition of TLR4 signaling and thereby the production of inflammatory cytokines (IL-1, IL-6, and TNFα), which is reflected by a lower risk of allergies, whereas consumption of saturated fats and omega-6 PUFA, a potential trigger for TLR4-induced inflammation, has been associated with a higher risk of allergies. In addition, PUFA supplementation during pregnancy was associated with a reduction in allergic outcomes after birth (57, 58), but not when it was supplemented to infants (8, 59–61), suggesting that pregnancy is an important time that influences the development of the immune system.

Supplementation of maternal diet with pre-/probiotics

Probiotics are living microorganisms which, when administered in adequate amounts, confer a health benefit to the host. They generally exist of Lactobacillus, Bifidobacterium, or Escherichia species, which are commonly found in a normal microbiota. Prebiotics are mostly dietary fibers that are non-digestible food ingredients and beneficially affect the host's health by selectively stimulating the growth and/or activity of some genera of microorganisms in the colon, generally lactobacilli and bifidobacteria.

Intestinal microbiota strongly influence the maturation of the immune system (62) and

particularly the development of immune tolerance, because they affect the Th1/Th2/Th17/Treg balance. The microbiota composition is modulated by dietary components that help shaping and timing of the composition of the early microbiome (63, 64). In addition, microbiota can be transmitted directly into the uterus during fetal development, passage through the birth canal or during cesarean-section, breastfeeding, and when providing care to the offspring (65, 66).

Food supplements, which are often termed functional foods, have been used to alter, modify, and reinstate pre-existing intestinal microbiota (67). Supplementation of prebiotics, probiotics, and synbiotics (68–74), as well as PUFA (58, 69, 75–77) during pregnancy and breastfeeding, may reduce eczema in infants. This is further supported by preclinical studies, which indicated that supplementing the maternal diet with specific pre- or probiotics affects milk composition (78) and that supplementing non-digestible oligosaccharides diminished allergic disease in offspring (79–81). This may, in part, be linked to the production of SCFA by the intestinal microbiota (82–86). Even though maternal diet during pregnancy and breastfeeding can modulate the prevalence of allergy in the offspring, the potential role of breastfeeding in allergy prevention is still under discussion, as it seems to be linked to variations in breast milk composition rather than to breastfeeding per se (53, 87).

Effects of consumption of raw milk and the farming environment

Most of our current knowledge on the effects of environment and diet on epigenetics and early life immune function is based on epidemiological findings, which indicate that children who grow up on farms have a decreased risk of developing allergies, especially asthma. Allergies are multifactorial, Th2-driven diseases that are triggered by gene-environment interactions. Environmental factors can interact with genes involved in asthma and allergy development via epigenetic mechanisms, such as DNA methylation and histone modifications. These epigenetic mechanisms can regulate gene expression by modifying the accessibility of the DNA to transcription enzymes without altering the DNA nucleotide sequence (30, 33). In addition to the consumption of raw cow's milk (22–29), contact with livestock and animal feed along with other farm-related exposures have shown independent protective effects, indicating that a farm/country lifestyle can contributes to a reduced risk of asthma and allergies in children (25, 27, 88–90). Interestingly, the timing of these exposures seems to be crucial, with the strongest effects observed for exposures that occurred in utero and during the first year of life (23, 91, 92). Since the protective "farm effect" was demonstrated to sustain into adult life (25), effects might be mediated via epigenetic inheritance/regulation.

Several epigenome wide-association studies concerning allergies have been performed and reviewed (30). These studies showed that allergic disease is accompanied by changing

DNA methylation patterns in Th2, Th1, Th17, Th9, and Treg subsets in the affected tissues. DNA methylation changes by demethylation and increased FoxP3+ regulatory T cell numbers in peripheral blood mononuclear cells were shown in 4.5-year-old farm children (93). These regulatory T cell numbers were negatively associated with doctor-diagnosed asthma. It remains to be seen if these changes also precede the onset of allergic disease and can be predictive for allergy development, but questions remain as to how are these epigenetic changes induced. It has been suggested that the epigenome is affected by the farm environment. The first indication for a potential role of epigenetic regulation in the protective "farm effect" was provided by Slaats et al. who demonstrated that DNA methylation of the promoter region of CD14 in placentas of mothers living on farms was lower compared to mothers not living on a farm (94). These lower DNA methylation levels were reflected in higher CD14 mRNA expression levels (95). Interestingly, a higher expression of the CD14 gene was also observed in farmers' children (96). Prenatal farm exposure was also associated with increased gene expression of other innate immune receptors, such as TLR5, TLR7, TLR8, and TLR9, at birth (97, 98) and TLR2 and TLR4 in farm-raised children at school age (95, 96). Maternal exposure to farm environments increases the number of T regulatory (Treg) cells in the cord blood of infants, which is associated with decreased Th2 cytokines and may be linked to demethylation at the FOXp3 promoter (99). Whether epigenetic inheritance is underlying these effects requires further investigation. Further evidence that the farm environment affects the epigenome was provided by a pilot study which showed hypermethylation of genes related to IgE regulation and Th2 differentiation in cord blood from farmers' as compared to non-farmers' children (100). Interestingly, at least part of the protective effect triggered by those factors has been ascribed to the farm bacteria, for instance, Acinetobacter lwoffii (101, 102), with a pivotal contribution of downstream epigenetic mechanisms, specifically histone modifications (103).

Milk components

Human milk contains a unique combination of lipids, proteins, carbohydrates, vitamins, and minerals and thereby provides an ideal source of nutrition for the healthy growth and development of a newborn (104). However, human milk is more than nutrition as it also contains bioactive components that can modulate the immune system, such as immunoglobulins, lactoferrin, human milk oligosaccharides (HMO), long-chain fatty acids, and anti-inflammatory cytokines (18, 105, 106). Most of the immunologically relevant components in breast milk are also found in bovine milk (18). Several key components of breast milk that are not present at high enough levels in bovine milk are added to infant formula to provide the crucial nutrients needed. These include prebiotics or even single HMO like 2'-fucosyllactose (as an alternative to the complex mixture of HMO in breast milk), lactoferrin, PUFA, vitamins, and minerals.

Non-digestible milk oligosaccharides

One of the major differences between human breast milk and bovine milk is the amount and diversity of the HMO, i.e., complex, non-digestible oligosaccharides (107, 108). The HMO in breast milk constitutes about 20% of the milk saccharides next to the major carbohydrate in milk, lactose. Human breast milk contains ~5–15 mg/ml of these non-digestible HMO, consisting of up to 200 or more unique structures. In contrast, bovine milk only contains a few of these oligosaccharides, at much lower levels. One injected, HMO survive passage and digestion through the stomach and small intestine and reach the colon, where they are fermented into SCFA like acetate, butyrate, and propionate (107, 108). In addition, they shape the microbiota by selectively enhancing the growth of bifidobacteria and lactobacilli. These SCFAs serve as an energy source for colonic intestinal tissue and shape the interactions between the host and its gut microbiota. Furthermore, SCFA reduces intestinal pH, limit outgrowth of Enterobacteriaceae, and support intestinal barrier function. HMO is the key factor in shaping the development of immunity and early microbiota after birth. HMO have effects on microbiota and infections (107, 108). Of these, 2'-fucosyllactose is the HMO that is most abundantly present in breast milk and has therefore been chosen as the first HMO that was introduced in infant nutrition in 2018.

Prebiotics are non-digestible oligosaccharides like galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), and have widely been used in infant nutrition to mimic the bifidogenic- and SCFA-inducing effect of HMO. There is some evidence that prebiotic oligosaccharides in infant nutrition may prevent eczema in infants (109–112). It is not clear if these effects also extend to the prevention of other allergic diseases, as only one study to date has reported the effects of prebiotics on asthma and food allergy (113). For probiotics, effects are also seen when they are added in infant nutrition (68). As can be seen in detail in Lomax and Calder (114), several studies have reported that infant formula supplemented with prebiotics have a trend toward or even a significant preventive effect on the occurrence of gastrointestinal infections. Trends toward decreased fever episodes, antibiotic use, and upper respiratory tract infections (URTI) have been described. Two studies, by Bruzzese et al. and Arslanoglu et al. and performed with scGOS/lcFOS, supplemented very young infants from early after birth for 6–12 months (115, 116). Both studies showed a significant reduction in gastroenteritis (115) and a reduction in the total number of infections (116). A study from Westerbeek et al., in which scGOS/lcFOS were combined with acidic oligosaccharides (pAOS) showed a non-significant tendency toward fewer serious infections (117). This study was, however, conducted over a shorter time period, and the infants were preterm. In two other studies infants were older than 6 months (118, 119) were supplemented with oligofructose, one did not show an effect on diarrhea, whilst the latter observed a protective effect against diarrhea. Since these components and their effects have been reviewed in detail previously, we will not address them in detail here, and will instead, only focus on their potential epigenetic and long-lasting immune health effects.

Bioactive components besides non-digestible oligosaccharides

Both human milk and bovine milk contain many other bioactive components that can modulate immune function [reviewed in (18, 19, 105–107)]. The components in human and in bovine milk that can be isolated in large quantities have largely been studied as separate entities, because they are potential infant nutrition ingredients. Several of these components, such as transforming growth factor-β (TGF-β) (120), bovine lactoferrin (121–124), bovine alkaline phosphatase (19, 125), bovine osteopontin (126, 127), and the milk fat globular membrane (MFGM) (128), as well as milk exosomes (39), have been linked to immunological outcomes with varying levels of evidence (infection, allergy). Another milk component that may have more sustained immunological effects are bovine IgG antibodies. Where IgA is the predominant immunoglobulin isotype in breast milk, bovine milk has a larger amount of IgG (129). Bovine milk IgG (bIgG) has been shown to bind to aeroallergens (130) as well as to respiratory pathogens such as respiratory syncytial virus (RSV), and can inhibit infection of human cells with human RSV (131). Through the formation of immune complexes, bIgG can enhance RSV-specific T cell responses (132). Similarly, bovine colostrum, which is a rich source of IgG can prevent the infection of mice with RSV (133). Different from adaptive immunity, innate immunity was until recently believed to lead to immune memory. However, vaccination studies have shown that after vaccination—that is associated with cross-protection to other pathogens—the innate immune response is increased to the vaccine, but also other pathogens (134, 135). The mechanism of this was elucidated in several mechanistic studies and was shown to be dependent on epigenetic modification of monocytes and macrophages (136–139). Even though epigenetic modification was not directly shown, bovine IgG can induce trained immunity in monocytes (140). In addition to possibly preventing some of the epigenetic modifications induced by infection with respiratory viruses, which would be the result of the lower prevalence of respiratory tract infections (21), bovine IgG may also directly modify subsequent innate immune responses in infants.

(Epigenetic) Effects of human breast milk and bovine milk on allergy outcomes later in life

Several epigenome wide-association studies on allergies have been performed, as reviewed elsewhere (30). These studies have shown that allergic disease is accompanied by changing DNA methylation patterns in Th2, Th1, Th17, Th9, and Treg subsets in affected tissues. The epigenetic mechanism behind T cell subset differentiation is strongly affected by essential micronutrients (folate, vitamins B2, B6, and B12, methionine choline, and betaine) (141), bioactive food components (tea polyphenols, genistein from soybean, isothiocyanates from plant foods, curcumin, and curcumin-derived synthetic analogs) (142), total diet (fiber, protein, fat, and hormones) (143), ethanol, and carbohydrates (144). Dietary compounds, especially vitamin D, folate, and zinc, also have the potency to interfere with

DNA methylation and thereby steer the Th1-Th2 balance. In addition to these effects on DNA methylation, prenatal supplementation with PUFA or maternal levels of folate, and microbiota-derived SCFA have been associated with changes in histone acetylation patterns at important T cell differentiation regulating genes (Box 2). After birth, these immunomodulatory dietary components are also transferred to the newborn via breast milk.

Epigenetic effects of breastfeeding, raw milk, and exposure to the farming environment in early life

As already mentioned, the mechanisms underlying the anti-allergic effects of human milk are most probably complex, as human milk contains not only nutritional substances but also functional molecules including polysaccharides, cytokines, proteins, and other components forming a real biological system which can modulate and shape the innate and adaptive immune responses of the infant in very early life (104, 145). If and how those components affect the epigenetic status of the growing child and what consequences this has for allergy development need to be addressed in future studies. Considering the observations made about farm milk (see below), as well as indications that breastfeeding may be capable of changing DNA methylation patterns in the offspring (146), such studies are justified.

Epigenetic modulation of the Foxp3 gene by farm milk was demonstrated in an animal model. In this study, exposure to raw, unprocessed, cow's milk for 8 days, increased histone acetylation of Foxp3 in splenocyte-derived CD4+ T cells compared to processed milk exposure (147). In the same study, mice were subjected to an ovalbumin-induced food allergy model after milk exposure and, interestingly, histone acetylation of Th2 genes was lower in raw milk-pretreated mice compared to processed milk-pretreated mice. These mice also showed a reduction in food allergic symptoms (147). As for farm exposure, exposure to raw milk in the first year of life was also associated with changes in gene expression of the innate immune receptors (98). Moreover, it was demonstrated that a polymorphism in the CD14 gene influenced the protective effect of raw cow milk consumption on allergic diseases (148). DNA demethylation and increased Foxp3+ in the regulatory T cell numbers in the peripheral blood mononuclear cells of 4.5 year-old children were also shown in farm children (93). These regulatory T cell numbers were negatively associated with doctor-diagnosed asthma. It remains to be seen if these changes also precede the onset of allergic disease and can be predictive of allergy development.

There is evidence that the epigenome is affected by the farming environment. The first indication for a potential role of epigenetic regulation in the protective "farm effect" was provided by Slaats et al. who demonstrated that DNA methylation of the promoter region of CD14 in placentas of mothers living on a farm was lower compared to mothers not

living on a farm (94). These lower DNA methylation levels were reflected in higher CD14 mRNA expression levels (95). Interestingly, a higher expression of the CD14 gene was also observed in the children of farmers (96). Prenatal farm exposure was also associated with increased gene expression of other innate immune receptors, such as TLR5, TLR7, TLR8, and TLR9, at birth (97, 98) and TLR2 and TLR4 in farm-raised children at school age (91, 96). Maternal exposure to farming environments increased the number of Treg cells in the cord blood of infants, which is associated with decreased Th2 cytokines and may be linked to demethylation at the Foxp3 promoter (50). Whether epigenetic inheritance is the underlying cause of these effects requires further research. Additional evidence that the farm environment affects the epigenome was provided by a pilot study that showed DNA hypermethylation of genes related to IgE regulation and Th2 differentiation in cord blood from the children of farmers as compared to the children of non-farmers (100).

Epigenetic effects of miRNA containing extracellular vesicles (exosomes)

Interestingly, both human and cow's milk contain extracellular vesicles, or exosomes, that are resistant to the acidic environment in the stomach and RNAses in the GI tract. These exosomes contain a variety of especially immune function-related microRNAs (miRNAs). miRNAs represent short noncoding RNA molecules that control 40-60% of the total gene expression by inducing mRNA degradation and/or post-transcriptional inhibition of translation. As a result, specific miRNA can silence selective gene expression. The expression of a single gene can be regulated by several miRNAs, and likewise a single miRNA can regulate over 100 genes (32, 149). This activity thereby constitutes an epigenetic mechanism by which nutritional factors can influence immune activity or the induction of tolerance by affecting the Th1-Th2 balance. Bovine milk exosomes are taken up by human macrophages (150) and epithelial cells (151,152), exosomes become systemically available in the body of laboratory animals upon oral delivery (153), and bovine miRNA are detectible in the blood after drinking pasteurized milk (154). However, systemic availability could not be demonstrated for breast milk derived exosomes (155) or vegetable derived miRNA (156). Breast milk-derived exosomes were described in 2007 to enhance Treg development *in vitro* (157). Based on miRNA content, bovine milk exosomes contain immunoregulatory miRNAs, like miRNA155, that are involved in the development of Tregs and are thought to play a role in the effect of raw milk consumption on asthma (39). In addition to allergy, orally delivered bovine milk exosomes ameliorate arthritis in a murine model (158), and recent evidence also links milk exosomes to prevention of necrotizing enterocolitis and intestinal damage in *in vitro* and *in vivo* investigations (159,160), These studies suggest that miRNAs in human and raw bovine milk exosomes may have epigenetic effects in infants Interestingly, both human and cow's milk contain extracellular vesicles, or exosomes, that are resistant to the acidic environment in the stomach and RNAses in the GI tract. These exosomes contain a variety of especially immune function-related microRNAs (miRNAs). miRNAs represent short noncoding RNA molecules that control 40–60% of the total gene expression by inducing mRNA degradation and/or post-transcriptional inhibition of translation. As a result, specific miRNA can silence selective gene expression. The expression of a single gene

can be regulated by several miRNAs, and likewise, a single miRNA can regulate over 100 genes (32, 149). This activity thereby constitutes an epigenetic mechanism by which nutritional factors can influence immune activity or the induction of tolerance by affecting the Th1-Th2 balance. Bovine milk exosomes are taken up by human macrophages (150) and epithelial cells (151, 152), exosomes become systemically available in the body of laboratory animals upon oral delivery (153), and bovine miRNA are detectible in the blood after drinking pasteurized milk (154). However, systemic availability could not be demonstrated for breast milk derived exosomes (155) or vegetable derived miRNA (156). Breast milk-derived exosomes were described in 2007 to enhance Treg development in vitro (157). Based on miRNA content, bovine milk exosomes contain immunoregulatory miRNAs, like miRNA155, that are involved in the development of Tregs and are thought to play a role in the effect of raw milk consumption on asthma (39). In addition to allergy, orally delivered bovine milk exosomes ameliorated arthritis in a murine model (158), and recent evidence also links milk exosomes to the prevention of necrotizing enterocolitis and intestinal damage in in vitro and in vivo investigations (159, 160). These studies suggest that miRNAs in human and raw bovine milk exosomes may have epigenetic effects in infants.

Epigenetic effects of SCFA

Several studies have implicated the SCFA butyrate, propionate, and acetate as epigenetic modifiers of early life immunity, especially in the development of asthma (161). In addition to regulating Treg differentiation and histone acetylation, SCFAs can induce effector T cell differentiation in secondary lymphoid organs by inhibiting endogenous HDAC activity independent of activation of G-protein-coupled receptor (GPCR). In more detail, SCFA can modulate diverse cell processes by two mechanisms, either via interacting with the GPCR (GPR43, GPR41, GPR109A) on the plasma membrane or following a receptor-independent entrance to the cells (162). SCFA entry occurs through passive diffusion or actively by the involvement of two transporters, namely, monocarboxylate transporter 1 (MCT1/SLa16a1) and sodium-coupled monocarboxylate transporter 1 (SMCT1/SLc5a8). These receptors and transporter molecules are widely present in immune and non-immune cells (162, 163). This effect is highly pronounced for butyrate and to a lesser extent for propionate and acetate (164–166). HDAC inhibition allows HATs activity leading to histone hyperacetylation and subsequently an altered gene expression (37) which might, for instance, result in the proliferation of Treg cells (167–169). The significance of this mechanism is illustrated by the fact that bovine, but not human, milk triglycerides contain a relatively high concentration of the SCFA butyrate (18). Altogether, present evidence implies that HDAC inhibitory activity of SCFA might be cell and tissue dependent, and the gene expression pattern is related to the cellular stage and other environmental signals. If bovine milk consumption is associated with decreased allergy prevalence, does this also mean that milk components can affect epigenetic mechanisms? There is no in vivo evidence that the induction of SCFA by sialyllactose when ingested in bovine milk, but sialyllactose has been reported to induce SCFA production in in vitro fecal microbiota cultures (170) and may thus affect histone acetylation in infants. A high fiber diet (resulting in SCFA production in the colon) or direct feeding of SCFA has been shown

to prevent airway inflammation in animal models (84, 85), and SCFA levels in fecal samples of children associated inversely with sensitization to aeroallergens (171, 172).

In addition to allergies, intestinal immunity can also be influenced by microbiota-derived metabolites. For example, tryptophan metabolites can act as aryl hydrocarbon receptor (AhR) ligands, inducing IL-22 and antibacterial peptide production (173), SCFA can directly support the intestinal epithelial barrier, and bile acids can also be metabolized by the microbiota and influence intestinal barrier function and immunity (174). Two studies reported a decreased risk of wheezing in infants because of high maternal dairy intake (175, 176). Taken together, alterations in the local cellular microenvironment and the microbiome (56) allow milk to induce epigenetic changes in both maternal and neonatal nutrition-mediated genes, which can ultimately affect immune programming in the offspring (177).

Conclusions

This review summarizes current knowledge on the potential effects of human and bovine milk on neonatal immunity and epigenetic programming and its possible consequences on the development of allergies in early childhood and beyond (see Figure 1).

Breast milk is the food of choice for newborns and infants. When breast milk is not sufficiently available, cow's milk based formula is the best alternative, and thus cow's milk has become an integral part of early life diet.

Several epidemiological studies that have shown that exposure to a farm environment as well as to raw/unprocessed cow's milk in the prenatal period and early childhood is associated with protection against the development of asthma and other allergies later in life. Many cow's milk components have been shown to have similar effects on human immune cells as their breast milk counterparts.

Some of the molecular pathways that may explain the association between the consumption of raw milk asthma and allergy may be linked to epigenetics. Epigenetic mechanisms like DNA methylation, but also histone modifications, and non-classical epigenetics represented by miRNA may all contribute to the effects induced by raw cow's milk.

However, milk and dairy products are subject to industrial processing to ensure microbiological safety. As a result, milk proteins can be denatured, and lose their functional activity. In addition, glycation of milk proteins is thought to increase the risk of developing cow's milk allergy, illustrating that preserving milk proteins and preventing glycation may be important innovations to help prevent allergies.

Based on what is currently known on immunological and epigenetic effects that can be exerted by

human and different types of bovine milk, future research should focus on enhancing the functional (immunological as well as epigenetic) activity of milk components in early life nutrition, and on establishing epigenetic markers of immunological responses to milk. These could be especially important for diagnostic purposes and assessing the risk of developing CMA. Knowledge gathered during studies on the epigenetic effects of milk can be used in the future to drive the development of preventive or therapeutic anti-allergic strategies based on components that affect epigenetic mechanisms.

Finally, the continuation of epidemiologic and mechanistic studies on the effects of the components of breast and bovine milk on human immune function and health will increase our knowledge and help in finding potential applications that may help prevent allergies in the neonatal period

References

1. Hales CN, Barker DJ. Type 2 (non-insulin-dependent) diabetes mellitus: The thrifty phenotype hypothesis. Diabetologia. (1992) 35:595-601. doi: 10.1007/BF00400248
2. de Rooij SR, Painter RC, Phillips DI, Osmond C, Michels RP, Godsland IF, et al. Impaired insulin secretion after prenatal exposure to the dutch famine. Diabetes Care. (2006) 29:1897-901. doi: 10.2337/dc06-0460
3. Lumey LH, Khalangot MD, Vaiserman AM. Association between type 2 diabetes and prenatal exposure to the ukraine famine of 1932-33: A retrospective cohort study. Lancet Diabetes Endocrinol. (2015) 3:787-94. doi: 10.1016/S2213-8587(15)00279-X
4. Li C, Lumey LH. Exposure to the chinese famine of 1959-61 in early life and long-term health conditions: A systematic review and meta-analysis. Int J Epidemiol. (2017) 46:1157-70. doi: 10.1093/ije/dyx013
5. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci U S A. (2008) 105:17046-9. doi: 10.1073/pnas.0806560105
6. Prescott SL. Early nutrition as a major determinant of 'immune health': Implications for allergy, obesity and other noncommunicable diseases. Nestle Nutr Inst Workshop Ser. (2016) 85:1-17. doi: 10.1159/000439477
7. Harb H, Alashkar Alhamwe B, Acevedo N, Frumento P, Johansson C, Eick L, et al. Epigenetic modifications in placenta are associated with the child's sensitization to allergens. Biomed Res Int. (2019) 2019:1315257. doi: 10.1155/2019/1315257
8. Acevedo N, Frumento P, Harb H, Alashkar Alhamwe B, Johansson C, Eick L, et al. Histone acetylation of immune regulatory genes in human placenta in association with maternal intake of olive oil and fish consumption. Int J Mol Sci. (2019) 20:1060. doi: 10.3390/ijms20051060
9. Prescott SL. Early-life environmental determinants of allergic diseases and the wider pandemic of inflammatory noncommunicable diseases. J Allergy Clin Immunol. (2013) 131:23-30. doi: 10.1016/j.jaci.2012.11.019
10. Koletzko B, Brands B, Grote V, Kirchberg FF, Prell C, Rzehak P, et al. Long-term health impact of early nutrition: The power of programming. Ann Nutr Metab. (2017) 70:161-9. doi: 10.1159/000477781
11. Georgountzou A, Papadopoulos NG. Postnatal innate immune development: From birth to adulthood. Front Immunol. (2017) 8:957. doi: 10.3389/fimmu.2017.00957
12. West CE, D'Vaz N, Prescott SL. Dietary immunomodulatory factors in the development of immune tolerance. Curr Allergy Asthma Rep. (2011) 11:325-33. doi: 10.1007/s11882-011-0200-0
13. Torow N, Marsland BJ, Hornef MW, Gollwitzer ES. Neonatal mucosal immunology. Mucosal Immunol. (2017) 10:5-17. doi: 10.1038/mi.2016.81
14. McDade TW. Early environments and the ecology of inflammation. Proc Natl Acad Sci U S A. (2012) 109 Suppl 2:17281-8. doi: 10.1073/pnas.1202244109
15. Brugman S, Perdijk O, van Neerven RJ, Savelkoul HF. Mucosal immune development in early life: Setting the stage. Arch Immunol Ther Exp. (2015) 63:251-68. doi: 10.1007/s00005-015-0329-y

16. Agostoni C, Braegger C, Decsi T, Kolacek S, Koletzko B, Michaelsen KF, et al. Breast-feeding: A commentary by the espghan committee on nutrition. J Pediatr Gastroenterol Nutr. (2009) 49:112-25. doi: 10.1097/MPG.0b013e31819f1e05
17. Victora CG, Bahl R, Barros AJ, Franca GV, Horton S, Krasevec J, et al. Breastfeeding in the 21st century: Epidemiology, mechanisms, and lifelong effect. Lancet. (2016) 387:475-90. doi: 10.1016/S0140-6736(15)01024-7
18. van Neerven RJ, Knol EF, Heck JM, Savelkoul HF. Which factors in raw cow's milk contribute to protection against allergies? J Allergy Clin Immunol. (2012) 130:853-8. doi: 10.1016/j.jaci.2012.06.050
19. Abbring S, Hols G, Garssen J, van Esch BCAM. Raw cow's milk consumption and allergic diseases - the potential role of bioactive whey proteins. Eur J Pharmacol. (2019) 843:55-65. doi: 10.1016/j.ejphar.2018.11.013
20. Perdijk O, van Splunter M, Savelkoul HFJ, Brugman S, van Neerven RJJ. Cow's milk and immune function in the respiratory tract: Potential mechanisms. Front Immunol. (2018) 9:143. doi: 10.3389/fimmu.2018.00143
21. Loss G, Depner M, Ulfman LH, van Neerven RJ, Hose AJ, Genuneit J, et al. Consumption of unprocessed cow's milk protects infants from common respiratory infections. J Allergy Clin Immunol. (2015) 135:56-62. doi: 10.1016/j.jaci.2014.08.044
22. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, et al. The protective effect of farm milk consumption on childhood asthma and atopy: The gabriela study. J Allergy Clin Immunol. (2011) 128:766-73 e4. doi: 10.1016/j.jaci.2011.07.048
23. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, et al. Exposure to farming in early life and development of asthma and allergy: A cross-sectional survey. Lancet. (2001) 358:1129-33. doi: 10.1016/S0140-6736(01)06252-3
24. Brick T, Hettinga K, Kirchner B, Pfaffl MW, Ege MJ. The beneficial effect of farm milk consumption on asthma, allergies, and infections: From meta-analysis of evidence to clinical trial. J Allergy Clin Immunol Pract. (2020) 8:878-89 e3. doi: 10.1016/j.jaip.2019.11.017
25. von Mutius E, Vercelli D. Farm living: Effects on childhood asthma and allergy. Nat Rev Immunol. (2010) 10:861-8. doi: 10.1038/nri2871
26. Sozanska B, Pearce N, Dudek K, Cullinan P. Consumption of unpasteurized milk and its effects on atopy and asthma in children and adult inhabitants in rural poland. Allergy. (2013) 68:644-50. doi: 10.1111/all.12147
27. Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR, et al. Not all farming environments protect against the development of asthma and wheeze in children. J Allergy Clin Immunol. (2007) 119:1140-7. doi: 10.1016/j.jaci.2007.01.037
28. Waser M, Michels KB, Bieli C, Floistrup H, Pershagen G, von Mutius E, et al. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across europe. Clin Exp Allergy. (2007) 37:661-70. doi: 10.1111/j.1365-2222.2006.02640.x
29. Perkin MR, Strachan DP. Which aspects of the farming lifestyle explain the inverse association with childhood allergy? J Allergy Clin Immunol. (2006) 117:1374-81. doi: 10.1016/j.jaci.2006.03.008

30. Potaczek DP, Harb H, Michel S, Alhamwe BA, Renz H, Tost J. Epigenetics and allergy: From basic mechanisms to clinical applications. Epigenomics. (2017) 9:539-71. doi: 10.2217/epi-2016-0162
31. Alaskhar Alhamwe B, Khalaila R, Wolf J, von Bulow V, Harb H, Alhamdan F, et al. Histone modifications and their role in epigenetics of atopy and allergic diseases. Allergy Asthma Clin Immunol. (2018) 14:39. doi: 10.1186/s13223-018-0259-4
32. Baskara-Yhuellou I, Tost J. The impact of micrornas on alterations of gene regulatory networks in allergic diseases. Adv Protein Chem Struct Biol. (2020) 120:237-312. doi: 10.1016/bs.apcsb.2019.11.006
33. Alashkar Alhamwe B, Alhamdan F, Ruhl A, Potaczek DP, Renz H. The role of epigenetics in allergy and asthma development. Curr Opin Allergy Clin Immunol. (2020) 20:48-55. doi: 10.1097/ACI.0000000000000598
34. Martino DJ, Prescott SL. Silent mysteries: Epigenetic paradigms could hold the key to conquering the epidemic of allergy and immune disease. Allergy. (2010) 65:7-15. doi: 10.1111/j.1398-9995.2009.02186.x
35. Suarez-Alvarez B, Rodriguez RM, Fraga MF, Lopez-Larrea C. DNA methylation: A promising landscape for immune system-related diseases. Trends Genet. (2012) 28:506-14. doi: 10.1016/j.tig.2012.06.005
36. Grozinger CM, Schreiber SL. Deacetylase enzymes: Biological functions and the use of small-molecule inhibitors. Chem Biol. (2002) 9:3-16. doi: 10.1016/s1074-5521(02)00092-3
37. Eberharter A, Becker PB. Histone acetylation: A switch between repressive and permissive chromatin. Second in review series on chromatin dynamics. EMBO Rep. (2002) 3:224-9. doi: 10.1093/embo-reports/kvf053
38. Verdone L, Caserta M, Di Mauro E. Role of histone acetylation in the control of gene expression. Biochem Cell Biol. (2005) 83:344-53. doi: 10.1139/o05-041
39. Melnik BC, John SM, Carrera-Bastos P, Schmitz G. Milk: A postnatal imprinting system stabilizing foxp3 expression and regulatory t cell differentiation. Clin Transl Allergy. (2016) 6:18. doi: 10.1186/s13601-016-0108-9
40. Janson PC, Winerdal ME, Winqvist O. At the crossroads of t helper lineage commitment-epigenetics points the way. Biochim Biophys Acta. (2009) 1790:906-19. doi: 10.1016/j.bbagen.2008.12.003
41. Wilson CB, Rowell E, Sekimata M. Epigenetic control of t-helper-cell differentiation. Nat Rev Immunol. (2009) 9:91-105. doi: 10.1038/nri2487
42. Tripathi SK, Lahesmaa R. Transcriptional and epigenetic regulation of t-helper lineage specification. Immunol Rev. (2014) 261:62-83. doi: 10.1111/imr.12204
43. Makar KW, Perez-Melgosa M, Shnyreva M, Weaver WM, Fitzpatrick DR, Wilson CB. Active recruitment of DNA methyltransferases regulates interleukin 4 in thymocytes and t cells. Nat Immunol. (2003) 4:1183-90. doi: 10.1038/ni1004
44. Lal G, Zhang N, van der Touw W, Ding Y, Ju W, Bottinger EP, et al. Epigenetic regulation of foxp3 expression in regulatory t cells by DNA methylation. J Immunol. (2009) 182:259-73. doi: 10.4049/jimmunol.182.1.259
45. Baron U, Floess S, Wieczorek G, Baumann K, Grutzkau A, Dong J, et al. DNA demethylation in the

human foxp3 locus discriminates regulatory t cells from activated foxp3(+) conventional t cells. Eur J Immunol. (2007) 37:2378-89. doi: 10.1002/eji.200737594

46. Hirahara K, Vahedi G, Ghoreschi K, Yang XP, Nakayamada S, Kanno Y, et al. Helper t-cell differentiation and plasticity: Insights from epigenetics. Immunology. (2011) 134:235-45. doi: 10.1111/j.1365-2567.2011.03483.x
47. Onodera A, Kokubo K, Nakayama T. Epigenetic and transcriptional regulation in the induction, maintenance, heterogeneity, and recall-response of effector and memory th2 cells. Front Immunol. (2018) 9:2929. doi: 10.3389/fimmu.2018.02929
48. Oestreich KJ, Weinmann AS. Transcriptional mechanisms that regulate t helper 1 cell differentiation. Curr Opin Immunol. (2012) 24:191-5. doi: 10.1016/j.coi.2011.12.004
49. Kim LK, Esplugues E, Zorca CE, Parisi F, Kluger Y, Kim TH, et al. Oct-1 regulates il-17 expression by directing interchromosomal associations in conjunction with ctcf in t cells. Mol Cell. (2014) 54:56-66. doi: 10.1016/j.molcel.2014.02.004
50. Begin P, Nadeau KC. Epigenetic regulation of asthma and allergic disease. Allergy Asthma Clin Immunol. (2014) 10:27. doi: 10.1186/1710-1492-10-27
51. Paparo L, Nocerino R, Bruno C, Di Scala C, Cosenza L, Bedogni G, et al. Randomized controlled trial on the influence of dietary intervention on epigenetic mechanisms in children with cow's milk allergy: The epicma study. Sci Rep. (2019) 9:2828. doi: 10.1038/s41598-019-38738-w
52. World Health Organization. Infant and young child nutrition. Global strategy on infant and young child feeding (2002). https://www.who.int/nutrition/topics/infantfeeding_recommendation/en/ [Accessed April, 2020].
53. Munblit D, Peroni DG, Boix-Amoros A, Hsu PS, Van't Land B, Gay MCL, et al. Human milk and allergic diseases: An unsolved puzzle. Nutrients. (2017) 9. doi: 10.3390/nu9080894
54. Prentice S. They are what you eat: Can nutritional factors during gestation and early infancy modulate the neonatal immune response? Front Immunol. (2017) 8:1641. doi: 10.3389/fimmu.2017.01641
55. Abrams EM, Chan ES. It's not mom's fault: Prenatal and early life exposures that do and do not contribute to food allergy development. Immunol Allergy Clin North Am. (2019) 39:447-57. doi: 10.1016/j.iac.2019.06.001
56. Amarasekera M, Prescott SL, Palmer DJ. Nutrition in early life, immune-programming and allergies: The role of epigenetics. Asian Pac J Allergy Immunol. (2013) 31:175-82. doi:
57. Dunstan JA, Mori TA, Barden A, Beilin LJ, Taylor AL, Holt PG, et al. Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: A randomized, controlled trial. J Allergy Clin Immunol. (2003) 112:1178-84. doi: 10.1016/j.jaci.2003.09.009
58. Gunaratne AW, Makrides M, Collins CT. Maternal prenatal and/or postnatal n-3 long chain polyunsaturated fatty acids (lcpufa) supplementation for preventing allergies in early childhood. Cochrane Database Syst Rev. (2015) CD010085. doi: 10.1002/14651858.CD010085.pub2
59. D'Vaz N, Meldrum SJ, Dunstan JA, Lee-Pullen TF, Metcalfe J, Holt BJ, et al. Fish oil supplementation in early infancy modulates developing infant immune responses. Clin Exp Allergy. (2012) 42:1206-16. doi: 10.1111/j.1365-2222.2012.04031.x

60. Schindler T, Sinn JK, Osborn DA. Polyunsaturated fatty acid supplementation in infancy for the prevention of allergy. Cochrane Database Syst Rev. (2016) 10:CD010112. doi: 10.1002/14651858.CD010112.pub2
61. Harb H, Irvine J, Amarasekera M, Hii CS, Kesper DA, Ma Y, et al. The role of pkczeta in cord blood t-cell maturation towards th1 cytokine profile and its epigenetic regulation by fish oil. Biosci Rep. (2017) 37. doi: 10.1042/BSR20160485
62. Nauta AJ, Ben Amor K, Knol J, Garssen J, van der Beek EM. Relevance of pre- and postnatal nutrition to development and interplay between the microbiota and metabolic and immune systems. Am J Clin Nutr. (2013) 98:586S-93S. doi: 10.3945/ajcn.112.039644
63. Lynch SV, Boushey HA. The microbiome and development of allergic disease. Curr Opin Allergy Clin Immunol. (2016) 16:165-71. doi: 10.1097/ACI.0000000000000255
64. Palmer DJ, Huang RC, Craig JM, Prescott SL. Nutritional influences on epigenetic programming: Asthma, allergy, and obesity. Immunol Allergy Clin North Am. (2014) 34:825-37. doi: 10.1016/j.iac.2014.07.003
65. Hollingsworth JW, Maruoka S, Boon K, Garantziotis S, Li Z, Tomfohr J, et al. In utero supplementation with methyl donors enhances allergic airway disease in mice. J Clin Invest. (2008) 118:3462-9. doi: 10.1172/JCI34378
66. Riiser A. The human microbiome, asthma, and allergy. Allergy Asthma Clin Immunol. (2015) 11:35. doi: 10.1186/s13223-015-0102-0
67. Pandey KR, Naik SR, Vakil BV. Probiotics, prebiotics and synbiotics- a review. J Food Sci Technol. (2015) 52:7577-87. doi: 10.1007/s13197-015-1921-1
68. Cuello-Garcia CA, Brozek JL, Fiocchi A, Pawankar R, Yepes-Nunez JJ, Terracciano L, et al. Probiotics for the prevention of allergy: A systematic review and meta-analysis of randomized controlled trials. J Allergy Clin Immunol. (2015) 136:952-61. doi: 10.1016/j.jaci.2015.04.031
69. Garcia-Larsen V, Ierodiakonou D, Jarrold K, Cunha S, Chivinge J, Robinson Z, et al. Diet during pregnancy and infancy and risk of allergic or autoimmune disease: A systematic review and meta-analysis. PLoS Med. (2018) 15:e1002507. doi: 10.1371/journal.pmed.1002507
70. West CE. Probiotics for allergy prevention. Benef Microbes. (2016) 7:171-9. doi: 10.3920/BM2015.0073
71. Enomoto T, Sowa M, Nishimori K, Shimazu S, Yoshida A, Yamada K, et al. Effects of bifidobacterial supplementation to pregnant women and infants in the prevention of allergy development in infants and on fecal microbiota. Allergol Int. (2014) 63:575-85. doi: 10.2332/allergolint.13-OA-0683
72. Rautava S, Isolauri E. The development of gut immune responses and gut microbiota: Effects of probiotics in prevention and treatment of allergic disease. Curr Issues Intest Microbiol. (2002) 3:15-22. doi:
73. Rautava S, Luoto R, Salminen S, Isolauri E. Microbial contact during pregnancy, intestinal colonization and human disease. Nat Rev Gastroenterol Hepatol. (2012) 9:565-76. doi: 10.1038/nrgastro.2012.144
74. Wickens K, Black PN, Stanley TV, Mitchell E, Fitzharris P, Tannock GW, et al. A differential effect of 2 probiotics in the prevention of eczema and atopy: A double-blind, randomized, placebo-controlled

trial. J Allergy Clin Immunol. (2008) 122:788-94. doi: 10.1016/j.jaci.2008.07.011

75. Miles EA, Calder PC. Can early omega-3 fatty acid exposure reduce risk of childhood allergic disease? Nutrients. (2017) 9. doi: 10.3390/nu9070784
76. Willemsen LEM. Dietary n-3 long chain polyunsaturated fatty acids in allergy prevention and asthma treatment. Eur J Pharmacol. (2016) 785:174-86. doi: 10.1016/j.ejphar.2016.03.062
77. Sausenthaler S, Koletzko S, Schaaf B, Lehmann I, Borte M, Herbarth O, et al. Maternal diet during pregnancy in relation to eczema and allergic sensitization in the offspring at 2 y of age. Am J Clin Nutr. (2007) 85:530-7. doi: 10.1093/ajcn/85.2.530
78. Azagra-Boronat I, Tres A, Massot-Cladera M, Franch A, Castell M, Guardiola F, et al. Lactobacillus fermentum cect5716 supplementation in rats during pregnancy and lactation affects mammary milk composition. J Dairy Sci. (2020) 103:2982-92. doi: 10.3168/jds.2019-17384
79. Hogenkamp A, Knippels LM, Garssen J, van Esch BCAM. Supplementation of mice with specific nondigestible oligosaccharides during pregnancy or lactation leads to diminished sensitization and allergy in the female offspring. J Nutr. (2015) 145:996-1002. doi: 10.3945/jn.115.210401
80. Hogenkamp A, Thijssen S, van Vlies N, Garssen J. Supplementing pregnant mice with a specific mixture of nondigestible oligosaccharides reduces symptoms of allergic asthma in male offspring. J Nutr. (2015) 145:640-6. doi: 10.3945/jn.114.197707
81. van Vlies N, Hogenkamp A, Thijssen S, Dingjan GM, Knipping K, Garssen J, et al. Effects of short-chain galacto- and long-chain fructo-oligosaccharides on systemic and local immune status during pregnancy. J Reprod Immunol. (2012) 94:161-8. doi: 10.1016/j.jri.2012.02.007
82. Mischke M, Plosch T. More than just a gut instinct-the potential interplay between a baby's nutrition, its gut microbiome, and the epigenome. Am J Physiol Regul Integr Comp Physiol. (2013) 304:R1065-9. doi: 10.1152/ajpregu.00551.2012
83. Gray LE, O'Hely M, Ranganathan S, Sly PD, Vuillermin P. The maternal diet, gut bacteria, and bacterial metabolites during pregnancy influence offspring asthma. Front Immunol. (2017) 8:365. doi: 10.3389/fimmu.2017.00365
84. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, et al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med. (2014) 20:159-66. doi: 10.1038/nm.3444
85. Thorburn AN, McKenzie CI, Shen S, Stanley D, Macia L, Mason LJ, et al. Evidence that asthma is a developmental origin disease influenced by maternal diet and bacterial metabolites. Nat Commun. (2015) 6:7320. doi: 10.1038/ncomms8320
86. Torow N, Hornef MW. The neonatal window of opportunity: Setting the stage for life-long host-microbial interaction and immune homeostasis. J Immunol. (2017) 198:557-63. doi: 10.4049/jimmunol.1601253
87. Fujimura T, Lum SZC, Nagata Y, Kawamoto S, Oyoshi MK. Influences of maternal factors over offspring allergies and the application for food allergy. Front Immunol. (2019) 10:1933. doi: 10.3389/fimmu.2019.01933
88. Remes ST, Iivanainen K, Koskela H, Pekkanen J. Which factors explain the lower prevalence of atopy amongst farmers' children? Clin Exp Allergy. (2003) 33:427-34. doi: 10.1046/j.1365-

2222.2003.01566.x

89. Riedler J, Eder W, Oberfeld G, Schreuer M. Austrian children living on a farm have less hay fever, asthma and allergic sensitization. Clin Exp Allergy. (2000) 30:194-200. doi: 10.1046/j.1365-2222.2000.00799.x
90. von Ehrenstein OS, von Mutius E, Illi S, Baumann L, Bohm O, von Kries R. Reduced risk of hay fever and asthma among children of farmers. Clin Exp Allergy. (2000) 30:187-93. doi: 10.1046/j.1365-2222.2000.00801.x
91. Ege MJ, Bieli C, Frei R, van Strien RT, Riedler J, Ublagger E, et al. Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. J Allergy Clin Immunol. (2006) 117:817-23. doi: 10.1016/j.jaci.2005.12.1307
92. Douwes J, Cheng S, Travier N, Cohet C, Niesink A, McKenzie J, et al. Farm exposure in utero may protect against asthma, hay fever and eczema. Eur Respir J. (2008) 32:603-11. doi: 10.1183/09031936.00033707
93. Lluis A, Depner M, Gaugler B, Saas P, Casaca VI, Raedler D, et al. Increased regulatory t-cell numbers are associated with farm milk exposure and lower atopic sensitization and asthma in childhood. J Allergy Clin Immunol. (2014) 133:551-9. doi: 10.1016/j.jaci.2013.06.034
94. Slaats GG, Reinius LE, Alm J, Kere J, Scheynius A, Joerink M. DNA methylation levels within the cd14 promoter region are lower in placentas of mothers living on a farm. Allergy. (2012) 67:895-903. doi: 10.1111/j.1398-9995.2012.02831.x
95. Joerink M, Oortveld MA, Stenius F, Rindsjo E, Alm J, Scheynius A. Lifestyle and parental allergen sensitization are reflected in the intrauterine environment at gene expression level. Allergy. (2010) 65:1282-9. doi: 10.1111/j.1398-9995.2010.02328.x
96. Lauener RP, Birchler T, Adamski J, Braun-Fahrlander C, Bufe A, Herz U, et al. Expression of cd14 and toll-like receptor 2 in farmers' and non-farmers' children. Lancet. (2002) 360:465-6. doi: 10.1016/S0140-6736(02)09641-1
97. Roduit C, Wohlgensinger J, Frei R, Bitter S, Bieli C, Loeliger S, et al. Prenatal animal contact and gene expression of innate immunity receptors at birth are associated with atopic dermatitis. J Allergy Clin Immunol. (2011) 127:179-85, 85 e1. doi: 10.1016/j.jaci.2010.10.010
98. Loss G, Bitter S, Wohlgensinger J, Frei R, Roduit C, Genuneit J, et al. Prenatal and early-life exposures alter expression of innate immunity genes: The pasture cohort study. J Allergy Clin Immunol. (2012) 130:523-30 e9. doi: 10.1016/j.jaci.2012.05.049
99. Schaub B, Liu J, Höppler S, Schleich I, Huehn J, Olek S, et al. Maternal farm exposure modulates neonatal immune mechanisms through regulatory T cells. J Allergy Clin Immunol. 2009 Apr;123(4):774-82.e5. doi: 10.1016/j.jaci.2009.01.056.
100. Michel S, Busato F, Genuneit J, Pekkanen J, Dalphin JC, Riedler J, et al. Farm exposure and time trends in early childhood may influence DNA methylation in genes related to asthma and allergy. Allergy. (2013) 68:355-64. doi: 10.1111/all.12097
101 Conrad ML, Ferstl R, Teich R, Brand S, Blumer N, Yildirim AO, et al. Maternal tlr signaling is required for prenatal asthma protection by the nonpathogenic microbe acinetobacter lwoffii f78. J Exp Med. (2009) 206:2869-77. doi: 10.1084/jem.20090845

102. Hagner S, Harb H, Zhao M, Stein K, Holst O, Ege MJ, et al. Farm-derived gram-positive bacterium staphylococcus sciuri w620 prevents asthma phenotype in hdm- and ova-exposed mice. Allergy. (2013) 68:322-9. doi: 10.1111/all.12094
103. Brand S, Teich R, Dicke T, Harb H, Yildirim AO, Tost J, et al. Epigenetic regulation in murine offspring as a novel mechanism for transmaternal asthma protection induced by microbes. J Allergy Clin Immunol. (2011) 128:618-25 e1-7. doi: 10.1016/j.jaci.2011.04.035
104. Ballard O, Morrow AL. Human milk composition: Nutrients and bioactive factors. Pediatr Clin North Am. (2013) 60:49-74. doi: 10.1016/j.pcl.2012.10.002
105. Verhasselt V. Oral tolerance in neonates: From basics to potential prevention of allergic disease. Mucosal Immunol. (2010) 3:326-33. doi: 10.1038/mi.2010.25
106. Boix-Amoros A, Collado MC, Van't Land B, Calvert A, Le Doare K, Garssen J, et al. Reviewing the evidence on breast milk composition and immunological outcomes. Nutr Rev. (2019). doi: 10.1093/nutrit/nuz019
107. Triantis V, Bode L, van Neerven RJJ. Immunological effects of human milk oligosaccharides. Front Pediatr. (2018) 6:190. doi: 10.3389/fped.2018.00190
108. Bode L. Human milk oligosaccharides: Every baby needs a sugar mama. Glycobiology. (2012) 22:1147-62. doi: 10.1093/glycob/cws074
109. Osborn DA, Sinn JK. Prebiotics in infants for prevention of allergy. Cochrane Database Syst Rev. (2013) CD006474. doi: 10.1002/14651858.CD006474.pub3
110 . Cuello-Garcia C, Fiocchi A, Pawankar R, Yepes-Nunez JJ, Morgano GP, Zhang Y, et al. Prebiotics for the prevention of allergies: A systematic review and meta-analysis of randomized controlled trials. Clin Exp Allergy. (2017) 47:1468-77. doi: 10.1111/cea.13042
111 . Eigenmann PA. Evidence of preventive effect of probiotics and prebiotics for infantile eczema. Curr Opin Allergy Clin Immunol. (2013) 13:426-31. doi: 10.1097/ACI.0b013e3283630bad
112 . de Moura PN, Rosario Filho NA. The use of prebiotics during the first year of life for atopy prevention and treatment. Immun Inflamm Dis. (2013) 1:63-9. doi: 10.1002/iid3.8
113. Arslanoglu S, Moro GE, Schmitt J, Tandoi L, Rizzardi S, Boehm G. Early dietary intervention with a mixture of prebiotic oligosaccharides reduces the incidence of allergic manifestations and infections during the first two years of life. J Nutr. (2008) 138:1091-5. doi: 10.1093/jn/138.6.1091
114. Lomax AR, Calder PC. Prebiotics, immune function, infection and inflammation: A review of the evidence. Br J Nutr. (2009) 101:633-58. doi: 10.1017/s0007114508055608
115. Bruzzese E, Volpicelli M, Squeglia V, Bruzzese D, Salvini F, Bisceglia M, et al. A formula containing galacto- and fructo-oligosaccharides prevents intestinal and extra-intestinal infections: An observational study. Clin Nutr. (2009) 28:156-61. doi: 10.1016/j.clnu.2009.01.008
116. Arslanoglu S, Moro GE, Boehm G. Early supplementation of prebiotic oligosaccharides protects formula-fed infants against infections during the first 6 months of life. J Nutr. (2007) 137:2420-4. doi: 10.1093/jn/137.11.2420
117. Westerbeek EA, van den Berg JP, Lafeber HN, Fetter WP, Boehm G, Twisk JW, et al. Neutral and acidic oligosaccharides in preterm infants: A randomized, double-blind, placebo-controlled trial. Am J Clin Nutr. (2010) 91:679-86. doi: 10.3945/ajcn.2009.28625

118 . Duggan C, Penny ME, Hibberd P, Gil A, Huapaya A, Cooper A, et al. Oligofructose-supplemented infant cereal: 2 randomized, blinded, community-based trials in peruvian infants. Am J Clin Nutr. (2003) 77:937-42. doi: 10.1093/ajcn/77.4.937

119. Waligora-Dupriet AJ, Campeotto F, Nicolis I, Bonet A, Soulaines P, Dupont C, et al. Effect of oligofructose supplementation on gut microflora and well-being in young children attending a day care centre. Int J Food Microbiol. (2007) 113:108-13. doi: 10.1016/j.ijfoodmicro.2006.07.009

120. Khaleva E, Gridneva Z, Geddes DT, Oddy WH, Colicino S, Blyuss O, et al. Transforming growth factor beta in human milk and allergic outcomes in children: A systematic review. Clin Exp Allergy. (2019) 49:1201-13. doi: 10.1111/cea.13409

121. Manzoni P, Rinaldi M, Cattani S, Pugni L, Romeo MG, Messner H, et al. Bovine lactoferrin supplementation for prevention of late-onset sepsis in very low-birth-weight neonates: A randomized trial. JAMA. (2009) 302:1421-8. doi: 10.1001/jama.2009.1403

122. Manzoni P, Stolfi I, Messner H, Cattani S, Laforgia N, Romeo MG, et al. Bovine lactoferrin prevents invasive fungal infections in very low birth weight infants: A randomized controlled trial. Pediatrics. (2012) 129:116-23. doi: 10.1542/peds.2011-0279

123. King JC, Jr., Cummings GE, Guo N, Trivedi L, Readmond BX, Keane V, et al. A double-blind, placebo-controlled, pilot study of bovine lactoferrin supplementation in bottle-fed infants. J Pediatr Gastroenterol Nutr. (2007) 44:245-51. doi: 10.1097/01.mpg.0000243435.54958.68

124 . Chen K, Chai L, Li H, Zhang Y, Xie HM, Shang J, et al. Effect of bovine lactoferrin from iron-fortified formulas on diarrhea and respiratory tract infections of weaned infants in a randomized controlled trial. Nutrition. (2016) 32:222-7. doi: 10.1016/j.nut.2015.08.010

125. Abbring S, Ryan JT, Diks MAP, Hols G, Garssen J, van Esch BCAM. Suppression of food allergic symptoms by raw cow's milk in mice is retained after skimming but abolished after heating the milk - a promising contribution of alkaline phosphatase. Nutrients. (2019) 11:1499. doi: 10.3390/nu11071499

126. Lonnerdal B, Kvistgaard AS, Peerson JM, Donovan SM, Peng YM. Growth, nutrition, and cytokine response of breast-fed infants and infants fed formula with added bovine osteopontin. J Pediatr Gastroenterol Nutr. (2016) 62:650-7. doi: 10.1097/MPG.0000000000001005

127. West CE, Kvistgaard AS, Peerson JM, Donovan SM, Peng YM, Lonnerdal B. Effects of osteopontin-enriched formula on lymphocyte subsets in the first 6 months of life: A randomized controlled trial. Pediatr Res. (2017) 82:63-71. doi: 10.1038/pr.2017.77

128. Timby N, Hernell O, Vaarala O, Melin M, Lonnerdal B, Domellof M. Infections in infants fed formula supplemented with bovine milk fat globule membranes. J Pediatr Gastroenterol Nutr. (2015) 60:384-9. doi: 10.1097/MPG.0000000000000624

129. Ulfman LH, Leusen JHW, Savelkoul HFJ, Warner JO, van Neerven RJJ. Effects of bovine immunoglobulins on immune function, allergy, and infection. Front Nutr. (2018) 5:52. doi: 10.3389/fnut.2018.00052

130 . Collins AM, Roberton DM, Hosking CS, Flannery GR. Bovine milk, including pasteurised milk, contains antibodies directed against allergens of clinical importance to man. Int Arch Allergy Appl Immunol. (1991) 96:362-7. doi: 10.1159/000235523

131. den Hartog G, Jacobino S, Bont L, Cox L, Ulfman LH, Leusen JH, et al. Specificity and effector functions of human rsv-specific igg from bovine milk. PLoS One. (2014) 9:e112047. doi: 10.1371/journal.pone.0112047
132. Nederend M, van Stigt AH, Jansen JHM, Jacobino SR, Brugman S, de Haan CAM, et al. Bovine igg prevents experimental infection with rsv and facilitates human t cell responses to RSV. Front Immunol. (2020) In press. doi:
133. Xu ML, Kim HJ, Wi GR, Kim HJ. The effect of dietary bovine colostrum on respiratory syncytial virus infection and immune responses following the infection in the mouse. J Microbiol. (2015) 53:661-6. doi: 10.1007/s12275-015-5353-4
134 . Aaby P, Roth A, Ravn H, Napirna BM, Rodrigues A, Lisse IM, et al. Randomized trial of bcg vaccination at birth to low-birth-weight children: Beneficial nonspecific effects in the neonatal period? J Infect Dis. (2011) 204:245-52. doi: 10.1093/infdis/jir240
135. Benn CS, Netea MG, Selin LK, Aaby P. A small jab - a big effect: Nonspecific immunomodulation by vaccines. Trends Immunol. (2013) 34:431-9. doi: 10.1016/j.it.2013.04.004
136. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, et al. Bacille calmette-guerin induces nod2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci U S A. (2012) 109:17537-42. doi: 10.1073/pnas.1202870109
137. Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. Cell Host Microbe. (2012) 12:223-32. doi: 10.1016/j.chom.2012.06.006
138. Cheng SC, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, et al. Mtor- and hif-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity. Science. (2014) 345:1250684. doi: 10.1126/science.1250684
139. Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajanirefah A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. Science. (2014) 345:1251086. doi: 10.1126/science.1251086
140. van Splunter M, van Osch TLJ, Brugman S, Savelkoul HFJ, Joosten LAB, Netea MG, et al. Induction of trained innate immunity in human monocytes by bovine milk and milk-derived immunoglobulin g. Nutrients. (2018) 10. doi: 10.3390/nu10101378
141. Marques AH, O'Connor TG, Roth C, Susser E, Bjorke-Monsen AL. The influence of maternal prenatal and early childhood nutrition and maternal prenatal stress on offspring immune system development and neurodevelopmental disorders. Front Neurosci. (2013) 7:120. doi: 10.3389/fnins.2013.00120
142. Claycombe KJ, Brissette CA, Ghribi O. Epigenetics of inflammation, maternal infection, and nutrition. J Nutr. (2015) 145:1109S-15S. doi: 10.3945/jn.114.194639
143. Choi SW, Friso S. Epigenetics: A new bridge between nutrition and health. Adv Nutr. (2010) 1:8-16. doi: 10.3945/an.110.1004
144 . Paparo L, di Costanzo M, di Scala C, Cosenza L, Leone L, Nocerino R, et al. The influence of early life nutrition on epigenetic regulatory mechanisms of the immune system. Nutrients. (2014) 6:4706-19. doi: 10.3390/nu6114706

145. Rajani PS, Seppo AE, Jarvinen KM. Immunologically active components in human milk and development of atopic disease, with emphasis on food allergy, in the pediatric population. Front Pediatr. (2018) 6:218. doi: 10.3389/fped.2018.00218
146. Hartwig FP, Loret de Mola C, Davies NM, Victora CG, Relton CL. Breastfeeding effects on DNA methylation in the offspring: A systematic literature review. PLoS One. (2017) 12:e0173070. doi: 10.1371/journal.pone.0173070
147. Abbring S, Wolf J, Ayechu-Muruzabal V, Diks MAP, Alashkar Alhamwe B, Alhamdan F, et al. Raw cow's milk reduces allergic symptoms in a murine model for food allergy - a potential role for epigenetic modifications. Nutrients. (2019) 11:1721. doi: 10.3390/nu11081721
148. Bieli C, Eder W, Frei R, Braun-Fahrlander C, Klimecki W, Waser M, et al. A polymorphism in cd14 modifies the effect of farm milk consumption on allergic diseases and cd14 gene expression. J Allergy Clin Immunol. (2007) 120:1308-15. doi: 10.1016/j.jaci.2007.07.034
149. Cui J, Zhou B, Ross SA, Zempleni J. Nutrition, micrornas, and human health. Adv Nutr. (2017) 8:105-12. doi: 10.3945/an.116.013839
150. Izumi H, Tsuda M, Sato Y, Kosaka N, Ochiya T, Iwamoto H, et al. Bovine milk exosomes contain microrna and mrna and are taken up by human macrophages. J Dairy Sci. (2015) 98:2920-33. doi: 10.3168/jds.2014-9076
151. Wolf T, Baier SR, Zempleni J. The intestinal transport of bovine milk exosomes is mediated by endocytosis in human colon carcinoma caco-2 cells and rat small intestinal iec-6 cells. J Nutr. (2015) 145:2201-6. doi: 10.3945/jn.115.218586
152. Zempleni J, Sukreet S, Zhou F, Wu D, Mutai E. Milk-derived exosomes and metabolic regulation. Annu Rev Anim Biosci. (2019) 7:245-62. doi: 10.1146/annurev-animal-020518-115300
153. Munagala R, Aqil F, Jeyabalan J, Gupta RC. Bovine milk-derived exosomes for drug delivery. Cancer Lett. (2016) 371:48-61. doi: 10.1016/j.canlet.2015.10.020
154. Baier SR, Nguyen C, Xie F, Wood JR, Zempleni J. Micrornas are absorbed in biologically meaningful amounts from nutritionally relevant doses of cow milk and affect gene expression in peripheral blood mononuclear cells, hek-293 kidney cell cultures, and mouse livers. J Nutr. (2014) 144:1495-500. doi: 10.3945/jn.114.196436
155. Title AC, Denzler R, Stoffel M. Uptake and function studies of maternal milk-derived micrornas. J Biol Chem. (2015) 290:23680-91. doi: 10.1074/jbc.M115.676734
156. Link J, Thon C, Schanze D, Steponaitiene R, Kupcinskas J, Zenker M, et al. Food-derived xeno-micrornas: Influence of diet and detectability in gastrointestinal tract-proof-of-principle study. Mol Nutr Food Res. (2019) 63:e1800076. doi: 10.1002/mnfr.201800076
157. Admyre C, Johansson SM, Qazi KR, Filen JJ, Lahesmaa R, Norman M, et al. Exosomes with immune modulatory features are present in human breast milk. J Immunol. (2007) 179:1969-78. doi: 10.4049/jimmunol.179.3.1969
158. Arntz OJ, Pieters BC, Oliveira MC, Broeren MG, Bennink MB, de Vries M, et al. Oral administration of bovine milk derived extracellular vesicles attenuates arthritis in two mouse models. Mol Nutr Food Res. (2015) 59:1701-12. doi: 10.1002/mnfr.201500222
159. Li B, Hock A, Wu RY, Minich A, Botts SR, Lee C, et al. Bovine milk-derived exosomes enhance

goblet cell activity and prevent the development of experimental necrotizing enterocolitis. PLoS One. (2019) 14:e0211431. doi: 10.1371/journal.pone.0211431

160. Gao R, Zhang R, Qian T, Peng X, He W, Zheng S, et al. A comparison of exosomes derived from different periods breast milk on protecting against intestinal organoid injury. Pediatr Surg Int. (2019) 35:1363-8. doi: 10.1007/s00383-019-04562-6
161. Woo V, Alenghat T. Host-microbiota interactions: Epigenomic regulation. Curr Opin Immunol. (2017) 44:52-60. doi: 10.1016/j.coi.2016.12.001
162. Koh A, De Vadder F, Kovatcheva-Datchary P, Backhed F. From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. Cell. (2016) 165:1332-45. doi: 10.1016/j.cell.2016.05.041
163. Kim CH, Park J, Kim M. Gut microbiota-derived short-chain fatty acids, t cells, and inflammation. Immune Netw. (2014) 14:277-88. doi: 10.4110/in.2014.14.6.277
164. Kendrick SF, O'Boyle G, Mann J, Zeybel M, Palmer J, Jones DE, et al. Acetate, the key modulator of inflammatory responses in acute alcoholic hepatitis. Hepatology. (2010) 51:1988-97. doi: 10.1002/hep.23572
165. Kiefer J, Beyer-Sehlmeyer G, Pool-Zobel BL. Mixtures of scfa, composed according to physiologically available concentrations in the gut lumen, modulate histone acetylation in human ht29 colon cancer cells. Br J Nutr. (2006) 96:803-10. doi: 10.1017/bjn20061948
166. Sealy L, Chalkley R. The effect of sodium butyrate on histone modification. Cell. (1978) 14:115-21. doi: 10.1016/0092-8674(78)90306-9
167. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory t-cell generation. Nature. (2013) 504:451-5. doi: 10.1038/nature12726
168. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory t cells. Nature. (2013) 504:446-50. doi: 10.1038/nature12721
169. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, et al. The microbial metabolites, short-chain fatty acids, regulate colonic treg cell homeostasis. Science. (2013) 341:569-73. doi: 10.1126/science.1241165
170. Perdijk O, van Baarlen P, Fernandez-Gutierrez MM, van den Brink E, Schuren FHJ, Brugman S, et al. Sialyllactose and galactooligosaccharides promote epithelial barrier functioning and distinctly modulate microbiota composition and short chain fatty acid production in vitro. Front Immunol. (2019) 10:94. doi: 10.3389/fimmu.2019.00094
171. Roduit C, Frei R, Ferstl R, Loeliger S, Westermann P, Rhyner C, et al. High levels of butyrate and propionate in early life are associated with protection against atopy. Allergy. (2019) 74:799-809. doi: 10.1111/all.13660
172. Arrieta MC, Stiemsma LT, Dimitriu PA, Thorson L, Russell S, Yurist-Doutsch S, et al. Early infancy microbial and metabolic alterations affect risk of childhood asthma. Sci Transl Med. (2015) 7:307ra152. doi: 10.1126/scitranslmed.aab2271
173. Gao J, Xu K, Liu H, Liu G, Bai M, Peng C, et al. Impact of the gut microbiota on intestinal

immunity mediated by tryptophan metabolism. Front Cell Infect Microbiol. (2018) 8:13. doi: 10.3389/fcimb.2018.00013

174. Lee-Sarwar KA, Lasky-Su J, Kelly RS, Litonjua AA, Weiss ST. Gut Microbial-Derived Metabolomics of Asthma. Metabolites. 2020;10(3):97. Published 2020 Mar 6. doi:10.3390/metabo10030097
175. Chatzi L, Garcia R, Roumeliotaki T, Basterrechea M, Begiristain H, Iñiguez C, et al. Mediterranean diet adherence during pregnancy and risk of wheeze and eczema in the first year of life: Inma (spain) and rhea (greece) mother-child cohort studies. Br J Nutr. (2013) 110:2058-68. doi: 10.1017/s0007114513001426
176. Miyake Y, Sasaki S, Tanaka K, Hirota Y. Dairy food, calcium and vitamin d intake in preg nancy, and wheeze and eczema in infants. Eur Respir J. (2010) 35:1228-34. doi: 10.1183/09031936.00100609
177. Palmer AC. Nutritionally mediated programming of the developing immune system. Adv Nutr. (2011) 2:377-95. doi: 10.3945/an.111.000570

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CHAPTER

Short chain fatty acids (SCFA) inhibit activation of T lymphocytes and myeloid cells and induce innate immune tolerance

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Abstract

The intestinal microbiota contributes to gut immune homeostasis, where short-chain fatty acids (SCFAs) act as the major mediators. We aimed to elucidate the immunomodulatory effects of acetate, propionate, and butyrate. With that in mind, we sought to characterize the expression of SCFA receptors and transporters as well as SCFAs impact on the activation of different immune cells. Whereas all three SCFAs decreased TNF-a production in activated T cells, only butyrate and propionate inhibited IFN-γ, IL-17, IL-13, and IL-10 production. Butyrate and propionate inhibited the expression of the chemokine receptors CCR9 and CCR10 in activated T- and B cells, respectively. Similarly, butyrate and propionate were effective inhibitors of IL-1β, IL-6, TNF-a, and IL-10 production in myeloid cells upon LPS and R848 stimulation. Acetate was less efficient at inhibiting cytokine production except for IFN-a. Moreover, SCFAs inhibited the production of IL-6 and TNF-a in monocytes, mDC, and pDC whereas acetate effects were relatively more prominent in pDCs. In monocytes and mDCs, acetate was a less efficient inhibitor, but it was equally effective in inhibiting pDCs activation. We also studied the ability of SCFAs to induce trained immunity or tolerance. Butyrate and propionate - but not acetate - prevented TLR-mediated activation in SCFA-trained cells as demonstrated by a reduced production of IL-6 and TNF-a. Our findings indicate that butyrate and propionate are equally efficient in inhibiting the adaptive and innate immune response and did not induce trained immunity. The findings may be explained by differential SCFA receptor and transporter expression profiles of the immune cells.

Introduction

Short-Chain Fatty Acids (SCFAs) have a central role in the interplay between diet, microbiota, and host physiology. They are important players in maintaining gut homeostasis by promoting epithelial integrity and mucosal immunity, and recent evidence indicates that they are also crucial for regulating systemic immunity and preventing respiratory allergies [112-115]. The "Western diet" with low dietary fiber content that results in lower levels of intestinal SCFAs has been linked to many inflammatory disorders, including diabetes, certain autoimmune diseases, asthma, and allergies [116, 117]. In fact, dietary fiber consumption (as the precursors for SCFAs) was found to be inversely correlated to the risk of death from cardiovascular, cancer, infectious, and respiratory disorders [118].

SCFAs are metabolites produced by bacteria during the fermentation of dietary fibers as well as oligo- and polysaccharides. Complex plant-derived polysaccharides and milk oligosaccharides that are not digested in the small intestine reach the large intestine, where they are metabolized by the gut microbiota. Bacterial species within the phyla Bacteroidetes and Firmicutes present in the cecum and colon act as primary or secondary fermenters, producing acetate (C2), propionate (C3), and butyrate (C4), with a colonic molar ratio of around 3:1:1 respectively [24, 119, 120]. SCFAs, especially butyrate, are taken up and metabolized by the colonocytes as a preferred energy source over competing substrates such as glucose and glutamine [121]. In fact, most of the butyrate is metabolized locally, providing up to 60-70 percent of the energy supply for the epithelial cells in the colon [122]. The remainders pass through the intestinal epithelial cells and interact with the cells in the Gut-Associated Lymphoid Tissue (GALT) before entering the circulation. While colonocytes metabolize a small portion of propionate, the majority of it, along with acetate, is transported to the liver via the portal vein. In the liver, most of the propionate is metabolized by the hepatocytes. The remaining SCFAs, which mainly consists of acetate, circulate through the bloodstream and reach other organs such as the respiratory system, urinary tract, and central nervous system, where they can directly interact with cells and have a broad range of impact on other cells and tissues (reviewed in [22, 116, 123]).

SCFAs can exert their effects through two mechanisms: via direct effects on cellular biological processes upon internalization and indirectly via the engagement with membrane receptors [22, 27]. SCFAs diffuse passively into cells; however, a more significant part is actively transported into cells via two transporter molecules located on the cell membrane: MonoCarboxylate Transporter 1 (MCT-1) and Sodium-coupled MonoCarboxylate Transporter 1 (SMCT-1). The active transportation is essential for the SCFAs accumulation inside the cells and, subsequently, blockade of Histone Deacetylases (HDACs) activity, thereby directly influencing epigenetics and regulating gene expression [124]. Although HDACs blockade is generally considered permissive

for gene transcription, inhibitory or stimulatory consequences depend on various factors, including cell type and inflammatory context [125]. Another mechanism by which SCFAs modulate cells is through their interaction with G-Coupled Protein Receptors (GPCRs) on the cell membrane [126, 127]. Three GPCRs, GPR41, GPR43, and GPR109A, have been identified as being involved and able to bind to SCFAs with varying affinities. GPR41 (Free Fatty Acid Receptor 3 – FFAR3) has the highest affinity for propionate, followed by butyrate, whilst GPR43 (Free Fatty Acid Receptor 2 – FFAR2) interacts with acetate and propionate the most [128, 129]. GPR109A or the Niacin receptor exclusively binds to butyrate [127, 130]. The transduced signal via these receptors also has an inhibitory effect on the enzymatic activity of HDACs [131]. Besides regular G-proteins, all aforementioned GPCRs engage the alternative signaling pathway mediated by β-arrestin-2 to, e.g., inhibit the NF-κB signaling pathway and induce anti-inflammatory effects [132, 133].

Even though there is substantial data on SCFAs and their immunomodulatory effects, a comprehensive study covering the impact of all three SCFA on peripheral blood mononuclear cells is missing. The current study aimed to elucidate the direct and indirect effects of exposure to individual SCFAs on innate and adaptive immune function. To this aim, human PBMCs were treated with acetate, propionate, or butyrate and were stimulated *ex vivo* with various Toll-Like Receptors (TLRs) ligands including LPS and R848 to mimic bacterial and viral stimulations, respectively. We examined the effect of SCFAs on the production of cytokines by activated T- and B cells, as well as their potential to modulate the expression of tissue homing-associated chemokine receptors. Additionally, we assessed cytokine production by activated innate immune cells, namely monocytes, myeloid dendritic cells (mDCs), and plasmacytoid dendritic cells (pDCs) in the presence or absence of SCFAs. Furthermore, since SCFAs are known as potent HDAC inhibitors, we investigated their ability to epigenetically modify human monocytes toward training or tolerance induction in an *in vitro* innate immune training model.

Material & Methods

Study samples and PBMC isolation

The experiments were performed using buffy coats from the Sanquin blood bank in Nijmegen, the Netherlands, or freshly collected blood from several individuals after obtaining written consent. Blood samples from 6-8 individuals were used to stimulate innate and adaptive immune cells for PBMC work, whereas 11 buffy coats were used for the innate immune training model. The PBMCs were isolated by density gradient centrifugation using Ficoll Paque Plus (GE Healthcare, 17-1440-02). The Leucosep tubes (Greiner Bio-One, #227290) were filled with 15 mL of Ficoll and briefly centrifuged to move the Ficoll below the porous barrier. Blood samples or the buffy coats were diluted 1:1 with warm phosphate-buffered saline (PBS) (Gibco, #20012027) and then were pipetted

on top of the porous barrier. The tubes were then centrifuged, and the buffy layer above the porous barrier was poured into new 50 mL falcon tubes (Corning, #352070). The tubes were topped up with warm PBS and centrifuged to wash away the Ficoll and diluted plasma residuals. The cells were washed three times in total, resuspending the pellet before every wash. After washing and discarding the PBS, the cell pellets were resuspended in RPMI 1640 Glutamax (Gibco, #61870010) enriched with 10% FCS (Gibco, #10270106) and 1% penicillin/streptomycin (Gibco, #15140122). Cells were counted using the flow cytometer CytoFLEX LX (Beckman Coulter, #C11186), and appropriate dilutions for downstream processes were prepared.

SCFA receptors and transporters expression on immune cells

The expression of SCFA receptors (GPR41, GPR43, GPR109A) and transporters (MCT-1 & SMCT-1) on the cell membrane of innate and adaptive immune cells were studied. PBMC from three donors ($7.5x10^5$/well) were stained with antibodies for immune cell phenotyping to discriminate between T- and B lymphocytes, monocytes, mDCs, and pDCs. The expression of GPR41 (Invivogen, #PA5-25146), GPR43 (R&D system, #FAB10082R), and GPR109A (R&D system, #FAB2760T) were analyzed by flow cytometry. Similarly, antibodies against MCT-1 (R&D system, #FAB8275T) and SMCT-1 (R&D system, #FAB8398R) were used to screen the expression of SCFAs transporters on the aforementioned immune cells. The full antibody panel is summarized in Suppl. Table S1 & S2 for and Fig S1 describes the gating strategy.

Adaptive immune cell activation

SCFAs were evaluated for their potential to modulate the expression of tissue homing-associated chemokine receptors CCR9 and CCR10 on T and B lymphocytes, as well as their cytokine induction profile. $5x10^5$/well PBMCs were cultured on 48-well flat-bottom cell culture plates (Sigma-Aldrich, Costar 3548) containing 15 millimolar (mM) of either sodium acetate (Sigma-Aldrich, #S2889), sodium propionate (Sigma-Aldrich, #P1880), sodium butyrate (Sigma-Aldrich, #3034100), or RPMI 1640 as a negative control. For B cell activation, CpG (1.5 μg/mL) (Invivogen, #tlrl-2216) was used, and a combination of soluble anti-CD3 (a-CD3)(eBioscience, #14-0038-82)(1 μg/mL) and anti-CD28 (a-CD28)(BD, #555725)(1 μg/mL) was applied to activate T cells. The cell stimulation was done in the presence or absence of retinoic acid (RA, 1 μM) (Merck, #R2625) for its known ability to induce cell homing markers. After 48 hours of incubation, the plates were centrifuged, and 100 μL/well of the supernatants were transferred to a NUNC plate and stored at -20°C for Cytometric Bead Array (CBA) and cytokine measurement. Individual cytokine Flex-sets were used to measure the level of IL-13 (BD, #558450), IL-17 (BD, #562151), TNF-a (BD, #558273), IFN-γ (BD, #558269), and IL-10 (BD, #558274).

Then the cells were harvested and transferred to a 96-wells NUNC plate (ThermoFisher,

#267245) on ice for fluorescence-activated cell sorting (FACS) staining. Extracellular staining with a-CD4 (Biolegend, #300506) and a-CD19 (Biolegend, #302234) for selecting T- and B lymphocytes was performed, and the expression of homing markers was quantified by staining the cells with a-CCR9 (Biolegend, #358904) and a-CCR10 (Biolegend, #341506). In summary, the PBMCs were washed with cold FACS buffer (PBS supplemented with BSA (0.5% v/v), ethylenediaminetetraacetic acid (EDTA, 2.5 mM), and sodium azide (NaN3, 10% v/v)), and the supernatant was discarded after centrifugation. Subsequently, the cells were incubated with the antibody mixture (Suppl. Table S3) diluted in FACS buffer at 4°C in the dark for 20 minutes. Following that, the cells were washed twice with cold FACS buffer and resuspended in 200 µL of FACS buffer before being used for the measurement.

The samples were measured on CytoFLEX LX, and the generated flow cytometry data were analyzed with FlowJo (FlowJo LLC, v10). The analysis was carried out to determine the percentage of T- and B cells expressing CCR9 and CCR10 (Suppl. Fig S2). The experiment included two replicates, and the analysis employed the arithmetic mean of the replicates.

Innate immune cell activation

SCFAs were investigated for their capacity to modulate the innate immune response by assessing cytokine levels in the PBMC culture supernatant and intracellularly in monocytes, mDCs, and pDCs. PBMCs were seeded ($5x10^5$/well) in flat-bottom 48-wells culture plates (Sigma-Aldrich, Costar 3548) where acetate, propionate, or butyrate with a final concentration of 15 mM was added. The innate cells were stimulated with TLR4 ligand (LPS, 0.2 µg/mL)(Sigma-Aldrich, #L2880) or TLR7/8 ligand (R848, 1 µg/mL) (Sigma-Aldrich, #SML0196). Brefeldin A (0.1 µg/mL) (Invitrogen, #00-4506-51) was added to the wells to keep the produced cytokines inside the cell. The plates were incubated at 37°C with 5% CO_2 for 3 hours. After incubation, the cells were harvested, transferred to a 96-wells NUNC plate, and kept on ice for FACS antibody staining. Extracellular and intracellular FACS labeling of the markers was used for phenotyping the cells and determining IL-6 and TNF-a production. In summary, the cells were washed with cold FACS buffer and then incubated with an antibody mixture against extracellular markers (Suppl. Table S4). The plates were then incubated at 4°C in dark conditions for 20 minutes. Following live/dead staining with fixable viability dye 520 (FVD520) (Invitrogen, #65-0867-14), the cells were fixed, and the cell membrane was permeabilized according to the manufacturer's protocol using the IC fix/perm kit (Invitrogen, #88-8824-00). Then, antibodies against IL-6 (Biolegend, #501107) and TNF-a (Biolegend, #502916) were added to quantify the percentage of cells that were producing these cytokines. The samples were measured on CytoFLEX LX, and the generated flow cytometry data were analyzed with FlowJo (FlowJo LLC, v10). The innate immune cells were discriminated, and the percentage of IL-6 and TNF-a producing cells) was quantified (Suppl. Fig S3).

A second plate with the same conditions and stimuli but without BFA was incubated at 37°C for 24 hours to measure cytokine levels in the culture supernatant. The culture supernatant was collected after 24 hours and was stored at -20°C until CBA analysis. Individual cytokines Flex-sets were used for quantifying IL-1β (BD, #558279), IL-6 (BD, #558276), TNF-a (BD, #558273), IFN-a (BD, #560379), and IL-10 (BD, #558274). Each condition was replicated twice, and the arithmetic mean of the replicates was used for analysis.

Monocyte isolation and training

The training or tolerogenic properties of SCFAs on monocytes was investigated in an *in vitro* innate immune training model [46, 47, 134]. The EasySep Human Monocyte Isolation Kit (Stemcell technologies, #19359) was used in accordance with the manufacturer's protocol to isolate $CD14^+$ monocytes from donors' fresh PBMCs. In summary, the PBMCs were resuspended at a concentration of $5x10^7$ cells/mL in Monocyte Isolation Buffer (PBS containing 2% FBS and 1mM EDTA without Ca^{2+} or Mg^{2+}). Subsequently, 50 µL/mL of the isolation cocktail and then platelet removal cocktail were added, and the cells were incubated at room temperature for 5 minutes. Next, magnetic particles (50 µL/mL) were added, and the tubes were put in a magnet following 5 minutes of incubation. The negative fraction was collected and washed with the monocytes isolation buffer before being resuspended in the enriched RPMI 1640 (+1% gentamicin and 1% pyruvate). The cells were counted and checked for $CD14^+$ monocytes purity by staining with antibodies against CD14 (Biolegend, #301830) and CD3 (BD, #555334). All donors had a purity of >80% $CD14^+$ monocytes with <3% $CD3^+$ T cell contamination.

A flat-bottom 96-wells cell culture plate (Sigma-Aldrich, Costar 3596) was filled with isolated monocytes ($1x10^5$ monocytes/well) and was incubated at 37°C for two hours to allow the cells to settle down and adhere to the plate. After incubation, the cells were stimulated with either medium as a negative control, 100 µg/mL Whole Glucan Particles (WGP)(Invivogen, #tlrl-wgp) as a positive control, 5 mM of SCFAs (acetate, propionate, or butyrate), 0.1 µg/mL LPS, or 10 µg/mL R848 and were incubated at 37°C for 24 hours. The plate was washed twice with warm PBS the next day, and fresh RPMI medium enriched with 10% human pooled serum (Sigma-Aldrich, #H3667) was added. The medium was refreshed on day 4. During the resting phase, the monocytes were differentiated into macrophages which were stimulated on day 7 with TLR-ligands LPS (0.1 µg/mL), R848 (10 µg/mL), or Pam3CSK4 (10 µg/mL) (Invivogen, #tlrl-pms). The plate was incubated with stimuli at 37°C for 24 hours. On day 8, the supernatant was collected and stored at -20°C until CBA analysis. The CBA was performed using Flex-sets for measuring IL-6 (BD, #558276) and TNF-a (BD, #558273).

Statistical analysis

GraphPad Prism (version 9) was used for statistical analysis and preparing the charts. One-way ANOVA with Dunnett's post-test was used for comparing the conditions to the no SCFAs group within each stimulation. The innate immune training data was normalized and expressed as fold changes compared to the negative control (RPMI medium). Friedman test was used for pair-wise comparisons. The differences were considered significant when the p-value was <0.05 (*), <0.01 (**), <0.001 (***), or <0.0001 (****) as indicated in the graphs.

Results

Differential expression of SCFA receptors and transporters

The relative expression of SCFA receptors and transporters was evaluated on different immune cells from PBMCs. When individual cell types were examined, GPR41 was expressed at a higher level than other receptors, with monocytes and B cells showing relatively the highest expression levels (Fig 1A). GPR109A was abundant on monocytes, expressed at low levels on mDC and B cells but not detectable on T cells or pDC. The expression levels of GPR43 in mDC, pDC, and B cells were considerably higher than the expression of GPR109A. On monocytes, GPR43 expression was much lower compared to GPR109A and GPR41 expression. T cells did not express detectable levels of GPR43 or GPR109A and only expressed GPR41.

For the SCFA transporters, the presence of MCT-1 was only detected on T cells (Fig 1B). SMCT-1, on the other hand, is extensively expressed on monocytes, mDCs, pDCs, T-, and B lymphocytes as a key SCFAs membrane transporter. The highest levels of SMCT-1 expression were found in monocytes, DCs (both mDCs and pDCs), and B cells. In fact, SMCT-1 expression in B lymphocytes was more than 6-fold higher than the expression in T cells.

Overall, the expression patterns of SCFA receptors and transporters varied significantly across the cell types studied. Monocytes showed the highest expression of GPR41 and GPR109A, low levels of GPR43, considerable SMCT-1 with no detectable MCT-1 expression. mDCs express GPR41 and GPR43 much higher than GPR109A. While SMCT-1 was abundantly present on mDCs, MCT-1 was entirely absent. No GPR109A was detected on pDCs while GPR41 and GPR43 were equally expressed. pDCs highly expressed SMCT-1 with marginal MCT-1 expression. Although GPR41 is present on both T and B cells, the levels were considerably higher on B cells. Besides, B cells also express GPR43 and GPR109A while both receptors were absent on T cells. The MCT-1 molecule was detectable on T cells, and they also expressed SMCT-1, while on B cells, only SMCT-1 was present.

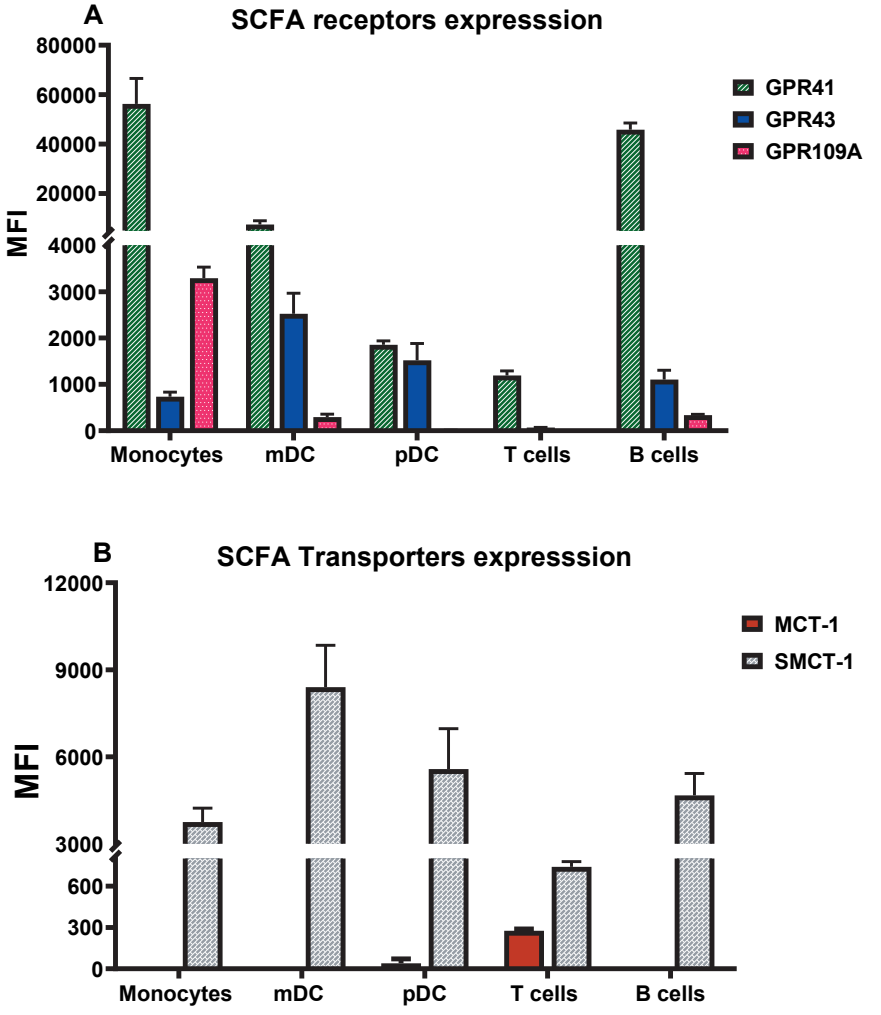

Fig 4. Differential expression pattern of SCFA receptors and transporters on PBMCs: The relative expression of SCFA receptors, GPR41, GPR43, and GPR109A (A) and SCFAs membrane transporters, MCT-1 and SMCT-1 (B) on monocytes, mDCs, pDCs, T- and B lymphocytes

Effects of SCFAs on cytokine release from adaptive immune cells

The production of cytokines was measured in the culture supernatant of PBMCs. Cells were incubated with SCFAs and with soluble a-CD3/a-CD28 antibodies (T-cell activation) or CpG (for B-cell activation) for 48h and cytokines were measured in the supernatants. Stimulation of T cells with a-CD3/a-CD28 resulted in the production of TNF-a, IFN-g, IL-17, IL-13, and IL-10, reflecting the determination of Th1, Th17, Th2, and Treg, respectively (Fig 2). All three SCFAs, with different potency, had an inhibitory effect on TNF-a production in activated T cells (Fig 2A). Similarly, butyrate and propionate, but not acetate, significantly decreased IL-17, IL-13, and IFN-γ levels after T cell stimulation

with soluble a-CD3/a-CD28 (Fig 2B-D). Stimulation with a-CD3/a-CD28 antibodies also resulted in the production of the anti-inflammatory cytokine, IL-10. Production of IL-10 was blocked in the cells treated with butyrate and propionate but not by acetate (Fig 2E). Stimulation of B cells with CpG did not induce substantial production of cytokines, with the exception of IL-10 that was inhibited by butyrate, propionate, and to a lesser extent by acetate (Fig 2A-E).

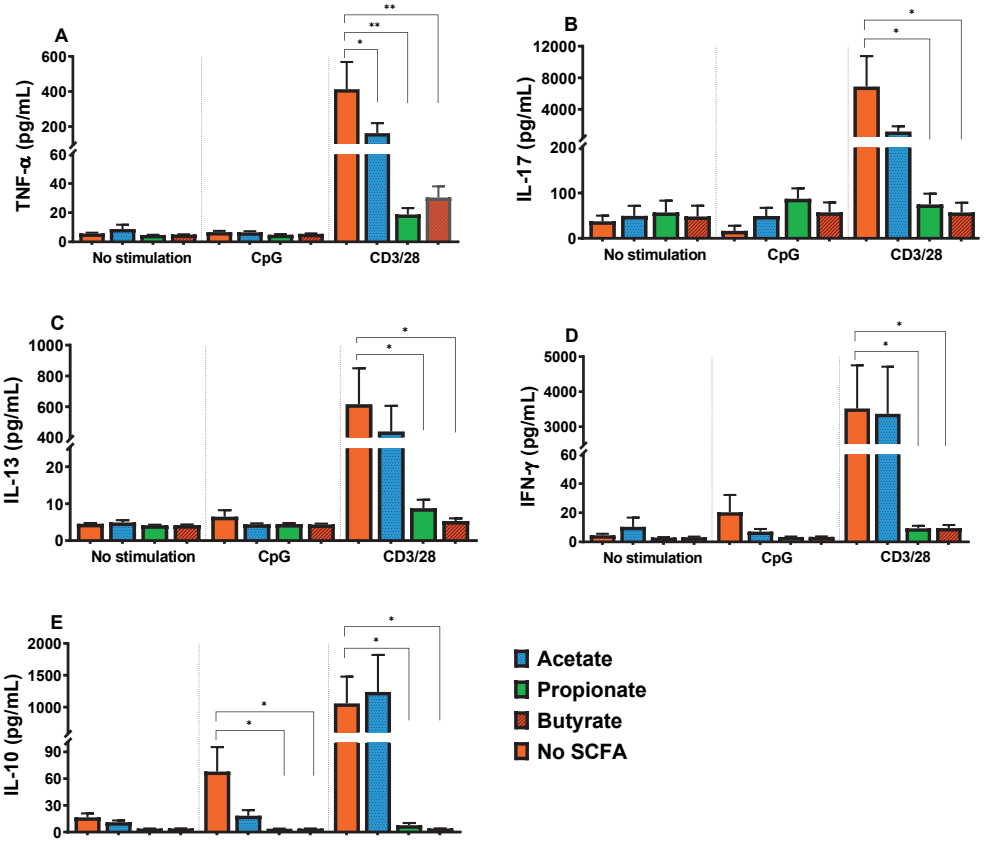

Fig 5. The effects of SCFAs on the production of cytokines from adaptive cells: The levels of TNF-a (A), IL-17 (B), IL-13 (C), INF-γ (D), and IL-10 (E) in the 48h culture supernatant of the PBMCs incubated for 48h with acetate, propionate, butyrate, or RPMI and stimulated with CpG or the combination of a-CD3/a-CD28 antibodies. The significance of differences is shown as p-value <0.05 (*), <0.01 (**), <0.001 (***), or <0.0001 (****).

The effects of SCFAs on the expression of homing receptors on B and T cells

In order to study the ability of SCFAs to modulate the expression of the tissue homing-associated chemokine receptors CCR9 and CCR10, PBMCs were co-incubated with individual SCFAs and T- and B-cell stimuli for 48 hours in the presence or absence of retinoic acid (RA). RA is known for its ability to upregulate CCR9 expression and homing to the small intestine [135]. CCR9 expression was only induced significantly after

activation of the T cells with a-CD3/a-CD28 in the presence of RA, and it was inhibited by butyrate and propionate (Fig 3A). The average of T cells expressing CCR9 declined from 20% in the no SCFAs group to 5% in the group of cells treated with butyrate and propionate. In contrast, stimulation of B cells by CpG increased (however trivial) CCR10 expression, which was not affected by the addition of RA (Fig 3B). This expression was inhibited by butyrate and propionate but not by acetate. None of the stimuli or SCFA resulted in the expression of CCR9 on B cells or CCR10 on T cells (data not shown).

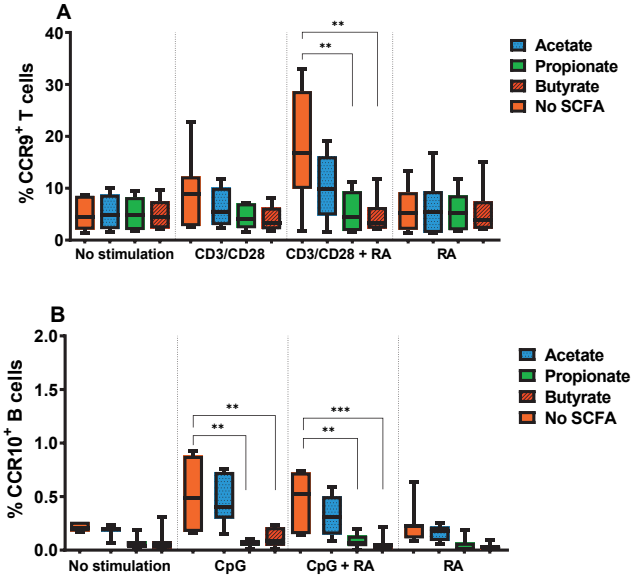

Fig 6. Expression of CCR9 and CCR10 in the presence of SCFAs: Expression of CCR9 in T cells cultured for 48h with acetate, propionate, butyrate, or RPMI and stimulated with a-CD3/a-CD28 in the presence of retinoic acid (RA) (A). Expression levels of CCR10 in B cells incubated for 48h with acetate, propionate, butyrate, or RPMI and activated by CpG in the presence or absence of RA (B). The significance of differences is shown as p-value <0.05 (*), <0.01 (**), <0.001 (***), or <0.0001 (****).

Effects of SCFAs on cytokines release from innate immune cells

To address the effects of the SCFA on stimulated innate immune cells (monocytes, mDC, pDC), PBMCs were incubated with TLR4 (LPS) and TLR7/8 (R848) agonists in the presence or absence of individual SCFAs for 24 hours. The production of IL-1β, TNF-a, IL-6, IL-10, and IFN-a was quantified in the culture supernatant, and the results were compared to the group treated with no SCFAs. Upon LPS and R848 stimulation, IL-1β, TNF-a, IL-6, and IL-10 production was induced, but IFN-a production was only induced after stimulation with R848 (Fig 4A-E). Following R848 stimulation, all three SCFAs were competent in decreasing IL-1β production in innate immune cells; however, the differences between conditions were not significant following LPS stimulation (Fig 4A). Butyrate and propionate both inhibited the production of IL-6 following stimulation

with LPS and R848, while acetate did not have an effect (Fig 4B). TNF-a production in the innate cells was significantly reduced by all three SCFAs upon stimulation with R848, whereas in the LPS stimulated group, this decrease was only observed in cells treated with butyrate and propionate, but not acetate (Fig 4C). As mentioned before, LPS did not induce the production of IFN-a in any of the groups, but on the contrary, stimulation with R848 resulted in IFN-a production, which was blocked by acetate and butyrate, but not propionate (Fig 4D). Finally, all three SCFAs significantly decreased IL-10 levels in LPS stimulated cells, while only butyrate inhibited this production in R848 treated cells. Nevertheless, treatment with acetate marginally increased the production of IL-10 in the R848 stimulated cells (Fig 4E). In summary, under those conditions where the addition of SCFA reduced cytokines released from stimulated cells, butyrate was the most potent inhibitor, followed by propionate. Except for a few stimulation conditions, the cytokine production in the acetate treated group was comparable to those without any SCFAs.

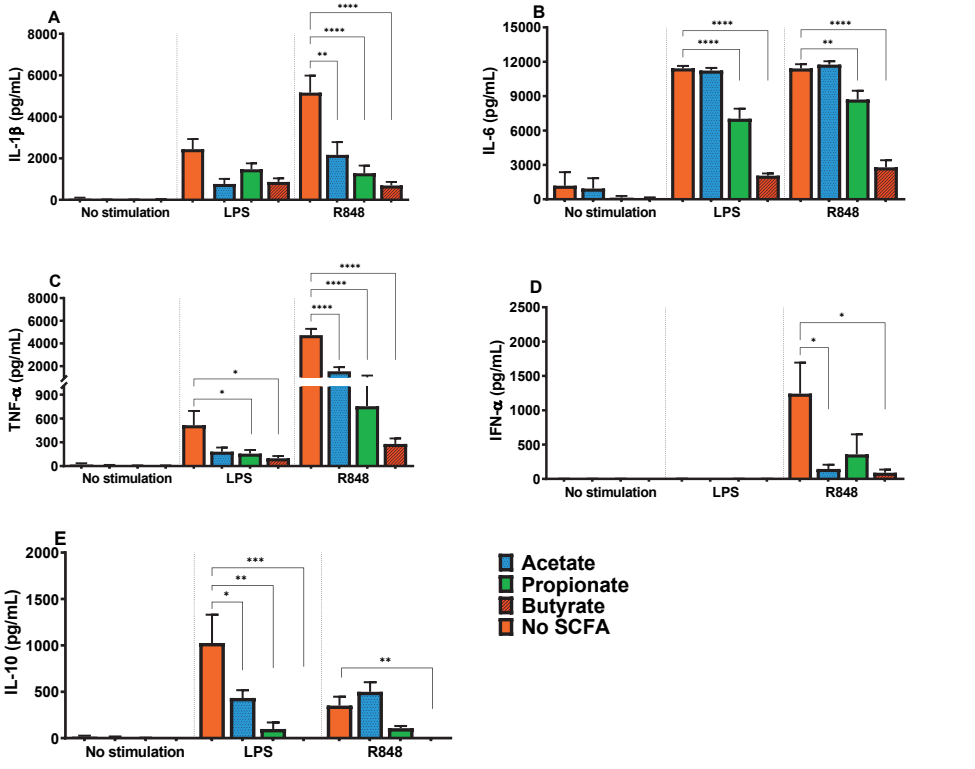

Fig 7. The effects of SCFAs on the production of cytokines in innate immune cells: The cytokine levels of IL-1β (A), IL-6 (B), TNF-a (C), IFN-a (D), and IL-10 (E) was measured in the 24h culture supernatant of PBMCs incubated with acetate, propionate, butyrate, or RPMI and activated with LPS or R848. The significance of differences is shown as p-value <0.05 (*), <0.01 (**), <0.001 (***), or <0.0001 (****).

The effects of SCFAs on IL-6 and TNF-a production in monocytes, mDCs, and pDCs

To measure the effects of the SCFA on stimulated monocytes, mDC, and pDC, PBMCs were incubated with or without the individual SCFAs and were stimulated with the TLR4 ligand LPS or the TLR7/8 ligand R848 for 3 hours. This assay was executed to characterize the effects of SCFAs on individual myeloid innate immune cells within PBMC. BFA was added to the culture to block cytokine excretion. Following stimulation, the cells were labeled with the fluorochrome-conjugated monoclonal antibodies to identify monocytes, mDC, and pDC; they were permeabilized, and the intracellular presence of IL-6 and TNF-a was detected by flow cytometry. Both LPS and R848 stimulation induced the production of IL-6 in more than 85% of monocytes. The IL-6 production was blocked in the group of cells treated with butyrate and propionate, but not acetate (Fig 5A). In fact, the percentage of IL-6 producing monocytes dropped to less than 40% in the butyrate and less than 45% in the propionate group, where acetate did not exert significant effects (Fig 5A). On the contrary, exposure to all three SCFAs significantly decreased the TNF-a production in monocytes induced by R848 (Fig 5B). R848 but not LPS stimulation induced TNF-a production in more than 80% of monocytes while acetate, propionate, and butyrate decreased this percentage to around 60, 30, and 35% respectively (Fig 5B). When mDCs were examined, it was noted that acetate, butyrate, and propionate potently inhibited the IL-6 production induced by both LPS and R848 (Fig 5C). The percentage of IL-6 producing mDCs declined from more than 50% to around 25% in acetate-treated cells and around 10% after butyrate and propionate treatment (Fig 5C). From the same mDCs, stimulation with LPS and R848 resulted in the production of TNF-a in more than 40% and 70% of cells, respectively. However, TNF-a positive cells were significantly decreased by butyrate to 25% in the LPS stimulated cells and less than 40% by butyrate and propionate in the cells activated by R848 (Fig 5D). R848 but not LPS induced the production of IL-6 and TNF-a in pDCs. However, IL-6 production was blocked efficiently by all three SCFAs (Fig 5E). The percentage of IL-6 producing pDCs decreased more than 4-fold from above 8% to less than 2% (Fig 5E). Moreover, more than 50% of pDCs responded to R848, but not LPS stimulation, and produced TNF-a. Nevertheless, the cytokine production was decreased by butyrate and propionate, and the levels dropped to 25% of cells. It is worth mentioning that acetate also lowered the percentage of TNF-a producing cells to less than 30%, but the difference was not quite significant (p-value=0.06) (Fig 5F).

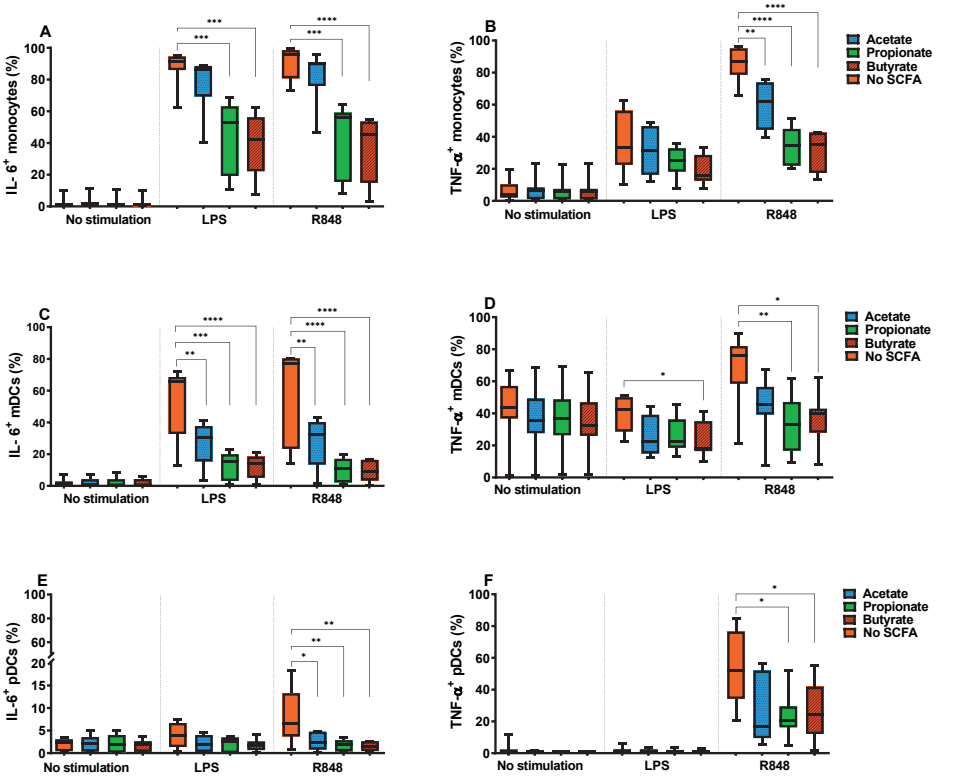

Fig 8. Intracellular production of IL-6 and TNF-a in innate immune cells: After three hours of PBMCs incubation with acetate, propionate, butyrate, or RPMI, the percentage of cells producing IL-6 and TNF-a upon stimulation with LPS or R848 was quantified. The IL-6 (A) or TNF-a (B) producing monocytes, IL-6 (C) or TNF-a (D) producing mDCs, and IL-6 (E) or TNF-a (F) producing pDCs are shown in the figure. The significance of differences is shown as p-value <0.05 (*), <0.01 (**), <0.001 (***), or <0.0001 (****).

Innate immune training by SCFAs

Using an *in vitro* trained immunity model [134], we investigated the ability of SCFAs to modulate the responsiveness of human monocytes to secondary stimulation. The monocytes were incubated/trained with individual SCFAs, WGP, or TLR ligands (LPS and R848) as tolerogenic controls for 24 hours. Thereafter, the monocytes were rested for 6 days and were stimulated with the TLR ligands PAM3CSK4 (TLR2/TLR1), LPS (TLR4), or R848 (TLR7/8). The monocytes trained with butyrate and propionate showed a decreased production of IL-6 and TNF-a upon stimulation with LPS, R848, and Pam3CSK4 compared to the negative control (RPMI) (Fig 6A-F). Training with acetate did not significantly lower IL-6 and TNF-a levels in any of the stimulations.

Training with the tolerogenic controls strongly inhibited IL-6 and TNF-a production in all cases, in a similar manner to butyrate and propionate (Fig 6A-F), except IL-6 production in response to LPS stimulation. This could be explained by the high variability of responses

in this group of participants (Fig 6C). In our hands, training with WGP induced slightly higher IL-6 and TNF-a production than the RPMI control, but the differences did not reach statistical significance (Fig 6A-F)

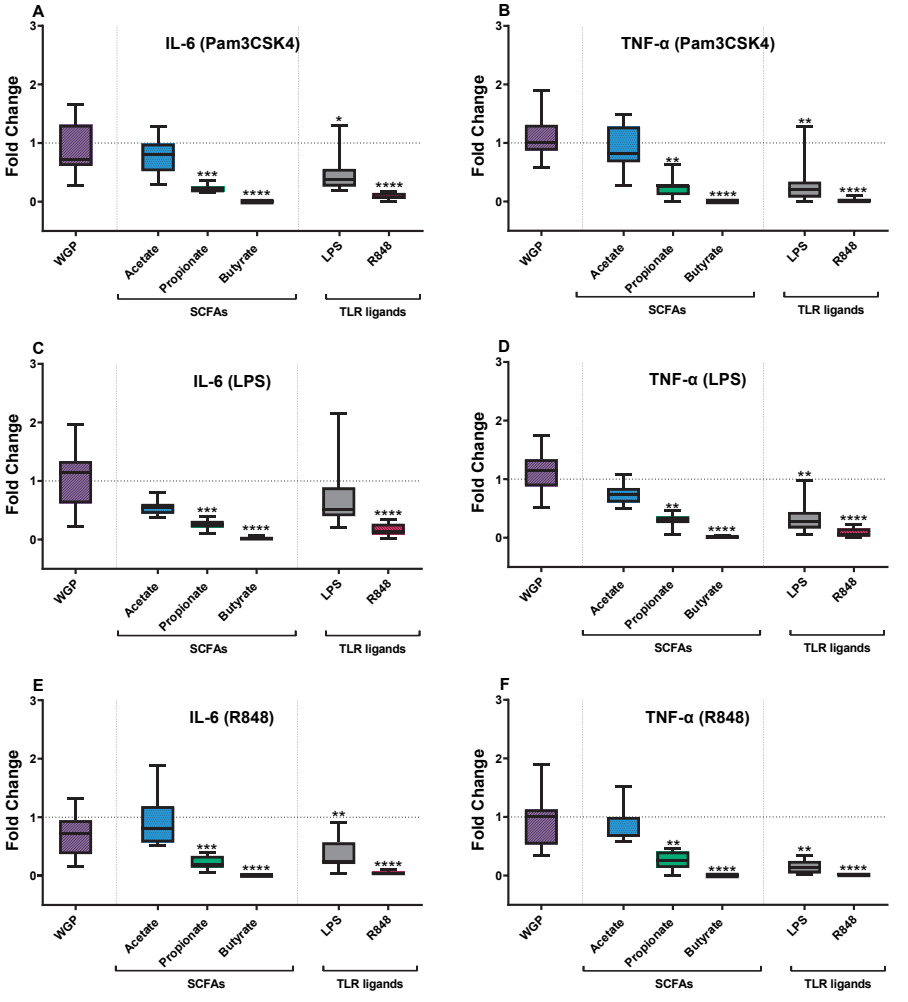

Fig 9. The effects of SCFASs training on monocytes responsiveness: Monocytes were treated for 24 hours with WGP, different SCFAs, and tolerogenic TLR ligands (LPS or R848) and then rested for 6 days before being stimulated with TLR ligands. The production of IL-6 in monocytes stimulated with Pam3CSK4 (A), LPS (C), or R848 (E) as well as TNF-a upon stimulation with Pam3CSK4 (B), LPS (D), or R848 (F) are expressed as fold changes compared to untrained cell (RPMI) which are expressed as 1. The average (Range) of cytokines in the RPMI controls were 2734 (632 - 5103 pg/mL), 328 (85 - 873 pg/mL), 5174 (2199 - 10957 pg/mL), 1371 (825 - 2377 pg/mL), 9001 (3037 - 30641 pg/mL), and 1941 (914 - 4929 pg/mL) for conditions A to F, respectively. The Boxes represent 50% of the data, and the line is the median value where upper and lower whiskers present upper and lower 25% of the data, respectively. The significance of differences is shown as p-value <0.05 (*), <0.01 (**), <0.001 (***), or <0.0001 (****).

Discussion

In this study, we studied the effects of individual SCFAs on the activation of innate (monocytes, mDC, pDC) and adaptive (T and B) immune cells, as well as the ability to induce immune training on monocytes. Here, we show that butyrate and propionate were the most potent tested SCFAs at inhibiting activation of monocytes, mDC, B- and T-cells. Noteworthy, activation of pDC was inhibited by all three SCFAs, including acetate, which could be explained by the differential expression profiles of SCFA receptors on these cells. In addition, although GPRs have some downstream signaling pathways in common with Dectin-1, the receptor through which b-glucans induce innate immune training, SCFAs did not induce innate immune training.

SCFAs are well-known for their ability to maintain local and systemic homeostasis and to prevent excessive inflammatory responses to commensal bacteria and dietary components [27, 112]. However, to our knowledge, no studies to date have addressed the effects of all three most common SCFAs on innate as well as adaptive immune cell types in a single study, as most studies focus on single SCFA or single cell type. This approach enabled us to not only investigate the overall impacts of SCFAs but also to compare the effects of SCFA on individual cell types linked to the expression of their receptors.

Acetate, propionate, and butyrate contain 2, 3, and 4 carbon atoms, respectively, produced by colonic bacteria via the glycolytic pathway or pentose phosphate pathway [22]. While colonocytes consume the majority of butyrate as their source of energy, propionate and acetate enter the portal vein and are taken to the liver. Hepatocytes in the liver metabolize propionate, and therefore, acetate is the SCFA with the highest concentrations in the peripheral blood [120]. We speculate that even though SCFA concentrations measured in the circulation are relatively low, their concentration might be closer to the luminal SCFA concentrations in the intestinal mucosa where they first encounter immune cells. Interestingly, in some studies, no obvious effects were observed when using low concentrations of acetate and propionate [23, 136, 137]. In this regard, acetate, propionate, and butyrate were shown to decrease LPS-stimulated TNFα release from neutrophils at a concentration as high as 30 mM [138]. Considering this, the SCFAs concentrations applied in this study enabled us to observe significant effects while maintaining relatively good cell viability. After 3 or 24 hours of incubation with SCFAs, the viability of T- and B-cells, as well as monocytes, was still > 80%. However, incubation of monocytes with 5mM of butyrate for 24 hours resulted in a reduction to 60% of viable cells (Suppl. Fig S4).

SCFAs can exert their effects by directly inhibiting the enzymatic effects of HDACs upon cell entry or indirectly by signaling via GPR receptors. Butyrate and propionate inhibited HDAC activity in effector and regulatory T cells independently of GPR41

and GPR43 [139]. Furthermore, butyrate and propionate have been shown to limit the development of dendritic cells (DCs) in mice. This effect has been observed due to HDACs inhibition following SMCT-1-mediated cell entry [124]. On the other hand, a growing body of research indicates that the direct involvement of GPR proteins is crucial for the SCFAs to exert their immunomodulatory effects. Colonic inflammation was suppressed by activating GPR109A [127], whereas the signal transduced via GPR41 and GPR43 promoted an inflammatory response in mouse intestinal epithelial cells [126]. It is unclear which mechanism is more prominent or whether both pathways are necessary for SCFAs to induce their effects.

Incubation with SCFAs inhibited the production of cytokines in activated T cells *ex vivo*. TNF-a, IL-13, IL-17, and IFN-γ levels were significantly lower in cells treated with butyrate and propionate compared to the absence of SCFAs (Fig 2). Butyrate was previously found to block HDAC activity in murine $CD4^+$ T cells, resulting in cell anergy [140], while propionate was demonstrated to suppress T helper 2 (Th2) response and decrease IL-4, IL-5, and IL-17 levels in allergic murine lungs [141]. Similarly, in another study, butyrate decreased IFN-γ and IL-2 levels, thereby inhibiting cell proliferation in rat mesenteric lymph node lymphocytes [142]. Regulating the inflammatory cytokines produced by various T cell subsets, carried on effectively by SCFAs, is critical for preventing excessive inflammatory or allergy responses at mucosal surfaces. The decreased T cell activity observed following SCFA administration is consistent with differential expression of relevant transporter molecules. Although GPRs are not highly expressed on the T lymphocytes, these cells express MCT-1 and particularly SMCT-1, which was found to be essential for intracellular accumulation of butyrate and propionate and direct HDAC activity inhibition [124]. Additionally, an indirect effect via decreased DC capacity for naive T cell stimulation may partially account for the observations [143, 144].

Previously, SCFAs were shown to prevent allergic inflammation in animal models [115, 141]. Therefore, we were interested to know whether individual SCFAs might influence lymphocyte homing to distant tissues. Our findings indicate that SCFAs cannot stimulate homing to the respiratory or intestinal tract but rather inhibit the activation-induced expression of homing receptors. Both butyrate and propionate prevented the upregulation of the CCR9 expression in activated T cells. Likewise, the same two SCFAs suppressed the expression of CCR10 in activated B lymphocytes, albeit the overall expression was relatively low. CCR9 interaction with CCL25 directs intestinal intraepithelial lymphocytes and DCs to the intestine [145, 146], and CCR10 is required for tissue-specific migration to various mucosal tissues, including the airways [147]. The absence of GPR43 may thus explain why acetate does not affect T cell activity, whereas at the same time, the high availability of GPR41 and SMCT-1 may justify the observations for CCR10 expression in B lymphocytes. Although specific effects of SCFA on intestinal and respiratory tract homing

have not been described so far, an inhibitory effect of SCFAs on immune cells trafficking has been demonstrated previously. Butyrate downregulated Intercellular Adhesion Molecule 1 (ICAM-1) on monocytes [148], butyrate and propionate significantly decreased the expression of chemokine ligands CCL5, CXCL9, CXCL10, and CXCL11 in DCs [149], and propionate showed the ability to inhibit vascular cell adhesion molecule 1 (VCAM-1) and ICAM-1 expression [150]. These findings suggest that SCFAs exert tight control over immune cell migration by retaining them in draining lymph nodes where they can exhibit their effects. Additional research could be conducted to determine these applications for the treatment of inflammatory diseases.

Butyrate, and to a lesser extent, propionate, demonstrated a suppressive effect on inflammatory cytokines production from innate immune cells, while acetate was shown to be more selective in its impact. Proinflammatory IL-1β, IL-6, TNF-a, and IFN-a levels were elevated in PBMCs stimulated with TLR agonists (Fig 4). However, butyrate, followed by propionate, significantly reduced the production of all these cytokines, whereas acetate did so primarily for IFN-a, the cytokine typically produced by pDC upon viral(like) stimulation. Further experiments on the intracellular synthesis of IL-6 and TNF-a in innate immune cells confirmed and extended the results described for cytokine production in the culture supernatant. Monocytes and mDCs produced IL-6 and TNF-a in response to both LPS and R848, but pDCs only responded to R848 stimulation. Human pDCs do not possess TLR4; therefore, they do not react to LPS activation but do respond to pathogen-derived nucleic acids due to the presence of TLR7 and TLR9 [151-153]. Butyrate and propionate were able to inhibit IL-6 and TNF-a production in monocytes and mDCs in a similar fashion. Moreover, acetate also had significant effects by decreasing TNF-a in monocytes and IL-6 in mDCs, but this influence was less pronounced than those of butyrate and propionate. Subsequently, we tried to link these findings to the expression of SCFA receptors on different immune cells. Monocytes were shown to express high levels of GPR109A and GPR41, but not GPR43. As a result, significant effects from butyrate and propionate are expected, while acetate has essentially little effect. On the other hand, acetate showed inhibitory effects on mDCs and reduced the percentage of IL-6 producing cells by half, in accordance with the higher expression of GPR43 as the primary receptor of acetate on mDCs. The effect of acetate on pDC, though, was comparable to the effects of both butyrate and propionate, with a three- to a four-fold reduction of cytokine-producing cells. GPR41 and GPR43 are equally present on pDCs mediating the inhibitory effects of propionate and acetate, respectively, whereas high availability of SMCT-1 on pDCs may explain the butyrate effect in a comparable manner to T cells.

In agreement with our findings, reduction of LPS-induced TNF-a by SCFAs was previously demonstrated [138, 154]. Similarly, butyrate and propionate-treated cells produced less IL-6 and IL-12, where acetate treatment did not have the same effects on monocyte-

derived DCs [149]. Butyrate also showed the ability to suppress NF-κB activity and hence decrease IL-6 and TNF-a levels in mouse macrophages [155]. The cytokine suppressing effects were found to be related to SCFAs' ability to modulate NF-κB activity [156]. Upon NF-κB activation, depending on the stimuli and immunological context, a wide range of proinflammatory cytokines such as IL-6 and TNF-a are produced. Nevertheless, both butyrate and propionate were shown to effectively decrease NF-κB activity in PBMCs in a manner comparable to that of trichostatin A (TSA) [136].

Here, one remarkable result was found for pDCs. As the primary producers of the antiviral cytokine IFN-a, these cells generated detectable quantities of the cytokine after stimulation of unseparated PBMCs with the TLR7/8 ligand (R848) despite their lower prevalence in blood compared to monocytes (less than 0.5% compared to about 10% of PBMCs). pDCs do not express GPR109A and have a low level of GPR41 expression; however, GPR43 with a higher affinity for acetate is abundantly present on their cell membrane. Notably, IFN-a is the only cytokine that was reduced by acetate as effective as butyrate and propionate, which could be explained by higher GPR43 expression on pDCs.

Previously, low-concentration butyrate and propionate exposure was shown to increase the production of IL-10 in PBMCs and T cells, respectively [157, 158]. However, in our study, we observed a reduction in the synthesis of this cytokine when SCFAs were applied. The contradictory results could be explained by differences in the SCFA concentrations applied. It was previously demonstrated that adding sodium butyrate to human regulatory T cells (Tregs), the primary producers of IL-10, inhibited their proliferation *in vitro* while enhancing their inhibitory effect on other T cells [159]. The inhibition of IL-10 production reported in the current study is most likely due to decreased proliferative activity of Tregs exposed to higher concentrations of butyrate and propionate.

Apart from the dissimilar potential of SCFAs, the diversity of immunomodulatory effects could have been reflected from differential expression of receptor and transporter molecules on immune cells. Here we showed that the expression of GPR43, with the highest affinity for acetate followed by propionate, is relatively low in monocytes and lymphocytes while it is present on both mDCs and pDCs. As a result, one should anticipate that acetate administration will have little or no effect on monocytes and lymphocyte-associated cytokines but will substantially impact mDCs and pDCs. This is entirely consistent with our findings in the current study. GPR41, on the other hand, is abundantly expressed on monocytes, B cells, and mDCs examined. GPR41 is the primary receptor for propionate followed by butyrate, the same two SCFAs with the most significant effect on the aforementioned cell types. Furthermore, GPR109A, which exclusively interacts with butyrate, is not highly expressed on the immune cells (except monocytes), but in return, SMCT-1 availability may make up for that, as described earlier. Taken together, the lower

effects of acetate on lymphocytes and monocytes may be attributable in part to the low level of GPR43 expression on these cells, while the higher impact on mDCs and especially pDCs may confirm this notion.

Considering SCFAs' documented ability to activate extracellular signal-regulated kinases (ERK)1/2 [160] as well as inhibiting HDAC activity (hence alter gene expression), we hypothesized that these metabolites might prime monocytes towards trained immunity or tolerance. Modifying epigenome via HDAC modulation and metabolic rewiring was shown to be the underlying mechanism for both trained immunity and tolerance [161, 162]. As a result, we investigated SCFAs in an established *in vitro* trained immunity/tolerance induction model. Our findings indicate that butyrate and propionate modulate monocytes' responsiveness to TLR ligands upon secondary stimulation. It should be mentioned that for butyrate at 5mM - but not for propionate or acetate - reduced viability of the monocytes was noted after 24h incubation, which may in part explain these findings. Butyrate and propionate, but not acetate, lowered monocytes' response to Pam3CSK4, LPS, and R848 stimulation a week after training. In line with our findings, oral supplementation with butyrate decreased the trained immunity induced by oxLDL and β-glucan *in vivo* [163]. The findings can be explained by the differential expression pattern of SCFA receptors on monocytes similar to what was described for direct SCFAs stimulation. Monocytes with high GPR109A and GPR41 expression responded similarly to butyrate and propionate treatment but not to acetate, most likely due to the low availability of GPR43. In general, HDAC inhibition is regarded as a permissive factor for gene expression, which may result in increased gene expression. However, depending on the chromatin status and promoter, HDAC inhibition may also suppress gene expression [125]. Our observation on decreased IL-6 and TNF-a production in monocytes is in accordance with this statement. As previously stated, trained immunity and tolerance are two sides of the same coin, sharing epigenetic and metabolic alterations as the underlying mechanism [162]. In this study, however, we demonstrate the inhibitory effects of SCFAs on IL-6 and TNF-a production after secondary stimulation in a similar manner to responses of established tolerogenic controls in this training model. To confirm the tolerogenic properties of SCFAs, further in-depth research on altered epigenetic markers, signaling cascades, and cell metabolism are necessary.

Here we provide extensive evidence for the immunomodulatory effects of individual SCFA on human PBMCs. Both butyrate and propionate inhibited the production of lymphocyte cytokines in activated T cells and prevented the upregulation of the tissue homing markers expression in activated T- and B cells, thus reducing their potential to migrate to distant tissues. Whereas butyrate and propionate strongly inhibited the activation of monocytes and mDC, acetate administration had a selective effect on the TLR-mediated activation of pDC. Finally, butyrate and propionate inhibited the IL-6 and TNF-a secretion in a

trained immunity/tolerance setting, whereas acetate did not affect these responses. These dissimilar SCFAs findings could be due to differential expression of relevant receptors and transporters on different immune cells. Acetate is found in higher quantities in circulation and can reach the most remote organs. Furthermore, pDCs revealed the most pronounced response to acetate. Thus, it would be intriguing to investigate the effects of acetate on antiviral response regulation in pDCs in organs such as the respiratory tract in order to discover new therapeutic or prophylactic applications for this important SCFA.

References

1. Medzhitov, R. and C. Janeway, *Innate Immunity.* New England Journal of Medicine, 2000. **343**(5): p. 338-344.
2. Turvey, S.E. and D.H. Broide, *Innate immunity.* The Journal of allergy and clinical immunology, 2010. **125**(2 Suppl 2): p. S24-S32.
3. Fu, Y.L. and R.E. Harrison, *Microbial Phagocytic Receptors and Their Potential Involvement in Cytokine Induction in Macrophages.* Frontiers in immunology, 2021. **12**: p. 662063-662063.
4. Amarante-Mendes, G.P., et al., *Pattern Recognition Receptors and the Host Cell Death Molecular Machinery.* Frontiers in Immunology, 2018. **9**.
5. Collin, M., N. McGovern, and M. Haniffa, *Human dendritic cell subsets.* Immunology, 2013. **140**(1): p. 22-30.
6. Strober, W., *Chapter 47 - Overview: Mucosal Immunity and Infections*, in *Mucosal Immunology (Fourth Edition)*, J. Mestecky, et al., Editors. 2015, Academic Press: Boston. p. 945-948.
7. Holmgren, J. and C. Czerkinsky, *Mucosal immunity and vaccines.* Nature Medicine, 2005. **11**(4): p. S45-S53.
8. Brandtzaeg, P., *Mucosal Immunity: Induction, Dissemination, and Effector Functions.* Scandinavian Journal of Immunology, 2009. **70**(6): p. 505-515.
9. Williams, I.R. and R.L. Owen, *Chapter 13 - M Cells: Specialized Antigen Sampling Cells in the Follicle-Associated Epithelium*, in *Mucosal Immunology (Fourth Edition)*, J. Mestecky, et al., Editors. 2015, Academic Press: Boston. p. 211-229.
10. Govers, C., et al., *Ingestion, Immunity, and Infection: Nutrition and Viral Respiratory Tract Infections.* Frontiers in Immunology, 2022. **13**.
11. Hellings, P., M. Jorissen, and J.L. Ceuppens, *The Waldeyer's ring.* Acta Otorhinolaryngol Belg, 2000. **54**(3): p. 237-41.
12. Brandtzaeg, P., *Immunology of tonsils and adenoids: everything the ENT surgeon needs to know.* International Journal of Pediatric Otorhinolaryngology, 2003. **67**: p. S69-S76.
13. Savelkoul, H.F., et al., *Choice and Design of Adjuvants for Parenteral and Mucosal Vaccines.* Vaccines (Basel), 2015. **3**(1): p. 148-71.
14. Schulz, O., et al., *Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions.* J Exp Med, 2009. **206**(13): p. 3101-14.
15. Tezuka, H. and T. Ohteki, *Regulation of IgA Production by Intestinal Dendritic Cells and Related Cells.* Frontiers in Immunology, 2019. **10**.
16. Brandtzaeg, P., *Role of secretory antibodies in the defence against infections.* International Journal of Medical Microbiology, 2003. **293**(1): p. 3-15.
17. Brandtzaeg, P., *Induction of secretory immunity and memory at mucosal surfaces.* Vaccine, 2007. **25**(30): p. 5467-5484.
18. Bode, L., *The functional biology of human milk oligosaccharides.* Early Human Development, 2015. **91**(11): p. 619-622.
19. Maslowski, K.M. and C.R. Mackay, *Diet, gut microbiota and immune responses.* Nature Immunology,

2011. **12**(1): p. 5-9.

20. Thursby, E. and N. Juge, *Introduction to the human gut microbiota.* The Biochemical journal, 2017. **474**(11): p. 1823-1836.
21. Krautkramer, K.A., et al., *Diet-Microbiota Interactions Mediate Global Epigenetic Programming in Multiple Host Tissues.* Molecular Cell, 2016. **64**(5): p. 982-992.
22. Tan, J., et al., *Chapter Three - The Role of Short-Chain Fatty Acids in Health and Disease*, in *Advances in Immunology*, F.W. Alt, Editor. 2014, Academic Press. p. 91-119.
23. Maslowski, K.M., et al., *Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43.* Nature, 2009. **461**(7268): p. 1282-1286.
24. Blaak, E.E., et al., *Short chain fatty acids in human gut and metabolic health.* Benef Microbes, 2020. **11**(5): p. 411-455.
25. Portela, A. and M. Esteller, *Epigenetic modifications and human disease.* Nature Biotechnology, 2010. **28**(10): p. 1057-1068.
26. Mörbe, U.M., et al., *Human gut-associated lymphoid tissues (GALT); diversity, structure, and function.* Mucosal Immunology, 2021. **14**(4): p. 793-802.
27. van der Hee, B. and J.M. Wells, *Microbial Regulation of Host Physiology by Short-chain Fatty Acids.* Trends in Microbiology, 2021.
28. Netea, Mihai G., J. Quintin, and Jos W.M. van der Meer, *Trained Immunity: A Memory for Innate Host Defense.* Cell Host & Microbe, 2011. **9**(5): p. 355-361.
29. Netea, M.G., et al., *Defining trained immunity and its role in health and disease.* Nature Reviews Immunology, 2020.
30. Velema, J.P., et al., *Childhood mortality among users and non-users of primary health care in a rural west African community.* Int J Epidemiol, 1991. **20**(2): p. 474-9.
31. Niobey, F., et al., *Risk factors for death caused by pneumonia in children younger than 1 year old in a metropolitan region of southeastern Brazil. A case-control study.* Revista de saude publica, 1992. **26**(4): p. 229-238.
32. Garly, M.-L., et al., *BCG scar and positive tuberculin reaction associated with reduced child mortality in West Africa: a non-specific beneficial effect of BCG?* Vaccine, 2003. **21**(21-22): p. 2782-2790.
33. Vaugelade, J., et al., *Non-specific effects of vaccination on child survival: prospective cohort study in Burkina Faso.* bmj, 2004. **329**(7478): p. 1309.
34. Kleinnijenhuis, J., et al., *Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes.* Proc Natl Acad Sci U S A, 2012. **109**(43): p. 17537-42.
35. Sun, J.C., *Re-educating natural killer cells.* The Journal of experimental medicine, 2010. **207**(10): p. 2049-2052.
36. Sun, J.C., J.N. Beilke, and L.L. Lanier, *Immune memory redefined: characterizing the longevity of natural killer cells.* Immunological reviews, 2010. **236**: p. 83-94.
37. Sun, J.C., J.N. Beilke, and L.L. Lanier, *Adaptive immune features of natural killer cells.* Nature, 2009. **457**(7229): p. 557-561.
38. Quintin, J., et al., *Candida albicans infection affords protection against reinfection via functional*

reprogramming of monocytes. Cell host & microbe, 2012. **12**(2): p. 223-232.

39. Saeed, S., et al., *Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity.* Science (New York, N.Y.), 2014. **345**(6204): p. 1251086-1251086.
40. Dou, Y., et al., *Influenza vaccine induces intracellular immune memory of human NK cells.* PLoS One, 2015. **10**(3): p. e0121258.
41. Sun, J.C., J.N. Beilke, and L.L. Lanier, *Adaptive immune features of natural killer cells.* Nature, 2009. **457**(7229): p. 557-61.
42. Hole, C.R., et al., *Induction of memory-like dendritic cell responses in vivo.* Nature communications, 2019. **10**(1): p. 2955-2955.
43. Katzmarski, N., et al., *Transmission of trained immunity and heterologous resistance to infections across generations.* Nature Immunology, 2021.
44. Rizzetto, L., et al., *Fungal Chitin Induces Trained Immunity in Human Monocytes during Cross-talk of the Host with Saccharomyces cerevisiae*.* Journal of Biological Chemistry, 2016. **291**(15): p. 7961-7972.
45. Bekkering, S., et al., *Oxidized Low-Density Lipoprotein Induces Long-Term Proinflammatory Cytokine Production and Foam Cell Formation via Epigenetic Reprogramming of Monocytes.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2014. **34**(8): p. 1731-1738.
46. van Splunter, M., et al., *Induction of Trained Innate Immunity in Human Monocytes by Bovine Milk and Milk-Derived Immunoglobulin G.* Nutrients, 2018. **10**(10): p. 1378.
47. Hellinga, A.H., et al., *In Vitro Induction of Trained Innate Immunity by bIgG and Whey Protein Extracts.* International Journal of Molecular Sciences, 2020. **21**(23): p. 9077.
48. Ifrim, D.C., et al., *Trained immunity or tolerance: opposing functional programs induced in human monocytes after engagement of various pattern recognition receptors.* Clinical and vaccine immunology : CVI, 2014. **21**(4): p. 534-545.
49. Cheng, S.-C., et al., *mTOR- and HIF-1α-mediated aerobic glycolysis as metabolic basis for trained immunity.* Science (New York, N.Y.), 2014. **345**(6204): p. 1250684-1250684.
50. Arts, R.J.W., et al., *Immunometabolic Pathways in BCG-Induced Trained Immunity.* Cell reports, 2016. **17**(10): p. 2562-2571.
51. Domínguez-Andrés, J., L.A.B. Joosten, and M.G. Netea, *Induction of innate immune memory: the role of cellular metabolism.* Current Opinion in Immunology, 2019. **56**: p. 10-16.
52. Foster, S.L., D.C. Hargreaves, and R. Medzhitov, *Gene-specific control of inflammation by TLR-induced chromatin modifications.* Nature, 2007. **447**(7147): p. 972-978.
53. Netea, M.G., et al., *Trained immunity: A program of innate immune memory in health and disease.* Science, 2016. **352**(6284).
54. Saroj, P., et al., *An overview on immunomodulation.* J Adv Sci Res, 2012. **3**(1): p. 7-12.
55. De Rosa, V., et al., *Nutritional control of immunity: Balancing the metabolic requirements with an appropriate immune function.* Seminars in Immunology, 2015. **27**(5): p. 300-309.
56. Prescott, S.L., *Early Nutrition as a Major Determinant of 'Immune Health': Implications for Allergy, Obesity and Other Noncommunicable Diseases.* Nestle Nutr Inst Workshop Ser, 2016. **85**: p. 1-17.
57. Childs, C.E., P.C. Calder, and E.A. Miles, *Diet and Immune Function.* Nutrients, 2019. **11**(8): p.

1933.
58. Venter, C., et al., *Nutrition and the Immune System: A Complicated Tango.* Nutrients, 2020. **12**(3): p. 818.
59. (EFSA), E.F.S.A., *Nutrition and Health Claims (Article 13 claims).* 2020.
60. Calder, P.C., *The role of marine omega-3 (n-3) fatty acids in inflammatory processes, atherosclerosis and plaque stability.* Mol Nutr Food Res, 2012. **56**(7): p. 1073-80.
61. Calder, P.C., *n–3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases.* The American Journal of Clinical Nutrition, 2006. **83**(6): p. 1505S-1519S.
62. Purssell, E., *Prevention and management of gastrointestinal infections in infants from a nutritional perspective.* J Fam Health Care, 2009. **19**(6): p. 200-3.
63. Bührer, C., H.S. Fischer, and S. Wellmann, *Nutritional interventions to reduce rates of infection, necrotizing enterocolitis and mortality in very preterm infants.* Pediatr Res, 2020. **87**(2): p. 371-377.
64. Calder, P.C. and S. Kew, *The immune system: a target for functional foods?* British Journal of Nutrition, 2002. **88**(S2): p. S165-S176.
65. Kollmann, T.R., et al., *Innate immune function by Toll-like receptors: distinct responses in newborns and the elderly.* Immunity, 2012. **37**(5): p. 771-783.
66. Förster-Waldl, E., et al., *Monocyte Toll-Like Receptor 4 Expression and LPS-Induced Cytokine Production Increase during Gestational Aging.* Pediatric Research, 2005. **58**(1): p. 121-124.
67. Schüller, S.S., et al., *Preterm neonates display altered plasmacytoid dendritic cell function and morphology.* Journal of Leukocyte Biology, 2013. **93**(5): p. 781-788.
68. Macpherson, A.J., M.G. de Agüero, and S.C. Ganal-Vonarburg, *How nutrition and the maternal microbiota shape the neonatal immune system.* Nature Reviews Immunology, 2017. **17**: p. 508.
69. Hurley, W.L. and P.K. Theil, *Perspectives on immunoglobulins in colostrum and milk.* Nutrients, 2011. **3**(4): p. 442-474.
70. Duijts, L., M.K. Ramadhani, and H.A. Moll, *Breastfeeding protects against infectious diseases during infancy in industrialized countries. A systematic review.* Maternal & child nutrition, 2009. **5**(3): p. 199-210.
71. Agarwal, S. and P.J. Busse, *Innate and adaptive immunosenescence.* Annals of Allergy, Asthma & Immunology, 2010. **104**(3): p. 183-190.
72. Shaw, A.C., et al., *Dysregulation of human Toll-like receptor function in aging.* Ageing Research Reviews, 2011. **10**(3): p. 346-353.
73. Pawelec, G., *Age and immunity: What is "immunosenescence"?* Experimental Gerontology, 2018. **105**: p. 4-9.
74. Schanzer, D., J. Vachon, and L. Pelletier, *Age-specific Differences in Influenza A Epidemic Curves: Do Children Drive the Spread of Influenza Epidemics?* American Journal of Epidemiology, 2011. **174**(1): p. 109-117.
75. Pera, A., et al., *Immunosenescence: Implications for response to infection and vaccination in older people.* Maturitas, 2015. **82**(1): p. 50-55.
76. Andersen-Civil, A.I.S., P. Arora, and A.R. Williams, *Regulation of Enteric Infection and Immunity by Dietary Proanthocyanidins.* Frontiers in Immunology, 2021. **12**.

77. Graham, D.Y., M.K. Estes, and L.O. Gentry, *Double-blind comparison of bismuth subsalicylate and placebo in the prevention and treatment of enterotoxigenic Escherichia coli-induced diarrhea in volunteers.* Gastroenterology, 1983. **85**(5): p. 1017-22.
78. Nabavi, S.F., et al., *Antibacterial Effects of Cinnamon: From Farm to Food, Cosmetic and Pharmaceutical Industries.* Nutrients, 2015. **7**(9): p. 7729-7748.
79. Ozogul, Y., et al., *Antimicrobial Impacts of Essential Oils on Food Borne-Pathogens.* Recent Patents on Food, Nutrition & Agriculture, 2015. **7**(1): p. 53-61.
80. Bovee-Oudenhoven, I.M.J., et al., *Diarrhea caused by enterotoxigenic Escherichia coli infection of humans is inhibited by dietary calcium.* Gastroenterology, 2003. **125**(2): p. 469-476.
81. Top, J., et al., *Low-calcium diet in mice leads to reduced gut colonization by Enterococcus faecium.* MicrobiologyOpen, 2019. **8**(12): p. e936-e936.
82. Wu, D., et al., *Nutritional Modulation of Immune Function: Analysis of Evidence, Mechanisms, and Clinical Relevance.* Frontiers in Immunology, 2019. **9**.
83. Cousins, R.J., *Nutritional regulation of gene expression.* The American Journal of Medicine, 1999. **106**(1, Supplement 1): p. 20-23.
84. Hanevik, K., et al., *The way forward for ETEC controlled human infection models (CHIMs).* Vaccine, 2019. **37**(34): p. 4794-4799.
85. Bekeredjian-Ding, I., et al., *Controlled Human Infection Studies: Proposals for guidance on how to design, develop and produce a challenge strain.* Biologicals, 2021. **74**: p. 16-23.
86. World Health organization, *Infant and Young Child Nutrition. Global Strategy on Infant and Young Child Feeding.* 2002.
87. Oddy, W.H., *The impact of breastmilk on infant and child health.* Breastfeed Rev, 2002. **10**(3): p. 5-18.
88. Haschke, F., N. Haiden, and S.K. Thakkar, *Nutritive and Bioactive Proteins in Breastmilk.* Ann Nutr Metab, 2016. **69 Suppl 2**: p. 17-26.
89. Bäckhed, F., et al., *Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life.* Cell Host Microbe, 2015. **17**(5): p. 690-703.
90. Demmelmair, H., et al., *Benefits of Lactoferrin, Osteopontin and Milk Fat Globule Membranes for Infants.* Nutrients, 2017. **9**(8).
91. Oddy, W.H. and F. Rosales, *A systematic review of the importance of milk TGF-β on immunological outcomes in the infant and young child.* Pediatric Allergy and Immunology, 2010. **21**(1-Part-I): p. 47-59.
92. van Neerven, R.J.J., et al., *Which factors in raw cow's milk contribute to protection against allergies?* Journal of Allergy and Clinical Immunology, 2012. **130**(4): p. 853-858.
93. Wu, S., et al., *Development of an annotated library of neutral human milk oligosaccharides.* J Proteome Res, 2010. **9**(8): p. 4138-51.
94. Wu, S., et al., *Annotation and structural analysis of sialylated human milk oligosaccharides.* Journal of proteome research, 2011. **10**(2): p. 856-868.
95. Tao, N., et al., *Bovine milk glycome.* J Dairy Sci, 2008. **91**(10): p. 3768-78.
96. Ebina, T., et al., *PREVENTION OF ROTAVIRUS INFECTION BY COW COLOSTRUM CONTAINING ANTIBODY AGAINST HUMAN ROTAVIRUS.* The Lancet, 1983. **322**(8357): p.

1029-1030.

97. Loss, G., et al., *Consumption of unprocessed cow's milk protects infants from common respiratory infections.* Journal of Allergy and Clinical Immunology, 2015. **135**(1): p. 56-62.e2.
98. C., B.F. and V.M. E., *Can farm milk consumption prevent allergic diseases?* Clinical & Experimental Allergy, 2011. **41**(1): p. 29-35.
99. Loss, G., et al., *The protective effect of farm milk consumption on childhood asthma and atopy: The GABRIELA study.* Journal of Allergy and Clinical Immunology, 2011. **128**(4): p. 766-773.e4.
100. van Neerven, J., *The effects of milk and colostrum on allergy and infection: Mechanisms and implications*. Vol. 4. 2014. 16-22.
101. Brick, T., et al., *The Beneficial Effect of Farm Milk Consumption on Asthma, Allergies, and Infections: From Meta-Analysis of Evidence to Clinical Trial.* The Journal of Allergy and Clinical Immunology: In Practice, 2020. **8**(3): p. 878-889.e3.
102. den Hartog, G., et al., *Specificity and Effector Functions of Human RSV-Specific IgG from Bovine Milk.* PLOS ONE, 2014. **9**(11): p. e112047.
103. Rump, J.A., et al., *Treatment of diarrhoea in human immunodeficiency virus-infected patients with immunoglobulins from bovine colostrum.* The clinical investigator, 1992. **70**(7): p. 588-594.
104. Lissner, R., H. Schmidit, and H. Karch, *A standard immunoglobulin preparation produced from bovine colostra shows antibody reactivity and neutralization activity against Shiga-like toxins and EHEC-hemolysin of Escherichia coli O157:H7.* Infection, 1996. **24**(5): p. 378-83.
105. Collins, A.M., et al., *Bovine Milk, including Pasteurised Milk, Contains Antibodies Directed against Allergens of Clinical Importance to Man.* International Archives of Allergy and Immunology, 1991. **96**(4): p. 362-367.
106. Ulfman, L.H., et al., *Effects of Bovine Immunoglobulins on Immune Function, Allergy, and Infection.* Frontiers in Nutrition, 2018. **5**(52).
107. Brooks, H.J., et al., *Potential prophylactic value of bovine colostrum in necrotizing enterocolitis in neonates: an in vitro study on bacterial attachment, antibody levels and cytokine production.* FEMS Immunology & Medical Microbiology, 2006. **48**(3): p. 347-354.
108. Casswall, T., et al., *Bovine anti-Helicobacter pylori antibodies for oral immunotherapy.* Scandinavian journal of gastroenterology, 2002. **37**(12): p. 1380-1385.
109. Naaber, P., et al., *Inhibition of adhesion of Clostridium difficile to Caco-2 cells.* FEMS Immunology & Medical Microbiology, 1996. **14**(4): p. 205-209.
110. An, M.J., et al., *Bovine colostrum inhibits nuclear factor κB–mediated proinflammatory cytokine expression in intestinal epithelial cells.* Nutrition Research, 2009. **29**(4): p. 275-280.
111. Nederend, M., et al., *Bovine IgG Prevents Experimental Infection With RSV and Facilitates Human T Cell Responses to RSV.* Frontiers in immunology, 2020. **11**: p. 1701-1701.
112. Corrêa-Oliveira, R., et al., *Regulation of immune cell function by short-chain fatty acids.* Clinical & translational immunology, 2016. **5**(4): p. e73-e73.
113. Frati, F., et al., *The Role of the Microbiome in Asthma: The Gut-Lung Axis.* International journal of molecular sciences, 2018. **20**(1): p. 123.
114. Roduit, C., et al., *High levels of butyrate and propionate in early life are associated with protection against*

atopy. Allergy, 2019. **74**(4): p. 799-809.

115. Yip, W., et al., *Butyrate Shapes Immune Cell Fate and Function in Allergic Asthma.* Frontiers in immunology, 2021. **12**: p. 628453-628453.
116. Thorburn, Alison N., L. Macia, and Charles R. Mackay, *Diet, Metabolites, and "Western-Lifestyle" Inflammatory Diseases.* Immunity, 2014. **40**(6): p. 833-842.
117. Manzel, A., et al., *Role of "Western diet" in inflammatory autoimmune diseases.* Current allergy and asthma reports, 2014. **14**(1): p. 404-404.
118. Park, Y., et al., *Dietary Fiber Intake and Mortality in the NIH-AARP Diet and Health Study.* Archives of Internal Medicine, 2011. **171**(12): p. 1061-1068.
119. Cummings, J.H., et al., *The effect of meat protein and dietary fiber on colonic function and metabolism II. Bacterial metabolites in feces and urine.* The American Journal of Clinical Nutrition, 1979. **32**(10): p. 2094-2101.
120. Cummings, J.H., et al., *Short chain fatty acids in human large intestine, portal, hepatic and venous blood.* Gut, 1987. **28**(10): p. 1221.
121. Roediger, W.E., *Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man.* Gut, 1980. **21**(9): p. 793-798.
122. Clausen, M.R. and P.B. Mortensen, *Kinetic studies on colonocyte metabolism of short chain fatty acids and glucose in ulcerative colitis.* Gut, 1995. **37**(5): p. 684.
123. Ratajczak, W., et al., *Immunomodulatory potential of gut microbiome-derived short-chain fatty acids (SCFAs).* Acta Biochim Pol, 2019. **66**(1): p. 1-12.
124. Singh, N., et al., *Blockade of dendritic cell development by bacterial fermentation products butyrate and propionate through a transporter (Slc5a8)-dependent inhibition of histone deacetylases.* Journal of Biological Chemistry, 2010. **285**(36): p. 27601-27608.
125. Reichert, N., M.-A. Choukrallah, and P. Matthias, *Multiple roles of class I HDACs in proliferation, differentiation, and development.* Cellular and Molecular Life Sciences, 2012. **69**(13): p. 2173-2187.
126. Kim, M.H., et al., *Short-Chain Fatty Acids Activate GPR41 and GPR43 on Intestinal Epithelial Cells to Promote Inflammatory Responses in Mice.* Gastroenterology, 2013. **145**(2): p. 396-406.e10.
127. Singh, N., et al., *Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis.* Immunity, 2014. **40**(1): p. 128-39.
128. Brown, A.J., et al., *The Orphan G Protein-coupled Receptors GPR41 and GPR43 Are Activated by Propionate and Other Short Chain Carboxylic Acids.* Journal of Biological Chemistry, 2003. **278**(13): p. 11312-11319.
129. Le Poul, E., et al., *Functional Characterization of Human Receptors for Short Chain Fatty Acids and Their Role in Polymorphonuclear Cell Activation.* Journal of Biological Chemistry, 2003. **278**(28): p. 25481-25489.
130. Thangaraju, M., et al., *GPR109A is a G-protein-coupled receptor for the bacterial fermentation product butyrate and functions as a tumor suppressor in colon.* Cancer research, 2009. **69**(7): p. 2826-2832.
131. Wu, J., et al., *Butyrate-induced GPR41 Activation Inhibits Histone Acetylation and Cell Growth.* Journal of Genetics and Genomics, 2012. **39**(8): p. 375-384.
132. Gao, H., et al., *Identification of β-Arrestin2 as a G Protein-Coupled Receptor-Stimulated*

Regulator of NF-κB Pathways. Molecular Cell, 2004. **14**(3): p. 303-317.

133. Chai, J.T., J.E. Digby, and R.P. Choudhury, *GPR109A and Vascular Inflammation.* Current Atherosclerosis Reports, 2013. **15**(5): p. 325.
134. Domínguez-Andrés, J., et al., *In vitro induction of trained immunity in adherent human monocytes.* STAR protocols, 2021. **2**(1): p. 100365-100365.
135. Takeuchi, H., et al., *Efficient Induction of CCR9 on T Cells Requires Coactivation of Retinoic Acid Receptors and Retinoid X Receptors (RXRs): Exaggerated T Cell Homing to the Intestine by RXR Activation with Organotins.* The Journal of Immunology, 2010. **185**(9): p. 5289.
136. Usami, M., et al., *Butyrate and trichostatin A attenuate nuclear factor κB activation and tumor necrosis factor α secretion and increase prostaglandin E2 secretion in human peripheral blood mononuclear cells.* Nutrition Research, 2008. **28**(5): p. 321-328.
137. Iraporda, C., et al., *Lactate and short chain fatty acids produced by microbial fermentation downregulate proinflammatory responses in intestinal epithelial cells and myeloid cells.* Immunobiology, 2015. **220**(10): p. 1161-1169.
138. Tedelind, S., et al., *Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease.* World journal of gastroenterology, 2007. **13**(20): p. 2826-2832.
139. Park, J., et al., *Short-chain fatty acids induce both effector and regulatory T cells by suppression of histone deacetylases and regulation of the mTOR-S6K pathway.* Mucosal Immunol, 2015. **8**(1): p. 80-93.
140. Fontenelle, B. and K.M. Gilbert, *n-Butyrate Anergized Effector CD4+ T Cells Independent of Regulatory T cell Generation or Activity.* Scandinavian Journal of Immunology, 2012. **76**(5): p. 457-463.
141. Trompette, A., et al., *Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis.* Nature Medicine, 2014. **20**(2): p. 159-166.
142. Cavaglieri, C.R., et al., *Differential effects of short-chain fatty acids on proliferation and production of pro- and anti-inflammatory cytokines by cultured lymphocytes.* Life Sciences, 2003. **73**(13): p. 1683-1690.
143. L. MILLARD, A., et al., *Butyrate affects differentiation, maturation and function of human monocyte-derived dendritic cells and macrophages.* Clinical & Experimental Immunology, 2002. **130**(2): p. 245-255.
144. Liu, L., et al., *Butyrate interferes with the differentiation and function of human monocyte-derived dendritic cells.* Cellular Immunology, 2012. **277**(1): p. 66-73.
145. Sumida, H., *Recent advances in roles of G-protein coupled receptors in intestinal intraepithelial lymphocytes.* Bioscience of microbiota, food and health, 2020. **39**(3): p. 77-82.
146. Pathak, M. and G. Lal, *The Regulatory Function of CCR9(+) Dendritic Cells in Inflammation and Autoimmunity.* Frontiers in immunology, 2020. **11**: p. 536326-536326.
147. Xiong, N., et al., *CCR10 and its ligands in regulation of epithelial immunity and diseases.* Protein & cell, 2012. **3**(8): p. 571-580.
148. Bohmig, G.A., et al., *n-butyrate downregulates the stimulatory function of peripheral blood-derived antigen-presenting cells: a potential mechanism for modulating T-cell responses by short-chain fatty acids.* Immunology, 1997. **92**(2): p. 234-43.

149. Nastasi, C., et al., *The effect of short-chain fatty acids on human monocyte-derived dendritic cells.* Scientific Reports, 2015. **5**(1): p. 16148.
150. Zapolska-Downar, D. and M. Naruszewicz, *Propionate reduces the cytokine-induced VCAM-1 and ICAM-1 expression by inhibiting nuclear factor-κ B (NF-κB) activation.* Journal of Physiology and Pharmacology, 2009. **60**(2): p. 123-131.
151. Hornung, V., et al., *Quantitative Expression of Toll-Like Receptor 1–10 mRNA in Cellular Subsets of Human Peripheral Blood Mononuclear Cells and Sensitivity to CpG Oligodeoxynucleotides.* The Journal of Immunology, 2002. **168**(9): p. 4531-4537.
152. Reizis, B., *Plasmacytoid Dendritic Cells: Development, Regulation, and Function.* Immunity, 2019. **50**(1): p. 37-50.
153. Kaisho, T., *Pathogen sensors and chemokine receptors in dendritic cell subsets.* Vaccine, 2012. **30**(52): p. 7652-7657.
154. Vinolo, M.A.R., et al., *Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils.* The Journal of Nutritional Biochemistry, 2011. **22**(9): p. 849-855.
155. Ohira, H., et al., *Butyrate Attenuates Inflammation and Lipolysis Generated by the Interaction of Adipocytes and Macrophages.* Journal of Atherosclerosis and Thrombosis, 2013. **20**(5): p. 425-442.
156. Segain, J.-P., et al., *Butyrate inhibits inflammatory responses through NFκB inhibition: implications for Crohn's disease.* Gut, 2000. **47**(3): p. 397-403.
157. Säemann, M.D., et al., *Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production.* The FASEB Journal, 2000. **14**(15): p. 2380-2382.
158. Smith, P.M., et al., *The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis.* Science, 2013. **341**(6145): p. 569-73.
159. Akimova, T., et al., *Histone/protein deacetylase inhibitors increase suppressive functions of human FOXP3+ Tregs.* Clinical Immunology, 2010. **136**(3): p. 348-363.
160. Seljeset, S. and S. Siehler, *Receptor-specific regulation of ERK1/2 activation by members of the "free fatty acid receptor" family.* Journal of Receptors and Signal Transduction, 2012. **32**(4): p. 196-201.
161. Quintin, J., et al., *Innate immune memory: towards a better understanding of host defense mechanisms.* Current Opinion in Immunology, 2014. **29**: p. 1-7.
162. Divangahi, M., et al., *Trained immunity, tolerance, priming and differentiation: distinct immunological processes.* Nature Immunology, 2021. **22**(1): p. 2-6.
163. Cleophas, M.C.P., et al., *Effects of oral butyrate supplementation on inflammatory potential of circulating peripheral blood mononuclear cells in healthy and obese males.* Scientific Reports, 2019. **9**(1): p. 775.
164. Victora, C.G., et al., *Breastfeeding in the 21st century: epidemiology, mechanisms, and lifelong effect.* The Lancet, 2016. **387**(10017): p. 475-490.
165. Chen, K., et al., *Effect of bovine lactoferrin from iron-fortified formulas on diarrhea and respiratory tract infections of weaned infants in a randomized controlled trial.* Nutrition, 2016. **32**(2): p. 222-227.
166. Abbring, S., et al., *Raw cow's milk consumption and allergic diseases – The potential role of bioactive whey proteins.* European Journal of Pharmacology, 2019. **843**: p. 55-65.
167. Esch, B.C.A.M.v., et al., *The Impact of Milk and Its Components on Epigenetic Programming of Immune*

Function in Early Life and Beyond: Implications for Allergy and Asthma. Frontiers in Immunology, 2020. **11**(2141).

168. Perdijk, O., et al., *Cow's Milk and Immune Function in the Respiratory Tract: Potential Mechanisms.* Frontiers in Immunology, 2018. **9**(143).
169. van Kempen, M.J.P., G.T. Rijkers, and P.B. van Cauwenberge, *The Immune Response in Adenoids and Tonsils.* International Archives of Allergy and Immunology, 2000. **122**(1): p. 8-19.
170. Lu, L.L., et al., *Beyond binding: antibody effector functions in infectious diseases.* Nature Reviews Immunology, 2017. **18**: p. 46.
171. Bournazos, S., A. Gupta, and J.V. Ravetch, *The role of IgG Fc receptors in antibody-dependent enhancement.* Nature Reviews Immunology, 2020. **20**(10): p. 633-643.
172. Hayes, J.M., et al., *Fc gamma receptors: glycobiology and therapeutic prospects.* Journal of inflammation research, 2016. **9**: p. 209-219.
173. Nimmerjahn, F. and J.V. Ravetch, *Fcγ receptors as regulators of immune responses.* Nature Reviews Immunology, 2008. **8**: p. 34.
174. Swisher, J.F.A. and G.M. Feldman, *The many faces of FcγRI: implications for therapeutic antibody function.* Immunological Reviews, 2015. **268**(1): p. 160-174.
175. Bruhns, P., *Properties of mouse and human IgG receptors and their contribution to disease models.* Blood, 2012. **119**(24): p. 5640-5649.
176. Bruhns, P., et al., *Specificity and affinity of human Fcγ receptors and their polymorphic variants for human IgG subclasses.* Blood, 2009. **113**(16): p. 3716-3725.
177. Chen, X., et al., *FcγR-Binding Is an Important Functional Attribute for Immune Checkpoint Antibodies in Cancer Immunotherapy.* Frontiers in immunology, 2019. **10**: p. 292-292.
178. Holgado, M.P., et al., *CD32 Ligation Promotes the Activation of CD4+ T Cells.* Frontiers in Immunology, 2018. **9**(2814).
179. Anania, J.C., et al., *The Human FcγRII (CD32) Family of Leukocyte FcR in Health and Disease.* Frontiers in Immunology, 2019. **10**(464).
180. Simister, N.E., *Placental transport of immunoglobulin G.* Vaccine, 2003. **21**(24): p. 3365-3369.
181. Ober, R.J., et al., *Visualizing the Site and Dynamics of IgG Salvage by the MHC Class I-Related Receptor, FcRn.* The Journal of Immunology, 2004. **172**(4): p. 2021-2029.
182. Qiao, S.-W., et al., *Dependence of antibody-mediated presentation of antigen on FcRn.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(27): p. 9337-9342.
183. Weflen, A.W., et al., *Multivalent immune complexes divert FcRn to lysosomes by exclusion from recycling sorting tubules.* Molecular biology of the cell, 2013. **24**(15): p. 2398-2405.
184. Mohapatra, S.S. and S. Boyapalle, *Epidemiologic, Experimental, and Clinical Links between Respiratory Syncytial Virus Infection and Asthma.* Clinical Microbiology Reviews, 2008. **21**(3): p. 495-504.
185. Régnier, S.A. and J. Huels, *Association Between Respiratory Syncytial Virus Hospitalizations in Infants and Respiratory Sequelae: Systematic Review and Meta-analysis.* The Pediatric Infectious Disease Journal, 2013. **32**(8): p. 820-826.
186. Mazur, N.I., et al., *Breast Milk Prefusion F Immunoglobulin G as a Correlate of Protection Against Respiratory Syncytial Virus Acute Respiratory Illness.* The Journal of Infectious Diseases, 2018. **219**(1):

p. 59-67.

187. Xu, M.L., et al., *The effect of dietary bovine colostrum on respiratory syncytial virus infection and immune responses following the infection in the mouse.* Journal of Microbiology, 2015. **53**(9): p. 661-666.
188. Miyake, Y., et al., *C-type Lectin MCL Is an FcRγ-Coupled Receptor that Mediates the Adjuvanticity of Mycobacterial Cord Factor.* Immunity, 2013. **38**(5): p. 1050-1062.
189. McLellan, J.S., et al., *Structure-Based Design of a Fusion Glycoprotein Vaccine for Respiratory Syncytial Virus.* Science, 2013. **342**(6158): p. 592-598.
190. Widjaja, I., et al., *Characterization of Epitope-Specific Anti-Respiratory Syncytial Virus (Anti-RSV) Antibody Responses after Natural Infection and after Vaccination with Formalin-Inactivated RSV.* Journal of Virology, 2016. **90**(13): p. 5965-5977.
191. Moerings, B.G.J., et al., *Continuous Exposure to Non-Soluble β-Glucans Induces Trained Immunity in M-CSF-Differentiated Macrophages.* Frontiers in immunology, 2021. **12**: p. 672796-672796.
192. Hjelm, F., et al., *Antibody-Mediated Regulation of the Immune Response.* Scandinavian Journal of Immunology, 2006. **64**(3): p. 177-184.
193. van Erp, E.A., et al., *Fc-Mediated Antibody Effector Functions During Respiratory Syncytial Virus Infection and Disease.* Frontiers in Immunology, 2019. **10**(548).
194. Tay, M.Z., K. Wiehe, and J. Pollara, *Antibody-Dependent Cellular Phagocytosis in Antiviral Immune Responses.* Frontiers in Immunology, 2019. **10**(332).
195. Underhill, D.M. and H.S. Goodridge, *Information processing during phagocytosis.* Nature reviews. Immunology, 2012. **12**(7): p. 492-502.
196. Boross, P., et al., *FcRγ-chain ITAM signaling is critically required for cross-presentation of soluble antibody-antigen complexes by dendritic cells.* Journal of immunology (Baltimore, Md. : 1950), 2014. **193**(11): p. 5506-5514.
197. Pennock, N.D., et al., *T cell responses: naive to memory and everything in between.* Advances in physiology education, 2013. **37**(4): p. 273-283.
198. Jaumouillé, V. and S. Grinstein, *Receptor mobility, the cytoskeleton, and particle binding during phagocytosis.* Current Opinion in Cell Biology, 2011. **23**(1): p. 22-29.
199. Lux, A., et al., *Impact of Immune Complex Size and Glycosylation on IgG Binding to Human FcγRs.* The Journal of Immunology, 2013. **190**(8): p. 4315.
200. Zhu, X., et al., *MHC class I-related neonatal Fc receptor for IgG is functionally expressed in monocytes, intestinal macrophages, and dendritic cells.* Journal of immunology (Baltimore, Md. : 1950), 2001. **166**(5): p. 3266-3276.
201. Kramski, M., et al., *Anti-HIV-1 antibody-dependent cellular cytotoxicity mediated by hyperimmune bovine colostrum IgG.* European journal of immunology, 2012. **42**(10): p. 2771-2781.
202. van Egmond, M., G. Vidarsson, and J.E. Bakema, *Cross-talk between pathogen recognizing Toll-like receptors and immunoglobulin Fc receptors in immunity.* Immunological Reviews, 2015. **268**(1): p. 311-327.
203. Rittirsch, D., et al., *Cross-Talk between TLR4 and FcγReceptorIII (CD16) Pathways.* PLOS Pathogens, 2009. **5**(6): p. e1000464.
204. Means, T.K., et al., *Human lupus autoantibody-DNA complexes activate DCs through cooperation of*

CD32 and TLR9. The Journal of clinical investigation, 2005. **115**(2): p. 407-417.

205. Bunk, S., et al., *Internalization and Coreceptor Expression Are Critical for TLR2-Mediated Recognition of Lipoteichoic Acid in Human Peripheral Blood.* The Journal of Immunology, 2010. **185**(6): p. 3708-3717.
206. Parcina, M., et al., *Staphylococcus aureus-Induced Plasmacytoid Dendritic Cell Activation Is Based on an IgG-Mediated Memory Response.* The Journal of Immunology, 2008. **181**(6): p. 3823-3833.
207. Boulé, M.W., et al., *Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes.* The Journal of experimental medicine, 2004. **199**(12): p. 1631-1640.
208. Lovgren, T., *Induction of interferon-alpha by immune complexes or liposomes containing systemic lupus erythematosus autoantigen-and Sjogren's syndrome autoantigen-associated RNA.* Arthritis Rheum, 2006. **54**: p. 1917-1927.
209. Miller, M.A., et al., *Frozen-State Storage Stability of a Monoclonal Antibody: Aggregation is Impacted by Freezing Rate and Solute Distribution.* Journal of Pharmaceutical Sciences, 2013. **102**(4): p. 1194-1208.
210. Netea, M.G. and J.W.M. van der Meer, *Trained Immunity: An Ancient Way of Remembering.* Cell Host & Microbe, 2017. **21**(3): p. 297-300.
211. Griffiths, C.D., et al., *IGF1R is an entry receptor for respiratory syncytial virus.* Nature, 2020. **583**(7817): p. 615-619.
212. Bekkering, S., et al., *Metabolic Induction of Trained Immunity through the Mevalonate Pathway.* Cell, 2018. **172**(1): p. 135-146.e9.
213. Lux, A. and F. Nimmerjahn. *Impact of Differential Glycosylation on IgG Activity*. 2011. New York, NY: Springer New York.
214. Pakkanen, R. and J. Aalto, *Growth factors and antimicrobial factors of bovine colostrum.* International Dairy Journal, 1997. **7**(5): p. 285-297.
215. Stelwagen, K., et al., *Immune components of bovine colostrum and milk1.* Journal of Animal Science, 2009. **87**(suppl_13): p. 3-9.
216. Ten Bruggencate, S.J., et al., *Dietary Milk-Fat-Globule Membrane Affects Resistance to Diarrheagenic Escherichia coli in Healthy Adults in a Randomized, Placebo-Controlled, Double-Blind Study.* The Journal of Nutrition, 2015. **146**(2): p. 249-255.
217. Tacket, C.O., et al., *Protection by Milk Immunoglobulin Concentrate against Oral Challenge with Enterotoxigenic Escherichia coli.* New England Journal of Medicine, 1988. **318**(19): p. 1240-1243.
218. Savarino, S.J., et al., *Prophylactic Efficacy of Hyperimmune Bovine Colostral Antiadhesin Antibodies Against Enterotoxigenic Escherichia coli Diarrhea: A Randomized, Double-Blind, Placebo-Controlled, Phase 1 Trial.* The Journal of Infectious Diseases, 2017. **216**(1): p. 7-13.
219. Davidson, G.P., et al., *PASSIVE IMMUNISATION OF CHILDREN WITH BOVINE COLOSTRUM CONTAINING ANTIBODIES TO HUMAN ROTAVIRUS.* The Lancet, 1989. **334**(8665): p. 709-712.
220. Shaw, A.L., et al., *Absorption and safety of serum-derived bovine immunoglobulin/protein isolate in*

healthy adults. Clinical and experimental gastroenterology, 2016. **9**: p. 365-375.

221. Arts, R.J.W., et al., *Long-term in vitro and in vivo effects of γ-irradiated BCG on innate and adaptive immunity.* Journal of Leukocyte Biology, 2015. **98**(6): p. 995-1001.
222. Heijden, C.D.C.C.v.d., et al., *Catecholamines Induce Trained Immunity in Monocytes In Vitro and In Vivo.* Circulation Research, 2020. **127**(2): p. 269-283.
223. Leentjens, J., et al., *The Effects of Orally Administered Beta-Glucan on Innate Immune Responses in Humans, a Randomized Open-Label Intervention Pilot-Study.* PLOS ONE, 2014. **9**(9): p. e108794.
224. van Splunter, M., et al., *Bovine Lactoferrin Enhances TLR7-Mediated Responses in Plasmacytoid Dendritic Cells in Elderly Women: Results From a Nutritional Intervention Study With Bovine Lactoferrin, GOS and Vitamin D.* Frontiers in Immunology, 2018. **9**(2677).
225. Troeger, C.E., et al., *Quantifying risks and interventions that have affected the burden of diarrhoea among children younger than 5 years: an analysis of the Global Burden of Disease Study 2017.* The Lancet Infectious Diseases, 2020. **20**(1): p. 37-59.
226. Qadri, F., et al., *Enterotoxigenic Escherichia coli in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention.* Clinical microbiology reviews, 2005. **18**(3): p. 465-483.
227. Kaper, J.B., J.P. Nataro, and H.L.T. Mobley, *Pathogenic Escherichia coli.* Nature Reviews Microbiology, 2004. **2**(2): p. 123-140.
228. Kollaritsch, H., M. Paulke-Korinek, and U. Wiedermann, *Traveler's Diarrhea.* Infectious Disease Clinics of North America, 2012. **26**(3): p. 691-706.
229. Foster, J.W., *Escherichia coli acid resistance: tales of an amateur acidophile.* Nature Reviews Microbiology, 2004. **2**(11): p. 898-907.
230. Lund, P., A. Tramonti, and D. De Biase, *Coping with low pH: molecular strategies in neutralophilic bacteria.* FEMS Microbiology Reviews, 2014. **38**(6): p. 1091-1125.
231. Nataro, J.P. and J.B. Kaper, *Diarrheagenic Escherichia coli.* Clinical microbiology reviews, 1998. **11**(1): p. 142-201.
232. Mirhoseini, A., J. Amani, and S. Nazarian, *Review on pathogenicity mechanism of enterotoxigenic Escherichia coli and vaccines against it.* Microbial Pathogenesis, 2018. **117**: p. 162-169.
233. Agustina, R., et al., *Randomized Trial of Probiotics and Calcium on Diarrhea and Respiratory Tract Infections in Indonesian Children.* Pediatrics, 2012. **129**(5): p. e1155.
234. Baqui, A.H., et al., *Simultaneous Weekly Supplementation of Iron and Zinc Is Associated with Lower Morbidity Due to Diarrhea and Acute Lower Respiratory Infection in Bangladeshi Infants.* The Journal of Nutrition, 2003. **133**(12): p. 4150-4157.
235. Black, R.E., et al., *Treatment of experimentally induced enterotoxigenic Escherichia coli diarrhea with trimethoprim, trimethoprim-sulfamethoxazole, or placebo.* Rev Infect Dis, 1982. **4**(2): p. 540-5.
236. van Hoffen, E., et al., *Characterization of the pathophysiological determinants of diarrheagenic Escherichia coli infection using a challenge model in healthy adults.* Sci Rep, 2021. **11**(1): p. 6060.
237. Levine, M.M., E.M. Barry, and W.H. Chen, *A roadmap for enterotoxigenic Escherichia coli vaccine development based on volunteer challenge studies.* Human Vaccines & Immunotherapeutics, 2019. **15**(6): p. 1357-1378.
238. Svedlund, J., I. Sjödin, and G. Dotevall, *GSRS—A clinical rating scale for gastrointestinal symptoms in*

patients with irritable bowel syndrome and peptic ulcer disease. Digestive Diseases and Sciences, 1988. **33**(2): p. 129-134.

239. Ouwehand, A.C., et al., *Lactobacillus acidophilus supplementation in human subjects and their resistance to enterotoxigenic Escherichia coli infection.* British Journal of Nutrition, 2014. **111**(3): p. 465-473.
240. Porter, C.K., et al., *A systematic review of experimental infections with enterotoxigenic Escherichia coli (ETEC).* Vaccine, 2011. **29**(35): p. 5869-85.
241. Tacket, C. and M. Levine, *Vaccines against enterotoxigenic Escherichia coli infections.* New generation vaccines, 2nd ed. Marcel Dekker, Inc., New York, NY, 1997: p. 875-883.
242. Pathirana, W.G.W., et al., *Faecal Calprotectin.* The Clinical biochemist. Reviews, 2018. **39**(3): p. 77-90.
243. Mantis, N.J., N. Rol, and B. Corthésy, *Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut.* Mucosal immunology, 2011. **4**(6): p. 603-611.
244. Corthesy, B., *Multi-Faceted Functions of Secretory IgA at Mucosal Surfaces.* Frontiers in Immunology, 2013. **4**(185).
245. Harro, C., et al., *Refinement of a human challenge model for evaluation of enterotoxigenic Escherichia coli vaccines.* Clinical and vaccine immunology : CVI, 2011. **18**(10): p. 1719-1727.
246. Zhang, W. and D.A. Sack, *Progress and hurdles in the development of vaccines against enterotoxigenic Escherichia coli in humans.* Expert Review of Vaccines, 2012. **11**(6): p. 677-694.
247. van Furth, R. and H. Beekhuizen, *Monocytes*, in *Encyclopedia of Immunology (Second Edition)*, P.J. Delves, Editor. 1998, Elsevier: Oxford. p. 1750-1754.
248. Qian, C. and X. Cao, *Dendritic cells in the regulation of immunity and inflammation.* Seminars in Immunology, 2018. **35**: p. 3-11.
249. Benn, C.S., et al., *A small jab – a big effect: nonspecific immunomodulation by vaccines.* Trends in Immunology, 2013. **34**(9): p. 431-439.
250. Giamarellos-Bourboulis, E.J., et al., *Activate: Randomized Clinical Trial of BCG Vaccination against Infection in the Elderly.* Cell, 2020. **183**(2): p. 315-323.e9.
251. Kleinnijenhuis, J., et al., *Bacille Calmette-Guérin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes.* Proceedings of the National Academy of Sciences, 2012. **109**(43): p. 17537.
252. Kleinnijenhuis, J., et al., *BCG-induced trained immunity in NK cells: Role for non-specific protection to infection.* Clinical Immunology, 2014. **155**(2): p. 213-219.
253. Brodin, P., et al., *Variation in the Human Immune System Is Largely Driven by Non-Heritable Influences.* Cell, 2015. **160**(1): p. 37-47.
254. Brodin, P. and M.M. Davis, *Human immune system variation.* Nature Reviews Immunology, 2017. **17**(1): p. 21-29.
255. Martino, D.J. and S.L. Prescott, *Silent mysteries: epigenetic paradigms could hold the key to conquering the epidemic of allergy and immune disease.* Allergy, 2010. **65**(1): p. 7-15.
256. Suarez-Alvarez, B., et al., *DNA methylation: a promising landscape for immune system-related diseases.* Trends in Genetics, 2012. **28**(10): p. 506-514.
257. Kim, Lark K., et al., *Oct-1 Regulates IL-17 Expression by Directing Interchromosomal Associations in Conjunction with CTCF in T Cells.* Molecular Cell, 2014. **54**(1): p. 56-66.

258. Kuo, M.-H. and C.D. Allis, *Roles of histone acetyltransferases and deacetylases in gene regulation.* BioEssays, 1998. **20**(8): p. 615-626.
259. Gregory, P.D., K. Wagner, and W. Hörz, *Histone Acetylation and Chromatin Remodeling.* Experimental Cell Research, 2001. **265**(2): p. 195-202.
260. Bannister, A.J. and T. Kouzarides, *Regulation of chromatin by histone modifications.* Cell research, 2011. **21**(3): p. 381-395.
261. Onodera, A., K. Kokubo, and T. Nakayama, *Epigenetic and Transcriptional Regulation in the Induction, Maintenance, Heterogeneity, and Recall-Response of Effector and Memory Th2 Cells.* Frontiers in Immunology, 2018. **9**.
262. Bégin, P. and K.C. Nadeau, *Epigenetic regulation of asthma and allergic disease.* Allergy, Asthma & Clinical Immunology, 2014. **10**(1): p. 27.
263. Baskara-Yhuellou, I. and J. Tost, *Chapter Six - The impact of microRNAs on alterations of gene regulatory networks in allergic diseases*, in *Advances in Protein Chemistry and Structural Biology*, R. Donev, Editor. 2020, Academic Press. p. 237-312.
264. Melnik, B.C., et al., *Milk: a postnatal imprinting system stabilizing FoxP3 expression and regulatory T cell differentiation.* Clinical and Translational Allergy, 2016. **6**(1): p. 18.
265. Georgountzou, A. and N.G. Papadopoulos, *Postnatal Innate Immune Development: From Birth to Adulthood.* Frontiers in Immunology, 2017. **8**.
266. Torow, N., et al., *Neonatal mucosal immunology.* Mucosal Immunology, 2017. **10**(1): p. 5-17.
267. McDade, T.W., *Early environments and the ecology of inflammation.* Proceedings of the National Academy of Sciences, 2012. **109**(Supplement 2): p. 17281-17288.
268. West, C.E., N. D'Vaz, and S.L. Prescott, *Dietary Immunomodulatory Factors in the Development of Immune Tolerance.* Current Allergy and Asthma Reports, 2011. **11**(4): p. 325-333.
269. Aagaard, K., et al., *The Placenta Harbors a Unique Microbiome.* Science Translational Medicine, 2014. **6**(237): p. 237ra65-237ra65.
270. Gil, A., et al., *Is there evidence for bacterial transfer via the placenta and any role in the colonization of the infant gut? – a systematic review.* Critical Reviews in Microbiology, 2020. **46**(5): p. 493-507.
271. Dunstan, J.A., et al., *Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: A randomized, controlled trial.* Journal of Allergy and Clinical Immunology, 2003. **112**(6): p. 1178-1184.
272. Gunaratne, A.W., M. Makrides, and C.T. Collins, *Maternal prenatal and/or postnatal n-3 long chain polyunsaturated fatty acids (LCPUFA) supplementation for preventing allergies in early childhood.* The Cochrane database of systematic reviews, 2015. **2015**(7): p. CD010085-CD010085.
273. Schaub, B., et al., *Maternal farm exposure modulates neonatal immune mechanisms through regulatory T cells.* Journal of Allergy and Clinical Immunology, 2009. **123**(4): p. 774-782.e5.
274. Fragkou, P.C., et al., *Impact of Early Life Nutrition on Children's Immune System and Noncommunicable Diseases Through Its Effects on the Bacterial Microbiome, Virome and Mycobiome.* Frontiers in Immunology, 2021. **12**.
275. Azagra-Boronat, I., et al., *Lactobacillus fermentum CECT5716 supplementation in rats during pregnancy and lactation affects mammary milk composition.* Journal of Dairy Science, 2020.

103(4): p. 2982-2992.

276. Hogenkamp, A., et al., *Supplementation of Mice with Specific Nondigestible Oligosaccharides during Pregnancy or Lactation Leads to Diminished Sensitization and Allergy in the Female Offspring.* The Journal of Nutrition, 2015. **145**(5): p. 996-1002.
277. Hogenkamp, A., et al., *Supplementing Pregnant Mice with a Specific Mixture of Nondigestible Oligosaccharides Reduces Symptoms of Allergic Asthma in Male Offspring.* The Journal of Nutrition, 2014. **145**(3): p. 640-646.
278. van Vlies, N., et al., *Effects of short-chain galacto- and long-chain fructo-oligosaccharides on systemic and local immune status during pregnancy.* Journal of Reproductive Immunology, 2012. **94**(2): p. 161-168.
279. Eberharter, A. and P.B. Becker, *Histone acetylation: a switch between repressive and permissive chromatin.* EMBO reports, 2002. **3**(3): p. 224-229.
280. Riedler, J., et al., *Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey.* The Lancet, 2001. **358**(9288): p. 1129-1133.
281. von Mutius, E. and D. Vercelli, *Farm living: effects on childhood asthma and allergy.* Nature Reviews Immunology, 2010. **10**(12): p. 861-868.
282. Sozańska, B., et al., *Consumption of unpasteurized milk and its effects on atopy and asthma in children and adult inhabitants in rural Poland.* Allergy, 2013. **68**(5): p. 644-650.
283. Ege, M.J., et al., *Not all farming environments protect against the development of asthma and wheeze in children.* Journal of Allergy and Clinical Immunology, 2007. **119**(5): p. 1140-1147.
284. Waser, M., et al., *Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe.* Clinical & Experimental Allergy, 2007. **37**(5): p. 661-670.
285. Perkin, M.R. and D.P. Strachan, *Which aspects of the farming lifestyle explain the inverse association with childhood allergy?* Journal of Allergy and Clinical Immunology, 2006. **117**(6): p. 1374-1381.
286. Schuijs, M.J., et al., *Farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells.* Science, 2015. **349**(6252): p. 1106-1110.
287. LEYNAERT, B., et al., *Does Living on a Farm during Childhood Protect against Asthma, Allergic Rhinitis, and Atopy in Adulthood?* American Journal of Respiratory and Critical Care Medicine, 2001. **164**(10): p. 1829-1834.
288. Kilpeläinen, M., et al., *Childhood farm environment and asthma and sensitization in young adulthood.* Allergy, 2002. **57**(12): p. 1130-1135.
289. Wlasiuk, G. and D. Vercelli, *The farm effect, or: when, what and how a farming environment protects from asthma and allergic disease.* Current Opinion in Allergy and Clinical Immunology, 2012. **12**(5): p. 461-466.
290. Arrieta, M.-C., et al., *Early infancy microbial and metabolic alterations affect risk of childhood asthma.* Science Translational Medicine, 2015. **7**(307): p. 307ra152-307ra152.
291. Khaleva, E., et al., *Transforming growth factor beta in human milk and allergic outcomes in children: A systematic review.* Clinical & Experimental Allergy, 2019. **49**(9): p. 1201-1213.
292. Manzoni, P., et al., *Bovine Lactoferrin Supplementation for Prevention of Late-Onset Sepsis in Very Low-Birth-Weight Neonates: A Randomized Trial.* JAMA, 2009. **302**(13): p. 1421-1428.
293. Manzoni, P., et al., *Bovine Lactoferrin Prevents Invasive Fungal Infections in Very Low Birth Weight*

Infants: A Randomized Controlled Trial. Pediatrics, 2012. **129**(1): p. 116-123.

294. King, J.C.J., et al., *A Double-Blind, Placebo-Controlled, Pilot Study of Bovine Lactoferrin Supplementation in Bottle-fed Infants.* Journal of Pediatric Gastroenterology and Nutrition, 2007. **44**(2): p. 245-251.
295. Lönnerdal, B., et al., *Growth, Nutrition, and Cytokine Response of Breast-fed Infants and Infants Fed Formula With Added Bovine Osteopontin.* Journal of Pediatric Gastroenterology and Nutrition, 2016. **62**(4): p. 650-657.
296. West, C.E., et al., *Effects of osteopontin-enriched formula on lymphocyte subsets in the first 6 months of life: a randomized controlled trial.* Pediatric Research, 2017. **82**(1): p. 63-71.
297. Timby, N., et al., *Infections in Infants Fed Formula Supplemented With Bovine Milk Fat Globule Membranes.* Journal of Pediatric Gastroenterology and Nutrition, 2015. **60**(3): p. 384-389.
298. Collins, A.M., et al., *Bovine milk, including pasteurised milk, contains antibodies directed against allergens of clinical importance to man.* Int Arch Allergy Appl Immunol, 1991. **96**(4): p. 362-7.
299. Zivkovic, A.M. and D. Barile, *Bovine Milk as a Source of Functional Oligosaccharides for Improving Human Health.* Advances in Nutrition, 2011. **2**(3): p. 284-289.
300. Koh, A., et al., *From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites.* Cell, 2016. **165**(6): p. 1332-1345.
301. Liu, P., et al., *The role of short-chain fatty acids in intestinal barrier function, inflammation, oxidative stress, and colonic carcinogenesis.* Pharmacological Research, 2021. **165**: p. 105420.
302. Bezkorovainy, A., *Probiotics: determinants of survival and growth in the gut.* The American Journal of Clinical Nutrition, 2001. **73**(2): p. 399s-405s.
303. Li, M., et al., *Pro- and anti-inflammatory effects of short chain fatty acids on immune and endothelial cells.* European Journal of Pharmacology, 2018. **831**: p. 52-59.
304. Tazoe, H., et al., *Roles of short-chain fatty acids receptors, GPR41 and GPR43 on colonic functions.* Journal of physiology and pharmacology : an official journal of the Polish Physiological Society, 2008. **59 Suppl 2**: p. 251-62.
305. Masui, R., et al., *G protein-coupled receptor 43 moderates gut inflammation through cytokine regulation from mononuclear cells.* Inflammatory Bowel Diseases, 2013. **19**(13): p. 2848-2856.
306. Halnes, I., et al., *Soluble Fibre Meal Challenge Reduces Airway Inflammation and Expression of GPR43 and GPR41 in Asthma.* Nutrients, 2017. **9**(1): p. 57.
307. Donohoe, Dallas R., et al., *The Warburg Effect Dictates the Mechanism of Butyrate-Mediated Histone Acetylation and Cell Proliferation.* Molecular Cell, 2012. **48**(4): p. 612-626.
308. Asarat, M., et al., *Short-Chain Fatty Acids Regulate Cytokines and Th17/Treg Cells in Human Peripheral Blood Mononuclear Cells in vitro.* Immunol Invest, 2016. **45**(3): p. 205-22.
309. Newman, J.D., et al., *Induction of the insulin receptor and other differentiation markers by sodium butyrate in the Burkitt lymphoma cell, Raji.* Biochemical and Biophysical Research Communications, 1989. **161**(1): p. 101-106.
310. Yamamoto, I., et al., *Interleukin-2-dependent augmentation of the anti-tnp antibody production by sodium butyrate in cultured murine splenic b cells.* International Journal of Immunopharmacology, 1997. **19**(6): p. 347-354.
311. Vinolo, M.A.R., et al., *Regulation of inflammation by short chain fatty acids.* Nutrients, 2011. **3**(10):

p. 858-876.
312. Schroeder, H.W. and L. Cavacini, *Structure and Function of Immunoglobulins.* The Journal of allergy and clinical immunology, 2010. **125**(2 0 2): p. S41-S52.
313. Forthal, D.N., *Functions of Antibodies.* Microbiology spectrum, 2014. **2**(4): p. 1-17.
314. Clark, M.R., et al., *Molecular basis for a polymorphism involving Fc receptor II on human monocytes.* The Journal of Immunology, 1989. **143**(5): p. 1731.
315. Ory, P.A., et al., *Characterization of polymorphic forms of Fc receptor III on human neutrophils.* The Journal of clinical investigation, 1989. **83**(5): p. 1676-1681.
316. Warmerdam, P.A., et al., *A single amino acid in the second Ig-like domain of the human Fc gamma receptor II is critical for human IgG2 binding.* The Journal of Immunology, 1991. **147**(4): p. 1338.
317. Bournazos, S., T.T. Wang, and J.V. Ravetch, *The Role and Function of Fcγ Receptors on Myeloid Cells.* Microbiology spectrum, 2016. **4**(6): p. 10.1128/microbiolspec.MCHD-0045-2016.
318. Ravetch, J.V., *Fc receptors: Rubor redux.* Cell, 1994. **78**(4): p. 553-560.
319. Shore, S.L., et al., *Detection of cell-dependent cytotoxic antibody to cells infected with herpes simplex virus.* Nature, 1974. **251**(5473): p. 350-352.
320. Jegerlehner, A., et al., *Influenza A Vaccine Based on the Extracellular Domain of M2: Weak Protection Mediated via Antibody-Dependent NK Cell Activity.* The Journal of Immunology, 2004. **172**(9): p. 5598.
321. Pearse, R.N., et al., *SHIP Recruitment Attenuates FcγRIIB-Induced B Cell Apoptosis.* Immunity, 1999. **10**(6): p. 753-760.
322. Ravetch, J.V. and L.L. Lanier, *Immune Inhibitory Receptors.* Science, 2000. **290**(5489): p. 84-89.
323. Langlet, C., et al., *CD64 Expression Distinguishes Monocyte-Derived and Conventional Dendritic Cells and Reveals Their Distinct Role during Intramuscular Immunization.* The Journal of Immunology, 2012. **188**(4): p. 1751-1760.
324. AK, M., et al., *Hyperimmune cow colostrum reduces diarrhoea due to rotavirus: a double-blind, controlled clinical trial.* Acta Paediatrica, 1995. **84**(9): p. 996-1001.
325. SARKER, S.A., et al., *Successful treatment of rotavirus diarrhea in children with immunoglobulin from immunized bovine colostrum.* The Pediatric Infectious Disease Journal, 1998. **17**(12): p. 1149-1154.
326. Marit, K., et al., *Anti-HIV-1 antibody-dependent cellular cytotoxicity mediated by hyperimmune bovine colostrum IgG.* European Journal of Immunology, 2012. **42**(10): p. 2771-2781.
327. Kelly, G.S., *Bovine colostrums: a review of clinical uses.* Altern Med Rev, 2003. **8**(4): p. 378-94.
328. Borchers, A.T., et al., *Respiratory syncytial virus--a comprehensive review.* Clinical reviews in allergy & immunology, 2013. **45**(3): p. 331-379.
329. Srikiatkhachorn, A. and T.J. Braciale, *Virus-specific CD8+ T lymphocytes downregulate T helper cell type 2 cytokine secretion and pulmonary eosinophilia during experimental murine respiratory syncytial virus infection.* The Journal of experimental medicine, 1997. **186**(3): p. 421-432.
330. Fernández, N., M. Renedo, and M. Sánchez Crespo, *FcγR receptors activate MAP kinase and up-regulate the cyclooxygenase pathway without increasing arachidonic acid release in monocytic cells.* European Journal of Immunology, 2002. **32**(2): p. 383-392.
331. Fernández, N., et al., *Activation of Monocytic Cells Through Fcγ Receptors Induces the Expression of*

Macrophage-Inflammatory Protein (MIP)-1α, MIP-1β, and RANTES. The Journal of Immunology, 2002. **169**(6): p. 3321-3328.

332. Jancar, S. and M.S. Crespo, *Human diseases associated with tissue immune complex deposition.* Trends in Immunology, 2005. **26**(1): p. 48-55.
333. Tsuboi, N., et al., *Human neutrophil Fcgamma receptors initiate and play specialized nonredundant roles in antibody-mediated inflammatory diseases.* Immunity, 2008. **28**(6): p. 833-846.
334. Gallo, P., R. Gonçalves, and D.M. Mosser, *The influence of IgG density and macrophage Fc (gamma) receptor cross-linking on phagocytosis and IL-10 production.* Immunology letters, 2010. **133**(2): p. 70-77.
335. Zhang, Y., et al., *Ligation of Fc gamma receptor IIB enhances levels of antiviral cytokine in response to PRRSV infection in vitro.* Veterinary Microbiology, 2012. **160**(3): p. 473-480.
336. Hayes, J.M., et al., *Glycosylation and Fc Receptors*, in *Fc Receptors*, M. Daeron and F. Nimmerjahn, Editors. 2014, Springer International Publishing: Cham. p. 165-199.
337. Dorshkind, K., E. Montecino-Rodriguez, and R.A.J. Signer, *The ageing immune system: is it ever too old to become young again?* Nature Reviews Immunology, 2009. **9**(1): p. 57-62.
338. van Splunter, M., et al., *Bovine Lactoferrin Enhances TLR7-Mediated Responses in Plasmacytoid Dendritic Cells in Elderly Women: Results From a Nutritional Intervention Study With Bovine Lactoferrin, GOS and Vitamin D.* Frontiers in immunology, 2018. **9**: p. 2677-2677.
339. Ciabattini, A., et al., *Vaccination in the elderly: The challenge of immune changes with aging.* Seminars in Immunology, 2018. **40**: p. 83-94.
340. Weinberger, B., *Vaccines for the elderly: current use and future challenges.* Immunity & ageing : I & A, 2018. **15**: p. 3-3.
341. Zbinden, D. and O. Manuel, *Influenza vaccination in immunocompromised patients: efficacy and safety.* Immunotherapy, 2014. **6**(2): p. 131-9.
342. Bosaeed, M. and D. Kumar, *Seasonal influenza vaccine in immunocompromised persons.* Human vaccines & immunotherapeutics, 2018. **14**(6): p. 1311-1322.
343. Kalafati, L., et al., *Innate Immune Training of Granulopoiesis Promotes Anti-tumor Activity.* Cell, 2020. **183**(3): p. 771-785.e12.
344. Byrne, K.A., C.L. Loving, and J.L. McGill, *Innate Immunomodulation in Food Animals: Evidence for Trained Immunity?* Frontiers in immunology, 2020. **11**: p. 1099-1099.
345. Rao, M.R., et al., *Serologic Correlates of Protection against Enterotoxigenic Escherichia coli Diarrhea.* The Journal of Infectious Diseases, 2005. **191**(4): p. 562-570.
346. Tobias, J., et al., *Preexisting antibodies to homologous colonization factors and heat-labile toxin in serum, and the risk to develop enterotoxigenic Escherichia coli-associated diarrhea.* Diagnostic Microbiology and Infectious Disease, 2008. **60**(2): p. 229-231.
347. McCall, M.B.B., et al., *Plasmodium falciparum Infection Causes Proinflammatory Priming of Human TLR Responses.* The Journal of Immunology, 2007. **179**(1): p. 162-171.
348. Rodrigues, J., et al., *Hemocyte differentiation mediates innate immune memory in Anopheles gambiae mosquitoes.* Science (New York, N.Y.), 2010. **329**(5997): p. 1353-1355.
349. Rusek, P., et al., *Infectious Agents as Stimuli of Trained Innate Immunity.* International Journal of Molecular Sciences, 2018. **19**(2): p. 456.

Supplementary Data

Table S1. Flow cytometry antibody panel for phenotyping immune cells and assessing the differential expression of SCFAs receptors

Antibody	Fluorochrome	Host/Isotype	Clone	Company	Catalog number
α-CD3	FITC	Mouse/ IgG1	UCHT1	Biolegend	300406
α-CD11c	BV421	Mouse/IgG1	3.9	Biolegend	301628
α-CD14	BV605	Mouse/IgG2b	63D3	Biolegend	367126
α-CD19	BV510	Mouse/IgG1	SJ25C1	BD	562947
α-CD123	PE-Cy7	Mouse/IgG1	6H6	Biolegend	306010
α-HLA-DR	APC-Cy7	Mouse/IgG2a	L243	Biolegend	307618
α-GPR41	Unconjugated AF790	Rabbit/IgG Goat/IgG	Polyclonal Polyclonal	Invivogen Invitrogen	PA5-25146 A11369
α-GPR43	AF647	Mouse/IgG2b	1000624	R&D systems	FAB10082R
α-GPR109A	AF594	Mouse/IgG2b	245106	R&D systems	FAB2760T

Table S2. Flow cytometry antibody panel for phenotyping immune cells and assessing the differential expression of SCFAs transporters

Antibody	Fluorochrome	Host/Isotype	Clone	Company	Catalog number
α-CD3	FITC	Mouse/ IgG1	UCHT1	Biolegend	300406
α-CD11c	BV421	Mouse/IgG1	3.9	Biolegend	301628
α-CD14	BV605	Mouse/IgG2b	63D3	Biolegend	367126
α-CD19	BV510	Mouse/IgG1	SJ25C1	BD	562947
α-CD123	PE-Cy7	Mouse/IgG1	6H6	Biolegend	306010
α-HLA-DR	APC-Cy7	Mouse/IgG2a	L243	Biolegend	307618
α-SMCT-1	AF647	Mouse/IgG1	903502	R&D systems	FAB8398R
α-MCT-1	AF594	Mouse/IgG2a	882616	R&D systems	FAB8275T

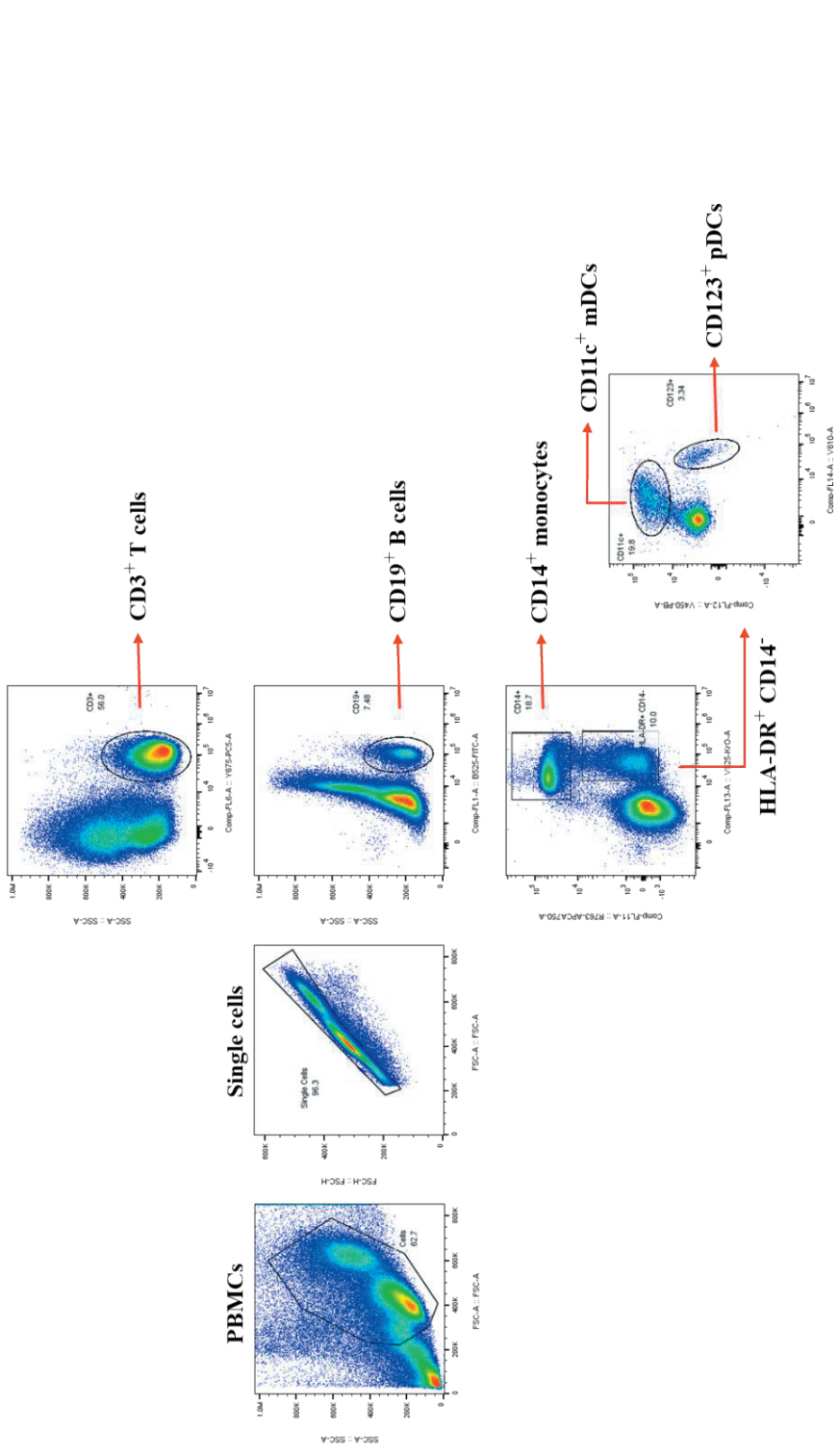

Fig S1. Gating strategy for identifying $CD3^+$ T cells, $CD19^+$ B cells, $CD14^+$ monocytes, $CD11c^+$ mDCs, and $CD123^+$ pDCs from PBMCs. The expression of SCFA receptors and transporters was determined for each cell type.

Table S3. Flow cytometry antibody panel for quantifying CCR9 and CCR10 in $CD3^+$ T cells and $CD19^+$ B cells

Antibody	Fluorochrome	Host/Isotype	Clone	Company	Catalog number
α-CD3	FITC	Mouse/IgG1	RPA-T4	Biolegend	300506
α-CD19	BV 421	Mouse/IgG1	HIB1	Biolegend	302234
α-CCR9	PE	Mouse/IgG2a	L053E8	Biolegend	358904
α-CCR10	APC	Hamster/IgG	6588-5	Biolegend	341506
Viability	DRAQ7	-	-	Cell Signaling	7406

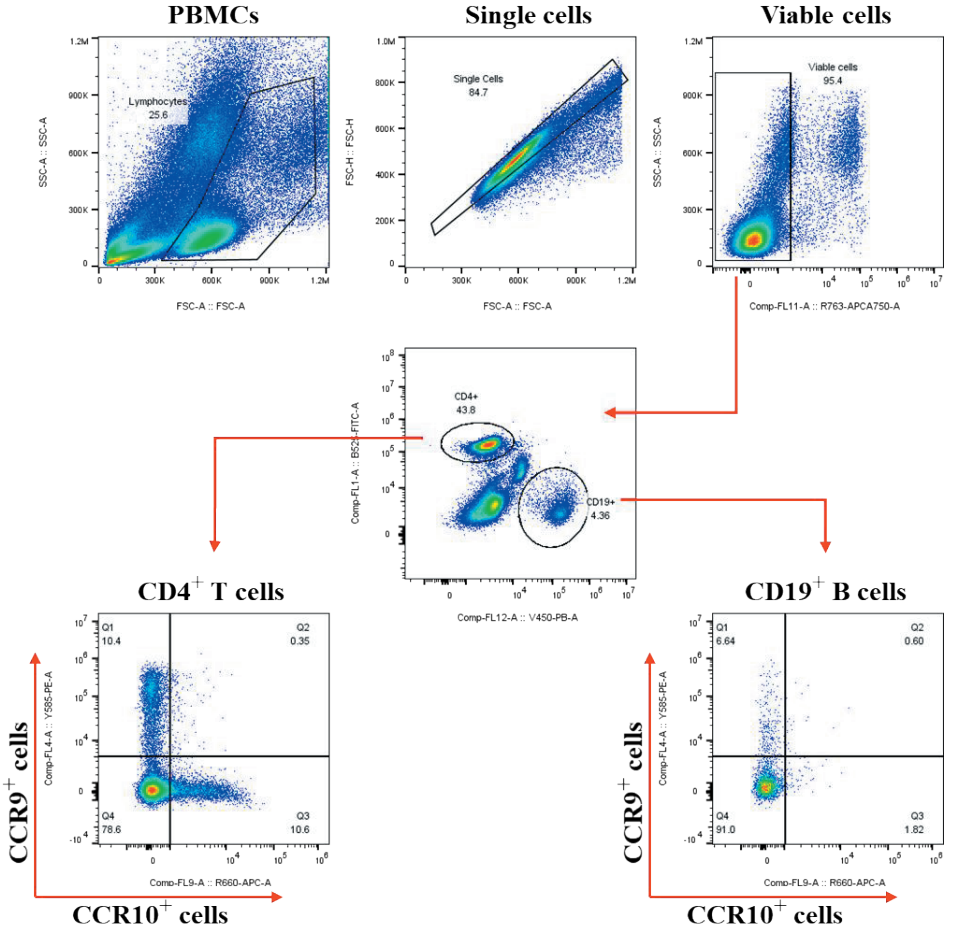

Fig S2. The gating strategy for identifying CD3+ T cells and CD19+ B cells in PBMCs and quantifying CCR9 and CCR10 expression within each cell type**.**

Table S4. Flow cytometry antibody panel for staining monocytes, mDCs, and pDCs and quantifying IL-6 and TNF-a levels

Antibody	Fluorochrome	Host/Isotype	Clone	Company	Catalog number
α-CD3	FITC	Mouse/IgG1	UCHT1	Biolegend	300406
α-CD11c	BV 421	Mouse/IgG1	3.9	Biolegend	301628
α-CD14	Percp-Cy5.5	Mouse/IgG1	HCD14	Biolegend	325622
α-CD19	FITC	Mouse/IgG1	SJ25C1	Biolegend	363008
α-CD20	FITC	Mouse/IgG2b	2H7	Biolegend	302304
α-CD56	FITC	Mouse/IgG1	HCD56	Biolegend	318304
α-HLA-DR	BV 510	Mouse/IgG2a	L243	Biolegend	307646
α-CD123	PE-Cy7	Mouse/IgG1	6H6	Biolegend	306010
α-IL-6	PE	Rat/IgG1	MQ2-13A5	Biolegend	501107
α-TNF-α	AF647	Mouse/IgG1	MAb11	Biolegend	502916

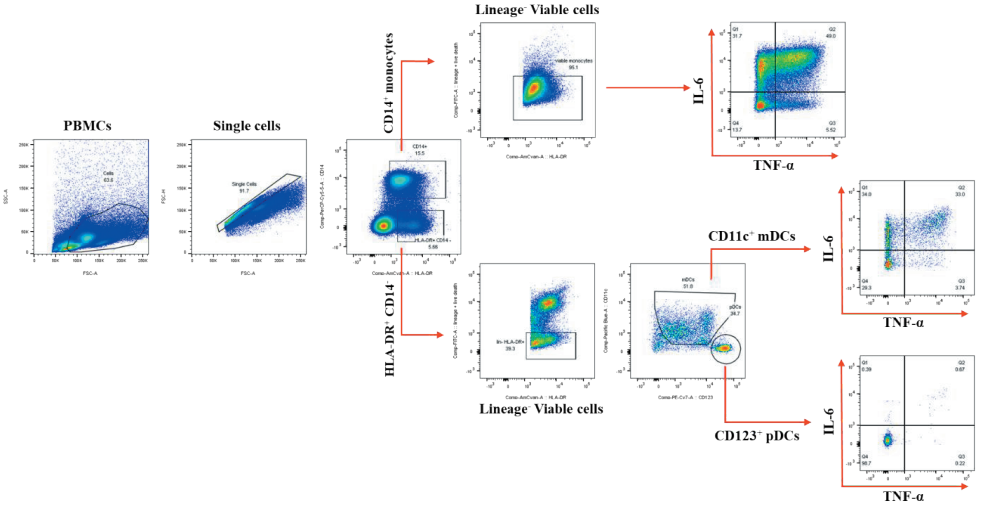

Fig S3. Gating strategy for discrimination of CD14⁺ monocytes, Cd11c⁺ mDCs, and CD123⁺ pDCs in PBMCs. The percentage of IL-6 and TNF-a producing cells was quantified within each population.

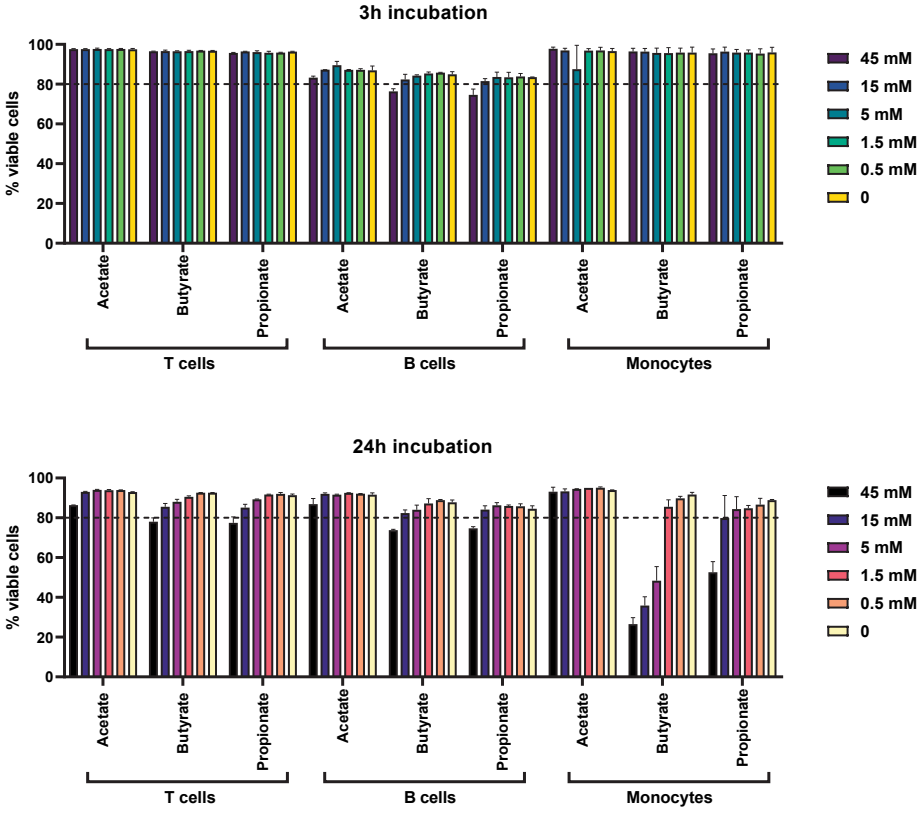

Fig S4. The % of viable T- and B cells, and monocytes after 3 or 24 hours incubation with different concentrations (45, 15, 5, 1.5, 0.5, and 0 mM) of acetate, butyrate, and propionate .

CHAPTER

Direct binding of bovine IgG-containing immune complexes to human monocytes and their putative role in innate immune training

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Abstract

Bovine milk IgG (bIgG) was shown to bind to and neutralize the human Respiratory Synovial Virus (RSV). In animal models, adding bIgG prevented experimental RSV infection and increased the number of activated T cells. This enhanced activation of RSV-specific T cells may be explained by receptor-mediated uptake and antigen presentation after binding of bIgG-RSV immune complexes (ICs) with FcγRs (primarily CD32) on human immune cells. This indirect effect of bIgG ICs on activation of RSV specific-T cells was confirmed previously in human T cell cultures. However, the direct binding of ICs to antigen-presenting cells has not been addressed. As bovine IgG can induce innate immune training, we hypothesized that this effect could be caused more efficiently by ICs. Therefore we characterized the expression of CD16, CD32, and CD64 on PBMCs, determined the optimal conditions to form ICs of bIgG with the RSV preF protein, and demonstrated the direct binding of these ICs to human $CD14^+$ monocytes. Similarly, bIgG complexed with a murine anti-bIgG mAb also bound efficiently to the monocytes. To evaluate if the ICs could induce innate immune training more efficiently than bIgG itself, the resulted ICs, as well as bIgG, were used in an *in vitro* innate immune training model. Training with the ICs containing bIgG and RSV preF protein - but not the bIgG alone - induced significantly higher TNF-a production upon LPS and R848 stimulation. However, the preF protein itself nonsignificantly increases cytokine production as well. This may be explained by its tropism to IGFR1, as IGF has been reported to induce innate immune training. Even so, these data suggest a role for IgG-containing ICs in inducing innate immune training after re-exposure to pathogens. However, as ICs of bIgG with a mouse anti-bIgG mAb did not induce this effect, further research is needed to confirm the putative role of bIgG ICs in enhancing innate immune responses *in vivo*.

Introduction

Newborns are susceptible to gastrointestinal and respiratory tract infections due to the immaturity of their immune system. Maternal immunoglobulin (Ig) G and IgA help to protect the infant from such infections. These immunoglobulins are transferred to the infant via the placenta during pregnancy and breastfeeding after birth [68, 69]. Although breastfeeding is recommended for the first 6 months of life by the World Health Organization (WHO) [86], many children are not breastfed and are dependent on bovine milk-based infant formula [164].

Bovine milk contains several immunomodulatory proteins that can support the immune system [106, 165-167]. Bovine IgG or bIgG is one of the major milk proteins that is thought to contribute to the protection against infections and allergies [92, 100, 168]. In the gastrointestinal tract or the tonsillar crypts in Waldeyer's ring, dietary components may come into direct contact with respiratory pathogens from the nasal cavity after swallowing [10, 169]. This implies that bIgG can directly encounter bacteria and viruses and form immune complexes (IC). After uptake into the mucosal tissue, these ICs can interact with receptors on immune cells such as neutrophils and macrophages, which phagocytose and eliminate the pathogen. Moreover, upon internalization of ICs, monocytes and Dendritic Cells (DCs) can process and present antigenic pathogen-derived peptides to T lymphocytes [102, 106].

IgG is known to interact with a conserved family of transmembrane glycoproteins known as Fc gamma receptors (FcγRs) [170, 171]. On human immune and non-immune cells, three classes of FcγRs are expressed with different affinity for IgG subclasses: high-affinity (10^{-9} M Kd) FcγRI (CD64), and low-affinity (10^{-6} M Kd) FcγRII (CD32) and FcγRIII (CD16) [172, 173]. The high-affinity CD64 is predominantly occupied by endogenous serum IgG monomers *in vivo* [173] and plays a critical role in Antibody-Dependent Cellular Phagocytosis (ADCP) in myeloid phagocytes [174]. IgG monomers do not bind to CD32, and only ICs comprising several IgGs bound to antigens can bind to and interact with these low-affinity receptors [175, 176]. Along with CD64, CD32 is essential for ADCP by neutrophils and macrophages and also in the process of antigen presentation to the naive T cells by DCs [177-179]. The lower affinity of CD32 for IgG monomers ensures that the antibodies' effector function is only initiated in the presence of a pathogen-derived antigen, preventing an aberrant immune response in the presence of normal levels of antibodies *in vivo*. CD16, another low-affinity FcγR, is primarily involved in eliminating infected cells via Antibody-Dependent Cellular Cytotoxicity (ADCC), mainly mediated by Natural Killer (NK) cells [177]. Apart from these classical FcγRs, it is known that the neonatal Fc receptor (FcRn) can interact with IgG. FcRn enables the transfer of maternal IgG to the fetus via the placenta, conferring passive immunity to the offspring [180]. FcRn

also mediates salvaging of internalized IgG from degradation through a pH-dependent cellular recycling mechanism [181]. Besides, it was demonstrated that FcRn is important for the internalization of IgG ICs - but not monomers - and the process of antigen presentation by Antigen Presenting Cells (APCs) [182, 183].

It has been established that bIgG binds to several human pathogens, including Respiratory Syncytial Virus (RSV) [102, 106, 111]. RSV is one of the most common causes of Respiratory Tract Infections (RTIs) in newborns, which also increases the risk of later-life health complications such as asthma [184, 185]. The F protein of RSV is crucial in binding to and infecting human cells, and breast milk preF protein-specific antibodies are a correlate of protection in infants [186]. Besides binding to and neutralizing human RSV, bIgG facilitates FcγRII-mediated internalization of bIgG-coated pathogens by human neutrophils, monocytes, and DCs [102]. Nederend et al. recently demonstrated that bIgG could neutralize RSV in an *in vitro* cellular infection model, as did Human Intravenous Immunoglobulin (IVIg) and the prophylactic RSV-specific monoclonal antibody, Palivizumab [111]. Interestingly, the authors showed that activation of RSV preF protein-specific $CD4^+$ and $CD8^+$ T cells was strongly enhanced in the presence of bovine IgG [111]. They concluded that the interaction between ICs and (activating) FcγRII on autologous monocytes resulted in higher antigen presentation and T cell activation. Moreover, bIgG was found to be protective against experimental RSV infection in mice [111]. Likewise, dietary supplementation of mice with bovine colostrum, a preparation very rich in bIgG, was shown to decrease RSV infection rates and increase the number of $CD69^+$, IFN-γ producing $CD8^+$ T cells [187].

Interestingly, bIgG may also contribute to the resistance against (viral) infections by inducing trained innate immunity in FcγR bearing monocytes [46, 47]. This mechanism leads to enhanced cytokine production of innate immune cells after stimulation with Toll-Like Receptor (TLR) ligands [53, 162]. In this concept, primary exposure to the training agent leads to a more robust secondary response to the same and related TLR stimulation. The underlying training mechanism for β-glucans - a compound with established training potential - was shown to be via the engagement of the Dectin-1 receptor and downstream signaling events, including the Raf-1 pathway [38, 51]. Following activation of the Dectin-1 receptor, epigenetic alterations in the cells occur by trimethylation of the H3K4 histone protein, a shift in cell metabolism from oxidative phosphorylation to aerobic glycolysis, and consequently a change in the responsiveness of the cells [49, 188]. bIgG has been demonstrated to induce innate immune training resulting in increased production of IL-6 and TNF-a in human monocytes *in vitro* upon TLR stimulation [46, 47].

As monomeric IgG does not interact with low-affinity IgG receptors, we hypothesized that the training effects of bIgG might be induced more efficiently by multimeric IgG

immune complexes. To address this question, we studied the direct binding of bovine IgG to human monocytes in the presence or absence of the RSV preF protein (preF) or anti-bIgG (a-bIgG) antibodies. We established optimal rations between bIgG and the RSV preF and a-bIgG for efficient binding and tested whether these immune complexes could induce innate immune training.

Material and Methods

PBMC isolation

PBMCs were isolated from buffy coats (Sanquin blood bank, Nijmegen) or fresh blood of donors collected at Wageningen University blood collection center after obtaining written consent. Gradient centrifugation on Ficoll Paque Plus (GE Healthcare, 17-1440-02) was used to isolate PBMCs. Ficoll (15 mL/tube) was transferred to Leucosep tubes (Greiner Bio-One, #227290), and the tubes were spun down briefly. Blood samples were added to the tubes after being diluted 1:1 with warm (37 °C) phosphate-buffered saline (PBS) (Gibco, #20012027). After centrifugation, the PBMCs fraction on top of the porous barrier was transferred to new 50 mL Falcon tubes (Corning, #352070). Warm PBS was added to wash the cells, and the tubes were spun down. The diluted plasma was discarded, and the cell pellet was resuspended after centrifugation. Following the third wash, the cells were resuspended in RPMI 1640 (Gibco, #61870010).

Reagents

Bovine Immunoglobulin G (bIgG) was isolated from bovine colostrum and provided by FrieslandCampina. Expression and purification of a DSCav1-like [189] prefusion-stabilized recombinant soluble RSV F protein (preF) were described previously [190]. Monoclonal anti-bovine IgG antibody (α-bIgG) (Sigma-Aldrich, #B6901) was used for bIgG IC formation. For bIgG detection by flow cytometry, AlexaFlour 647 conjugated goat anti-bovine IgG (Jackson ImmunoResearch, #101-605-165) was applied.

FcγR expression

The expression of various FcγRs was characterized on different immune cells within the PBMC fraction. PBMCs were stained with fluorochrome-conjugated antibodies (**Table 5**) for immune cell phenotyping. T- and B-cells, monocytes, mDCs, and pDCs were identified, and the FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) expression levels were measured. In brief, $1x10^6$ cells were plated in a NUNC plate (ThermoFisher, #267245) and washed with cold (4 °C) FACS buffer (PBS supplemented with 2.5 mM ethylenediaminetetraacetic acid (EDTA) and 0.05% sodium azide). The cells were then stained with the antibody mixture, and the plate was incubated for 30 minutes at 4°C in the dark. Then the cells were washed with two changes of cold FACS buffer, spinning and

discarding the supernatant after each wash. After resuspending the cells in FACS buffer, they were measured on CytoFLEX LX (Beckman Coulter, #C11186), and the generated data were analyzed using FlowJo (FlowJo LLC, v9). The gating strategy for selecting different cell subsets and assessing FcγR expression is described in the supplementary data (Suppl Fig S1).

Table 5. Antibody panel used for PBMCs phenotyping and assessing the expression of FcγRs

Antibody	Fluorochrome	Host/Isotype	Clone	Company	Catalog number
α-CD3	PE-Cy5	Mouse/ IgG1	UCHT1	BD	555334
α-CD11c	BV421	Mouse/IgG1	3.9	Biolegend	301628
α-CD14	APC-H7	mouse/IgG2b	MϕP9	BD	560180
α-CD19	FITC	Mouse/IgG1	HIB19	BD	555412
α-CD123	BV605	mouse/IgG2a	7G3	BD	564197
α-HLA-DR	BV510	mouse/IgG2a	L243	Biolegend	307646
α-CD64	APC	mouse/IgG1	10.1	Biolegend	305014
α-CD32	PerCp-Cy5.5	mouse/IgG2b	FUN-2	Biolegend	303216
α-CD16	PE	mouse/IgG1	B73.1	BD	332779

Detection of bIgG and bIgG-immune complexes bound to monocytes

Freshly isolated PBMCs were subjected to various concentrations of bIgG to confirm the binding of bIgG to the monocytes. PBMCs were incubated at 4°C for 20 minutes with bIgG (500, 50, 5, and 0 μg/mL) and then were stained with goat AlexaFlour 647 conjugated anti-bovine IgG (Jackson ImmunoResearch, #101-605-165) and anti-CD14 (Biolegend, #301830) for 30 minutes at 4°C in the dark. Next, the cells were washed twice with cold FACS buffer. FACS buffer was added, the plate was spun down, and the supernatant was discarded after each centrifugation. The cells were then resuspended in FACS buffer before being analyzed on a CytoFLEX LX flow cytometer. The data were analyzed using FlowJo, and the Median Fluorescence Intensity (MFI) of the bIgG signal was determined on the $CD14^+$ cells (Suppl Fig S2 for gating strategy).

To determine the optimal antibody: antigen ratio for the formation of large immune complexes (ICs), bIgG was titrated while keeping the concentration of the antigen constant. Increasing concentrations of bIgG were incubated with Respiratory Syncytial Virus (RSV) preF. As the first step and to dispose of antibody aggregates and obtain monomeric forms, the bIgG stock was spun down (17xg, RT, 15 minutes), and the supernatant was used for downstream experiments. bIgG at concentrations of 100, 30, 10, 3, 1, 0.3, 0.1, and 0 μg/mL were made using the serial dilution method and combined 1:1 with PreF protein (50 μg/mL) on a sterile NUNC plate. The Plate was wrapped in plastic foil and was pre-incubated at 37°C for 60 minutes to allow IC formation. After incubation, the plate was cooled down, and the mixture was exposed to freshly isolated PBMCs ($3x10^5$/well). The

plate was wrapped in foil and was incubated in the fridge (4°C) for 60 minutes to allow the binding of ICs to the cells. The cells were washed with cold FACS buffer to remove the unbound antibody/antigen residuals following the incubation. The cells were then stained with anti-bIgG (Jackson ImmunoResearch, #101-605-165) and also anti-CD14 antibody (Biolegend, #325606) for monocyte identification. The same bIgG concentrations but without preF protein (bIgG only) and preF protein alone were included as the experiment controls and background values. Cells were subsequently measured using a CytoFLEX LX flow cytometer, the data were processed using FlowJo, and graphs from the bIgG detection MFI were created using MS Excel (MS Office 365). The experiments were performed with two replicates of the same condition and were repeated with the blood of at least three different donors.

A similar approach was applied to identify the optimal ratio between bIgG and mouse anti-bovine IgG monoclonal antibody (a-bIgG) (Sigma-Aldrich, #B6901). The aim was to use a monoclonal antibody with a higher specificity against bIgG. Various bIgG concentrations (100, 30, 10, 3, 1, 0.3, 0.1, and 0 μg/mL) were incubated with two concentrations of a-bIgG (5 and 1 μg/mL) and the data were handled the same as RSV PreF protein as described above. The blood samples from at least three donors were used for the titration assays of bIgG and a-bIgG. The optimal antibody: antigen ratio determined in these assays was then utilized in subsequent innate immune training experiments.

Innate immune training

The ability of generated ICs for enhancing monocytes responses was evaluated in an *in vitro* innate immune training model [134]. PBMCs were isolated, $CD14^+$ monocytes were negatively selected and trained as described elsewhere [46, 47]. RPMI 1640 medium (Gibco, #A1049101) and 100 μg/mL of Whole Glucan Particle (WGP) (InvivoGen, #tlrl-wgp) were applied as the experiment negative and positive controls, respectively. The training was done with preF protein only (50 μg/mL), bIgG only (10 μg/mL), and their corresponding immune complexes (ICs) comprised of bIgG: preF as described earlier. In addition, a-bIgG only (5 μg/mL), bIgG only (3 μg/mL), and bIgG: a-bIgG immune complexes (ICs) were also used separately as the training compounds. After training and resting, the cells were stimulated with either 10 pg/mL of LPS (TLR4 ligand) (Sigma-Aldrich, #L2880) or 5 ng/mL of R848 (TLR7/8 ligand) (InvivoGen, #tlrl-r848). Cytometric Bead Array (CBA) and individual cytokines Flex-sets were used for measuring IL-6 (BD, #558276) and TNF-a (BD, #558273) in the culture supernatant of the cells (suppl Fig S3). The experiments were performed with the PBMCs isolated from 7-10 donors.

CBA data were analyzed by FCAP Array (BD Biosciences, v3.0) and then were transferred to GraphPad Prism (GraphPad Software, v9) for statistical analysis and preparing the

figures. The data were normalized and are expressed as fold changes relative to the untrained monocytes (control group-RPMI 1640). Wilcoxon matched-pairs signed-ranks test was used for head-to-head comparisons, and for multiple comparisons, the Friedman test was utilized to compare different groups with the control. The differences were considered significant when the p-value was <0.05 (*), or <0.01 (**), as indicated in the graphs.

Results

FcγR expression

To study the relative expression of FcγRs on immune cells, we determined the expression levels of the CD16, CD32, and CD64 on the surface of monocytes, mDC, pDC, and B- and T lymphocytes (Summarized in **Table 6**). FcγRIII (CD16) was highly expressed on a subset of mDCs (Fig 1A), whereas it was only present on a small percentage (less than 5%) of monocytes, and CD16 was not detected on T- and B cells and on pDCs (Fig 1A). While FcγRII (CD32) expression was high on monocytes and B lymphocytes, this receptor was not present on T cells and pDCs (Fig 1B). CD32 was also present on mDCs; however, the expression levels varied within the mDC subsets (Fig 1B). Monocytes highly expressed FcγRI (CD64), but in contrast, this receptor was absent on T- and B cells and pDCs (Fig 1C). The expression levels of CD64 on mDCs varied considerably (Fig 1C).

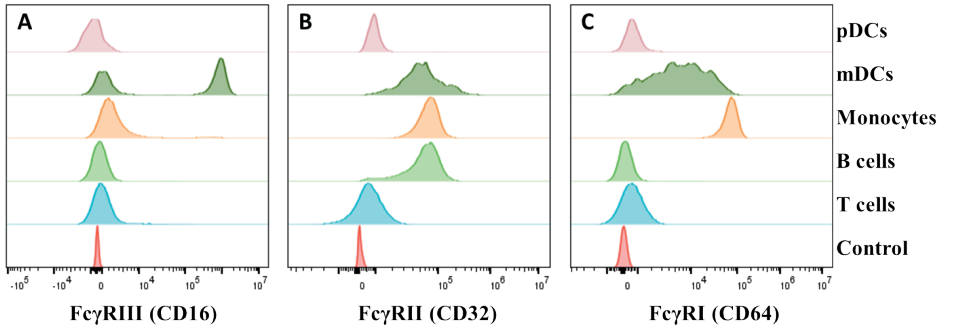

Fig 10. Histograms showing the relative expression of CD16 (A), CD32 (B), and CD64 (C) on T- and B lymphocytes, monocytes, mDCs, and pDCs cells within the PBMC fraction. See Supplementary Fig. S1 regarding the gating strategy for PBMC immunophenotyping.

Table 6. +, the receptor is expressed on the cell; -, the receptor is not expressed on the cell; +/-, the receptor is expressed on a subset of the cell; (+), different levels of receptor expression

Cell type	FcγRIII (CD16)	FcγRII (CD32)	FcγRI (CD64)
T cells	-	-	-
B cells	-	+	-
Monocytes	+/-	+	+
mDCs	+/-	+	(+)
pDCs	-	-	-

Direct binding of bIgG and bIgG-immune complexes to monocytes

To demonstrate binding of bovine IgG to human monocytes, a range of bIgG concentrations was allowed to bind to human PBMCs, and binding to monocytes was detected by flow cytometry using AlexaFlour 647-conjugated anti-bovine IgG antibody. Monocytes were selected since they highly express CD32, the same FcγR that bIgG was shown to bind [102]. Bovine IgG showed a dose-dependent binding to human monocytes, especially at high bIgG concentrations (Fig 2A-B). As we had previously noted the presence of some aggregated bIgG on Native-PAGE, this binding at high bIgG concentrations might be related to the aggregated bIgG. The presence of bIgG aggregates before centrifugation and their removal by centrifugation is shown in Suppl Fig S4.

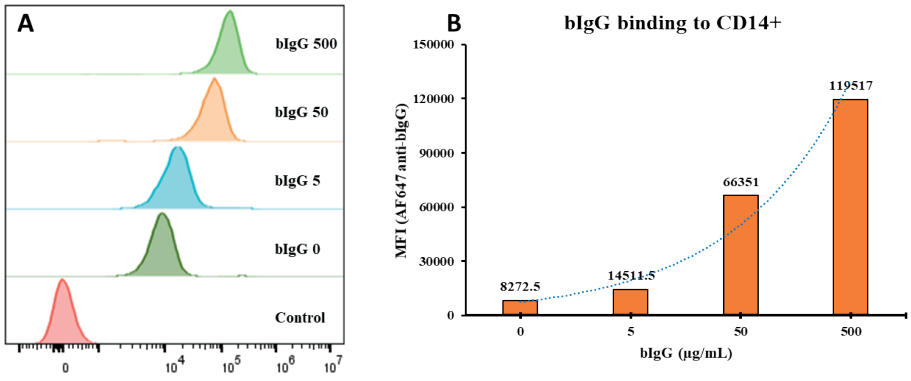

Fig 11. Histogram comparing the MFI of the bIgG signal on CD14⁺ monocytes: PBMCs were incubated with bIgG (500, 50, 5, or 0 µg/mL) for 20 minutes (A). A dose-dependent increase in the bIgG signal on the CD14⁺ monocytes was detected with an increase in the concentration of bIgG used (B).

To study if the binding to FcγRs on monocytes is increased by immune complexes, various concentrations of bIgG were preincubated with the RSV preF protein or with a monoclonal anti-bIgG to allow the formation of ICs before exposing them to the PBMCs. As shown in Fig 3A, we noted a dose-dependent binding of bIgG alone to the CD14⁺ monocytes. However, the combination of bIgG and preF protein (bIgG: preF) showed a higher binding, especially at lower bIgG concentrations used, suggesting that multivalent ICs are formed and bound to the monocytes (Fig 3A). Subtraction of the bIgG signal from the MFI of the bIgG: preF combination (DMFI) resulted in a bell-shaped curve suggestive of immune complex binding (Fig 3B). The DMFI curve had a peak at 10 µg/mL and 50 µg/mL for bIgG and PreF protein, respectively indicative of optimal ratio for the formation of large ICs. We observed comparable findings for additional donors tested, with the bell-shaped curves peaking at 10 µg/mL of bIgG (Suppl. Fig S5A-D) and the average maximum DMFI of about 17k (Fig 3B, Fig S5B & S5D).

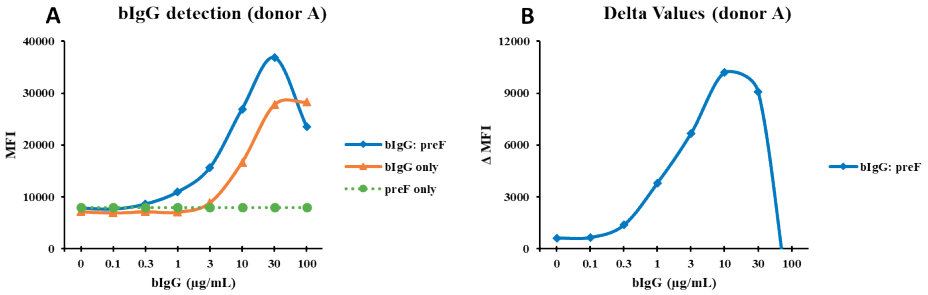

Fig 12. The curves drawn from MFI of the bIgG detection signal: CD14^{+} monocytes exposed to bIgG only (0-100 µg/mL), preF protein only (50 µg/mL), and the bIgG: preF ICs in one representative donor (donor A) and the MFI was used to generate the detection curve(A). The delta MFI (DMFI) was resulted when MFI of bIgG only signal was deducted from the MFI of bIgG: preF ICs signal (B).

As the window between the binding of bIgG: preF ICs and bIgG alone was relatively small, we tried a similar approach by using a monoclonal anti-bIgG (a-bIgG) in the hope that because of the high specificity, the peak of the binding would be at a lower bIgG concentration with even lower bIgG background binding. bIgG bound to human monocytes in a dose-dependent manner while a-bIgG alone did not show any binding (Fig 4A). However, the combination of bIgG and a-bIgG showed a strong increase in binding to the monocytes suggesting IC formation (Fig 4A). This was true for both a-bIgG concentrations that were used (5 and 1 µg/mL). After subtracting the MFI of bIgG alone from the MFI of the ICs, we obtained bell-shaped curves with a peak at bIgG 3 µg/mL (Fig 4B). Similar results were found for additional donors with the bell-shaped curves peaking at 3 µg/mL of bIgG (Suppl. Fig S6A-D) and the average maximum DMFI of about 40k (Fig 4B, Fig S6B & S6D). As the optimal concentrations for the formation of large ICs are at 3 and 5 µg/mL for bIgG and a-bIgG antibody respectively, these concentrations were used in innate immune training experiments.

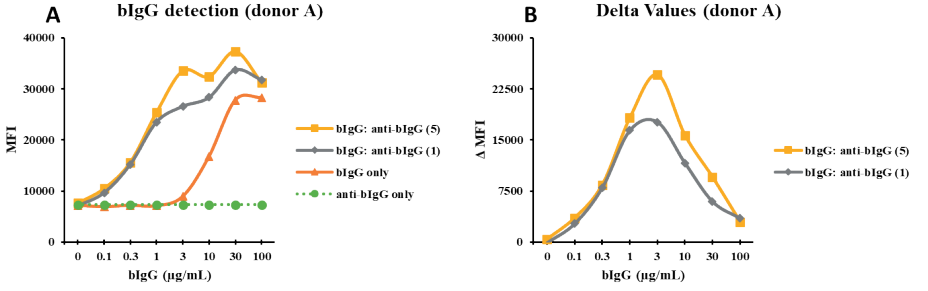

Fig 13. The curves drawn from MFI of the bIgG detection signal: the monocytes exposed to bIgG only (0-100 µg/mL), a-bIgG (5 µg/mL) only, a-bIgG only (1 µg/mL), and the bIgG: a-bIgG ICs in one representative donor (donor A) (A). The delta MFI (DMFI) was resulted when MFI of bIgG only signal was deducted from the MFI of bIgG: a-bIgG ICs signal (B).

bIgG-containing immune complexes and innate immune training

Based on the above findings, we used the optimal ratios of bovine IgG to RSV preF protein or a-bIgG to study if these immune complexes could induce innate immune training *in vitro* at concentrations at which bIgG itself had no effect in this model. The generated ICs were allowed to bind to freshly isolated $CD14^+$ monocytes for 24 hrs, after which the training compounds were removed by washing and resting for 6 days. After this resting period, the cells were stimulated with TLR4 (LPS) or TLR7/8 (R848) ligands. 24h supernatants were collected, and the levels of IL-6 and TNF-a were measured by CBA.

Whole Glucan Particles (WGP) were used as a positive control for training, as described by Moerings et al [191]. The cells trained with WGP produced a significantly higher amount of IL-6 and TNF-a in the culture supernatant upon re-stimulation with LPS (Fig 5A and 5B). We obtained similar results when stimulating the cells with R848, and the cells trained with WGP produced significantly more IL-6 (Fig 5C) and TNF-a (Fig 5D) than untrained cells.

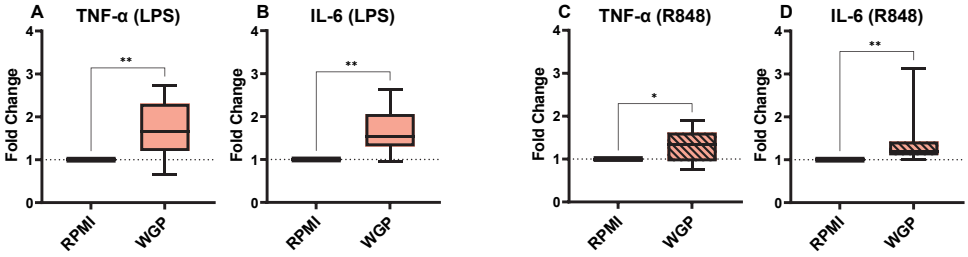

Fig 14. The fold changes in TNF-a (A) and IL-6 (B) production of the cells trained with WGP and stimulated with LPS in comparison to untrained control. The TNF-a (C) and IL-6 (D) production fold changes for WGP trained monocytes after stimulation with R848. The average (Range) of cytokines in the RPMI controls were 1805 (719 - 4825 pg/mL), 3181 (1044 - 7232 pg/mL), 3592 (1839 - 7434 pg/mL), and 9047 (3732 - 14230 pg/mL) for conditions A to D, respectively. The Boxes represent 50% of the data, and the line is the median value where upper and lower whiskers present upper and lower 25% of the data, respectively. The significance of differences is shown as p-value <0.05 (*) and <0.01 (**).

Similarly, IL-6 and TNF-a production levels were determined in supernatants of restimulated monocytes that were trained with RSV preF protein only (50 μg/mL), bIgG only (10 μg/mL), or the ICs of the combination of these. The cells trained with bIgG: preF ICs showed significantly higher (3-4 fold) TNF-a levels compared to the untrained cells, both after stimulation with LPS (Fig 6A) and R848 (Fig 6C). TNF-a levels were not significantly increased in the cells trained with RSV preF protein only or bIgG alone, although some increase was seen in the preF protein group. Despite variation in the responses, all donors exposed to the ICs consistently produced higher levels of TNF-a compared to the untrained monocytes. Even though IL-6 levels were slightly increased after stimulation with the TLR4 ligand LPS (Fig 6B) or TLR7/8 ligand R848 (Fig 6D), this did not reach significance.

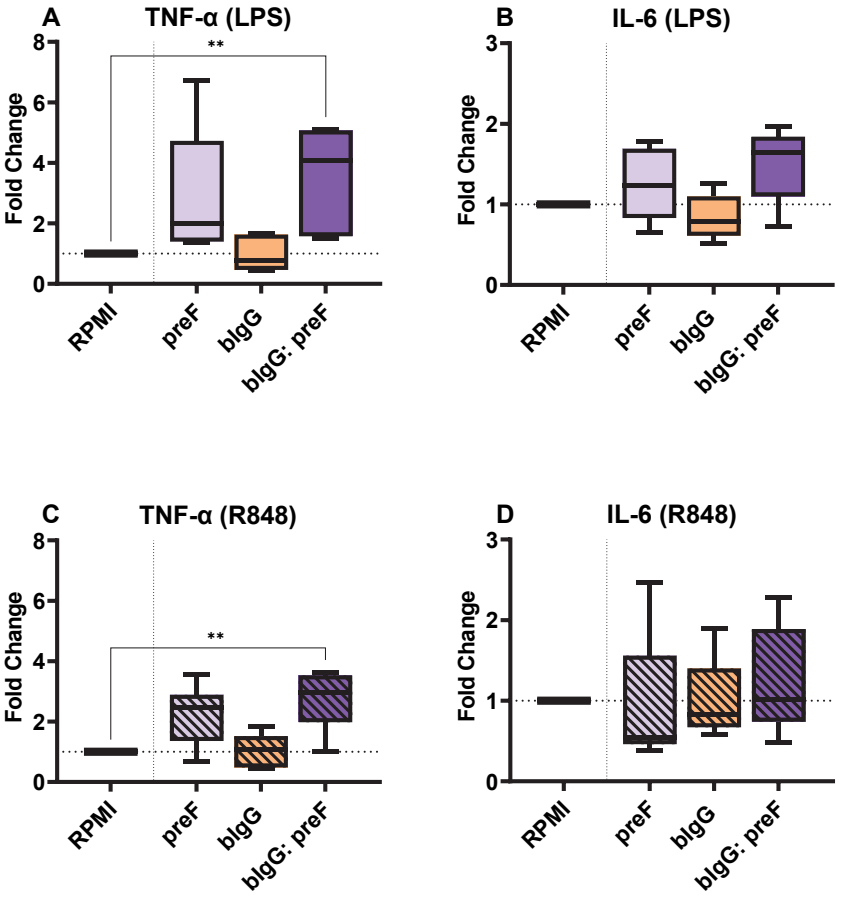

Fig 15. The fold changes in the TNF-α (A) and IL-6 (B) production in the cells trained with preF only (50 μg/mL) bIgG alone (10 μg/mL), or bIgG: preF ICs compared to untrained monocytes (RPMI) upon stimulation with LPS. Also, the TNF-α (C) and IL-6 (D) production fold changes compared to the RPMI control in the group of monocytes trained with single components or the bIgG: preF ICs after stimulation with and R848. The average (Range) of cytokines in the RPMI controls were 1791 (719 - 4825 pg/mL), 3522 (1044 - 7232 pg/mL), 3181 (1839 - 7434 pg/mL), and 9840 (3732 - 14230 pg/mL) for conditions A to D, respectively. The Boxes represent 50% of the data, and the line is the median value where upper and lower whiskers present upper and lower 25% of the data, respectively. The significance of differences is shown as p-value <0.05 (*) and <0.01 (**).

The same experimental setup was performed with bIgG: anti-bIgG ICs, evaluating the training potential of monoclonal anti-bIgG alone (5 μg/mL), bIgG alone (3 μg/mL), and the ICs resulted from combining both. As shown in Figure 7, contrary to the bIgG-RSV preF protein IC, no increases in IL-6 and TNF-a were noted. This holds true for stimulation with either LPS (Fig 7A) or R848 (Fig 7C). Similar to TNF-a, we did not observe any significant variation in the production of IL-6 in different conditions. Neither a-bIgG only, bIgG only, nor the ICs could enhance IL-6 production in monocytes after LPS (Fig 7B) or R848 (Fig 7D) stimulation.

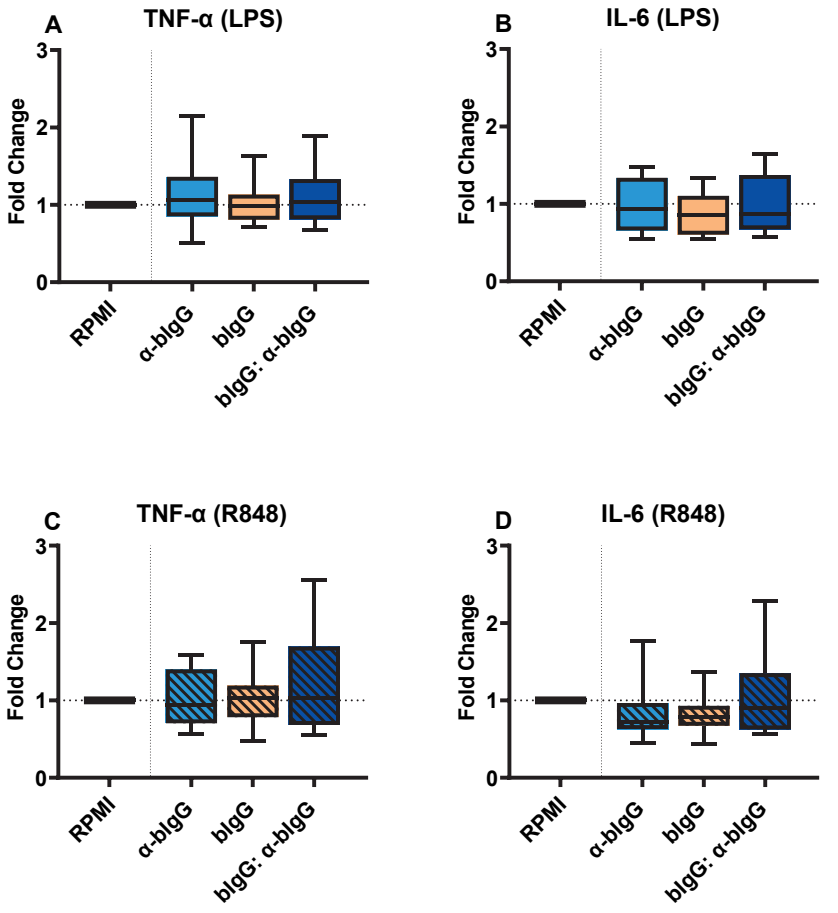

Fig 16. The fold changes in the production of TNF-α (A) and IL-6 (B) in the cells trained with α-bIgG only (5 μg/mL) only, bIgG alone (3 μg/mL), or bIgG: α-bIgG ICs compared to untrained monocytes (RPMI) upon stimulation with LPS and also TNF-α (C) and IL-6 (D) production after R848 stimulation. The average (Range) of cytokines in the RPMI controls were 1740 (719 - 4825 pg/mL), 3279 (1044 - 7232 pg/mL), 3508 (1839 - 7434 pg/mL), and 8961 (3732 - 14230 pg/mL) for conditions A to D, respectively. The Boxes represent 50% of the data, and the line is the median value where upper and lower whiskers present upper and lower 25% of the data, respectively.The significance of differences is shown as p-value <0.05 (*) and <0.01 (**).

Discussion

Here, we show that immune complexes comprising bIgG and RSV preF protein can induce innate immune training in human $CD14^+$ monocytes, while bIgG monomers did not have the same effects. We established and optimized an experimental system for detecting the direct binding of ICs to human monocytes. Using that system, we determined the optimal antibody: antigen ratio for the formation of ICs between bIgG and RSV preF protein as well as bIgG: a-bIgG and tested them on the *in vitro* innate immune training model.

The FcγRII (CD32) family consisting of CD32a, CD32b, and CD32c are key IgG receptors expressed by various leukocytes. While CD32a and CD32c have a Tyrosine-based Activation Motif (ITAM) on their C-terminal cytoplasmic tail mediating the activator signal, CD32b contains Tyrosine-based Inhibitory Motif (ITIM) [179]. CD32 expression has been identified on monocytes, DCs, B cells, as well as neutrophils, eosinophils, basophils, and mast cells [170, 179]. Monocytes/macrophages and DCs eminently express CD32a with an ITAM motif but also CD32b, while B cells exclusively possess the inhibitory CD32b [179]. The interplay between the activator and inhibitory signal regulates the antibody effector functions, including B cell IgG responses, APC maturation, and antigen presentation [192, 193]. CD32 expression on monocytes and DCs is essential for their distinct functions, including ADCP for clearing the pathogens, also known as FcγRII-dependent phagocytosis [194, 195]. Following the phagocytosis of antibody-opsonized targets, the capacity of DCs for activating naive T cells increases [196]. DCs present the antigen epitopes on Major Histocompatibility Complex class II (MHC-II) molecules, upregulate the expression of costimulatory molecules such as CD80/86 to interact with their T cells counterpart (CD28), and produce cytokines to drive T cell differentiation [197]. However, monomeric forms of IgG do not bind to low-affinity receptors such as CD32. Binding to and crosslinking multiple neighboring CD32 is essential for both FcγR-mediated phagocytosis and initiating the signal via the receptors' ITAM motif [198]. In fact, signaling cascades are activated by immunoreceptor aggregation rather than ligand-induced changes in receptor conformation [198]. In other words, the size matters when it comes to the level of IC binding and interaction with the low-affinity FcγRs, and only large multivalent ICs can bind to CD32 and induce the effector function [176, 199]. Large ICs can be formed when an optimal antibody: antigen ratio is present in the environment. When either the antibody or the antigen is in excess, large ICs are not formed, the interaction with CD32 does not occur, and hence, the antibody's effector function is weak [170]. As a result, the concentration-dependent binding of immune complexes to receptors typically results in a bell shaped curve, as confirmed in this paper.

RSV-specific IgG in the breast milk was shown to correlate with protection against RSV acute respiratory infection in the first 6 months of life [186]. Similarly, bovine milk IgG (bIgG) binds to and neutralizes RSV, a major human pathogen associated with respiratory tract infections [102]. Besides, bIgG was shown to interact with the FcγRII on human immune cells, which is essential for exerting the antibody effector functions [102, 111]. The interaction of bIgG with human immune cells conferred protection against experimental RSV infection in mice and also increased activation of RSV-specific human T cells [111]. The augmented T cell activity at low RSV preF protein levels is an effect resulting from the direct interaction of antigen-bound bIgG (ICs) with APCs. The interaction of IC with APCs enhances antigen presentation to RSV-specific T cells. However, the direct binding of bIgG ICs to APCs has not been shown before. In the current study, we succeeded in

generating bIgG and RSV preF protein ICs and demonstrated their direct binding to $CD14^+$ monocytes (Fig 3). The bell-shaped curves on DMFI results indicate that large ICs were formed and bound to CD32 on monocytes. The findings are especially important since we show the binding of bIgG ICs to the APC, an intermediate step linking the previously shown bIgG and preF binding with the consequent enhanced T cell response.

The method described here can be applied in future studies as a proxy to identify the optimal bIgG: antigen ratio for IC formation for additional pathogenic molecules and can also be used to detect IC binding on other immune cells such as neutrophils and DCs. We selected monocytes to study bIgG ICs binding for a number of reasons. Monocytes and B cells both express a high level of CD32 and the results from $CD19^+$ B cells support the findings from $CD14^+$ cells on optimal bIgG: antigen ratios (data not shown). However, unlike B cells that only express inhibitory CD32b, monocytes eminently express CD32a, which is essential for studying the cell-activating properties of bIgG ICs. This includes the innate immune training model applied in this research, which also has been optimized for monocytes.

It should be noted that the study did not address the relevance of the FcRn receptor in bIgG binding to monocytes because the binding of bovine IgG to human monocytes was reported to be mainly CD32 dependent [102]. However, FcRn is expressed on monocytes, macrophages, and DCs [200], and its role in IgG IC-mediated antigen presentation has been demonstrated [182, 183]. As blocking with anti-CD32/CD16 antibodies [102] or anti-CD16/32/64 [201] cannot completely block binding of bIgG to monocytes and granulocytes, a role for FcRn cannot be excluded.

Concurrent engagement and crosstalk between FcγRs and Pattern Recognition Receptors (PRRs) are critical for identifying and eliminating the pathogen [202, 203]. TLRs and C-type Lectin Receptors (CLRs) are among the PRRs that were found to be involved in this crosstalk which is necessary for the induction of inflammatory mediators such as IL-6 and TNF-a [202, 203]. On the other hand, trained immunity is mediated by the involvement of CLRs, such as Dectin-1 as it was demonstrated for β-1, 3-(D)-glucan derived from *C. albicans* [38]. After training, the quality of cell responses improves, as evidenced by increased IL-6 and TNF-a production in response to TLR re-stimulation [39]. This trained immunity results in enhanced innate immune responses to a wide array of TLR signals, resulting in improved protection against infection. Given that bIgG has been demonstrated to possess training abilities and interact with FcγRs, we hypothesized that these receptors would play a role in monocyte training. Immune complexes internalized via the engagement of FcγRs can stimulate endosomal or cytoplasmic PRRs like TLRs to further activate the cells [204-208]. If the assumption holds true, ICs may be more potent training-inducing components than bIgG alone.

Although the training effects of bIgG have been demonstrated previously [46, 47], the relevance of ICs in the training effects has not been studied. CD64 receptors on monocytes freshly isolated from human blood are occupied by human serum IgG, leaving no room for bIgG monomers to bind. Monomeric forms of bIgG, on the other hand, do not interact with the low-affinity CD32. Therefore, if we assume that the training potential of bIgG is (partly) exerted via interacting with FcγRs, the engagement of bIgG ICs and CD32 is critical. The ICs can crosslink several receptors and induce a much stronger effector signal. Surprisingly, although no antigen was added to the bIgG preparations and therefore no ICs are expected, the training effects were still evident in the previous studies [46, 47]. The explanation could be the within bIgG stock itself. IgG molecules tend towards aggregation, particularly after (long) storage in the freezer [209]. It is likely that the antibody aggregates present in the bIgG stock have mimicked IC properties and could be responsible for the previously observed training effects of bIgG alone, especially since they used high concentrations of the IgG.

We exposed monocytes to bIgG monomers and bIgG: preF ICs as the training agents to address this hypothesis. The experiment's positive control, WGP (a Dectin-1 agonist with established training potential [191]), ensured the validity of the model system used. Interestingly, incubation with the ICs increased the TNF-a production in the cells upon TLR stimulation (Fig 6). Pair-wise comparisons showed that IC-trained cells produced 2-4 times higher TNF-a than the untrained cells upon LPS and R848 stimulation. Remarkably, the training effect was not seen for the monomeric forms of the bIgG. This is in line with the fact that the bIgG alone concentration used is too low to induce innate immune training.

Given the increased TNF-a response, it appears that there is a general increase in the vigilance of monocyte. The heightened response was observed towards not only TLR7/8 activation with R848 but also LPS. The ICs contained RSV preF protein as a viral protein. Interestingly, higher TNF-a levels were also produced by IC-trained cells after stimulation of TLR4 with LPS, a compound found in the membrane of gram-negative bacteria. A documented aspect of trained immunity is an increase in the responsiveness of the cells to the same but also homologous stimuli [210]. Nevertheless, further research on cells' epigenetic changes and metabolic pathways is required to substantiate this notion.

The monocytes trained with bIgG: preF ICs also produced a relatively higher IL-6 than the untrained cells in response to LPS stimulation (Fig 6B). However, the changes did not reach statistical significance, probably due to higher variation in the response of the donors. In fact, TNF-a was previously described as a better indicator of innate immune training by bIgG than IL-6 [47]. However, the RSV preF protein alone seemed to induce some increase in production of TNF-a and IL-6 in these assays, although this did not

reach significance. Interestingly, the preF protein was recently shown to bind to Insulin-Like Growth Factor 1 (IGFR1) for cellular entry [211]. IGF, the natural ligand for this receptor, has been shown to induce innate immune training [212], which can explain why the preF protein by itself has an effect on this model system as well. Besides, viruses and viral proteins may be internalized given their antigenic nature, without the involvement of antibodies. When they are internalized, they can activate cytoplasmic or endosomal PRRs, inducing inflammatory responses. This fact could also partly explain why monocytes treated with RSV preF alone released more cytokines. More investigation is needed to determine what we described is the reason or whether a contaminant in the preparation caused the effect.

Contrary to our expectations, incubation with IC consisting of bIgG with a murine a-bIgG monoclonal antibody (mAb) did not induce monocyte training (Fig 7). The optimal ratio for bIgG and the a-bIgG antibody to form ICs was identified, and even the average of maximum DMFI for the a-bIgG ICs on the three donors tested was more than twice the level of the preF ICs (40k vs. 17k). This difference, in theory, should give a bigger window for the effects of a-bIgG ICs than preF ICs, which was not the case in practice. A possible explanation of these findings is that lower bIgG concentrations were used to generate ICs with a-bIgG than RSV preF protein (3 vs. 10 ug/mL). In addition, it is known that murine IgG does not bind efficiently to human CD32 [175]. This could result in ICs that consist of bIgG, of which the Fc region is mostly blocked by the murine mAb. As a result, in a-bIgG ICs, fewer bIgG molecules may be available to interact with CD32 on the monocytes, which may not result in efficient crosslinking and uptake of the IC. As the binding of the IC was detected with a polyclonal anti bIgG, higher levels of binding were detected in the FACS analysis of these IC. Another highly likely explanation for no training effects could be the absence of the antigen. IgG bound to antigen likely sends a different signal via FcγRs than an antibody linked to another antibody, possibly due to different IgG Fc glycosylation patterns between the different antibodies used [172, 213]. Besides, when the ICs that contain antigens are internalized, other endosomal or cytoplasmic PRRs such as CLRs and TLRs may become activated and synergistically complement the FcγRs signal, as discussed earlier. However, further investigation is necessary before ascertaining these claims.

In conclusion, we established a method for detecting the direct binding of bIgG-containing ICs to monocytes by flow cytometry. Our results also indicate that bIgG: preF ICs can induce monocyte training *in vitro*. The effects could be at least partly mediated by the interaction of bIgG ICs with the CD32 receptors on the monocytes, as this interaction was shown before. However, to formally prove the putative role of bIgG ICs in trained immunity, this has to be investigated with more antigen-bIgG ICs and should be extended to also include human IgG-antigen ICs.

References

1. Macpherson, A.J., M.G. de Agüero, and S.C. Ganal-Vonarburg, *How nutrition and the maternal microbiota shape the neonatal immune system.* Nature Reviews Immunology, 2017. **17**: p. 508.
2. Hurley, W.L. and P.K. Theil, *Perspectives on immunoglobulins in colostrum and milk.* Nutrients, 2011. **3**(4): p. 442-474.
3. World Health organization, *Infant and Young Child Nutrition. Global Strategy on Infant and Young Child Feeding.* 2002.
4. Victora, C.G., et al., *Breastfeeding in the 21st century: epidemiology, mechanisms, and lifelong effect.* The Lancet, 2016. **387**(10017): p. 475-490.
5. Chen, K., et al., *Effect of bovine lactoferrin from iron-fortified formulas on diarrhea and respiratory tract infections of weaned infants in a randomized controlled trial.* Nutrition, 2016. **32**(2): p. 222-227.
6. Abbring, S., et al., *Raw cow's milk consumption and allergic diseases – The potential role of bioactive whey proteins.* European Journal of Pharmacology, 2019. **843**: p. 55-65.
7. Esch, B.C.A.M.v., et al., *The Impact of Milk and Its Components on Epigenetic Programming of Immune Function in Early Life and Beyond: Implications for Allergy and Asthma.* Frontiers in Immunology, 2020. **11**(2141).
8. Ulfman, L.H., et al., *Effects of Bovine Immunoglobulins on Immune Function, Allergy, and Infection.* Frontiers in Nutrition, 2018. **5**(52).
9. van Neerven, R.J.J., et al., *Which factors in raw cow's milk contribute to protection against allergies?* Journal of Allergy and Clinical Immunology, 2012. **130**(4): p. 853-858.
10. Perdijk, O., et al., *Cow's Milk and Immune Function in the Respiratory Tract: Potential Mechanisms.* Frontiers in Immunology, 2018. **9**(143).
11. van Neerven, J., *The effects of milk and colostrum on allergy and infection: Mechanisms and implications*. Vol. 4. 2014. 16-22.
12. van Kempen, M.J.P., G.T. Rijkers, and P.B. van Cauwenberge, *The Immune Response in Adenoids and Tonsils.* International Archives of Allergy and Immunology, 2000. **122**(1): p. 8-19.
13. Govers, C., et al., *Ingestion, Immunity, and Infection: Nutrition and Viral Respiratory Tract Infections.* Frontiers in Immunology, 2022. **13**.
14. den Hartog, G., et al., *Specificity and Effector Functions of Human RSV-Specific IgG from Bovine Milk.* PLOS ONE, 2014. **9**(11): p. e112047.
15. Lu, L.L., et al., *Beyond binding: antibody effector functions in infectious diseases.* Nature Reviews Immunology, 2017. **18**: p. 46.
16. Bournazos, S., A. Gupta, and J.V. Ravetch, *The role of IgG Fc receptors in antibody-dependent enhancement.* Nature Reviews Immunology, 2020. **20**(10): p. 633-643.
17. Hayes, J.M., et al., *Fc gamma receptors: glycobiology and therapeutic prospects.* Journal of inflammation research, 2016. **9**: p. 209-219.
18. Nimmerjahn, F. and J.V. Ravetch, *Fcγ receptors as regulators of immune responses.* Nature Reviews Immunology, 2008. **8**: p. 34.
19. Swisher, J.F.A. and G.M. Feldman, *The many faces of FcγRI: implications for therapeutic antibody*

function. Immunological Reviews, 2015. **268**(1): p. 160-174.
20. Bruhns, P., *Properties of mouse and human IgG receptors and their contribution to disease models.* Blood, 2012. **119**(24): p. 5640-5649.
21. Bruhns, P., et al., *Specificity and affinity of human Fcγ receptors and their polymorphic variants for human IgG subclasses.* Blood, 2009. **113**(16): p. 3716-3725.
22. Chen, X., et al., *FcγR-Binding Is an Important Functional Attribute for Immune Checkpoint Antibodies in Cancer Immunotherapy.* Frontiers in immunology, 2019. **10**: p. 292-292.
23. Holgado, M.P., et al., *CD32 Ligation Promotes the Activation of CD4+ T Cells.* Frontiers in Immunology, 2018. **9**(2814).
24. Anania, J.C., et al., *The Human FcγRII (CD32) Family of Leukocyte FcR in Health and Disease.* Frontiers in Immunology, 2019. **10**(464).
25. Simister, N.E., *Placental transport of immunoglobulin G.* Vaccine, 2003. **21**(24): p. 3365-3369.
26. Ober, R.J., et al., *Visualizing the Site and Dynamics of IgG Salvage by the MHC Class I-Related Receptor, FcRn.* The Journal of Immunology, 2004. **172**(4): p. 2021-2029.
27. Qiao, S.-W., et al., *Dependence of antibody-mediated presentation of antigen on FcRn.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(27): p. 9337-9342.
28. Weflen, A.W., et al., *Multivalent immune complexes divert FcRn to lysosomes by exclusion from recycling sorting tubules.* Molecular biology of the cell, 2013. **24**(15): p. 2398-2405.
29. Nederend, M., et al., *Bovine IgG Prevents Experimental Infection With RSV and Facilitates Human T Cell Responses to RSV.* Frontiers in immunology, 2020. **11**: p. 1701-1701.
30. Mohapatra, S.S. and S. Boyapalle, *Epidemiologic, Experimental, and Clinical Links between Respiratory Syncytial Virus Infection and Asthma.* Clinical Microbiology Reviews, 2008. **21**(3): p. 495-504.
31. Régnier, S.A. and J. Huels, *Association Between Respiratory Syncytial Virus Hospitalizations in Infants and Respiratory Sequelae: Systematic Review and Meta-analysis.* The Pediatric Infectious Disease Journal, 2013. **32**(8): p. 820-826.
32. Mazur, N.I., et al., *Breast Milk Prefusion F Immunoglobulin G as a Correlate of Protection Against Respiratory Syncytial Virus Acute Respiratory Illness.* The Journal of Infectious Diseases, 2018. **219**(1): p. 59-67.
33. Xu, M.L., et al., *The effect of dietary bovine colostrum on respiratory syncytial virus infection and immune responses following the infection in the mouse.* Journal of Microbiology, 2015. **53**(9): p. 661-666.
34. van Splunter, M., et al., *Induction of Trained Innate Immunity in Human Monocytes by Bovine Milk and Milk-Derived Immunoglobulin G.* Nutrients, 2018. **10**(10): p. 1378.
35. Hellinga, A.H., et al., *In Vitro Induction of Trained Innate Immunity by bIgG and Whey Protein Extracts.* International Journal of Molecular Sciences, 2020. **21**(23): p. 9077.
36. Netea, M.G., et al., *Trained immunity: A program of innate immune memory in health and disease.* Science, 2016. **352**(6284).
37. Divangahi, M., et al., *Trained immunity, tolerance, priming and differentiation: distinct immunological processes.* Nature Immunology, 2021. **22**(1): p. 2-6.
38. Quintin, J., et al., *Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes.* Cell host & microbe, 2012. **12**(2): p. 223-232.

39. Domínguez-Andrés, J., L.A.B. Joosten, and M.G. Netea, *Induction of innate immune memory: the role of cellular metabolism.* Current Opinion in Immunology, 2019. **56**: p. 10-16.
40. Miyake, Y., et al., *C-type Lectin MCL Is an FcRγ-Coupled Receptor that Mediates the Adjuvanticity of Mycobacterial Cord Factor.* Immunity, 2013. **38**(5): p. 1050-1062.
41. Cheng, S.-C., et al., *mTOR- and HIF-1α-mediated aerobic glycolysis as metabolic basis for trained immunity.* Science (New York, N.Y.), 2014. **345**(6204): p. 1250684-1250684.
42. McLellan, J.S., et al., *Structure-Based Design of a Fusion Glycoprotein Vaccine for Respiratory Syncytial Virus.* Science, 2013. **342**(6158): p. 592-598.
43. Widjaja, I., et al., *Characterization of Epitope-Specific Anti-Respiratory Syncytial Virus (Anti-RSV) Antibody Responses after Natural Infection and after Vaccination with Formalin-Inactivated RSV.* Journal of Virology, 2016. **90**(13): p. 5965-5977.
44. Domínguez-Andrés, J., et al., *In vitro induction of trained immunity in adherent human monocytes.* STAR protocols, 2021. **2**(1): p. 100365-100365.
45. Moerings, B.G.J., et al., *Continuous Exposure to Non-Soluble β-Glucans Induces Trained Immunity in M-CSF-Differentiated Macrophages.* Frontiers in immunology, 2021. **12**: p. 672796-672796.
46. Hjelm, F., et al., *Antibody-Mediated Regulation of the Immune Response.* Scandinavian Journal of Immunology, 2006. **64**(3): p. 177-184.
47. van Erp, E.A., et al., *Fc-Mediated Antibody Effector Functions During Respiratory Syncytial Virus Infection and Disease.* Frontiers in Immunology, 2019. **10**(548).
48. Tay, M.Z., K. Wiehe, and J. Pollara, *Antibody-Dependent Cellular Phagocytosis in Antiviral Immune Responses.* Frontiers in Immunology, 2019. **10**(332).
49. Underhill, D.M. and H.S. Goodridge, *Information processing during phagocytosis.* Nature reviews. Immunology, 2012. **12**(7): p. 492-502.
50. Boross, P., et al., *FcRγ-chain ITAM signaling is critically required for cross-presentation of soluble antibody-antigen complexes by dendritic cells.* Journal of immunology (Baltimore, Md. : 1950), 2014. **193**(11): p. 5506-5514.
51. Pennock, N.D., et al., *T cell responses: naive to memory and everything in between.* Advances in physiology education, 2013. **37**(4): p. 273-283.
52. Jaumouillé, V. and S. Grinstein, *Receptor mobility, the cytoskeleton, and particle binding during phagocytosis.* Current Opinion in Cell Biology, 2011. **23**(1): p. 22-29.
53. Lux, A., et al., *Impact of Immune Complex Size and Glycosylation on IgG Binding to Human FcγRs.* The Journal of Immunology, 2013. **190**(8): p. 4315.
54. Zhu, X., et al., *MHC class I-related neonatal Fc receptor for IgG is functionally expressed in monocytes, intestinal macrophages, and dendritic cells.* Journal of immunology (Baltimore, Md. : 1950), 2001. **166**(5): p. 3266-3276.
55. Kramski, M., et al., *Anti-HIV-1 antibody-dependent cellular cytotoxicity mediated by hyperimmune bovine colostrum IgG.* European journal of immunology, 2012. **42**(10): p. 2771-2781.
56. van Egmond, M., G. Vidarsson, and J.E. Bakema, *Cross-talk between pathogen recognizing Toll-like receptors and immunoglobulin Fc receptors in immunity.* Immunological Reviews, 2015. **268**(1): p. 311-327.

57. Rittirsch, D., et al., *Cross-Talk between TLR4 and FcγReceptorIII (CD16) Pathways.* PLOS Pathogens, 2009. **5**(6): p. e1000464.
58. Saeed, S., et al., *Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity.* Science (New York, N.Y.), 2014. **345**(6204): p. 1251086-1251086.
59. Means, T.K., et al., *Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9.* The Journal of clinical investigation, 2005. **115**(2): p. 407-417.
60. Bunk, S., et al., *Internalization and Coreceptor Expression Are Critical for TLR2-Mediated Recognition of Lipoteichoic Acid in Human Peripheral Blood.* The Journal of Immunology, 2010. **185**(6): p. 3708-3717.
61. Parcina, M., et al., *Staphylococcus aureus-Induced Plasmacytoid Dendritic Cell Activation Is Based on an IgG-Mediated Memory Response.* The Journal of Immunology, 2008. **181**(6): p. 3823-3833.
62. Boulé, M.W., et al., *Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes.* The Journal of experimental medicine, 2004. **199**(12): p. 1631-1640.
63. Lovgren, T., *Induction of interferon-alpha by immune complexes or liposomes containing systemic lupus erythematosus autoantigen-and Sjogren's syndrome autoantigen-associated RNA.* Arthritis Rheum, 2006. **54**: p. 1917-1927.
64. Miller, M.A., et al., *Frozen-State Storage Stability of a Monoclonal Antibody: Aggregation is Impacted by Freezing Rate and Solute Distribution.* Journal of Pharmaceutical Sciences, 2013. **102**(4): p. 1194-1208.
65. Netea, M.G. and J.W.M. van der Meer, *Trained Immunity: An Ancient Way of Remembering.* Cell Host & Microbe, 2017. **21**(3): p. 297-300.
66. Griffiths, C.D., et al., *IGF1R is an entry receptor for respiratory syncytial virus.* Nature, 2020. **583**(7817): p. 615-619.
67. Bekkering, S., et al., *Metabolic Induction of Trained Immunity through the Mevalonate Pathway.* Cell, 2018. **172**(1): p. 135-146.e9.
68. Lux, A. and F. Nimmerjahn. *Impact of Differential Glycosylation on IgG Activity*. 2011. New York, NY: Springer New York.

Supplementary data

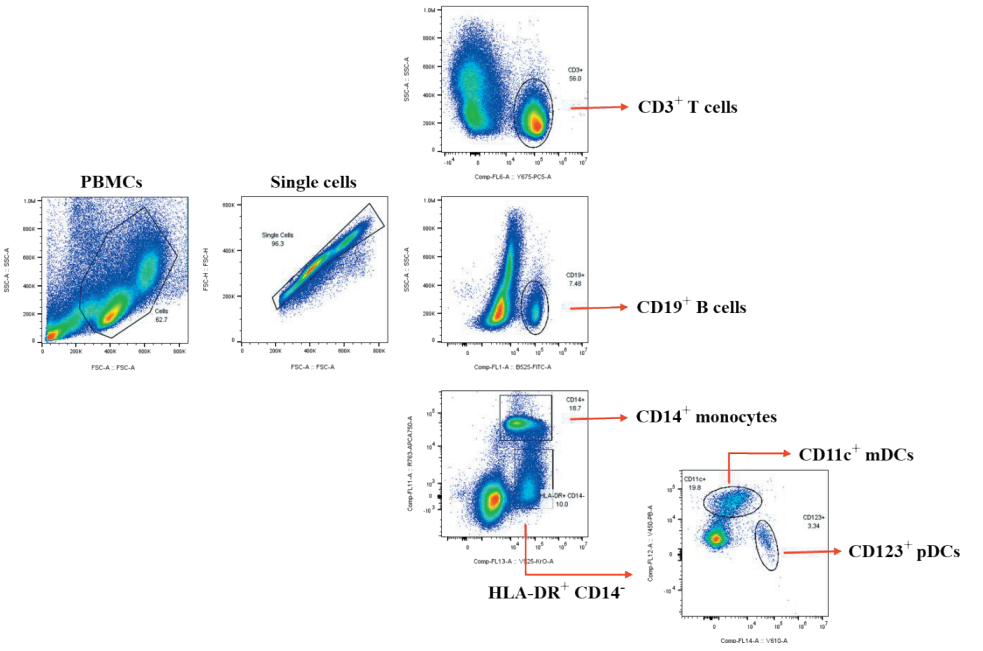

Fig S1. The gating strategy to identify T- and B cells, monocytes, mDC, and pDCs. Afterward, CD16, CD32, and CD64 expression levels were determined within each cell population.

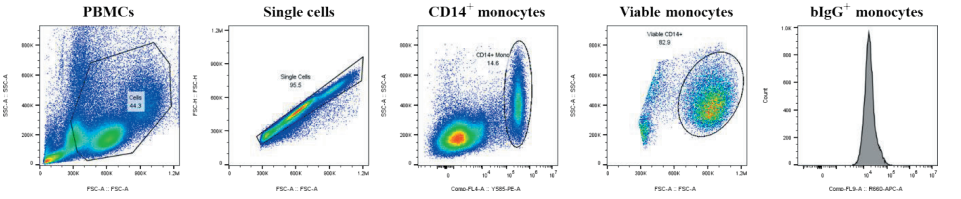

Fig S2. The gating strategy for selecting the CD14+ monocytes and quantifying the MFI of the bIgG signal.

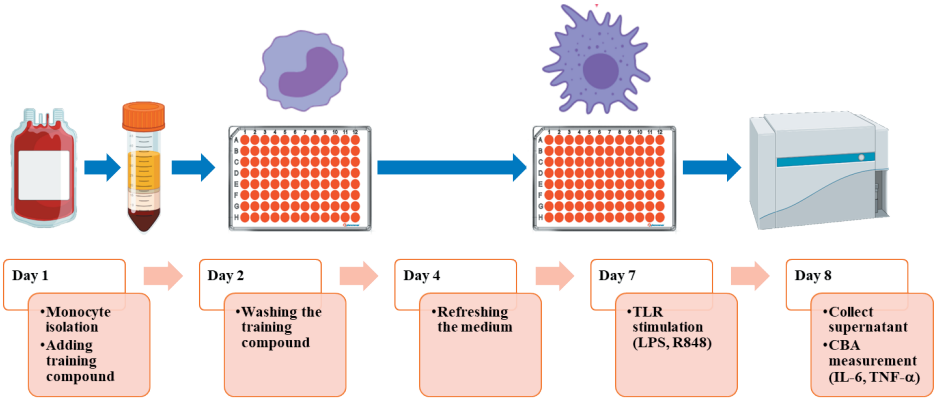

Fig S3. A schematic representation of the *in vitro* innate immune training model. CD14^{+} monocytes were isolated from the buffy coats, exposed to the training compounds for 24 hours, and rested for six days to differentiate into macrophages. Then the cells were stimulated for 24 hours with TLR ligands (LPS and R848), and the production of IL-6 and TNF-a was quantified in the culture supernatant of the cells.

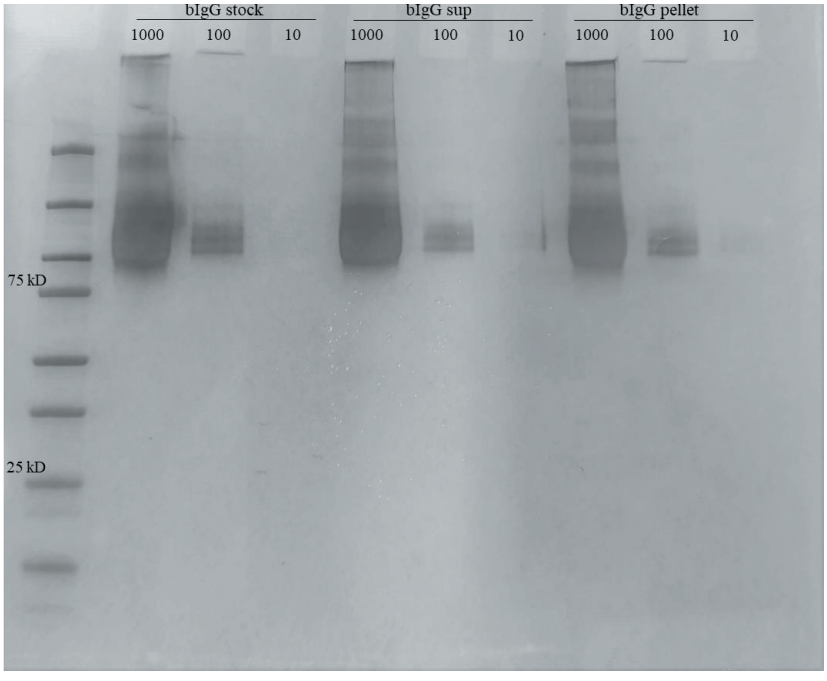

Fig S4. Native-PAGE gel from bIgG samples. The bIgG stock was spun down to remove the aggregates. Different concentrations (1000, 100, 10 µg/mL) of the stock itself, the supernatant after centrifugation, and the pellet were prepared in a non-reducing non-denaturing sample buffer (without SDS) and were loaded on the 4-15% 4–15% Mini-PROTEAN TGX Precast Protein Gel (Bio-RAD #456-1083). The gel was run at 110v for 75 min. The silver staining was used to stain the gel.

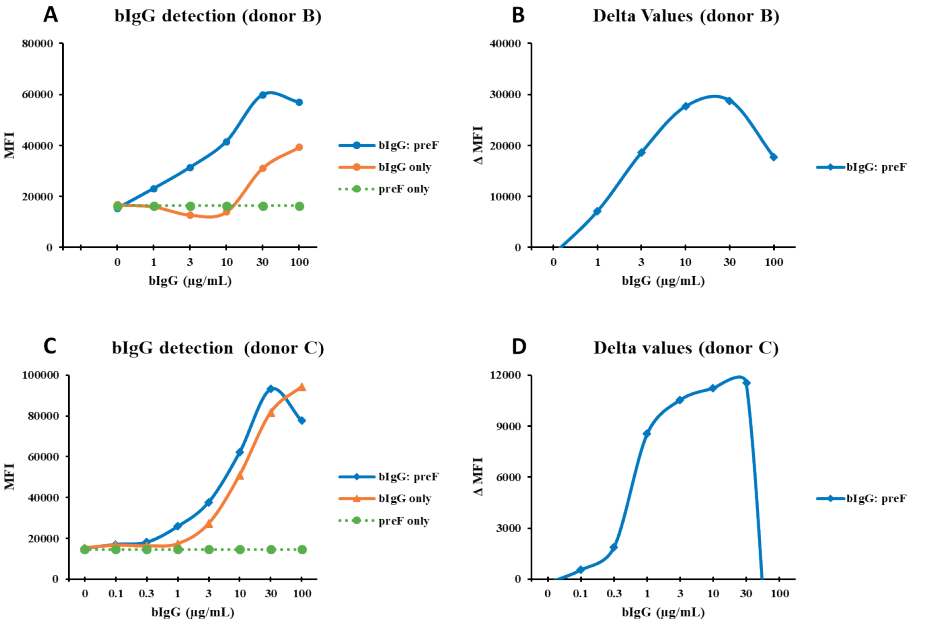

Fig S5. The curves drawn from MFI of the bIgG detection signal: The generation of bIgG detection curve for bIgG only, preF protein only, and the bIgG: preF ICs on monocytes of two selected donors (A & C). The delta MFI values resulted from the deduction of bIgG background values from the ICs curve (B & D).

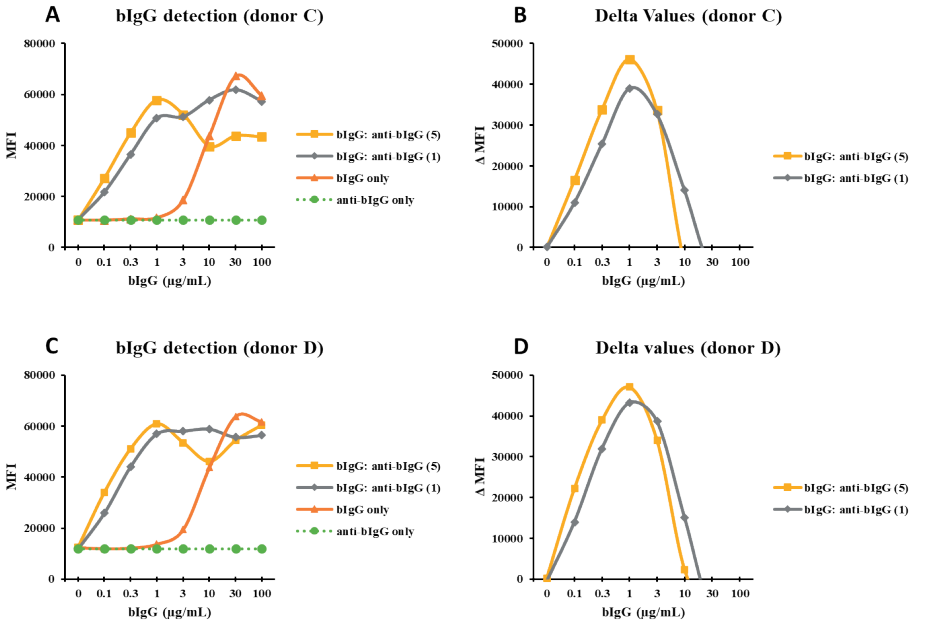

Fig S6. The curves drawn from MFI of the bIgG detection signal: bIgG detection curves were generated for bIgG only, a-bIgG (5 µg/mL) only, a-bIgG (1 µg/mL) only, and the ICs on monocytes of two selected donors (A & C). The delta MFI values resulted from the deduction of bIgG background values from the ICs curve (B & D).

CHAPTER

Dietary intervention with whey protein concentrate does not affect Toll-like receptor responses and gene expression patterns in peripheral blood mononuclear cells of healthy volunteers

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Manuscript in preparation

Abstract

Bovine milk contains components like bioactive proteins, carbohydrates, and phospholipids that possess immunomodulatory properties and affect the human immune system. These components may contribute to resistance to infections and allergies and modulate the immune system via various mechanisms. One putative mechanism is enhancing the innate immune response to secondary pathogen-related stimuli, a concept referred to as innate immune training. While milk components such as milk immunoglobulin G (IgG) have been shown to train human monocytes *in vitro*, evidence for *in vivo* innate immune training is scarce. To investigate if bovine IgG can also induce innate immune training *in vivo*, a human study was conducted using an IgG-rich whey protein concentrate (WPC). Healthy male volunteers consumed a high dose (n=19) or a low dose of WPC (n=10) or were placed in the placebo group (n=19). Blood samples were taken prior to and after two weeks of nutritional intervention. Peripheral blood mononuclear cells (PBMC) were isolated and activated with TLR ligands: LPS, flagellin, and whole diarrheagenic *E. coli*, and the production of IL-6 and TNF-a by monocytes, myeloid DCs, and plasmacytoid DCs was evaluated. In addition, RNA was isolated, and differential gene expression (DGE) analysis was performed on participants' PBMCs to determine the influence of WPC on intervention-induced changes in gene expression patterns. The two-week dietary intervention with WPC did not affect monocytes and mDCs' *ex vivo* response to TLR agonists. Furthermore, after 14 days of WPC consumption, there was no significant difference between PBMCs gene expression pattern of the placebo and the WPC high dose group. The data indicate that oral ingestion of WPC did not enhance the immune responses of young, healthy male participants.

Introduction

Milk is a complex fluid containing hundreds of components that support healthy growth and development, including proteins, lipids, carbohydrates, and micronutrients. Besides its nutritional values, several milk components have been shown to have immunomodulatory effects [214, 215]. In their intact bioactive forms, these components influence physiological processes at multiple levels and locations and can, for example, modulate innate and adaptive immune responses, as well as microbiota composition, ultimately contributing to immune health [100, 106, 168]. In combination with the extensive consumption of bovine milk and the use of milk in early life nutrition, milk or its components are attractive candidates for nutritional intervention strategies.

Immunologically active components of the bovine milk were shown to decrease infection and allergy incidence [3-5]. Milk-fat-globule membrane (MFGM) ingestion increased the resistance to diarrheagenic *E. coli* in healthy adults [216], and fortifying infant formula with bovine milk lactoferrin was shown to reduce the incidence of diarrhea and respiratory tract infections in weaned infants [165]. Moreover, consumption of bovine immunoglobulins from milk or colostrum of immunized cows decreased enterotoxigenic *E.coli* (ETEC)-induced diarrhea [217, 218] as well as rotavirus infection [96, 219]. In addition, infants consuming raw cow's milk have a reduced incidence of common respiratory infections [97], whereas TGF-b and other components present in raw bovine milk, contribute to establishing a regulatory environment that decreases T helper 2 (Th2) responses associated with allergic reactions [92].

Interestingly, milk-derived IgG has been shown to improve the responsiveness of human monocytes *in vitro* when stimulated with Toll-like receptor (TLR) ligands [46, 47]. This phenomenon is known as innate immune training or trained immunity. In this concept, monocytes are primed to have an increased response to pathogen-derived TLR ligands after the primary exposure to a training-inducing component [29, 53]. These changes are linked to epigenetic reprogramming of the cells at the chromatin organization level, including DNA methylation and a shift in the cellular metabolism [29]. The last two studies mentioned above used raw milk, purified bIgG, and whey protein concentrate (WPC) high in bIgG as training agents *in vitro,* leading to enhanced IL-6 and TNF-a production after stimulation with the TLR ligands LPS (TLR 4) and R848 (TLR7/8) [46, 47]. Bovine IgG is not thought to reach circulation after ingestion [220], but it interacts with immune cells in the gastrointestinal tract mucosa and the tonsillar crypts in Waldeyer's ring. However, it is not clear if the *in vitro* findings can be extrapolated to enhanced monocyte responses and gene expression patterns *in vivo* after nutritional intervention with food ingredients rich in bovine IgG.

We have recently shown that primary infection of healthy volunteers with attenuated enterotoxigenic *E. coli* protected from reinfection with the same diarrheagenic *E.coli* strain, accompanied by an increase in monocyte and dendritic cell responses to the homologous *E. coli*, as well as to other TLR ligands. The data suggest that primary diarrheagenic *E.coli* infection may induce innate immune training *in vivo* (Porbahaie et al. in prep).

A recent study was conducted on the impact of consuming a whey protein concentrate (WPC) on diarrheagenic E.coli infection using the same E.coli challenge model (Ulfman et al. 2022). WPC (Vivinal MFGM) is a whey protein concentrate containing bioactive whey proteins (including high levels of bovine IgG), MFGM, and phospholipids. For the current study, blood samples were analyzed from a randomly selected subgroup of the participants of the study mentioned above. To assess if nutritional intervention with WPC can enhance immune responses in vivo, as previously seen for primary diarrheagenic E. coli infection, we examined whether dietary intervention with bovine IgG-rich WPC improves monocyte and dendritic cell (DC) response to diarrheagenic E. coli and other TLR stimuli ex vivo. In addition, we studied whether nutritional intervention with WPC influenced the gene expression patterns in Peripheral Blood Mononuclear Cells (PBMCs) of the participants by isolating RNA and performing differential gene expression (DGE) analysis.

Material and Methods

Study design, participants, and specimens

The study protocol was approved by the Medical Ethics Committee (METC) of Brabant, Tilburg, the Netherlands (July 2019), and registered as NL66645.028.18. In addition, the study was recorded with the Netherlands Trial Register as NTR7613.

The main study enrolled 120 healthy male volunteers (age 18-55 years) who met all of the inclusion criteria and none of the exclusion criteria (supplementary data). Participants were randomly assigned to one of three treatment groups (n=40 per group) throughout this double-blind, parallel 4-week intervention trial: control hydrolyzed whey product (placebo), high dose, or low dose of the study product, WPC. The participants were instructed to maintain their usual physical activity and food intake while limiting their calcium intake to a maximum of 500 mg/day. Participants ingested the study product twice daily for four weeks: the high dose group (23 g/serving of WPC), the low dose group (11 g/serving of WPC supplemented with 12 g/serving control whey hydrolysate), and the placebo group (23 gr/serving of control whey hydrolysate). The participants were orally challenged with 1E10 CFU of a live attenuated *E. coli* strain E1392/75-2A on day 14 of the study. A separate paper discusses the evaluation of clinical effects following the infectious challenge (Ulfman et al. 2022).

The present research examined a randomly chosen subpopulation of the main study (Ulfman et al. 2022). Randomization was done by using ResearchManager software (ResearchManager, The Netherlands). Blood samples were taken from 48 participants (placebo n=19, WPC low dose n=10, WPC high dosage n=19) at baseline (day 1) and on day 14 of the trial, shortly prior to the infectious challenge. The aim was to evaluate the *ex vivo* response of PBMCs to TLR ligands and PBMCs gene expression analyses.

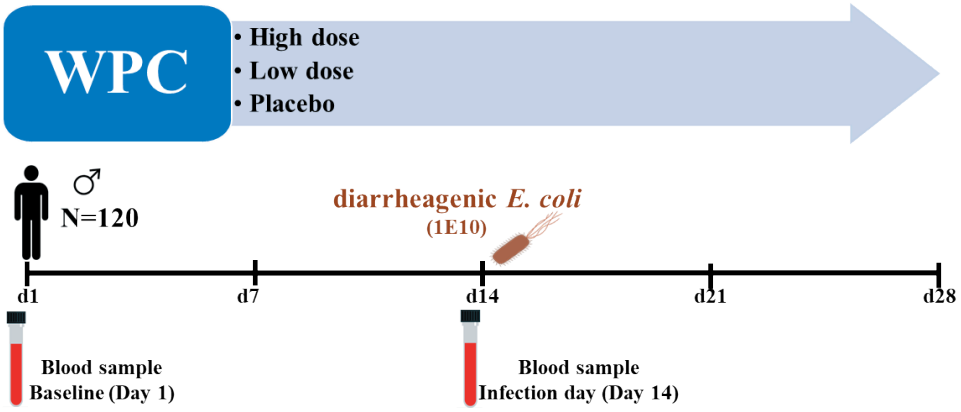

Fig. 17. Graphical presentation of the study events: 120 healthy male volunteers were included in the main study. They were randomized into three dose groups: WPC high dose, WPC low dose, and placebo. After two weeks of product consumption, they were orally challenged with 1E10 CFU of diarrheagenic E.coli strain E1392/75-2A. Blood samples were collected on day 1 (baseline) and day 14 (challenge day) from 48 randomly selected participants for the current study.

Ex vivo stimulation of monocytes, mDC, and pDC by diarrheagenic *E.coli* and TLR-ligands

PBMC isolation

On days 1 (baseline) and 14 (challenge), participants' whole blood was taken in BD Vacutainer CPT™ tubes (Becton Dickinson 362761, Franklin Lakes, NJ, USA). To isolate PBMCs, the tubes were directly centrifuged (1800xg, 25 minutes, room temperature) in a swinging bucket rotor. The upper interphase containing the buffy layer and plasma was then washed with 40 mL of warm PBS and centrifuged at 250g (7 minutes, RT). After repeating the washing procedure twice, the cells were resuspended in 1 mL of RPMI-1640 and prepared for cell counting. Following plating (2E6 cells/well) in 12-well plates (Costar CL3513, Sigma-Aldrich, St. Louis, MO, USA), the PBMCs were stimulated with medium (RPMI-1640) or TLR ligands: LPS (200 ng/mL - Sigma L2880, Sigma-Aldrich, St. Louis, MO, USA), flagellin (500 ng/mL - Invivogen tlrl-s) or the whole diarrheagenic *E. coli* strain E1392/75-2A (1E7 CFU/well). Brefeldin A (BFA) (Invitrogen 00-4506-51, Carlsbad, CA, USA) was used to inhibit cytokine excretion from the cells, and the plates were incubated for 3 hours at 37°C with 5% CO2.

FACS staining

Following a 3-hour incubation, the cells were harvested and labeled with fluorochrome-conjugated antibodies against extracellular markers for PBMC phenotyping (Table 1. [Panel1]). The cells were incubated with the first antibody mixture diluted in FACS buffer (PBS+5% BSA+ 2mM EDTA) for 30 minutes, wrapped in aluminum foil on ice (4°C). Following that, the dead cells were stained with eFluor 520 Fixable Viability Dye (eBioscience 65-0867-14, San Diego, CA, USA).

Table 7. Antibodies panel used for PBMCs immunological assay

Antibody	Panel	Fluorochrome	Host	Isotype	Light chain	Clone	Company	Catalog number
α-CD3	1	FITC	mouse	IgG1	κ	UCHT1	Biolegend	300406
α-CD11c	1	BV 421	mouse	IgG1	κ	3.9	Biolegend	301628
α-CD14	1	Percp-Cy5.5	mouse	IgG1	κ	HCD14	Biolegend	325622
α-CD19	1	FITC	mouse	IgG1	κ	SJ25C1	Biolegend	363008
α-CD20	1	FITC	mouse	IgG2b	κ	2H7	Biolegend	302304
α-CD56	1	FITC	mouse	IgG1	κ	HCD56	Biolegend	318304
α-HLA-DR	1	BV 510	mouse	IgG2a	κ	L243	Biolegend	307646
α-CD123	1	PE-Cy7	mouse	IgG1	κ	6H6	Biolegend	306010
α-IL-6	2	PE	rat	IgG1	κ	MQ2-13A5	Biolegend	501107
α-TNF-α	2	AF647	mouse	IgG1	κ	MAb11	Biolegend	502916

Company affiliation is Biolegend (San Diego, CA, USA)

After cell fixation and membrane permeabilization with IC fix/perm kit (Invitrogen, #88-8824-00, Carlsbad, CA, USA), the intracellular production of IL-6 and TNF-a was assessed by staining the cells with flow cytometry antibodies included in the second antibody mixture (Table 1. [Panel2]). The supplementary data contains the stepwise staining procedure. The stained samples were then measured on a Beckman Coulter Cytoflex LX, and the data were analyzed using FlowJo v10 (FlowJo LLC, Ashland, OR, USA). The supplementary data describe the gating strategy used to identify different cell types and cytokine production profiles. Paired sample t-test was performed to compare the baseline and day14 responses within each group, and differences were declared significant when the p-value was <0.05. The statistical analysis was performed using GraphPad Prism (8.0.1), and the graphs were generated with the same program.

RNA extraction and sequencing

RNA sequencing (RNA-seq) was performed on lysed frozen PBMCs from 48 randomly selected intervention study participants (19 in the placebo group, 10 in the low dose group,

and 19 in the high dose group). These individuals were the same as those subjected to *ex vivo* PBMC analysis. Each participant had paired samples taken at baseline and on day 14 of the research prior to infection with diarrheagenic *E. coli*, totaling 96 samples. On the study day, the PBMCs were lysed using buffer RLT (Qiagen 79216, Germantown, MD, USA) and stored frozen until the RNA extraction day. On that day, the samples were thawed, and total RNA from the cells was extracted according to the manufacturer's procedure using the RNeasy mini kit (Qiagen 74106, Germantown, MD, USA). Following that, Implen NanoPhotometer N60/N50 was used to quantify the extracted RNA, and the quality and integrity of the RNA samples were checked using the Agilent 2200 TapeStation system (Agilent, Santa Clara, CA, USA) according to the manufacturer's protocol. RNA samples with an RNA integrity number (RIN) of 8 were used for library preparation. Library construction and sequencing were performed by Novogene (Milton Road, Cambridge, UK), where 2x150 bp RNA-seq reads were obtained using Illumina sequencing using a strand-aware library preparation technique.

Differential Gene Expression analysis (DGE)

The sequence data was used to perform Differential Gene Expression analysis (DGE) at NIZO (NIZO, Ede, The Netherlands) using a custom bioinformatics pipeline. First, quality control of the raw sequencing data was performed using Fast QC (v.0.11.9) and MultiQC (v.1.9). Reads were then pre-processed using fastp (v.0.20.0) using a sliding window quality score-based trimming (window width 4 bases, minimum window Q score 15). Reads less than 60 bp long after quality trimming were removed. Surviving high-quality reads were pseudo-aligned with Kallisto (v.0.46.10) to the human transcriptome (Ensemble release 101, GRCh38), taking into account the strandedness of the library (e.g., using the '--rf-stranded' flag) and using 50 bootstraps and the GC bias correction ('--bias' flag). For diagnostic purposes, reads from a subset of samples were aligned to the human genome (Ensemble release 101, GRCh38) using STAR (v.2.7.5a). The resulting alignments were imported to SeqMonk (v.1.47.1) to obtain an overview of the proportion of reads aligning to introns, exons, mtDNA, and rRNA.

DGE analysis was performed using custom scripts written in R (v.4.0.2). The biomaRt (v.2.46.0) package was used to obtain and match gene and transcript identifiers (using the 101 release of Ensembl, same as the transcriptome version used as a reference in the pseudoalignment). The tximport (v.1.18.0) package was used to import data from Kallisto output files into R (v. 4.0.2).

The DGE analysis was implemented as 3 different workflows based on 3 different R packages for DGE: DESeq2 (v.1.30.0), edgeR (v.3.32.0), and sleuth (v.0.30). DESeq2 and edgeR aggregate transcript counts to gene-level before performing per-gene statistical

tests. Sleuth performs per-transcript statistical tests before aggregating the resulting p-values to gene-level. In all 3 flows, multiple comparison adjustment was performed on p-values using the Benjamini-Hochberg FDR procedure. Adjusted p-values smaller than 0.05 were considered significant. All 3 DGE workflows rely on likelihood ratio tests (LRTs) comparing the goodness of fit of a" full" model against that of a" reduced" model, where the full model contains the factor of interest while the reduced model does not. To exemplify, for the main outcome of interest in this analysis (i.e., group-specific changes in time, showing differential trends between the Placebo group and the High Dose group), the full model was " ~ time + group + group: time" while the reduced model was " ~ time + group". Thus, the full model included the interaction between the fixed effects of 'time' and 'group' (group: time), while the reduced model did not. For the secondary analyses focusing on the effect of time on gene expression (i.e., non-group-specific effect), the full model was " ~ subject + time" while the reduced model was " ~ subject".

The results of all 3 DGE workflows were integrated and compared. Genes found to be significant by all 3 workflows were regarded as being differentially expressed with very high confidence, while genes found by only one workflow were regarded as likely spurious. Results were visually summarized using Venn and Euler diagrams to assess the degree of consensus in the results of the 3 workflows. The tidyverse (v.1.30) package was used for data wrangling. The ggplot2 (v.3.3.2) and ComplexHeatmap (v.2.6.2) packages were used for visualizations.

Results

Baseline characteristics of the participants

In the GIGA study (Ulfman et al. 2022.), a total of 120 healthy male volunteers were stratified and randomized into one of the three different study groups; placebo, low dose WPC, and high dose WPC. The current study used a randomly selected sample of 48 out of the 120 participants in the GIGA trial. The age and BMI of research participants did not differ statistically between the groups. Table 2 summarizes the baseline characteristics of these 48 subjects.

Table 2. Baseline characterization of the study participants

Variable		Placebo	WPC low dose	WPC high dose
Number		19	10	19
Age	Mean (SD)	36.29 (11.6)	34.16 (11.93)	33.7 (9.98)
BMI (Kg/m^2)	Mean (SD)	24.43 (2.24)	23.85 (2.93)	24.02 (2.81)

Ex vivo stimulation of monocytes, mDC, and pDC by diarrheagenic E.coli and TLR ligands

To study the effects of WPC consumption on the innate immune cell response, PBMCs were isolated from freshly drawn blood samples on day 1 (baseline) and on day 14 and were stimulated with either fixed whole diarrheagenic *E.coli* strain E1392/75-2A, LPS (TLR4 ligand), flagellin (TLR5 ligand), or with RPMI medium as the negative control. Flow cytometry analysis was used to measure intracellular IL-6 and TNF-α production in monocytes, mDCs, and pDCs (Supp Fig. S1). The results are given as a percentage of all viable monocytes or mDCs that were double-positive (producing both cytokines), or as a percentage of all IL-6 producing cells (any IL-6 positive), or as a percentage of all TNF-α producing cells (any TNF-α positive). The responses of the three study groups were compared before and after two weeks of dietary intervention.

In all three groups, diarrheagenic *E.coli* stimulation of PBMCs isolated on the baseline and day 14 resulted in a comparable percentage of IL-6 and TNF-α positive (double-positive) monocytes (Fig. 2A) and mDCs (Fig. 3A). When comparing individual donors' responses (% of double-positive cells) to *E.coli* stimulation at the baseline and day 14, individual responses varied between donors. This observation holds true for both monocytes (Fig. 2B) and mDCs (Fig. 3B). Similar results were obtained for any IL-6 positive and any TNF-α positive monocytes and mDCs (data not shown) before and after product consumption. The findings indicate no significant differences in the percentage of any IL-6 or TNF-α positive monocytes (Fig. 2C-D) or any IL-6 or TNF-α positive mDCs (Fig. 3C-D) following diarrheagenic *E.coli* stimulation in any of the study groups.

As with *E. coli*, stimulation of PBMCs with TLR ligands did not increase cytokine responses in the intervention groups. Although some donors displayed different responses between baseline and day 14 were observed, these changes were not consistent across groups and cell types (Supp Fig. S2A-F) and occurred primarily in the placebo group. Moreover, the stimulation of PBMCs did not significantly affect the percentage of IL-6 and TNF-α producing pDCs evaluated on the baseline compared to day 14 (Supp Fig. S2G-I), although it should be mentioned that responses in pDCs to the stimuli utilized were very low. Overall, these results indicate that the nutritional intervention with WPC did not modify the responses of monocyte, mDC, or pDC to *ex vivo* stimulation.

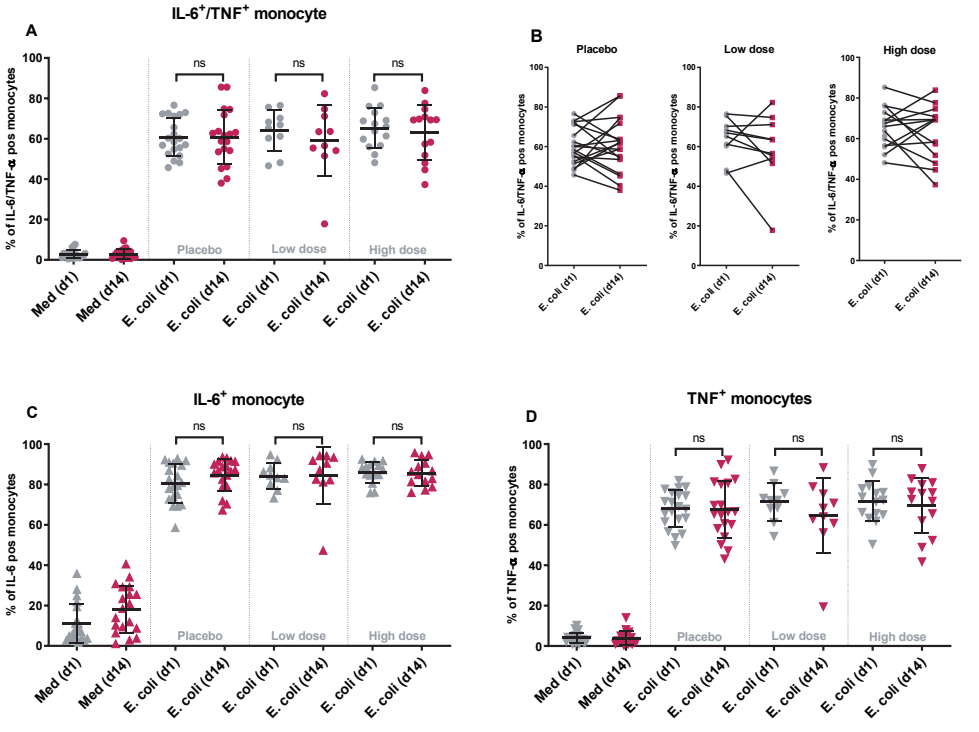

Fig. 2. The changes in the percentage of double-positive and single-positive monocytes upon stimulation with diarrheagenic *E.coli*: Following product consumption for two weeks, the percentage of monocytes producing IL-6 and TNF-α simultaneously did change significantly after an *ex vivo* stimulation with diarrheagenic *E.coli* (1E7 CFU) when comparing WPC high dose with placebo or WPC low dose group (A). The participants' individual responses between day 1 and day 14 after stimulation with *E. coli* are shown in different dose groups (B). Similarly, when comparing the percentage of any IL-6 (C) or any TNF-α (D) producing cells, no significant variation in the responses between WPC high dose and placebo or WPC low dose was noted.

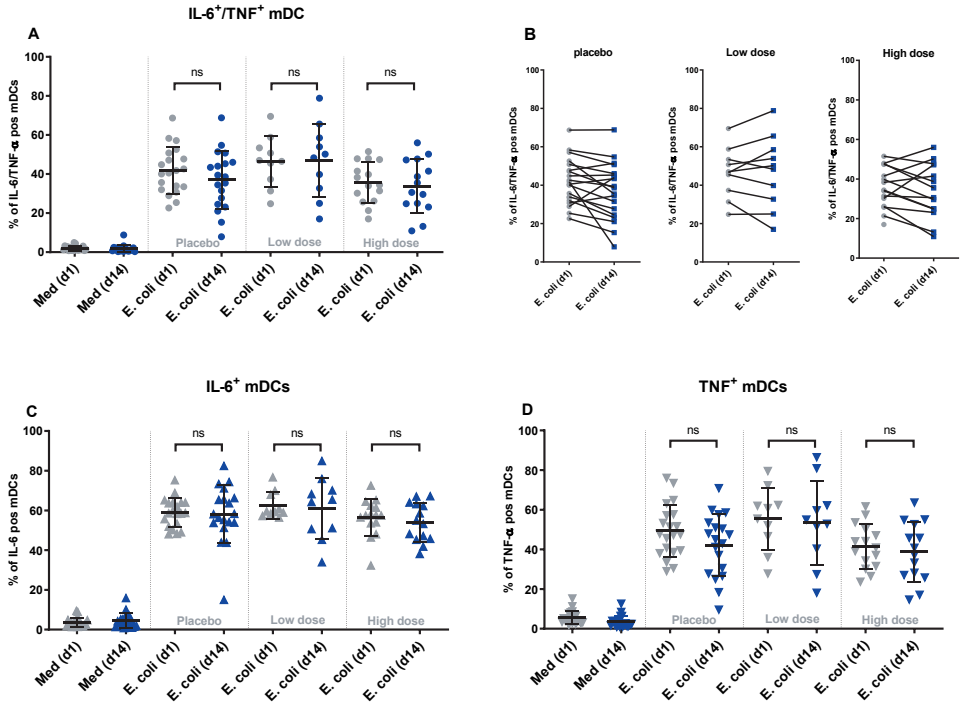

Fig. 3. The changes in the percentage of double-positive and single-positive mDCs upon stimulation with diarrheagenic *E.coli*: After two weeks of product consumption, the percentage of mDCs producing IL-6 and TNF-α simultaneously did not show any significant change after *ex vivo* stimulation with diarrheagenic *E.coli* (1E7 CFU) in any of the WPC dose groups (A). The participants' individual responses between day 1 and day 14 after stimulation with E. coli are shown in different dose groups (B). Looking at the % of any IL-6 producing (C) or any TNF-α producing mDCs (D), no significant difference between WPC dose groups was concluded.

Gene Expression analysis

In addition to *ex vivo* stimulation of PBMCs, mRNA was isolated from these cells at both timepoints and was sequenced to analyze the gene expression. Changes in gene expression patterns of PBMC from all three study groups were investigated with two goals in mind: to identify impacts of the dietary intervention (group-specific differential gene expression (DGE)) and to detect changes over time irrespective of the dietary intervention (baseline vs. day 14).

An exploratory data analysis of the sequenced mRNA samples was initially performed on the gene counts obtained from the pseudoalignment to detect any potential outliers and discern global trends in the data. To investigate and visualize the variation and patterns of our dataset in a single figure, results obtained from the various immunological analyses were used to conduct a two-dimensional Principal Component Analysis (PCA). The PCA indicates the amount of variation retained by each principal component (PC1 scores

on the x-axis and PC2 scores on the y-axis). For the differences in the DGE, the amount of variation explained by PC1 and PC2 is 10.6% and 8.1%, respectively. No differences were observed on the PCA plots between any of the study groups at day 14 (Fig. 4A), and apparent differences between baseline and day 14 were seen in all study groups (Fig. 4B). This indicates a time-dependent difference in PBMCs' gene expression in all groups that was not linked to the nutritional intervention since the samples from the placebo group showed the same trend.

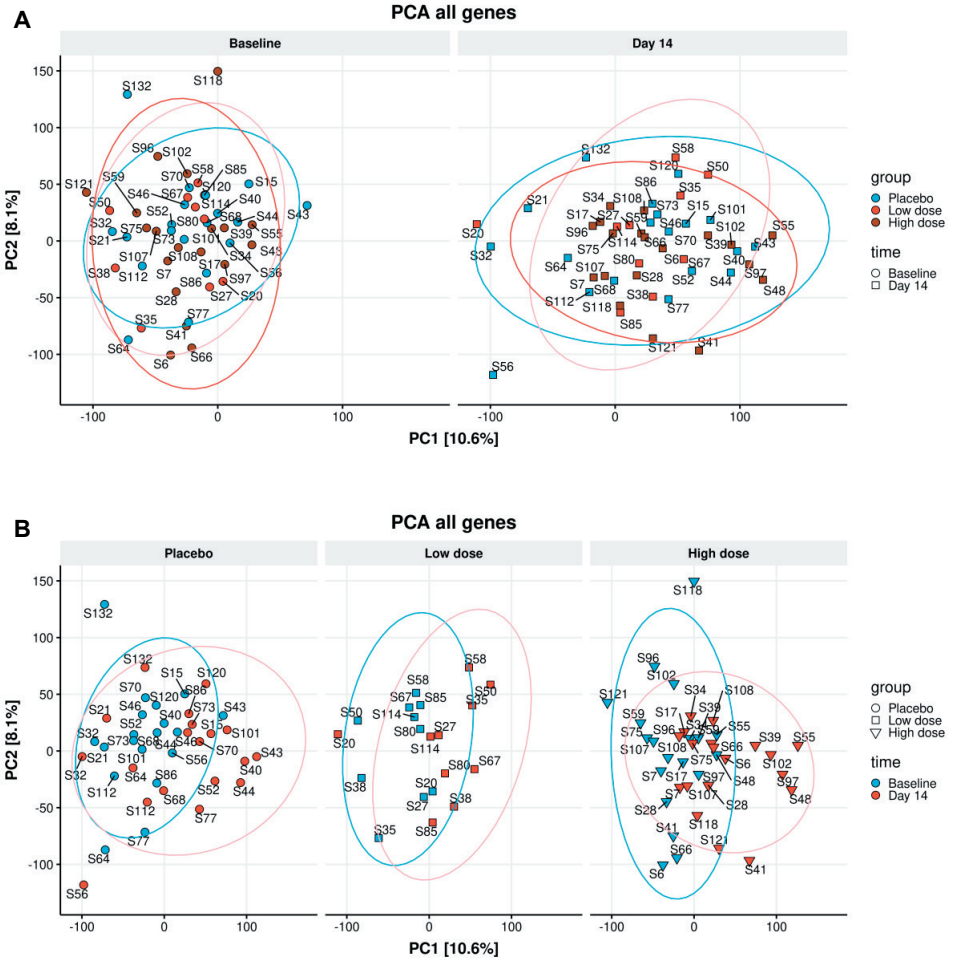

Fig. 4. Principle Component Analysis (PCA) for DGE: The differences in the DGE of all individuals on the baseline and all individuals on day 14 (A) of placebo, WPC low dose, and WPC high dose, no statistically significant gene expression difference in three dose groups was identified (A). Besides, the gene expression pattern of all participants in two time points did not lead to a clear difference (B).

Differential gene expression (DGE)

The PBMCs' gene expression was studied to identify any group-specific changes in gene expression after consuming the study product. All samples had more than 30 million raw sequencing read pairs (range 30-38 million read pairs). Around 26,000 genes had enough counts to be assessed using DGE analysis. The analysis focusing on the primary goal of identifying group-specific DGE, did not find any genes that were (significantly) differentially expressed in any of the three groups at day 14. These results indicate that no effects of nutritional intervention on gene expression patterns were present. No genes were found to display group-specific DGE by either sleuth- or DESeq2- analysis, and only three genes were found to be differentially expressed by edgeR analysis. At a closer inspection, in all three genes, the effect was found to be spurious, outlier-driven (2 of them shown in Fig. 5).

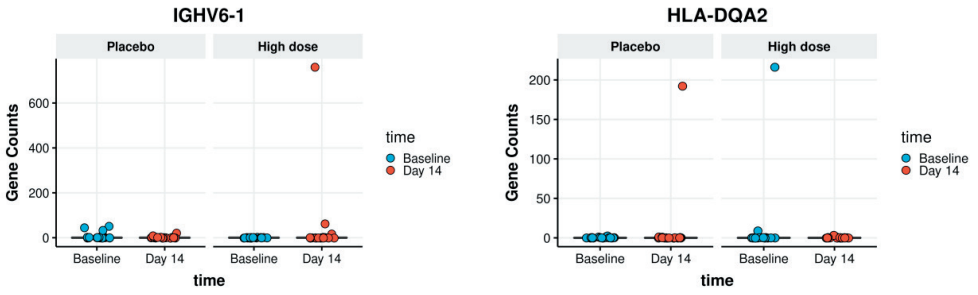

Fig. 5. Outlier-driven spurious findings of group-specific DGE genes: Boxplots showing outlier-driven spurious findings of 2 group-specific DGE genes. The boxplot itself (showing the interquartile range of each group) is compressed to the line by the extreme amplitude of a few outlier values: 1 outlier in the high dose group for the IGHV6-1 gene; 2 outliers for the HLADQA2 gene: 1 in the placebo group and 1 in the high dose group.

As a secondary goal, DGE analyses were also performed to identify genes that changed expression between baseline and day 14 of the study. Two analyses were performed, one focusing only on high dose group participants and the other focusing on the placebo group. The analyses found 3863 genes that were differentially expressed over time in the high dose group (Fig. 7A) and 241 genes in the placebo group (Fig. 7B). These numbers refer to high confidence genes found to be significant by the consensus of all 3 DGE flows: sleuth, DESeq2, and edgeR.

This indicates that even though no group-specific changes in gene expression were noted, in the high dose group compared to the placebo group, more genes were significantly changed in time. The list of (consensus) differentially expressed genes (in time) in the high dose group was extracted to investigate why this is the case. Then all the genes that were differentially expressed in the placebo group were removed from the list, even if non-consensus (e.g., even if found in just 1 of the 3 DGE workflows). The remaining 650 genes

in the list were examined. In all cases, these genes showed the same trend in the placebo group as in the high dose group, without reaching statistical significance (Four selected genes are shown in Supp Fig. 2A-D as examples). Overall, this means that although a higher number of genes were differentially expressed in the high dose group, the differences were not significant and this differences are not linked to the ingestion of the study product.

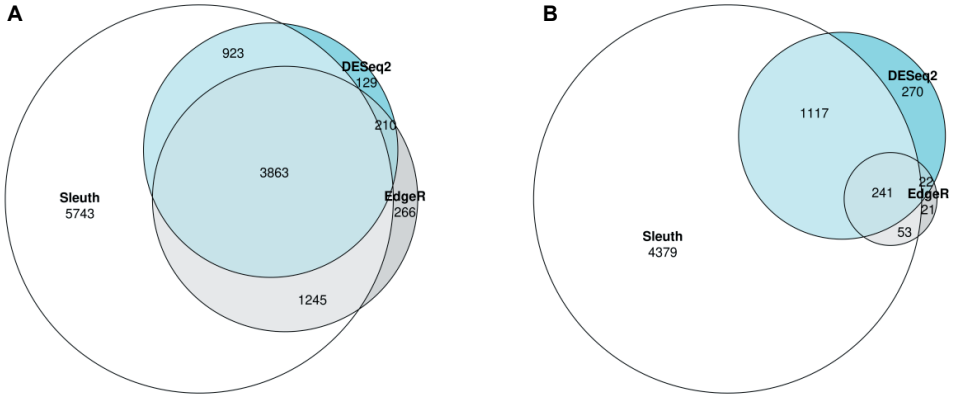

Fig. 7. Euler diagram of the DGE results from the three different workflows: Three different workflows, namely Sleuth, DESeq2, and EdgeR were applied to look at changes in PBMCs gene expression over time (baseline Vs. day 14) in the participants of the high dose group (A) and the placebo group (B). In the high dose group, 3863 genes were differentially expressed by all three workflows (A). The number of differentially expressed genes in the placebo was 241 when looking at the genes found by all three workflows.

Discussion

The present study shows that a two-week dietary intervention with a whey protein concentrate (WPC) compared to placebo had no effect on the myeloid innate immune cells response to TLR ligand-induced activation and did not induce differential gene expression patterns in PBMCs of the study participants.

We recently demonstrated that a primary *in vivo* challenge of healthy volunteers with diarrheagenic *E.coli* bacteria strain E1392/75-2A at doses as low as 1E6 CFU resulted in protection against secondary infection three weeks later (Porbahaie et al. in prep). The primary infection primed monocytes and mDCs for an increased IL-6 and TNF-α production after restimulation with *E. coli* or TLR ligands *ex vivo*. This enhanced innate immune response could be due to *in vivo* innate immune training.

Although innate immune training has been extensively studied *in vitro* and several components showed the effect, little evidence exists for *in vivo* applications [42, 221, 222].

In vitro training of monocytes has been demonstrated using raw milk, bovine milk IgG (bIgG) as a purified molecule, or whey protein preparations including WPC [46, 47]. Interestingly, depletion of bIgG from the whey protein extracts did not entirely eliminate the training effect, indicating the presence of (an)other active ingredient(s) in the whey preparation. The current study product is whey protein concentrate (WPC) from raw bovine milk, the same product applied in the previously mentioned *in vitro* study [47]. WPC is rich in bioactive components, including whey proteins (e.g., immunoglobulins, Milk Fat Globule Membrane proteins) and phospholipids. It was processed mildly enough to ensure that bIgG and other proteins were not denatured (based on internal validation). Therefore, we investigated whether the *in vitro* training results can be translated into *in vivo* immune effects on monocytes after WPC oral ingestion.

Prior to and after a two-week nutritional intervention in healthy adult male volunteers, PBMCs were isolated and stimulated with diarrheagenic *E.coli* bacteria as well as with TLR ligands. No changes in the cytokine response of monocytes, mDCs, and pDCs were seen between the study groups that is indicative of no training effects *ex vivo*. Nevertheless, we must keep in mind that the study participants were healthy male adults (18-55 yr) with no known medical conditions based on the study inclusion criteria. Arts et al., similar to our findings, were unable to replicate the *in vitro* results for trained immunity induced by BCG vaccination *in vivo* [221], and oral ingestion of β-glucan did not enhance the innate immune response in humans [223]. These studies were performed in a comparable group of people comprised of healthy adults ranging between 20-34 years old. However, the immune system competence is different in infants, the elderly, and immunocompromised individuals. TLR function, for example, is impaired in the context of aging, resulting in a decreased innate immune response and increased susceptibility to bacterial and viral infections [72]. In elderly women, it was demonstrated that ingestion of bovine lactoferrin (bLF) could partially restore TLR7/8 responsiveness in pDC [224]. Therefore, even though we could not demonstrate an effect of WPC on innate immune responses in healthy male adults, it is possible that elderly subjects might benefit from the dietary intervention, although future studies are required to substantiate this notion.

As it is well established that dietary components can influence cells' gene expression locally or systemically [14, 15], we also evaluated the effect of the nutritional intervention on gene expression in PBMCs of the participants. The intervention did not induce differential group-specific gene expression patterns of the PBMCs of the volunteers in any of the study groups. As a result, no downstream analyses could be performed (e.g., Gene Set Enrichment Analysis or Gene Ontology Enrichment Analysis). The same line of reasoning discussed above can also explain the gene expression findings. The gene expression regulatory machinery in healthy young individuals is sufficiently competent to maintain homeostasis. This is not entirely true in immunocompromised individuals, who most likely may benefit

from nutritional intervention. However, further research in specific populations is needed to validate this.

Interestingly, a time effect on gene expression pattern was noted in all groups regardless of the study product. Between baseline and day 14, large-scale changes in gene expression were seen in all study participants. These alterations occurred regardless of the study group and were observed in both the placebo and high dose groups. Further in-depth analysis of these findings revealed no statistically significant difference, and the observed trend was comparable in the placebo and high dose groups. A possible explanation for the time effect observed is that the study's dietary restriction guidelines resulted in these changes in gene expression. All study participants followed identical guidelines that began concurrently with the intervention study and resulted in considerable changes in dietary habits and may explain the findings.

The data presented here in a subgroup of participants of the GIGA study reflects the findings on the clinical effect of WPC on diarrheagenic *E. coli* infection symptom score and microbiota composition (Ulfman et al. 2022). After the two-week dietary intervention, participants of the GIGA study were orally inoculated with 1E10 CFU of live-attenuated diarrheagenic *E. coli* strain E1392/75-2A. On days 11-18 and two weeks later, on day 28, the progression of clinical diarrheal symptoms was evaluated. The clinical outcomes regarding diarrhea incidence, stool frequency, and gastrointestinal discomfort (Gastrointestinal Rating Score, GSRS) were comparable in all three study groups and could not demonstrate an effect of the nutritional intervention. Although participants experienced clinically mild diarrhea following the oral challenge, no dose group influence on diarrhea outcomes was observed. Finally, when the intervention effects on microbiota composition were studied, a similar time-dependent effect on microbiota was noted across all study groups. These findings corroborate our results on gene expression and *ex vivo* PBMC stimulation on a subset of the study population.

In conclusion, in the current study, we could not demonstrate an effect of WPC ingestion on either myeloid cells' responsiveness to TLR agonists or the gene expression pattern of PBMCs. However, people with lower immunocompetence, such as newborns and the elderly, could possibly benefit from such nutritional interventions. As previously demonstrated, these populations have reduced innate immune responses and may benefit from immune enhancement. However, prospective studies should look into the effects of *in vitro* tested potential compounds in certain populations to substantiate this notion and provide additional insight into this area.

References

1. Pakkanen, R. and J. Aalto, *Growth factors and antimicrobial factors of bovine colostrum.* International Dairy Journal, 1997. **7**(5): p. 285-297.
2. Stelwagen, K., et al., *Immune components of bovine colostrum and milk1.* Journal of Animal Science, 2009. **87**(suppl_13): p. 3-9.
3. van Neerven, J., *The effects of milk and colostrum on allergy and infection: Mechanisms and implications*. Vol. 4. 2014. 16-22.
4. Perdijk, O., et al., *Cow's Milk and Immune Function in the Respiratory Tract: Potential Mechanisms.* Frontiers in Immunology, 2018. **9**(143).
5. Ulfman, L.H., et al., *Effects of Bovine Immunoglobulins on Immune Function, Allergy, and Infection.* Frontiers in Nutrition, 2018. **5**(52).
6. Ten Bruggencate, S.J., et al., *Dietary Milk-Fat-Globule Membrane Affects Resistance to Diarrheagenic Escherichia coli in Healthy Adults in a Randomized, Placebo-Controlled, Double-Blind Study.* The Journal of Nutrition, 2015. **146**(2): p. 249-255.
7. Chen, K., et al., *Effect of bovine lactoferrin from iron-fortified formulas on diarrhea and respiratory tract infections of weaned infants in a randomized controlled trial.* Nutrition, 2016. **32**(2): p. 222-227.
8. Tacket, C.O., et al., *Protection by Milk Immunoglobulin Concentrate against Oral Challenge with Enterotoxigenic Escherichia coli.* New England Journal of Medicine, 1988. **318**(19): p. 1240-1243.
9. Savarino, S.J., et al., *Prophylactic Efficacy of Hyperimmune Bovine Colostral Antiadhesin Antibodies Against Enterotoxigenic Escherichia coli Diarrhea: A Randomized, Double-Blind, Placebo-Controlled, Phase 1 Trial.* The Journal of Infectious Diseases, 2017. **216**(1): p. 7-13.
10. Ebina, T., et al., *PREVENTION OF ROTAVIRUS INFECTION BY COW COLOSTRUM CONTAINING ANTIBODY AGAINST HUMAN ROTAVIRUS.* The Lancet, 1983. **322**(8357): p. 1029-1030.
11. Davidson, G.P., et al., *PASSIVE IMMUNISATION OF CHILDREN WITH BOVINE COLOSTRUM CONTAINING ANTIBODIES TO HUMAN ROTAVIRUS.* The Lancet, 1989. **334**(8665): p. 709-712.
12. Loss, G., et al., *Consumption of unprocessed cow's milk protects infants from common respiratory infections.* Journal of Allergy and Clinical Immunology, 2015. **135**(1): p. 56-62.e2.
13. van Neerven, R.J.J., et al., *Which factors in raw cow's milk contribute to protection against allergies?* Journal of Allergy and Clinical Immunology, 2012. **130**(4): p. 853-858.
14. van Splunter, M., et al., *Induction of Trained Innate Immunity in Human Monocytes by Bovine Milk and Milk-Derived Immunoglobulin G.* Nutrients, 2018. **10**(10): p. 1378.
15. Hellinga, A.H., et al., *In Vitro Induction of Trained Innate Immunity by bIgG and Whey Protein Extracts.* International Journal of Molecular Sciences, 2020. **21**(23): p. 9077.
16. Netea, M.G., et al., *Defining trained immunity and its role in health and disease.* Nature Reviews Immunology, 2020.
17. Netea, M.G., et al., *Trained immunity: A program of innate immune memory in health and disease.* Science, 2016. **352**(6284).

18. Shaw, A.L., et al., *Absorption and safety of serum-derived bovine immunoglobulin/protein isolate in healthy adults.* Clinical and experimental gastroenterology, 2016. **9**: p. 365-375.
19. Arts, R.J.W., et al., *Long-term in vitro and in vivo effects of γ-irradiated BCG on innate and adaptive immunity.* Journal of Leukocyte Biology, 2015. **98**(6): p. 995-1001.
20. Hole, C.R., et al., *Induction of memory-like dendritic cell responses in vivo.* Nature communications, 2019. **10**(1): p. 2955-2955.
21. Heijden, C.D.C.C.v.d., et al., *Catecholamines Induce Trained Immunity in Monocytes In Vitro and In Vivo.* Circulation Research, 2020. **127**(2): p. 269-283.
22. Leentjens, J., et al., *The Effects of Orally Administered Beta-Glucan on Innate Immune Responses in Humans, a Randomized Open-Label Intervention Pilot-Study.* PLOS ONE, 2014. **9**(9): p. e108794.
23. Shaw, A.C., et al., *Dysregulation of human Toll-like receptor function in aging.* Ageing Research Reviews, 2011. **10**(3): p. 346-353.
24. van Splunter, M., et al., *Bovine Lactoferrin Enhances TLR7-Mediated Responses in Plasmacytoid Dendritic Cells in Elderly Women: Results From a Nutritional Intervention Study With Bovine Lactoferrin, GOS and Vitamin D.* Frontiers in Immunology, 2018. **9**(2677).

Supplementary data

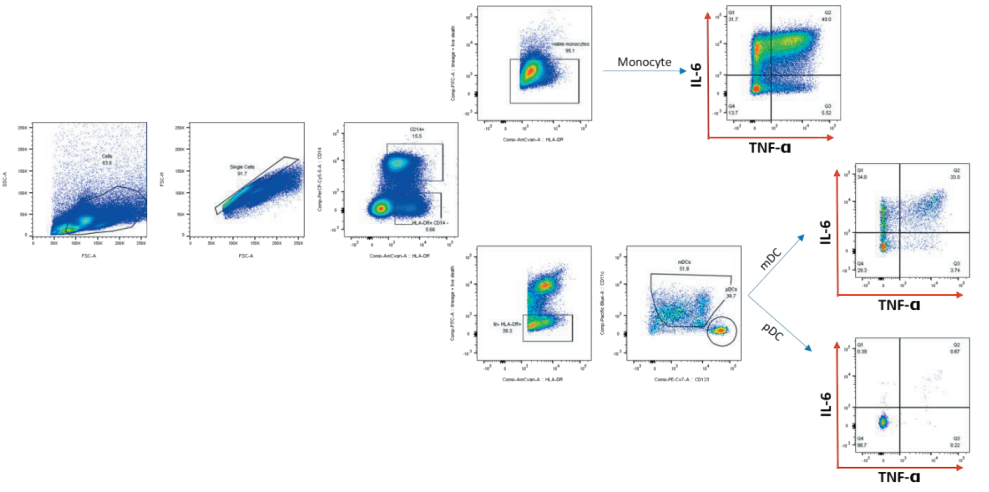

Fig. S1. Gating strategy for monocytes, mDCs, and pDCs phenotyping: PBMCs were selected in the FSC/SSC plot, and the duplets were gated out. HLA-DR^{+}CD14^{+} cells were selected as the monocytes, and dead cells were removed. CD3^{+}, CD19^{+},CD20^{+}, CD56^{+} and dead cells from the HLA-DR^{+}CD14^{-} population were excluded. From there, CD11c^{+} cells were considered as mDCs, and CD123^{+} cells were named pDCs. Within monocyte, mDC, and pDC populations, the percentage of cells that were producing IL-6, TNF-a or both cytokines were determined.

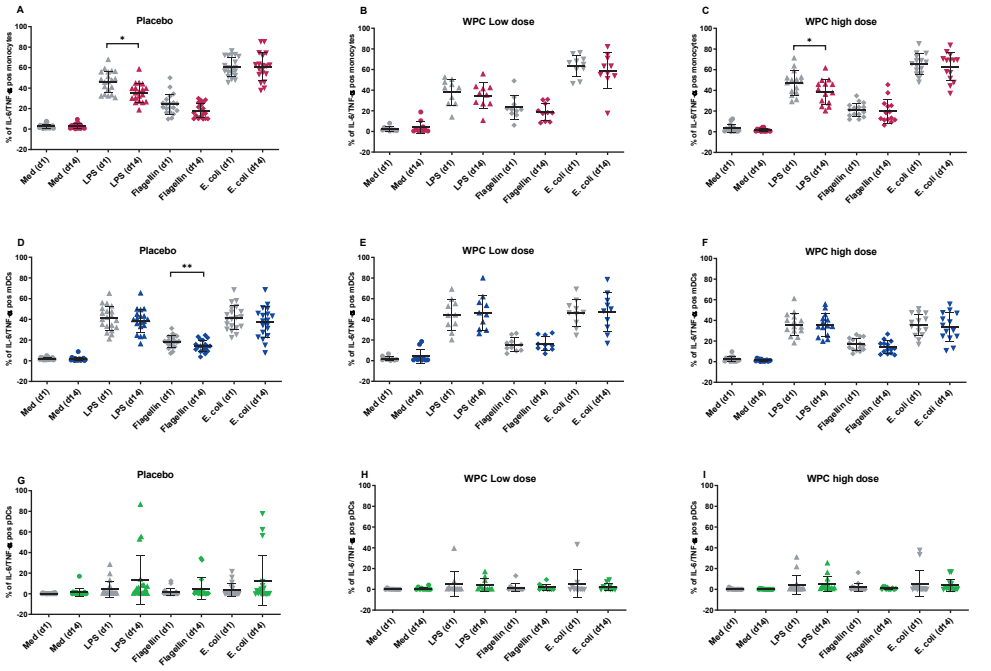

Fig. S2. The changes in the % of double-positive monocytes, mDCs, and pDCs following the stimulation with TLR ligands and *E. coli*: The percentage of monocytes (A-C), mDCs (D-F), and pDCs (G-I) simultaneously producing both IL-6 and TNF-α following the stimulation with LPS, flagellin, and whole E. coli bacteria were quantified. The comparison was made between each dose group's baseline and day 14 responses within each TLR stimulation.

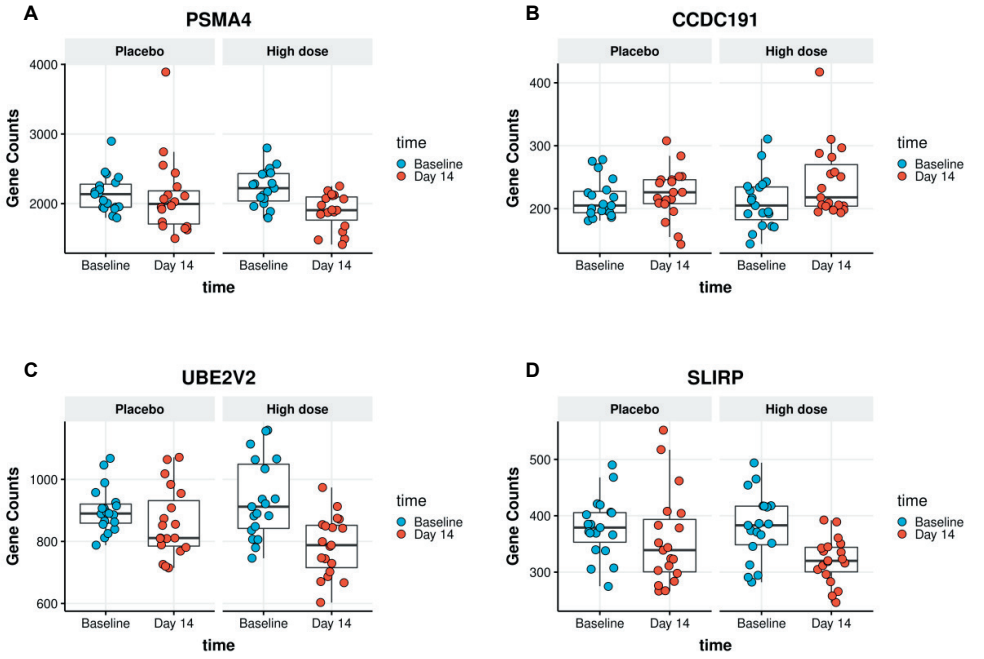

Fig. S3. An example of a gene showing the same trend in both Placebo and High Dose groups: yet not reaching statistical significance in the Placebo group.

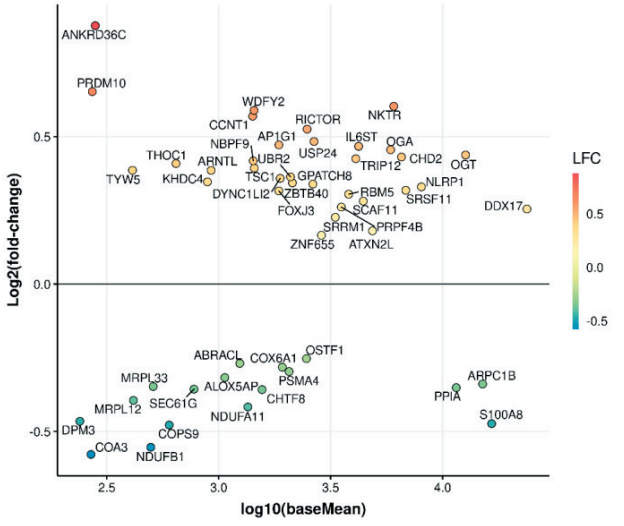

Fig. S4. MA plot showing the top 50 most differentially expressed genes in time in high dose group subjects (as determined by DESeq2-derived p-value): X-axis shows the mean expression levels of the genes; y-axis shows the fold-change in gene expression from Baseline to Day 14.

Study Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

Substantial:
1. Male
2. Age between 18 and 55 years.
3. BMI ≥18.5 and ≤30.0 kg/m2.
4. Healthy as assessed by the NIZO health questionnaire.

Procedural:
5. Ability to follow Dutch verbal and written instructions.
6. Availability of internet connection.
7. Signed informed consent.
8. Willing to accept disclosure of the financial benefit of participation in the study to the authorities concerned.
9. Willing to accept use of all encoded data, including publication, and the confidential use and storage of all data for at least 15 years.
10. Willing to comply with study procedures, including collection of stool and blood samples.
11. Willingness to abstain from high calcium containing products during the study.
12. Willingness to abstain from alcoholic beverages three days before, during and for 4 days after diarrheagenic *E. coli* challenge.
13. Willingness to abstain from medications that contain acetaminophen, aspirin, ibuprofen, and other nonsteroidal anti-inflammatory drugs, (OTC) antacids and antimotility agents (eg, loperamide) on the three days before, during and for 4 days after diarrheagenic *E. coli* challenge.
14. Willingness to abstain from probiotics and prebiotics/fibers starting from run-in and during the entire study.
15. Willingness to give up blood donation starting at run-in and during the entire study.

Study Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

Substantial:
1. Acute gastroenteritis in the 2 months prior to inclusion.
2. Any confirmed or suspected immunosuppressive or immunodeficient condition including human immunodeficiency virus infection (HIV).

3. Disease of the GI tract, liver, bile bladder, kidney, thyroid gland (self-reported), except for appendicitis.
4. History of microbiologically confirmed ETEC or cholera infection within 3 years prior to inclusion.
5. Symptoms consistent with Travelers' Diarrhea concurrent with travel to countries where ETEC infection is endemic (most of the developing world) within 3 years prior to inclusion, OR planned travel to endemic countries during the length of the study.
6. Vaccination for, or ingestion of cholera within 3 years prior to inclusion, including studies at NIZO.
7. Occupation involving handling of ETEC or *Vibrio cholerae* currently, or within 3 years prior to inclusion.
8. Vaccination for, or ingestion of ETEC or *E coli* heat labile toxin, including *E. coli* challenge studies at NIZO.
9. Evidence of current excessive alcohol consumption (>4 consumptions/day or >20 consumptions/week) or drug (ab)use, and not willing/able to stop this during the study.
10. Known allergy to the following antibiotics: ciprofloxacin, trimethoprim, sulfamethoxazole, and penicillins.
11. Reported average stool frequency of >3 per day or <1 per 2 days.
12. Use of antibiotics (up till 6 months prior to inclusion), norit, laxatives, cholestyramine, antacids H2 receptor antagonists or proton pump inhibitors (during 3 months prior to inclusion).
13. Use of immunosuppressive drugs (e.g. cyclosporine, azathioprine, systemic corticosteroids, antibodies).
14. Vegans.
15. Mental status that is incompatible with the proper conduct of the study.
16. A self-reported milk allergy, lactose intolerance or sensitivity to dairy ingredients.
17. Not having a general practitioner, not allowing disclosure of participation to the general practitioner or not allow to inform the general practitioner about abnormal results.
18. Participation in any clinical trial including blood sampling and/or administration of substances starting 1 month prior to study start and during the entire study.
19. Personnel of NIZO or FrieslandCampina, their partner and their first and second degree relatives.

Flow cytometry staining protocol

1. Harvest the cells from the culture plate and transfer the entire content of the wells (˜1mL) into a 1mL deep well NUNC plate.
2. Spin down the cells for 3 min 400g and discard the supernatant by flipping the plates

and discarding the medium in the sink.
3. Resuspend the cells in 200µL of FACS buffer and transfer them to a standard (500µL) NUNC plate (~2 x 10^6 /well).
4. Spin down for 3 min at 400g and discard the supernatant.
5. Add 50µL of (extracellular markers antibody mix + Fc Block) diluted in FACS buffer.
6. Wrap in aluminium foil and incubate on ice for 30 min.
7. Add 200µL FACS buffer.
8. Spin down for 3 min 400g and discard the supernatant.
9. Wash with 200µL of PBS 2 times (2X) and discard the supernatant after each wash.
10. Stain cells with 50µL of freshly-made FVD520 (diluted 400X) in PBS.
11. Wrap in aluminium foil and incubate in the fridge for 20 min (cold and dark).
12. Add 200µL FACS buffer.
13. Spin down for 3 min at 400g and discard the supernatant.
14. Resuspend in 100µL IC Fix buffer.
15. Incubate 45 min RT.
16. Add 100µL of Perm buffer.
17. Spin down for 3 min at 400g and discard the supernatant.
18. Wash cells twice (2X) with 200µL Perm buffer (spin down 3 min at 400g) and discard the supernatant after each wash.
19. Resuspend in 50µL intracellular antibody mix in perm buffer.
20. Wrap in aluminum foil and incubate in the fridge for 20 min (cold and dark).
21. Add 200µL Perm buffer.
22. Spin down for 3 min 400g and discard the supernatant.
23. Wash with 200µL of FACS buffer 2 times (2X) and discard the supernatant after each wash.
24. Resuspend pellet in 300µL FACS buffer and measure on the flow cytometer.

CHAPTER

Low doses of diarrhoeagenic *E. coli* induce enhanced monocyte and mDC responses and prevent development of symptoms after homologous rechallenge

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Abstract

The experimental challenge with attenuated enterotoxigenic *E. coli* strain E1392/75-2A prevents diarrhea upon a secondary challenge with the same bacteria. A dose-response pilot study was performed to investigate which immunological factors are associated with this protection. Healthy subjects were inoculated with increasing *E. coli* doses of 1E6-1E10 CFU, and three weeks later, all participants were rechallenged with the highest dose (1E10 CFU). Gastrointestinal discomfort symptoms were recorded, and stool and blood samples were analyzed. After the primary challenge, stool frequency, diarrhea symptom scores, and *E. coli*-specific serum IgG (IgG-CFA/II) titer increased in a dose-dependent manner. Fecal calprotectin and serum IgG-CFA/II response after primary challenge were delayed in the lower dose groups. Even though stool frequency after the secondary challenge was inversely related to the primary inoculation dose, all *E. coli* doses protected against clinical symptoms upon rechallenge. *Ex vivo* stimulation of PBMCs with *E. coli* just before the second challenge resulted in increased numbers of IL-6$^+$/TNF-α^+ monocytes and mDCs than before the primary challenge, without dose-dependency. These data demonstrate that primary *E. coli* infection with as few as 1E6 CFU protects against a high-dose secondary challenge with a homologous attenuated strain. Increased serum IgG-CFA/II levels and *E. coli*-induced mDC and monocyte responses after primary challenge suggest that protection against secondary *E. coli* challenges is associated with adaptive as well as innate immune responses.

Introduction

Diarrhea is prevalent worldwide and is one of the leading causes of death in all age groups, with children under five being the most impacted [225]. Foodborne diarrhea primarily affects inhabitants of developing countries with low sanitary standards and also affects visitors to these endemic areas, resulting in travelers' diarrhea. Etiologically, diarrheagenic *Escherichia coli* (DEC) - and most prominently enterotoxigenic *E. coli* (ETEC) - is the primary cause of these diarrheal diseases [226-228]. These ETEC strains can survive gastrointestinal digestion and transit successfully via the gastrointestinal tract to enter the intestine [229, 230]. These bacteria adhere to the intestine's gut mucosa, where the secreted toxins bind to their respective host cell receptors and eventually deregulate ion channels and induce diarrhea [227, 231, 232]. Due to the high mortality rates in children and the consequences in adults, including (temporary) impairment, ETEC infections have become a global health burden that must be addressed.

In the absence of a broadly effective approved vaccine against ETEC, improving host resistance to infection through dietary or pharmacological intervention may be a viable alternative strategy for reducing diarrhea. It has been demonstrated that dietary supplementation with calcium in the form of dairy products [80], calcium in combination with probiotics [233], and a combination of zinc and iron [234] reduce the occurrence and severity of diarrhea. Dietary components may prevent the pathogen from colonizing the gut [80], act as bactericidal agents [77], and/or enhance host immune responses. These examples imply the efficacy and benefit of dietary prevention strategies.

To evaluate the clinical efficacy of drugs or food ingredients in preventing diarrhea, infection challenge models have been employed in which healthy volunteers are challenged with (attenuated) live ETEC [77, 235]. One of the established models uses attenuated ETEC strain E1392/75-2A [80, 216, 236]. It is worth mentioning that although the strain applied in those studies does not produce enterotoxins, it induces diarrhea, making it a valuable model for studying mechanisms. It was shown that protection against ETEC strain E1392/75-2A provides approximately 75% protection against virulent enterotoxin-producing strains [237]. Therefore, this article refers to this strain as diarrheagenic *E. coli* (dia. *E. coli*) due to no enterotoxin production.

A recent study has addressed that a primary dia. *E. coli* inoculation protected the subjects against reinfection [236]. In this study, the primary challenge with a high dose of dia. *E. coli* strain E1392/75-2A (O6:H16) resulted in protection against the second challenge with the same bacteria three weeks later. However, it is unclear which immunological mechanisms confer protection and whether a low dose primary challenge results in decreased or absent protection. If this is the case, it provides a valid model for studying the

impact of drug and food components on long-term protection against *E. coli* infection and enables us to investigate the protection mechanisms following the second exposure.

To this aim, we conducted a dose-response pilot study using the dia. *E. coli* strain E1392/75-2A challenge model. The primary challenge in this model was performed with bacteria at doses ranging from 1E6 to 1E10 Colony-Forming Unit (CFU), and the second challenge was performed with the standard high dia. *E. coli* dose of 1E10 CFU. First, we aimed to identify the lowest dose capable of conferring protection against the second challenge. For this reason, clinical outcomes for stool frequency, stool consistency, fecal wet weight, and scores on the Gastrointestinal Symptoms Rating Scale (GSRS) [238] were quantified to monitor diarrhea progression. Furthermore, immune responses prior to and following the primary and secondary challenges were further characterized to elucidate the immunological mechanisms behind putative protection. Induction of serum IgG against dia. *E. coli*-specific Colonization Factor Antigen II (IgG-CFA/II) was followed, calprotectin, β-defensin, and secretory IgA (SIgA) levels in fecal water were determined, and *ex vivo* response of monocyte and dendritic cell to the *E. coli*, toll-like receptor 4 (TLR-4), and TLR-5 stimulation were evaluated.

Methods

Study design, participants, and specimens

The MIRRE study was designed as a randomized, double-blinded, parallel dose-response, 7-week infection challenge study in 30 healthy adult male volunteers. The selected participants' age was between 18 to 55 years, with a BMI ranging from 18.5-30 kg/m^2. The study was approved by the Medical Ethics Committee (METC) of Brabant (Tilburg, the Netherlands) and was conducted according to the Declaration of Helsinki and was registered at www.clinicaltrials.gov with identification number: NCT03596827 (First registration date 24/07/2018).

All participants were medically evaluated based on self-report following recruitment and provision of written informed consent. Participants were considered for participation if they met the inclusion criteria, and there were no indications of exclusion criteria (supplementary Fig S1). Subjects were stratified by age and BMI (as determined during pre-study screening) and then assigned randomly to one of five treatment groups (n=6 per group). Stratification and randomization of study participants, and blinding and labeling of dia. *E. coli* dosages, were coordinated by a scientist not involved in the project. Strata were defined manually in MS Excel using the individual data. Stratified randomization of participants to treatment group was performed using the "Research Manager" software (Research Manager, Deventer, The Netherlands). The researchers on the project team and the study participants were kept blind to the treatment assignment.

During a two-week acclimation period (Fig 1), participants were instructed to maintain their usual physical activity pattern and habitual food intake; however, they needed to reduce and standardize their dietary calcium intake (>500 mg/day) [80]. After a standard low-calcium dinner and an overnight fast, the participants were assigned to dosage groups. On study day 14, they were orally inoculated with the live-attenuated dia. *E. coli* strain E1392/75-2A (supplier: Acambis, Cambridge, UK) [80, 239]. Each group received a different dose (1E6, 1E7, 1E8, 1E9, or 1E10 CFU), and three weeks later (day 35), all participants were given the standard dose of 1E10 CFU. Participants first received a $NaHCO_3$ solution (100 mL 2% $NaHCO_3$) to neutralize the gastric acid. After 5 minutes, they received a fruit juice (100 mL, pH 7.4) containing the attenuated dia. *E. coli* strain at the doses mentioned earlier. Participants were requested to restrict their probiotics, medication, and alcohol intake for three days before and four days after challenges and record their clinical gastrointestinal symptoms daily using an online questionnaire. Multiple blood and fecal samples were taken for analysis at various time points. (Fig 1 and supplementary Fig S3).

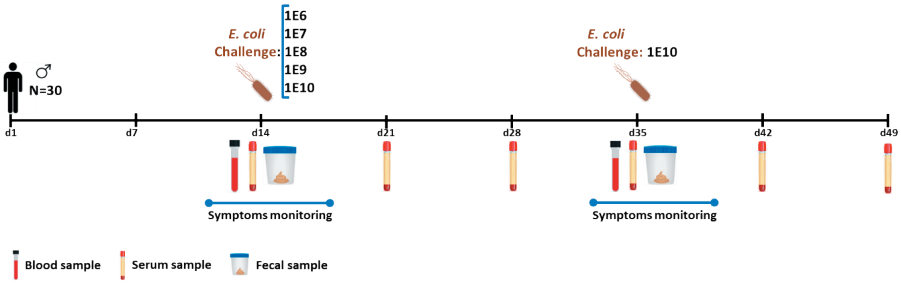

Fig 18. A graphical representation of the MIRRE study design: 30 male participants were recruited and randomized into five separate groups. On day 14, each group orally received different doses (1E6, 1E7, 1E8, 1E9, and 1E10 CFU) of dia. *E. coli* strain E1392/75-2A, and three weeks later on day 35, all participants were challenged with a dia. *E. coli* dose of 1E10 CFU. Three days before until four days after each challenge, participants were asked to report the clinical gastrointestinal symptoms. Blood and stool samples were collected for later analysis at multiple time points.

Online questionnaire

From day 11 till day 17 (interval I) and from day 32 till day 38 (interval II), participants had to report information on stool frequency (total number of stools per day) and stool consistency according to the Bristol Stool Scale (ranging from 1 as constipation to 7 as watery diarrhea). Moreover, participants were instructed to record the frequency and severity of symptoms by the validated Gastrointestinal Symptom Rating Scale (GSRS) [238]. The GSRS is a disease-specific scale of 15 questions related to five subdomains: diarrhea, abdominal pain, indigestion, reflux, and constipation. The GSRS has a seven-point graded Likert-type scale where 1 represents the absence of troublesome symptoms and 7 represents very troublesome symptoms. The later analysis of the GSRS score focused

on the GSRS total daily score, subdomain diarrhea (loose stools, increased passage of stools, and urgent defecation), and subdomain abdominal pain (abdominal discomfort, sucking sense epigastrium, nausea). These two subdomains were hypothesized to be affected the most by the dia. *E. coli* challenge. The GSRS total score ranges from 15-105, and the scores for the subdomains diarrhea and abdominal pain range from 3-21. The "Research Manager" program was used for data collection and management.

Collection and characterization of fecal samples

Fecal samples were collected a day before (day 12/13; day 33/34) and three days after each challenge (days 15-17; days 36–38). Participants were asked to collect all 24h stool samples in collection bags and freeze them on-site using the provided mini freezer. The frozen samples were transported to the analysis center, sorted, weighed, homogenized, aliquoted, and stored at -20°C until later analysis. Total fecal wet weight (24h pooled) was measured according to standard protocols (internal validated procedure), and the % fecal wet weight was quantified after the freeze-drying of the samples.

Quantification of intestinal immune biomarkers

The amount of calprotectin, β-defensin, and total secretory IgA (SIgA) in the participants' fecal water was measured by ELISA. Fecal water preparation and subsequent detection ELISA was performed with the provided reagents and according to the manufacturer protocol (ImmunDiagnostik, Bensheim, Germany; Calprotectin:K6927, β-defensin:K6500, SIgA:K8870). Two separate dilutions of the fecal water were used to assure in-range measurements, and the results are expressed as the amount of analyte per gram of fecal dry weight.

Measurement of IgG-CFA/II serum levels

On six timepoints during the study (days 14, 21, 28, 35, 42, and 49), blood samples were collected to measure serum specific IgG against CFA/II. For blood sample collection, Vacutainer serum tubes (Becton Dickinson 367895, Plymouth, United Kingdom) were used, and the sera were collected after centrifugation (2000xg, 10 min., RT). The centrifugation was done within 2 hours from the blood collection, and the resulting sera were stored at -80°C. The amount of CFA/II specific IgG was quantified by ELISA as described elsewhere [80]. A CFA/II-IgG-positive serum from a previous study was used as the standard curve [236], and values are expressed as Arbitrary Units (AU)/mL.

Activation of monocytes, mDC, and pDC by dia. *E. coli* and TLR-ligands

Just before bacteria inoculation (day 14 and day 35), blood samples from participants were collected in Vacutainer (K2-EDTA) tubes (Becton Dickinson 367525, Franklin Lakes, NJ, USA). The Peripheral Blood Mononuclear Cells (PBMCs) were isolated by density gradient centrifugation using Ficoll Paque Plus (GE Healthcare 17-1440-02, Chicago, IL,

USA). The isolated cells were seeded (2E6 cell/well) in 12-well plates (Costar CL3513, Sigma-Aldrich, St. Louis, MO, USA) and subsequently stimulated with either medium (RPMI-1640), 200 ng/mL of LPS (Sigma L2880, Sigma-Aldrich, St. Louis, MO, USA), 500 ng/mL of Flagellin (Invivogen tlrl-stfla, San Diego, CA, USA), or 1E7 CFU/well dia. *E. coli* (strain E1392/75-2A). Brefeldin A (BFA) (Invitrogen 00-4506-51, Carlsbad, CA, USA) was added to the wells to keep the produced cytokines inside the cells, and the plates were incubated for 3 hours at 37°C with 5% of CO_2. After the incubation, the cells were harvested and stained with fluorochrome-conjugated antibodies against extracellular markers for cell phenotyping (Table 1). Next, Fixable Viability Dye eFluor 520 (eBioscience 65-0867-14, San Diego, CA, USA) was applied as the live-dead marker. After cell fixation and membrane permeabilization, the production of IL-6 and TNF-α was determined intracellularly by staining the cells with flow cytometry antibodies (Table 1). The samples were measured on BD FACS CANTO II, and the generated flow cytometry data were analyzed with Flowjo v10 (FlowJo LLC, Ashland, OR, USA) (supplementary Fig S4).

Table 1. Antibodies panel used for PBMCs immunological assay

Antibody	Fluorochrome	Host	Isotype	Clone	Company	Catalog number
α-CD3	FITC	mouse	IgG1	UCHT1	Biolegend	300406
α-CD11c	BV 421	mouse	IgG1	3.9	Biolegend	301628
α-CD14	Percp-Cy5.5	mouse	IgG1	HCD14	Biolegend	325622
α-CD19	FITC	mouse	IgG1	SJ25C1	Biolegend	363008
α-CD20	FITC	mouse	IgG2b	2H7	Biolegend	302304
α-CD56	FITC	mouse	IgG1	HCD56	Biolegend	318304
α-HLA-DR	BV 510	mouse	IgG2a	L243	Biolegend	307646
α-CD123	PE-Cy7	mouse	IgG1	6H6	Biolegend	306010
α-IL-6	PE	rat	IgG1	MQ2-13A5	Biolegend	501107
α-TNF-α	AF647	mouse	IgG1	MAb11	Biolegend	502916

Company affiliation is Biolegend (San Diego, CA, USA)

Power calculation and statistical analysis

This research was designed as a pilot study. Since no information about the impact by bacterial doses was available, a specific setup, a so-called Design of Experiment (DOE: Plackett-Burman design (18)) with minimum and maximum challenge levels, was designed. Several doses were included with a calculated number of subjects in each group (n=6). By applying this DOE approach, it was expected to identify the dose-dependent trends and potentially a single dose optimum.

All data (except PBMCs work) were analyzed using repeated-measures Generalized Estimating Equations (GEE) model in a stepwise approach. In the GEE model, the dependent parameter, such as serum IgG levels of CFA/II, was associated with various

independent parameters, such as day and dose, as well as confounding factors, such as BMI, age, and start value. Moreover, non-linear functionalities of day and dose have been included to correct for these types of association. Fit of the model was assessed via Wald Chi-square. Only when the fit was significant the impact by the various independent parameters on the dependent parameter was confirmed. For statistical analysis of the PBMCs work, paired sample t-test was applied to compare the outcomes of d14 vs. d35 within each stimulation. The differences were considered significant for the whole dataset when the p-value was <0.05.

Results

Baseline characteristics of the participants

The baseline characteristics of the participants are summarized in Table 2. Healthy male participants (n=30) were randomized and divided into 5 study groups receiving different doses of dia. *E. coli* strain E1392/75-2A. Each group consisted of 6 participants; however, four individuals had withdrawn from the study at different time points indicated in Table 2 and described in supplementary Fig S1.

Table 2. Baseline characteristics of study participants.

Variable		Dose 1:	Dose 2:	Dose 3:	Dose 4:	Dose 5:
Dose		1E10	1E9	1E8	1E7	1E6
Number of participants		6 (1*)	6 (2*)	6 (1*)	6	6
Age	Mean (SD)	35.8 (15.2)	33.7 (10.6)	35.2 (13.1)	40.8 (7.7)	37.5 (10.9)
BMI (kg/m²)	Mean (SD)	22.9 (2.8)	23.8 (2.6)	24.7 (3.0)	24.4 (2.3)	23.8 (2.3)

* The number of individuals that have withdrawn from each group

Gastrointestinal symptom rating scale (GSRS)

The GSRS score was analyzed as the total daily score as well as for the subdomain diarrhea and subdomain abdominal pain at two time periods before and after each dia. *E. coli* inoculation. These analyses were done for interval I (days 12/13-17) and interval II (days 33/34-38).

After the primary challenge (interval I), GSRS domain diarrhea scores increased in a significant dose-dependent manner (P <0.05), with the score peak on day 15 (Fig 2A). GSRS domain abdominal pain (Fig 2B) and GSRS total daily score (Fig 2C) showed similar trends in the response; however, no significant dose-dependency was noted. During interval II, no major variation in any GSRS scores was recorded for neither of the primary doses compared to before the second challenge (Fig 2A-C).

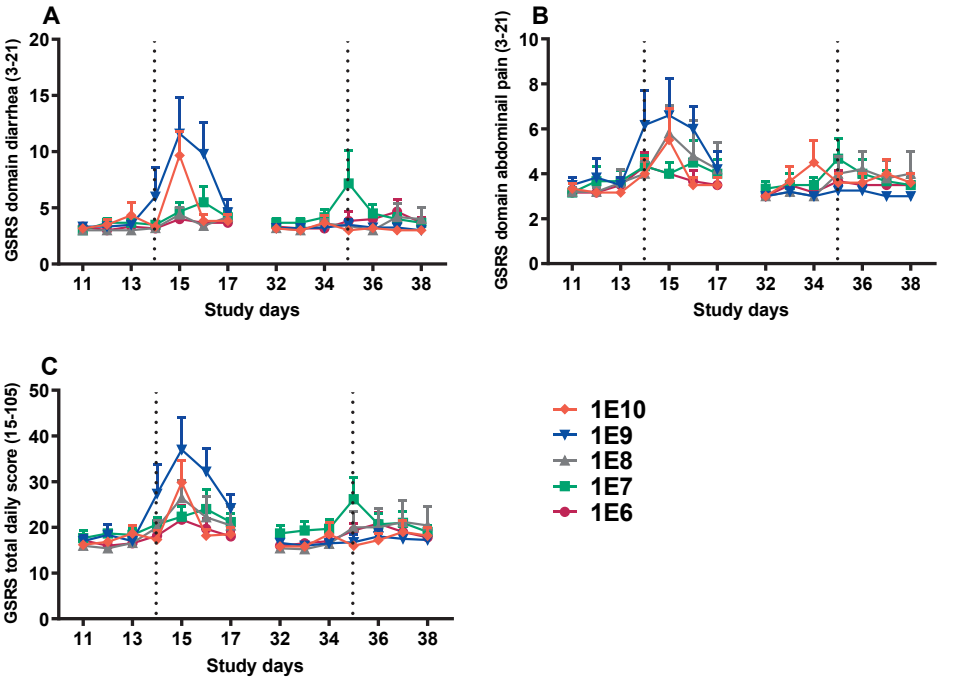

Fig 2. GSRS domain diarrhea (A), GSRS domain abdominal pain (B), and GSRS total daily score (C) before and after dia. *E. coli* challenges. The increase in GSRS domain diarrhea score was significantly dose-dependent (P<0.05) after the first challenge but not after the second one. Following the primary challenge, both GSRS domain abdominal pain and GSRS total daily score also increased, without dose-dependency, where an increase was not noted after the second challenge. The dotted lines represent challenge days 14 and 35 of the study, and each symbol represents the mean and one-sided SEM of the group.

Stool parameters

Stool parameters, including total and percentage fecal wet weight (%WW), stool frequency, and stool consistency, were determined for interval I (days 12/13-17) and interval II (days 33/34-38) during the study. An overall increase in total fecal wet weight and %WW was observed in interval I starting from day 15 for doses 1E9 and 1E10, where the increase was delayed for other doses by one day (Fig 3A & 3B). No significant dose-dependency in the responses was present, and all the groups returned to the baseline levels by day 17. In interval II, total fecal wet weight and %WW either showed no variations or decreased with no dose-dependency, and the highest levels did not surpass the maximum values of interval I. The baseline %WW values on days 13 and 34 were deducted from participants' maximum values during intervals I and II, respectively, and presented as delta % fecal wet weight (Δ%WW). This parameter demonstrated a non-significant dose-related increase in interval I (Fig 3C) and an inverted pattern during interval II (Fig 3D).

Moreover, following the primary dia. *E. coli* inoculation, an increase in the frequency of defecations (stools per day) of the participants with a significant dose-dependency (p<0.05)

was recorded (Fig 3E). In contrast, in interval II, the challenge dose was inversely related to the stool frequency ($p<0.05$), indicating a lower number of stools per day in participants of the groups which initially received higher doses of bacteria. Notably, all stool frequency scores in interval II were below the level of clinical diarrhea. Besides, there was no significant relationship between the primary bacteria doses and the stools' consistency based on Bristol Stool Score (BSS) in either of the intervals. This was true for both the average (Fig 3F) and maximum per day stool consistency score (supplementary Fig S2).

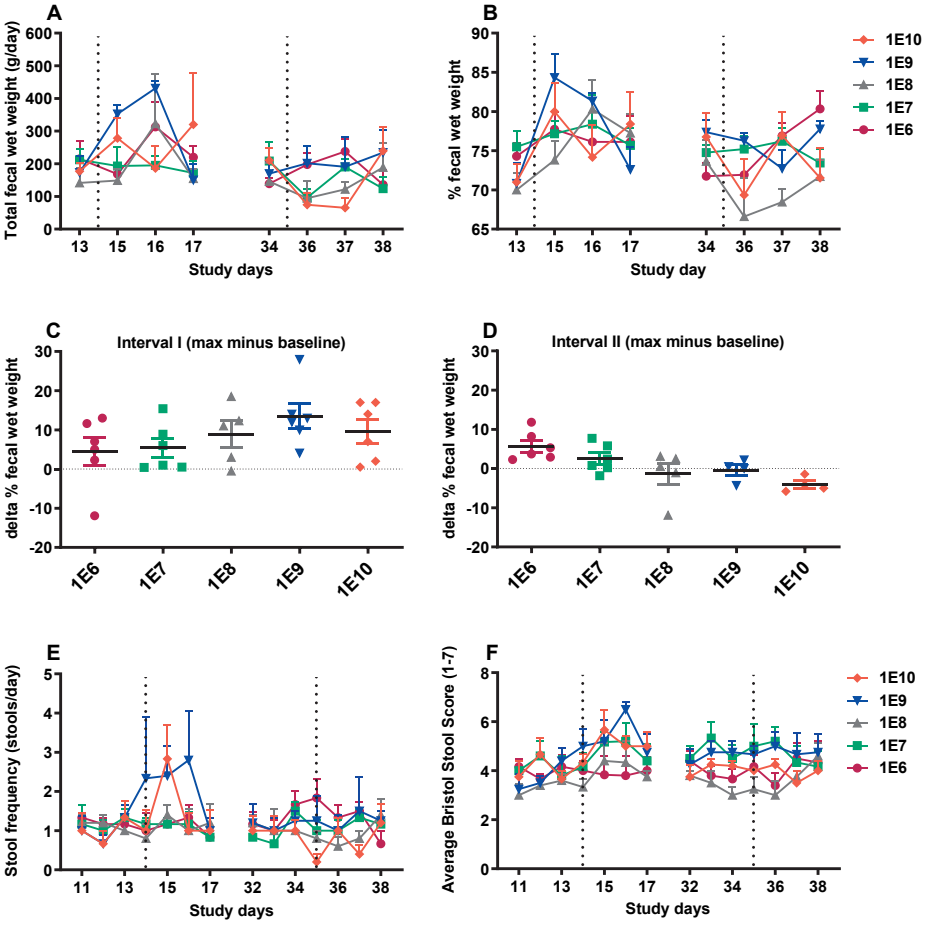

Fig 3. Total fecal wet weight (A), % of fecal wet weight (%WW) (B), delta % fecal wet weight (Δ%WW) in interval I (C), delta % fecal wet weight (Δ%WW) in interval II (D), stool frequency (E), and stool consistency (BSS) (F), before and after dia. *E. coli* challenges. The changes in total fecal wet weight, % fecal wet weight, and average Bristol Stool Score (BSS) were not dose-dependent after primary or secondary challenges. The stool frequency was positively related to the primary dia. *E. coli* dose ($p<0.05$) and negatively related to the dose after rechallenging ($p<0.05$). The Δ%WW increased relative to the primary dia. *E. coli* challenge dose in interval I and decreased in interval II; however, neither of the variations were statistically significant. The dotted lines represent challenges on day 14 and day 35 of the study, and each symbol represents the mean and SEM of the group.

Intestinal immune biomarkers

Three local intestinal immune markers: the inflammatory marker calprotectin, the antimicrobial peptide β-defensin, and total SIgA were analyzed in fecal water extracted from participants' stool samples collected in interval I (days 12/13-17) and interval II (days 33/34-38).

During interval I, a quick rise in calprotectin release was observed for the highest doses (1E10 and 1E9, and 1E8) compared to a delayed response in two lower doses (Fig 4A). Still, all primary dia. *E. coli* inoculation doses reached comparable levels by day 17. The response pattern was different in interval II, with no substantial increase in calprotectin levels. At rechallenge, only participants primarily inoculated with 1E6 and, to a lesser extent, 1E7 CFU showed a slight increase in calprotectin production, and the participants receiving higher primary doses were not affected. The primary infectious dose and the level of β-defensin did not show any relations in either of the intervals (Fig 4B), and generally, only minor changes in β-defensin production were recorded throughout the study. Likewise, fecal SIgA levels were not significantly correlated with the primary challenge dose (Fig 4C).

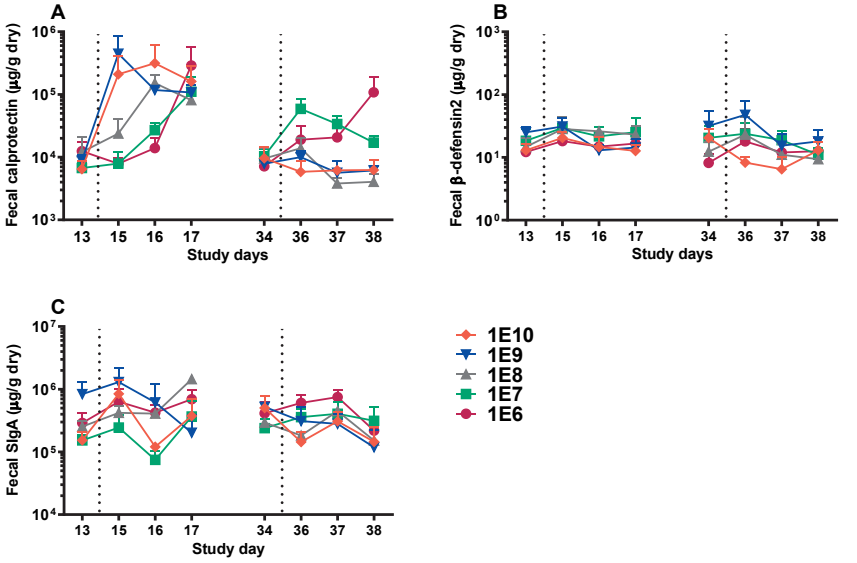

Fig 4. Fecal calprotectin (A), fecal β-defensin2 (B), and fecal total SIgA (C). Calprotectin production started on day 15 for doses 1E10 and 1E9, whereas for other doses, it started two days after the primary challenge (day 16). All doses reached similar levels on day 17 (A), and the levels returned to original status just before the second challenge and showed no major variations for any of the primary dose groups after the second challenge. β-defensin (B) and SIgA (C) production were not affected by various dia. *E. coli* doses during the study. The dotted lines represent challenges on day 14 and day 35 of the study, and each symbol represents the mean and one-sided SEM of the group.

Serum levels of IgG-CFA/II

Serum levels of IgG-CFA/II were measured at baseline and weekly for three weeks after each challenge as the study's primary immune parameter. Statistical analysis was performed to compare the absolute IgG levels for different doses over time during the study period (Fig 5).

The low baseline IgG-CFA/II antibody levels increased significantly ($p<0.05$) for all primary dia. *E. coli* doses throughout the study period. Moreover, different primary challenge doses had an overall significant ($p<0.05$) positive contribution to the IgG levels meaning the higher the dose, the higher the IgG level. This is illustrated by a positive association between primary dia. *E. coli* doses introduced at day 14 and IgG levels measured at day 35. Noticeably, the IgG-CFA/II levels did not increase further following the second challenge, except for the lowest dose group (1E6 CFU).

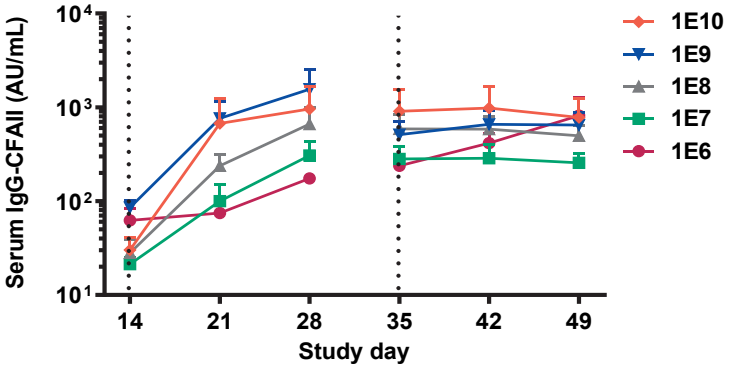

Fig 5. Serum IgG-CFA/II changes after the first and second challenges with dia. *E. coli*. The antibody levels increased significantly in all the groups and this increase was significantly dose-dependent. There was no significant increase in serum antibody levels after the second challenge in any doses except for 1E6. The dotted lines represent challenges on day 14 and day 35 of the study, and each symbol represents the mean and one-sided SEM of the group.

Ex vivo stimulation of monocytes, mDC, and pDC by dia. *E. coli* and TLR ligands

To address the innate immune system's capability to respond to *E. coli*, PBMCs isolated from the whole blood collected from the participants just before the challenge on day 14 and day 35 were stimulated with LPS (TLR4 ligand), flagellin (TLR5 ligand), or whole dia. *E. coli*. The percentage of IL-6 and TNF-α positive monocytes, myeloid dendritic cells (mDCs), and plasmacytoid dendritic cells (pDCs) was measured after a 3-hour *ex vivo* stimulation of the cells. These data are expressed as % of double-positive cells (producing both cytokines) or as % of all cells producing IL-6 or TNF-α (single positive). The data were analyzed separately for the different primary doses (supplementary Fig S6-7) and for participants of each challenge combined (Fig 6).

Stimulation of PBMCs with LPS, flagellin, and whole dia. *E. coli* bacteria resulted in a higher percentage of double-positive monocytes measured at day 35 compared with day 14 (Fig 6A). The background levels (RPMI medium group) were low on both day 14 and day 35, assuring the observed variations' validity. The same response pattern was observed for all different primary challenge dia. *E. coli* doses separately (supplementary Fig S6) and when all the doses were combined (Fig 6A). In fact, the average percentage of monocytes producing both IL-6 and TNF-α in response to dia. *E. coli* almost doubled from day 14 to day 35, and nearly all individuals showed an enhancement in response (Fig 6B). Following the monocyte's response pattern, stimulation of PBMCs with LPS, flagellin, and dia. *E. coli*, resulted in a significant increase in the percentage of double-positive mDCs. The observed response pattern was present for all dia. *E. coli* primary doses (supplementary Fig S7) and was significant when all primary doses were combined (Fig 6C). Nearly all individuals had an increase in double-positive mDCs upon dia. *E. coli* stimulation, with an average percentage increase of 1.5 times (Fig 6D). Analysis of IL-6 or TNF-α single-positive cells confirmed the pattern observed for double-positive cells (supplementary Fig S5). pDCs did not respond strongly to any of the stimuli, and changes in the percentage of double-positive pDCs were not significant (supplementary Fig S8).

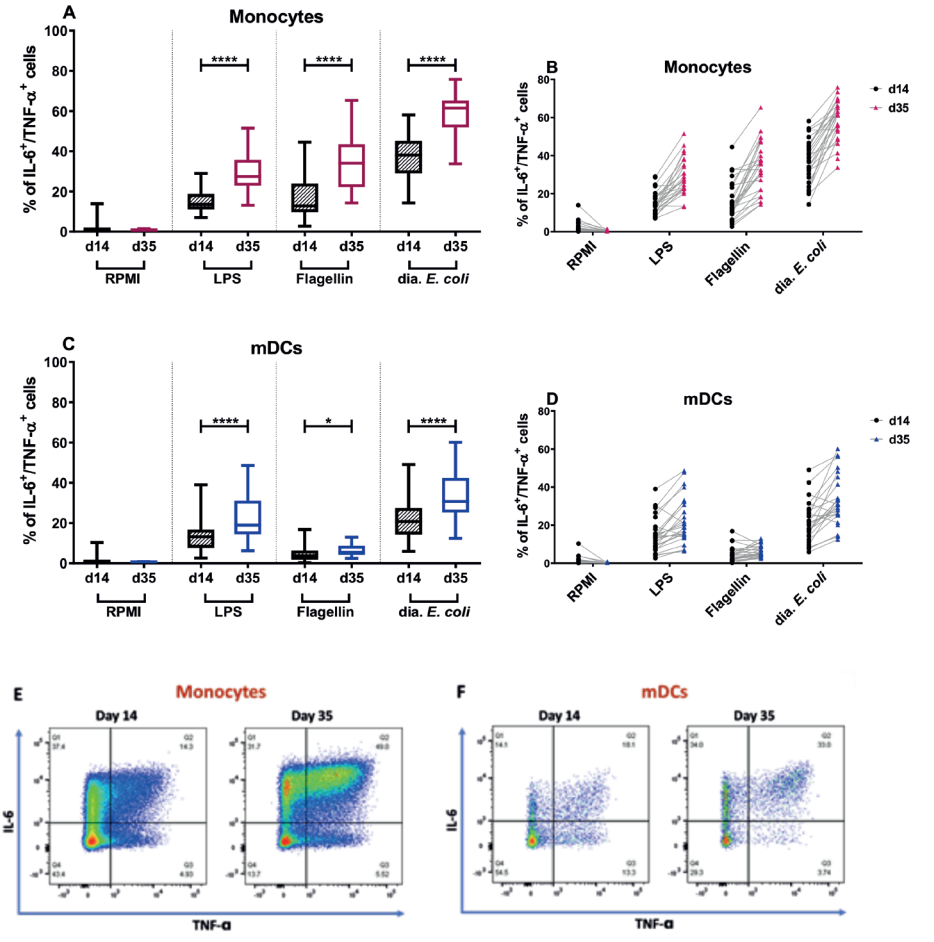

Fig 6. Variations in double-positive (IL-6 and TNF-α positive) monocytes and mDCs after dia. *E. coli* and TLR stimulation on days 14 and 35. A significant increase in the percentage of monocytes that were simultaneously producing IL-6 and TNF-α was seen after ex vivo stimulation with LPS (200 ng/mL), flagellin (500 ng/mL), or dia. E. coli (1E7 CFU/well) on day 35, comparing to day 14 (A). The average percentage of double-positive monocytes was almost doubled after stimulation with all stimuli in contrast to the RPMI control (B). Following the stimulation with the same stimuli, mDCs showed a significant increase in IL-6 and TNF-α production after exposure to LPS, flagellin, and dia. E. coli (C). Just about all participants showed an increase in double-positive mDCs after dia. E. coli and LPS stimulation, with an average percentage increase of almost 1.5 times (D). Data are represented in Whisker-plots with a median, 25% and 75 % quartile. * p<0.05; ** p<0.01; *** p<0.001,**** p<0.0001. A visual representation of changes in the percentage of IL-6 and TNF-α producing monocytes (E) and mDCs (F) of a representative participant (#10) on day 14 compared to day 35. The percentage of double-positive (top-right quadrant) monocytes increased from 14.3% to 49% (E), whereas double-positive mDCs had an increase from 18.1% on day 14 to 33% on day 35 (F) in this selected participant.

Correlation analysis

Statistical analysis was performed to analyze whether serum IgG-CFA/II level before the second challenge (day 35) was correlated with protection against clinical symptom

after the challenge. The analysis included the correlation between IgG-CFA/II levels and the following parameters: total fecal weight, % fecal wet weight, stool consistency, stool frequency, GSRS total, GSRS diarrhea, and GSRS abdominal pain. The analyses were based on using all individual data absolute values of IgG-CFA/II level on day 35 in relation to clinical symptoms on days 35-38. Overall, the analysis results suggest no statistically significant correlation between IgG-CFA/II levels and any clinical symptoms. Following the secondary challenge, a similar approach was applied to check the correlation between the monocytes and mDCs double-positive cells with the clinical outcomes. Comparable to IgG-CFA/II analysis outcomes, no significant correlation was observed between the monocyte or mDC double-positive cells and clinical symptoms (data not shown).

Discussion

Here we demonstrate that exposing healthy volunteers to diarrhoeagenic *E. coli* strain E1392/75-2A dosages as low as 1E6 CFU during a primary infection challenge resulted in clinical protection against a second challenge with a high dose of the same bacteria. This protection was accompanied by increased serum anti-CFA/II IgG levels and enhanced monocyte and mDC responses to *ex vivo* stimulation. However, these enhanced innate and adaptive immune parameters did not correlate significantly with protection to rechallenge.

Well-established and standardized oral infection challenge models are needed to demonstrate the clinical and immunological effects of drug and dietary interventions in preventing infections. Several ETEC challenge models have been described and applied for this reason [237, 240]. In this regard, supplementation with dietary calcium and milk-fat-globule membrane (MFGM) was shown to improve *in vivo* resistance to dia. *E. coli* strain E1392/75-2A -the same strain used in the current study- and Bismuth Subsalicylate ingestion reduced the diarrhea incidence caused by ETEC strain H10407 [77, 80, 216]. While these models aim to prevent primary infection by enhancing passive immunity, we aimed to study correlates of protection to reinfection with the same pathogen using a two-tier dia. *E. coli* challenge model. In this model, varying primary challenge doses of dia. *E. coli* strain E1392/75-2A is followed by a secondary challenge with a high dose of the same bacteria. The *E. coli* strain E1392/75-2A is a well-characterized live-attenuated variant of the ETEC strain O6:H16 that has a spontaneous deletion of enterotoxin-encoding genes but continues to produce CFA/II. Despite the induction of mild and transient gastrointestinal symptoms upon primary challenge, which makes it acceptable to use in human challenge studies, the protection against this strain was evidenced to provide 75% protection (scored as reduction of diarrhea incidence) against wild-type enterotoxin producing strains [237, 241].

Recently, van Hoffen et al. demonstrated that a primary challenge with standard high

dose (1E10 CFU) of dia. *E. coli* strain E1392/75-2A largely protected the participants from reinfection with the same pathogen [236]. The protection was accompanied by an increased serum IgG-CFA/II response. A higher primary dose of 5E10 CFU, on the other hand, did not affect the severity of clinical symptoms or the anti-CFA/II antibody response following the second challenge. Association analyses could not confirm a direct correlation between serum IgG-CFA/II titers and the intensity of clinical symptoms, and therefore, it could not be concluded that IgG-CFA/II serum levels provided protection against reinfection.

Because dia. *E. coli* primary challenge doses of 1E10 CFU or higher conferred total protection, identifying immune parameters that contributed to the protection against reinfection was not possible. The current study was designed as a dose-response pilot study to identify the underlying immunological mechanisms linked to correlates of protection. After the primary challenge, the lowest two doses of dia. *E. coli* did not induce clinical symptoms but increasing the bacteria dose resulted in clinical symptoms. This was illustrated by a significant dose-dependent increase in stool frequency and GSRS score in the subdomain diarrhea (Fig 3E & 2A). These parameters serve as primary indicators of diarrhea occurrence and were reported to increase following the inoculation of participants with high doses of dia. *E. coli* E1392/75-2A [216, 236]. Similarly, GSRS domain abdominal pain and GSRS daily total score presented a consistent but not significant trend towards increasing for the two highest doses. This is apparent from the kinetic pattern of the responses (Fig 2B & 2C). Moreover, when focusing on the variations in Δ%WW -as a clear indicator of changes in fecal wet weight related to diarrhea- it is quite illustrative that the challenge dose directs the severity of diarrhea after primary challenge (Fig 3C). Interestingly, there is a putative trend that higher doses protect better against the clinical symptoms, although all tested doses in primary challenge confer protection at rechallenge (Fig 3D).

After primary or secondary challenges, the analysis of β-defensin and total SIgA levels in fecal water showed no significant dia. *E. coli*-induced dose-dependent protein production (Fig 4B & 4C). However, the highest two doses of 1E10 and 1E9 significantly induced fecal calprotectin on day 15 (Fig 4A), the first day after the primary challenge, which is in line with previous reports [216, 239]. Calprotectin is a calcium-binding protein serving as a key marker of acute intestinal inflammation resulting from the influx of leukocytes, mainly neutrophils, into the gut as a response to infection [242]. Calprotectin induction in the fecal extract of participants exposed to low levels of dia. *E. coli* (1E6 and 1E7 CFU), and to a lesser extent, in those exposed to 1E8 CFU, did not occur until day 17, three days after the primary challenge. (Fig 4A). It is known that dia. *E. coli* strain E1392/75-2A only temporarily colonizes the gut and is typically cleared within 14 days after challenge with 1E10 CFU [80]. A possible explanation for the delayed dose-dependent induction of

calprotectin at low infectious dosages observed here may be that the low level of infection allows a temporary expansion of the bacteria, reaching a certain threshold level to result in calprotectin production before the infection is cleared.

A dose-dependent increase of CFA/II-specific IgG levels in the serum was noted on day 28 of the study (Fig 5). This was especially obvious when comparing the two highest doses of 1E9 and 1E10 to the lower doses. The observed dose-response pattern in CFA/II-specific IgG levels was consistent with previous findings reviewed by Porter et al., indicating that lower inoculation doses may lead to lower antibody response after primary challenge [240]. Contrary to our expectations, rechallenging with a high dose of dia. *E. coli* 1E10 CFU did not result in severe clinical symptoms in any of the dose groups tested. Even at the lowest dose (1E6 CFU), there were almost no reports of severe diarrhea or gastrointestinal symptoms (Fig 2). In fact, after rechallenge, all GSRS scores and stool parameters, including total and relative fecal wet weight, as well as stool frequency and consistency markers, showed minor fluctuations, indicating that all participants were protected from diarrhea regardless of the primary dia. *E. coli* challenge dose. Likewise, no intestinal inflammation was detected, as evidenced by the absence of an apparent increase in calprotectin levels in the subjects' fecal extracts (Fig 6A). After the second challenge, only those who received the lowest primary dia. *E. coli* doses of 1E6 and 1E7 experienced a slight increase in calprotectin levels. A possible explanation for the low inflammatory responses could be the exclusion of dia. *E. coli* by secretory IgA in the gut and, consequently, less interaction of the bacteria with mucosal epithelial receptors [243, 244].

Upon rechallenge, serum IgG-CFA/II level hardly increased further in any of the groups (Fig 6), suggesting attainment to a steady-state what was reported before for dose 1E10 of the same dia. *E. coli* strain [236] and for ETEC strain H10407 [245]. In our study, only two patients from the dose group 1E6 who did not respond to the primary challenge showed an increase in IgG-CFA/II levels following the second challenge. This finding suggests that, like calprotectin, a minimal inoculation dose is required to induce an IgG-CFA/II response, which may vary between participants. Later association analyses could not reveal a significant correlation between serum IgG-CFA/II level on day 35 and any clinical symptoms after the second challenge, similar to previous observations. To our knowledge, although systemic and local parameters have been measured in dia. *E. coli* challenge studies, no direct correlation between serum IgG/IgA or fecal IgA titer and clinical protection has been documented yet [240, 246]. Despite no documented correlations, the role of pathogen-specific IgG and IgA and, most importantly, SIgA -which facilitates bacteria clearance from the gut- in establishing protection against dia. *E. coli* may not be disregarded and needs to be addressed in future studies.

The relative relevance of the innate immune system in protection against dia. *E. coli*

reinfection has not been previously addressed. As two key innate immune cells, monocytes and mDCs are among the first cells to respond to pathogen translocation across the epithelial barrier by releasing pro-inflammatory mediators such as IL-6 and TNF-α. Their cytokine secretion profile and antigen presentation to naïve lymphocytes are critical for inducing host defense mechanisms and regulating both innate and adaptive immune responses [247, 248]. Interestingly, we observed an increased response of monocytes and mDCs to dia. *E. coli* -as well as to LPS and flagellin- three weeks after the primary challenge, regardless of the initial challenge dose used (Fig 6 & supplementary Fig S5-7). Given a 3-week period between the primary challenge and the increased monocyte and mDC response, a priming effect on these cells by the first challenge does not seem likely. An alternative explanation can be that the primary challenge with dia. *E. coli* leads to trained immunity.

The concept of trained immunity is based on observations that individuals who received the Bacillus Calmette–Guérin (BCG) vaccine develop cross-protection against unrelated pathogens [249, 250]. Netea and colleagues verified the findings and not only demonstrated the training ability of some other microbial components such as β-glucan but also described epigenetic modifications, e.g., increased H3K4 trimethylation as the underlying mechanism [29, 53, 251, 252]. Monocytes undergo an inheritable alteration in their gene expression pattern following training by potential components [39, 43]. After training, these cells exhibit an enhanced nonspecific response upon secondary TLR-mediated activation by the same and similar pathogens, as evidenced by increased pro-inflammatory cytokine production. While trained immunity was initially demonstrated in monocytes and later natural killer (NK) cells, new research indicates that other myeloid cells, including dendritic cells, may also be trained to elicit an increased response to repeated stimulation [42]. Therefore, the increased number of activated monocytes and mDCs after the primary challenge presented in this study suggests that dia. *E. coli* strain E1392/75-2A may induce trained immunity *in vivo*.

This hypothesis is supported by the fact that the enhanced responses were not limited to the applied dia. *E. coli* strain. Apart from the applied strain, the augmented number of IL-6 and TNF-α producing monocytes and mDCs were recorded upon the stimulation with TLR ligands, namely LPS (from *E. coli* strain O55:B5) and flagellin (from *Salmonella typhimurium*). This indicates that the enhanced innate response to the dia. *E. coli* is also extended towards unrelated pathogens. To which extent and for how long these effects will last is currently unknown.

This study links local and systemic immune parameters to clinical gastrointestinal symptoms following dia. *E. coli* infection to explain the immunological factors related to clinical protection against reinfection in this challenge mode. Even though no dose-dependent correlation between immune markers and clinical symptoms was noted, we

speculate that the observed protection may have resulted from the participation of multiple immunological components. Both humoral immunity -mainly via serum anti-CFA/II IgG and SIgA- and innate immunity -via the enhanced activity of monocytes and mDCs- may play a role in the clinical protection against infection.

In conclusion, our data suggest that low-dose dia. *E. coli* exposure can protect against reinfection through modulating adaptive and innate immune responses. The lower IgG and calprotectin responses induced by primary challenge, as well as the trend in % fecal wet weight at rechallenge, may be used to further establish this two-tier dia. *E. coli* challenge model as a model to explore the impact of drugs and food components on antibacterial immunity.

References

1. Troeger, C.E., et al., *Quantifying risks and interventions that have affected the burden of diarrhoea among children younger than 5 years: an analysis of the Global Burden of Disease Study 2017.* The Lancet Infectious Diseases, 2020. **20**(1): p. 37-59.
2. Qadri, F., et al., *Enterotoxigenic Escherichia coli in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention.* Clinical microbiology reviews, 2005. **18**(3): p. 465-483.
3. Kaper, J.B., J.P. Nataro, and H.L.T. Mobley, *Pathogenic Escherichia coli.* Nature Reviews Microbiology, 2004. **2**(2): p. 123-140.
4. Kollaritsch, H., M. Paulke-Korinek, and U. Wiedermann, *Traveler's Diarrhea.* Infectious Disease Clinics of North America, 2012. **26**(3): p. 691-706.
5. Foster, J.W., *Escherichia coli acid resistance: tales of an amateur acidophile.* Nature Reviews Microbiology, 2004. **2**(11): p. 898-907.
6. Lund, P., A. Tramonti, and D. De Biase, *Coping with low pH: molecular strategies in neutralophilic bacteria.* FEMS Microbiology Reviews, 2014. **38**(6): p. 1091-1125.
7. Nataro, J.P. and J.B. Kaper, *Diarrheagenic Escherichia coli.* Clinical microbiology reviews, 1998. **11**(1): p. 142-201.
8. Mirhoseini, A., J. Amani, and S. Nazarian, *Review on pathogenicity mechanism of enterotoxigenic Escherichia coli and vaccines against it.* Microbial Pathogenesis, 2018. **117**: p. 162-169.
9. Bovee-Oudenhoven, I.M.J., et al., *Diarrhea caused by enterotoxigenic Escherichia coli infection of humans is inhibited by dietary calcium.* Gastroenterology, 2003. **125**(2): p. 469-476.
10. Agustina, R., et al., *Randomized Trial of Probiotics and Calcium on Diarrhea and Respiratory Tract Infections in Indonesian Children.* Pediatrics, 2012. **129**(5): p. e1155.
11. Baqui, A.H., et al., *Simultaneous Weekly Supplementation of Iron and Zinc Is Associated with Lower Morbidity Due to Diarrhea and Acute Lower Respiratory Infection in Bangladeshi Infants.* The Journal of Nutrition, 2003. **133**(12): p. 4150-4157.
12. Graham, D.Y., M.K. Estes, and L.O. Gentry, *Double-blind comparison of bismuth subsalicylate and placebo in the prevention and treatment of enterotoxigenic Escherichia coli-induced diarrhea in volunteers.* Gastroenterology, 1983. **85**(5): p. 1017-22.
13. Black, R.E., et al., *Treatment of experimentally induced enterotoxigenic Escherichia coli diarrhea with trimethoprim, trimethoprim-sulfamethoxazole, or placebo.* Rev Infect Dis, 1982. **4**(2): p. 540-5.
14. Ten Bruggencate, S.J., et al., *Dietary Milk-Fat-Globule Membrane Affects Resistance to Diarrheagenic Escherichia coli in Healthy Adults in a Randomized, Placebo-Controlled, Double-Blind Study.* The Journal of Nutrition, 2015. **146**(2): p. 249-255.
15. van Hoffen, E., et al., *Characterization of the pathophysiological determinants of diarrheagenic Escherichia coli infection using a challenge model in healthy adults.* Sci Rep, 2021. **11**(1): p. 6060.
16. Levine, M.M., E.M. Barry, and W.H. Chen, *A roadmap for enterotoxigenic Escherichia coli vaccine development based on volunteer challenge studies.* Human Vaccines & Immunotherapeutics, 2019. **15**(6): p. 1357-1378.
17. Svedlund, J., I. Sjödin, and G. Dotevall, *GSRS—A clinical rating scale for gastrointestinal symptoms in*

patients with irritable bowel syndrome and peptic ulcer disease. Digestive Diseases and Sciences, 1988. **33**(2): p. 129-134.

18. Ouwehand, A.C., et al., *Lactobacillus acidophilus supplementation in human subjects and their resistance to enterotoxigenic Escherichia coli infection.* British Journal of Nutrition, 2014. **111**(3): p. 465-473.
19. Porter, C.K., et al., *A systematic review of experimental infections with enterotoxigenic Escherichia coli (ETEC).* Vaccine, 2011. **29**(35): p. 5869-85.
20. Tacket, C. and M. Levine, *Vaccines against enterotoxigenic Escherichia coli infections.* New generation vaccines, 2nd ed. Marcel Dekker, Inc., New York, NY, 1997: p. 875-883.
21. Pathirana, W.G.W., et al., *Faecal Calprotectin.* The Clinical biochemist. Reviews, 2018. **39**(3): p. 77-90.
22. Mantis, N.J., N. Rol, and B. Corthésy, *Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut.* Mucosal immunology, 2011. **4**(6): p. 603-611.
23. Corthesy, B., *Multi-Faceted Functions of Secretory IgA at Mucosal Surfaces.* Frontiers in Immunology, 2013. **4**(185).
24. Harro, C., et al., *Refinement of a human challenge model for evaluation of enterotoxigenic Escherichia coli vaccines.* Clinical and vaccine immunology : CVI, 2011. **18**(10): p. 1719-1727.
25. Zhang, W. and D.A. Sack, *Progress and hurdles in the development of vaccines against enterotoxigenic Escherichia coli in humans.* Expert Review of Vaccines, 2012. **11**(6): p. 677-694.
26. van Furth, R. and H. Beekhuizen, *Monocytes*, in *Encyclopedia of Immunology (Second Edition)*, P.J. Delves, Editor. 1998, Elsevier: Oxford. p. 1750-1754.
27. Qian, C. and X. Cao, *Dendritic cells in the regulation of immunity and inflammation.* Seminars in Immunology, 2018. **35**: p. 3-11.
28. Benn, C.S., et al., *A small jab – a big effect: nonspecific immunomodulation by vaccines.* Trends in Immunology, 2013. **34**(9): p. 431-439.
29. Giamarellos-Bourboulis, E.J., et al., *Activate: Randomized Clinical Trial of BCG Vaccination against Infection in the Elderly.* Cell, 2020. **183**(2): p. 315-323.e9.
30. Netea, M.G., et al., *Trained immunity: A program of innate immune memory in health and disease.* Science, 2016. **352**(6284).
31. Netea, M.G., et al., *Defining trained immunity and its role in health and disease.* Nature Reviews Immunology, 2020.
32. Kleinnijenhuis, J., et al., *Bacille Calmette-Guérin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes.* Proceedings of the National Academy of Sciences, 2012. **109**(43): p. 17537.
33. Kleinnijenhuis, J., et al., *BCG-induced trained immunity in NK cells: Role for non-specific protection to infection.* Clinical Immunology, 2014. **155**(2): p. 213-219.
34. Saeed, S., et al., *Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity.* Science (New York, N.Y.), 2014. **345**(6204): p. 1251086-1251086.
35. Katzmarski, N., et al., *Transmission of trained immunity and heterologous resistance to infections across generations.* Nature Immunology, 2021.
36. Hole, C.R., et al., *Induction of memory-like dendritic cell responses in vivo.* Nature communications, 2019. **10**(1): p. 2955-2955.

Supporting information

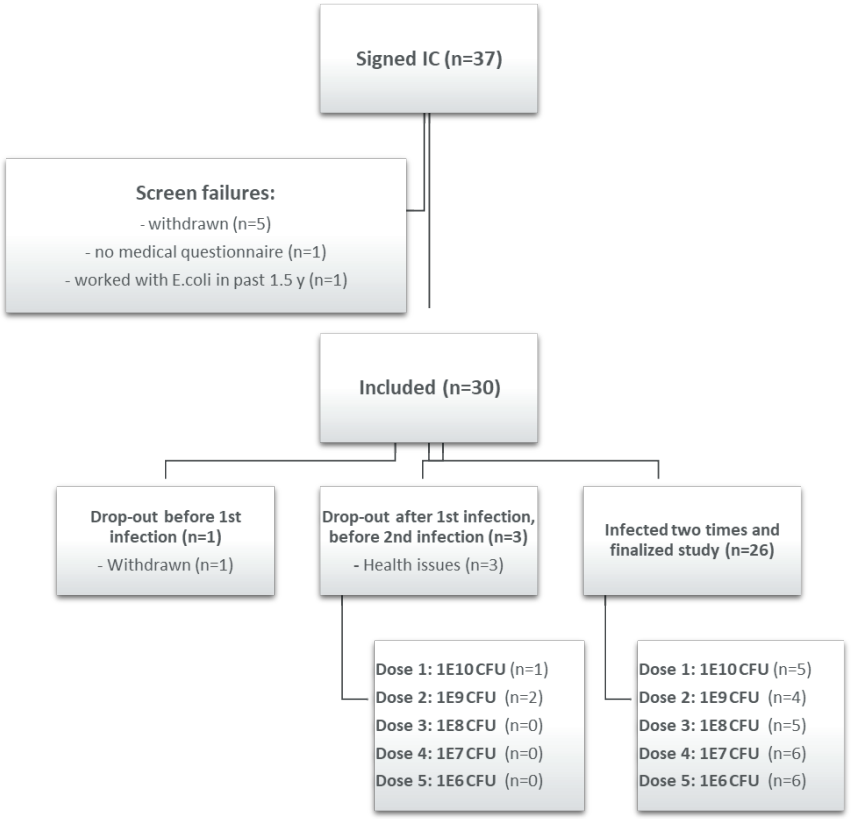

Figure S1. Recruitment and execution diagram of MIRRE study. The participants who signed the informed consent were further assessed for inclusion and exclusion criteria. The main inclusion criteria included sex, age, BMI, health condition, and the willingness to comply with all study procedures. The exclusion criteria were: current or previous underlying gastrointestinal disease; confirmed *E. coli* or cholera infection within 3 years prior to inclusion; diarrhea symptoms with a history of travel to *E. coli* endemic regions in past 3 years; vaccination for or ingestion of *E. coli* or cholera within 3 years before inclusion; known allergy to antibiotics; reported average stool frequency of <1 or >3 per day; use of antibiotics, activated charcoal, laxatives up till 6 months before inclusion; use of gastric acid suppression medication within 3 months before inclusion; current excessive alcohol consumption or drug (ab)use; vegans. Regarding the drop-out participant, One participant was dropped out of the study before the first infection and three others have withdrawn from the study before the second infection because of health issues. From those three, one participant had respiratory tract infections (not related to the study) and the second one experienced gastrointestinal symptoms before the second infection (not related to the study). One of the dropped out participants had serious adverse event (SAE) because of hospitalization due to the risk of dehydration. This subject suffered from diarrhea, nausea, vomiting, fainting and an overall feeling of malaise one day after the *E. coli* inoculation. The SAE was reported online to the national authority (CCMO) within 7 days after the reporting to the study team. The subject was hospitalized for 7 days, because of flu-like symptoms. The medical investigator assessed that the SAE was probably related to the study intervention, and that the SAE was completely resolved after discharge from the hospital. The medical investigator also consulted the general practitioner of this subject. It appeared that there were pre-existing factors that were probably triggered by the infection, which may have contributed to the need for (prolonged) hospitalization of this subject.

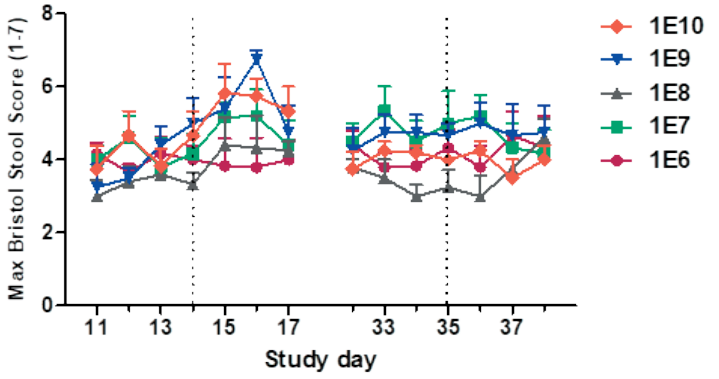

Figure S2. Maximum daily Bristol Stool score. The changes in maximum Bristol stool score (stool consistency score) after primary infection or after reinfection were not dependent on the bacterial dose used during primary infection. The dotted lines represent infection days 14 and 35 of the study, and each symbol represents the mean and one-sided SEM of the group.

Study days (period 1)	Pre-screening	1-10	11	12	13	14	15	16	17	18-20	21	22-27	28
Informed consent and screening													
Restriction for specific medicine intake													
Restriction for alcohol intake													
Standard evening meal													
Overnight fast													
Infection (attenuated ETEC)													
Data safety monitoring board													
Collection 24h fecal samples													
Collection blood samples													
BSS, Stool frequency, GSRS score (online)													
Restricted dietary guidelines & dairy intake													
Registration medication intake & compliance (online)													

Study days (period 2)		29-31	32	33	34	35	36	37	38	39-41	42	43-48	49
Restriction for specific medicine intake													
Restriction for alcohol intake													
Standard evening meal													
Overnight fast													
Infection (attenuated ETEC)													
Data safety monitoring board													
Collection 24h fecal samples													
Collection blood samples													
BSS, Stool frequency, GSRS score (online)													
Restricted dietary guidelines & dairy intake													
Registration medication intake & compliance (online)													

Figure S3. Schematic presentation of the events and measurements. A graphical representation of various steps that were taken daily before and after the first and the second infection.

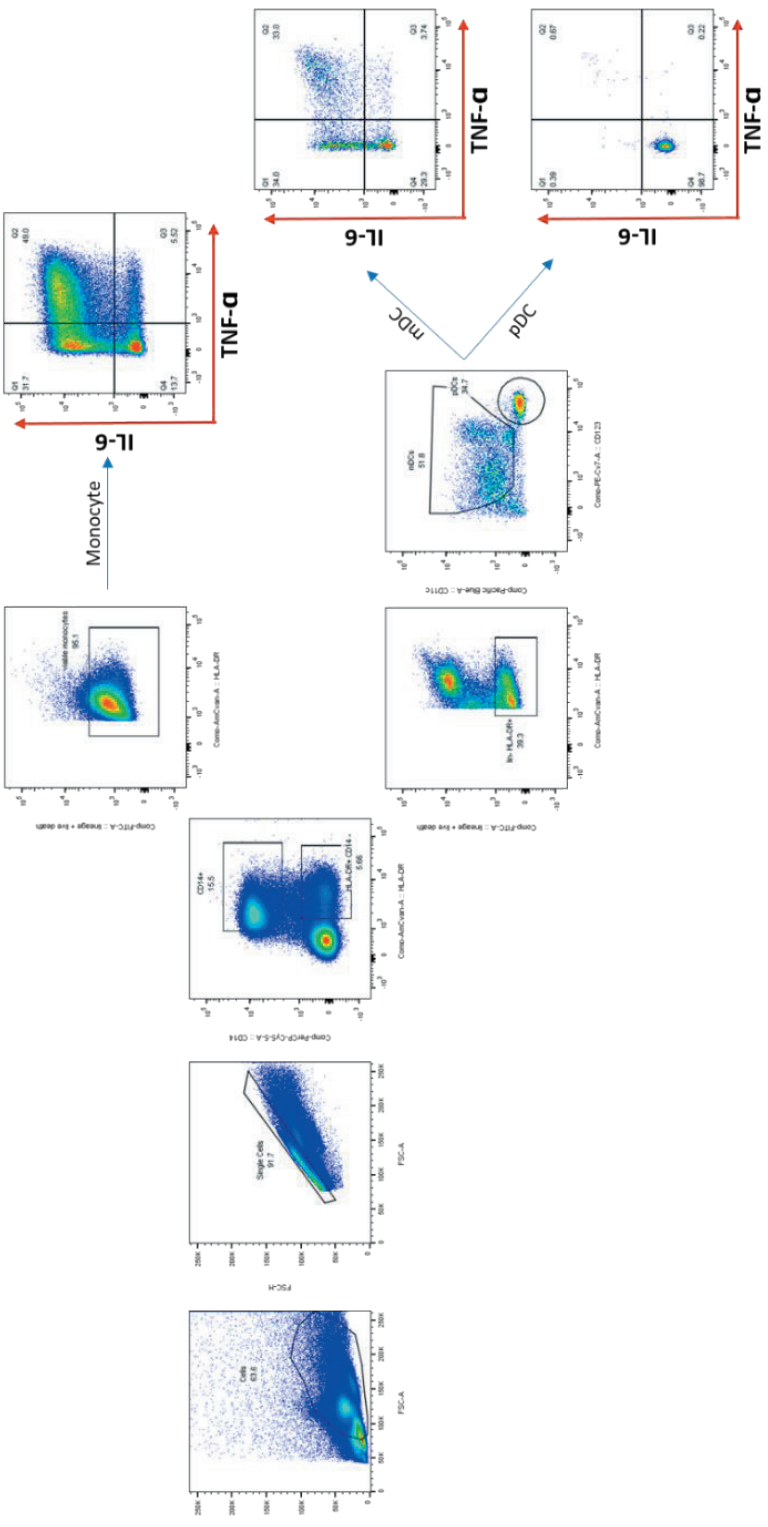

Figure S4. Gating strategy for selecting monocytes, mDCs, and pDCs. In the FSC/SSC plot cells within the PBMC region were selected. The duplets were gated out and the HLA-DR$^+$CD14$^+$ cells were considered as the monocytes. From the HLA-DR$^+$CD14$^-$ population CD3$^+$, CD19$^+$,CD20$^+$ and CD56$^+$ cells were excluded. In the remaining population, CD11c$^+$ cells were considered as mDCs and CD123$^+$ cells were named pDCs. Within monocyte, mDC, and pDC populations cells that were producing IL-6, TNF-a or both cytokines were determined.

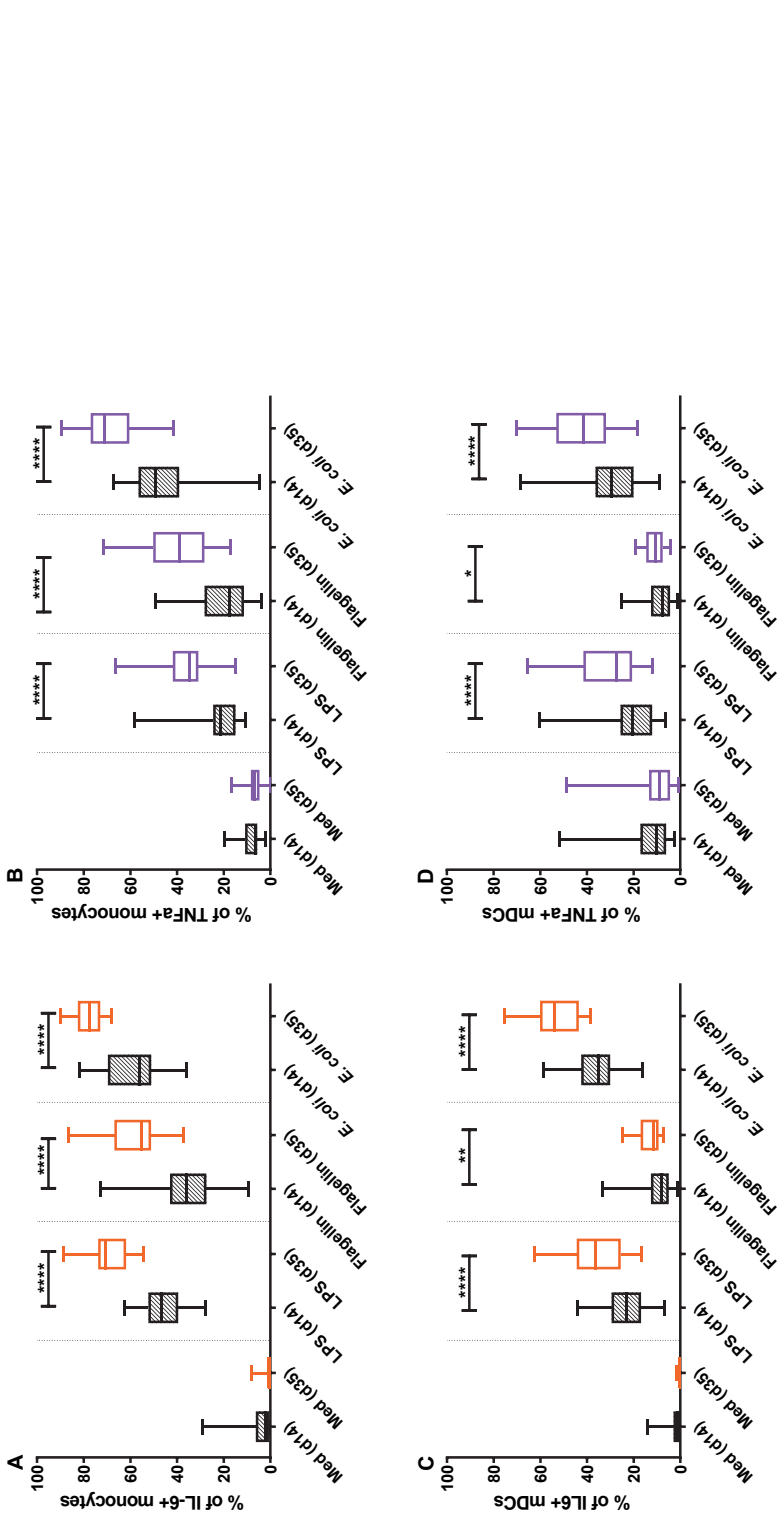

Figure S5. The variation in the percentage of all the cells that were producing IL-6 or TNF-a (single positives) after *ex vivo* stimulation. The cells were stimulated *ex vivo* with either medium (RPMI-1640), 200 ng/mL of LPS, 500 ng/mL of Flagellin, or 1E7 CFU/well of *E. coli* (strain E1392/75-2A). The percentage of IL-6$^+$ as well as TNF-a$^+$ monocytes increased significantly on day 35 compared to day 14. In mDCs, only LPS and *E. coli* increased the percentage of IL-6$^+$ cells on day 35 and the significant increase in TNF-a$^+$ mDCs only occurred after ETEC stimulation on day 35. Data are shown in Whisker-plots with a median, 25% and 75 % quartile. * p<0.05; ** p<0.01; *** p<0.001.

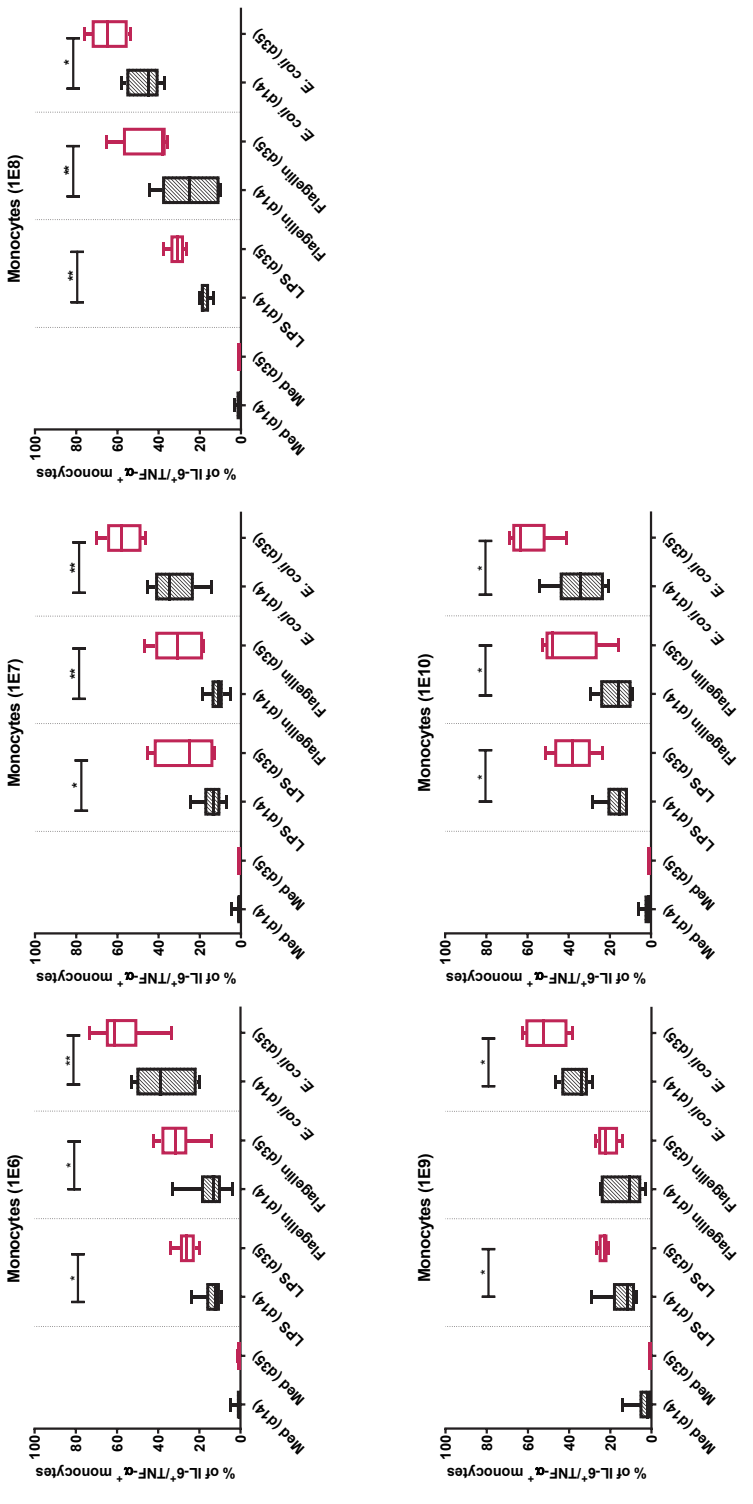

Figure S6. Variation in the percentage of double-positive monocytes within different primary infection group doses. After *ex vivo* stimulation of PBMCs with either medium (RPMI-1640), 200 ng/mL of LPS, 500 ng/mL of Flagellin, or 1E7 CFU/well of *E. coli* (strain E1392/75-2A), the percentage of double-positive monocytes increased in all dose groups. This increase was significant after stimulation with flagellin and *E. coli* in group dose 1E6 and 1E7, flagellin in group dose 1E8, and following LPS, flagellin, ETEC stimulation in group 1E10. Data are shown in Whisker-plots with a median, 25% and 75 % quartile. * p<0.05; ** p<0.01; *** p<0.001.

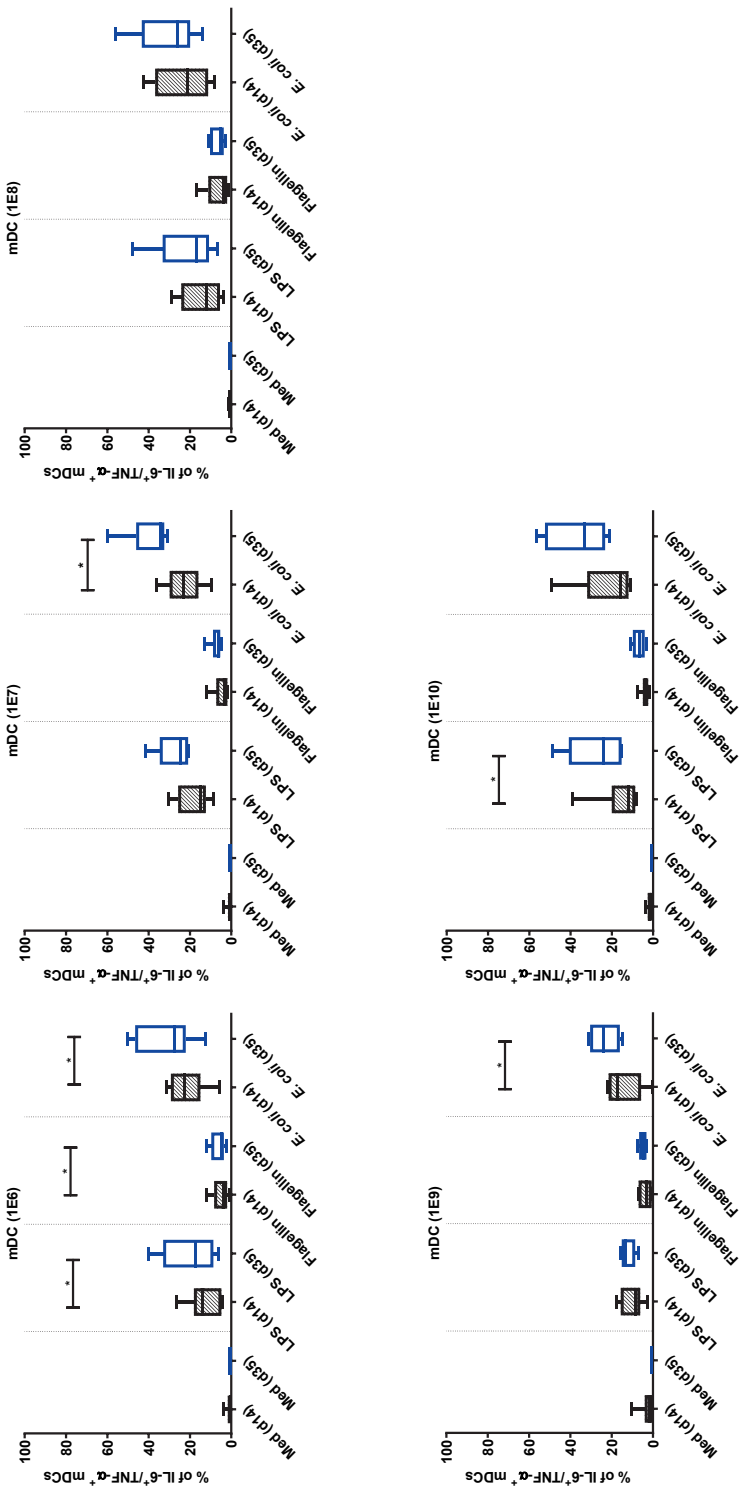

Figure S7. Variation in the percentage of double-positive mDCs within different primary infection group doses. After ex vivo stimulation of PBMCs with either medium (RPMI-1640), 200 ng/mL of LPS, 500 ng/mL of Flagellin, or 1E7 CFU/well of E. coli (strain E1392/75-2A), the percentage of double-positive mDCs increased in all dose groups. This increase was significant after stimulation with E. coli in group dose 1E7. Data are shown in Whisker-plots with a median, 25% and 75 % quartile. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

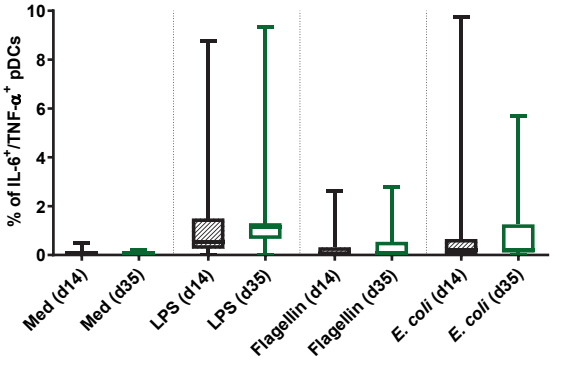

Figure S8. Variations in double-positive (IL-6 and TNF-α positive) pDCs after ETEC and TLR stimulation on days 14 and 35. Ex vivo stimulation of pDCs with either medium (RPMI-1640), 200 ng/mL of LPS, 500 ng/mL of Flagellin, or 1E7 CFU/well of E. coli (strain E1392/75-2A), did not result in an increased percentage of double-positive pDCs. The Amount of IL-6 and TNF-α production in pDCs were marginal in all challenge doses and upon all different stimuli and were not significantly different between base line and day 21 of the study.

CHAPTER

General discussion

Food and diet have a significant impact on our health and immune competence. Deficiency in several micronutrients such as vitamins and minerals leads to decreased immune function. On the other hand, immunomodulation by nutrition or supplementation of the food with immunomodulatory components can provide immune support to individuals with an immature immune or impaired immune system. Newborns, the elderly, and people with immunodeficiency do not have fully competent immune systems. Therefore, they may benefit from the supplementation of their diet with food components that can support their immune system. To substantiate the dietary components' beneficial effects and define the mechanisms involved, the potential food ingredients need to be studied *in vitro* and subsequently *in vivo* to substantiate the presumed health effects.

In this thesis, we aimed to investigate the immunomodulatory properties of selected (bovine) milk components and metabolites induced by them. For this purpose we:

- Reviewed the current knowledge on the ability of bioactive milk components to epigenetically program the immune function and implications on allergy and asthma (**Chapter 2**)

- Investigated the impact of individual SCFAs direct exposure on the function and response of innate and adaptive immune cells to stimulation (**Chapter 3**)

- Studied the binding of bovine milk IgG (bIgG) immune complexes (ICs) to human monocytes and the effector function exerted from the interaction of bIgG ICs with FcγRs on human monocytes (**Chapter 4**)

- Tested the immune-supportive impact of WPC consumption in enhancing the responsiveness of monocytes and DCs and possibly modulating PBMC gene expression in a human challenge model (**Chapter 5**)

- Optimized a human diarrheagenic *E. coli* challenge model for studying the effects of food ingredients and looked into the immunological mechanisms that brought protection at rechallenge (**Chapter 6**)

Impact of bioactive milk components on human epigenetics

The review presented in **Chapter 2**, which is pertinent to this thesis, focuses on the impacts of bioactive components in human and bovine milk, as well as environmental factors (e.g., farming, milk processing, and bacterial exposure). These factors can have an effect in early life and beyond, contributing to the epigenetic mechanisms underlying the persistent programming of immune functions and allergic diseases.

There is considerable heterogeneity in humans' immunological parameters and their response to stimulation. These variances exist even among identical twins, indicating a significant impact of non-heritable influences [253]. In fact, non-heritable factors contribute more to immune parameter adaptation than genetically transmitted traits. Comprehensive studies on monozygotic twins revealed that non-heritable variables account for more than half (61%) of the variations noted in immune cell frequency and serum proteins [253]. Non-heritable influences - colloquially referred to as environmental factors - include exposure to infectious agents and vaccines as well as any relevant external influence that is not genetically determined. The environment can modify the phenotypical characteristics of immune cells through *de novo* mutations and stochastic epigenetic alterations [254]. Epigenetic modifications may, to some extent, help to explain why individuals respond differently to comparable stimuli.

DNA methylation, histone modifications, and RNA interference via microRNA (miRNAs) are all examples of epigenetic modifications that heritably affect gene expression without changing the genomic sequences (Fig. 1). The addition of a methyl group to DNA (methylation) has been shown to influence gene expression, for instance, in the promoter regions of cytokines that steer T cells differentiation [255, 256]. Activated STAT6 proteins bind to the GATA3 gene locus in naive $CD4^+$ T cells, promoting Th2 development. Rapid demethylation during Th2 differentiation results in increased production of IL-4 and IL-13, which is a hallmark of allergy development [257]. Histone modifications like acetylation, methylation, and phosphorylation modify the accessibility of DNA for transcription factors as sequence-specific DNA-binding factors. Histone Acetyltransferases (HATs) promote euchromatin formation, which is typically considered permissive for transcription of genes, whereas Histone Deacetylases (HDACs) catalyze the more condensed heterochromatin status. The interplay between HATs and HDACs determines the histone acetylation balance and regulates the expression of genes for e.g., pro- and anti-inflammatory cytokines [258-260]. Histone modifications are also critical for T cell differentiation since demethylation of H3K9 and methylation of H3K27 have been linked to Th2 differentiation [257], and phosphorylation of STAT6 signal transducers results in the production of IL-4 producing Th2 cells [261]. In this context, asthmatic individuals were shown to have a lower HDAC:HAT ratio [262]. Besides, miRNAs found in biological fluids and extracellular vesicles have the potential to help degrade mRNA and/or inhibit post-transcriptional translation [263]. Based on this, miRNAs in milk exosomes can selectively silence gene expression and influence Foxp3 expressing regulatory T cells (Treg) [264].

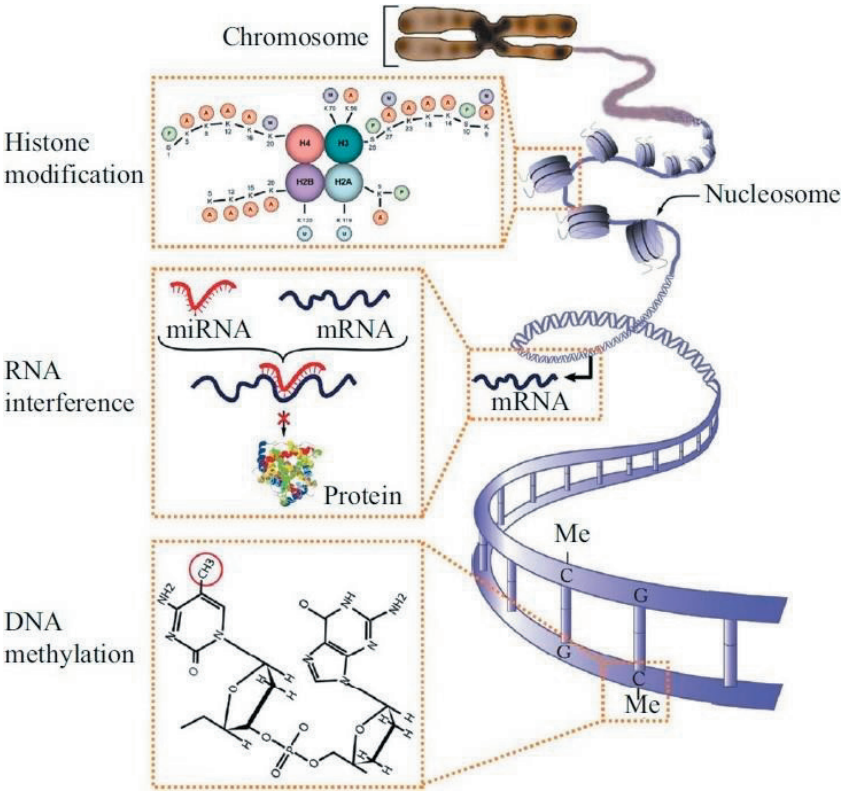

Fig. 19. Schematic representation of epigenetic modifications: DNA methylation, histone modifications, and RNA interference are all different mechanisms of epigenetic modification. DNA methylation is the addition of a methyl group to the cytosine nucleic acid on the promotor loci of a specific gene on the DNA. Histone modifications include acetylation, methylation, and phosphorylation of mainly H3 and H4, resulting in a more relaxed or condensed chromatin. RNA-mediated gene silencing occurs when micro RNA (miRNA) binds to the transcript mRNA and inhibits the RNA translation. various epigenetic modifications might be dependent on the exposure to foods and other compounds from the environment. (Figure adapted from Kim et al., Pulm. Circ. 2011)

Epigenetic changes begin during the intrauterine period when the mother's diet and prenatal antigen exposure imprint the fetus with long-lasting effects. During gestation, the mucosal immune system in the gastrointestinal (GI) tract is fully formed, and the aforementioned elements can have an impact on its development [265-268]. It is suggested that bacteria can be transmitted directly into the uterus via the placenta during fetal growth, contributing to immune system development [269], although this hypothesis has recently been challenged [270]. Consumption of omega-3 Polyunsaturated Fatty Acid (PUFA) by pregnant mothers has been linked to a lower incidence of allergies after birth [271, 272], demonstrating the impact of maternal diet on fetal immune system development. Also, the number of regulatory T cells (Treg) in a newborn's cord blood increases due to maternal exposure to farm environments, which is connected to lower Th2 cytokines and may be linked to demethylation at the FOXp3 promoter [273].

Following birth, factors like breastfeeding, early life nutrition, and environmental factors continue to shape the child's immune system. Nutrition contributes to further development of the mucosal system by influencing the composition of the (gut) microbiota which is associated with protection against infection as well as the development of non-communicable diseases, including asthma [274]. Adding specific pre- or probiotics to the mother's diet affects milk composition [275], and supplementing nondigestible oligosaccharides has been reported to reduce allergic disease in neonates [276-278]. The gut microbiota ferments Human Milk Oligosaccharides (HMOs) and Nondigestible Polysaccharides (NDPs) to produce Short-Chain Fatty Acids (SCFAs). SCFAs are well-known epigenetic modifiers that can inhibit the activity of HDACs, leading to histone hyperacetylation and subsequently influencing the gene expression patterns of the (immune) cells [279].

Epidemiological evidence indicates that growing up on a farm reduces a child's risk of developing allergies, particularly asthma [99, 101, 280-285]. These protective effects persisted into adulthood, indicating an inherited epigenetic change. The beneficial farm effects may be independently attributed to microbial exposure on the farm [286-289] as well as to the consumption of raw bovine milk, which is a rich source of immunomodulatory components [92, 166, 168]. Although breast milk is the preferred diet for newborns, bovine milk contains the majority of the bioactive components and functional molecules that modulate the infant's immune system [92]. Bovine milk does not contain a high concentration of nondigestible oligosaccharides and Human Milk Oligosaccharides (HMOs), which are abundant in human breast milk. HMOs, promote the growth of beneficial species of gut bacteria like Bifidobacteria that produce SCFAs. Prebiotics and HMOs are added to the infant formula to provide enough nondigestible oligosaccharides. Interestingly, SCFA levels in children's feces inversely correlate with sensitization to aeroallergens [114, 290]. Apart from HMOs, bovine milk contains other immunomodulatory components such as Transforming Growth Factor-β (TGF-β) [291], bovine lactoferrin [165, 292-294], bovine osteopontin [295, 296], and the Milk Fat Globular Membrane (MFGM) [297]. Bovine IgG (bIgG) is another significant bioactive component of bovine milk that has been shown to bind to aeroallergens [298] and respiratory pathogens such as Respiratory Syncytial Virus (RSV) [102, 111], hence potentially contributing to the protection against allergies and infections. bIgG was also shown to be capable of inducing innate immune training in human monocytes *in vitro* [46, 47]. In fact, the induction of innate immune training or trained immunity that we discuss extensively throughout this thesis requires affecting the epigenome. As stated earlier, miRNA from milk extracellular vesicles have the ability to function post-transcriptionally and selectively inhibit the translation of specific genes. Immunoregulatory miRNAs, such as miRNA155, are found in bovine milk exosomes and are hypothesized to play a role in the development of Tregs which are thought to play a role in the effect of raw milk

consumption on asthma [264]. Altogether, milk contains components that can influence epigenetic and induce non-DNA encoded variations in immune cell gene expression patterns and alter immune competence against allergies and infections.

Differential expression of SCFA receptors and inhibitory effects on immune cells

In early life, SCFAs are mainly produced after fermentation of HMOs in the breast milk or the oligosaccharides found in bovine milk (mainly 3'-Sialyllactose) in the case of formula feeding [299]. Commensal colonic bacteria, especially Bifidobacteria, possess the enzymatic machinery to ferment complex carbohydrates and produce SCFAs [300]. Acetate, propionate, and butyrate are the main SCFAs that serve as mediators of the interaction between microbiota and the host, with implications for health and disease [22, 24, 27]. Colonocytes and hepatocytes utilize a large proportion of butyrate and propionate respectively to generate energy [22]. SCFAs contribute significantly to the maintenance of local intestinal homeostasis by improving the function of the intestinal epithelial barrier [301], preventing the expansion of pathogenic bacteria [302], and educating mucosal immune cells [112]. SCFAs, however, have been found to be effective epigenetic modifiers, suggesting that their effects may extend well beyond the gut. The current scientific literature on SCFAs exposure reports contradictory effects on pro- and anti-inflammatory responses. While the majority of the literature indicates that SCFAs are anti-inflammatory, certain studies demonstrate the contrary [21, 303]. These contentious effects could be attributed to differences in receptor binding capabilities, varying local concentrations, and the influence of the immunological milieu and inflammatory status of the cells [303].

In **Chapter 3**, we investigated how direct exposure to individual SCFAs alters the functionality and response of activated innate and adaptive immune cells. Most importantly, whether the response to various SCFAs can be explained by differential expression of relevant receptors and transporters. Two key mechanisms by which SCFAs affect cells include accumulation inside the cytoplasm following passage through membrane transporters or signaling via membrane-associated G-coupled proteins (GPRs). It is not known at present whether SCFAs use these mechanisms separately or synergistically to induce their effects.

Apart from passive diffusion, SCFAs are actively transported into the cells via MCT-1 and SMCT-1 transporter molecules [22]. In **Chapter 3**, we demonstrated that the MCT-1 molecule was absent on the majority of immune cells tested. The expression of MCT-1 on pDCs was low; however, this receptor was expressed on the cell membrane of T lymphocytes. On the other hand, the SMCT-1 molecule was abundantly present on immune cells, with the highest levels found on DCs followed by monocytes and B cells. There is no indication that SMCT-1 or MCT-1 have a selective function, and it appears

that these transporters facilitate the passage of all SCFAs equally. Although additional research is needed to support this statement and define the priority of transporters for individual SCFAs, it seems that all tested immune cells types possess the machinery required to internalize SCFAs. Following their accumulation within cells, SCFAs operate as HDAC inhibitors (HDACi), allowing histone acetylation to occur primarily on H3 and H4 histones [21]. It is not yet fully understood which specific HDACs mediate the regulation of the inflammatory response of SCFAs in immune cells. However, accumulation of SCFAs with direct involvement of membrane transporters has been demonstrated to decrease the production of proinflammatory cytokines such as IL-6 and TNF-a, which is thought to be related to suppression of NF-κB activity [136, 155, 156].

SCFAs can also affect cell biological activities and influence gene expression through interacting with membrane-bound GPRs [129, 130, 304]. Similar to the direct influence of SCFAs upon cell entry, signaling via GPRs may lead to inhibition of HDACs and, therefore, regulation of gene expression [22, 130, 131]. The literature is again divided between the studies showing anti-inflammatory effects [155, 305, 306] and those which evidenced the proinflammatory cytokine generation after SCFAs interaction with GPRs [154, 303]. However, in the *in vitro* settings that we applied in **Chapter 3,** the suppressive effects of SCFAs on the release of inflammatory mediators were prominent.

The overall expression of GPR41 with a higher capacity to interact with propionate followed by butyrate was relatively higher than two other major receptors on the tested immune cells. The larger effects of propionate and butyrate can be explained in part by this higher expression. We observed that these two SCFAs were rather equally efficient in suppressing the production of TLR ligand-induced innate immunity associated cytokines such as IL-6 and TNF-a. The percentage of IL-6 and TNF-a producing monocytes, mDCs, and pDCs was reduced following treatment with butyrate and propionate and after stimulation with LPS and R848. The addition of either propionate or butyrate was demonstrated to reduce the production of the same two cytokines [155], with the effects being attributed to inhibition of NF-κB activity [136]. Except for pDCs, expression levels of GPR43, the major acetate receptor, were substantially lower than GPR41 in all immune cells tested. pDCs express comparable levels of GPR43 and GPR41, implying that these cells will be more reactive to acetate. In line with this assumption, the inhibitory effect of acetate on IL-6 and TNF-a production in activated pDCs was relatively higher than in monocytes and mDCs. Furthermore, acetate administration significantly affected R848-induced IFN-a production, a cytokine produced mainly by pDCs. GPR109A was found on monocytes but also on mDCs and B cells, albeit at considerably lower levels. Since this receptor exclusively interacts with butyrate, the significant influence of butyrate on monocytes may be explained in part by the high expression of GPR109A. However, among all SCFAs, butyrate exposure had the most pronounced effects, with the ability

to significantly lower the production of proinflammatory cytokines such as IL-1β, IL-6, and TNF-a from immune cells. This may imply that a considerable portion of butyrate's influence is exerted via its interaction with GPR41 or that direct suppression of cellular activities following its accumulation in cells may play a role. The latter activity appears to be critical, as butyrate inhibits HDACs while increasing the activity of HATs, hence influencing cell proliferation and gene expression [307]. After all, it appears that various factors, including the physiological state of the cell (cancerous vs. healthy) and the SCFA content, influence the mechanism by which SCFAs influence cell functions.

Individual SCFAs had a similar pattern of suppressive effects on T cell-associated cytokines as on innate cytokines. After incubation with propionate and butyrate, the production of IFN-γ, IL-17, and IL-13, which are signature cytokines of Th1, Th17, and Th2, respectively, was efficiently suppressed in activated T cells. The majority of studies targeted the effects of SCFAs on a subset of T lymphocytes such as Treg [308]. However, our data suggest that SCFAs utilize a mechanism that influences general T cell subset populations. Butyrate and propionate were both equally effective at lowering cytokine levels, but acetate had no such impact. As previously noted, T lymphocytes express relevant SCFA receptors and transporters, implying that the SCFA effects are partly mediated by direct contact of these metabolites with T cells. Fontenelli et al. showed that n-butyrate directly inhibited HDAC activity in $CD4^+$ T cells resulting in functional unresponsiveness of the cells [140]. Furthermore, after direct incubation of T cells with butyrate, a decrease in IFN-γ and IL-2 production was seen, which resulted in reduced T cell proliferation [142]. While aforementioned studies exposed SCFAs to isolated T cells, SCFAs were reported to be able to indirectly reduce the stimulatory capacity of DCs for activation of T cells [143, 144]. Incubation with SCFAs had a clear effect on activated T cells, but the results on B cells were less obvious. B lymphocytes express considerable levels of SCFAs receptors and transporters. Despite the fact that these molecules were present on the membrane of B cells at a much higher level than on T cells, no major influences on B cell functionality were seen. Research focusing on the effects of SCFAs on B cells are limited and *in vitro* studies suggest the regulatory effects of butyrate on antibody production. SCFAs influence B cell epigenetics by suppressing histone deacetylation and increasing antibody production during B cell differentiation into plasma cells [309, 310].

In **Chapter 3**, we further demonstrated that both propionate and butyrate but not acetate could influence activation-induced B and T cell migration to various tissues. In activated B cells, propionate and butyrate effectively reduced the expression of the mucosal tissue homing-associated chemokine receptor CCR10. The same two SCFAs reduced CCR9 expression in T cells, which directs the homing of intestinal intraepithelial lymphocytes to the intestine. CCR9 expression was elevated in activated T lymphocytes in the presence of Retinoic Acid (RA), which has previously been shown to enhance CCR9 expression

[135]. The available data on the impact of SCFAs on immune cell trafficking indicates that butyrate and propionate may reduce the recruitment of immune cells to the periphery. Butyrate reduced the expression of Intercellular Adhesion Molecule 1 (ICAM-1) on monocytes [148], whereas propionate inhibited Vascular Cell Adhesion Molecule 1 (VCAM-1) and ICAM-1 expression [150], and both SCFAs decreased CCL5, CXCL9, CXCL10, and CXCL11 levels in DCs [149]. Taken together, the data indicate that SCFAs maintain homeostasis not just by inhibiting inflammatory responses within cells but also by closely regulating immune cell migration to distant tissues.

As previously noted, inducing trained immunity requires epigenetic changes and metabolic reprogramming of cells. Apart from affecting cell metabolism, SCFAs have been shown to be potent inhibitors of HDAC activity, hence affecting the epigenome [22, 311]. However, as demonstrated in **Chapter 3**, SCFAs did not induce trained immunity but rather decreased monocytes' responsiveness to TLR activation *in vitro*. A week after incubation with butyrate and propionate but not acetate, monocytes responded less strongly to Pam3CSK4, LPS, and R848 stimulation than the control group, as reflected by decreased IL-6 and TNF-a production. This set of TLR ligands was selected to mimic and cover both gram-positive and negative bacteria as well as viral stimulation. The cytokine production results after stimulating several different TLR ligands corroborated each other, demonstrating that butyrate and propionate induce universal unresponsiveness in monocytes. Monocytes highly express GPR41 and GPR109 with reactivity to propionate and butyrate, respectively, and this implies that these two metabolites can profoundly impact immune reactivity. Although HDAC inhibition results in acetylation of histones which is generally considered permissive for gene expression, it seems that depending on the chromatin status and promoter region, HDAC inhibition may also suppress the expression of specific genes [125]. On the other hand, epigenetic modification can lead to innate immune tolerance, which is another aspect of immune cell compartment adaptation [162]. Exposure of macrophages to high concentrations of LPS epigenetically enforced tolerance and thereby prevented the expression of inflammatory genes like IL-6 [52]. In **Chapter 3,** we showed that butyrate and propionate acted in a comparable manner to LPS and R848 stimulation that were taken along as tolerance-inducing controls. We cannot confidently claim that butyrate and propionate can induce innate immune tolerance as the suppressive effects of SCFAs could have occurred via a different and at the present unknown mechanism. Further research on the epigenetic markers and metabolism of the cells upon exposure to SCFAs may shed light on this aspect.

The role of bIgG-containing ICs in inducing trained immunity

The humoral immune response is mediated by the production of antibodies, and the function of each antibody isotype is determined by its distinct structural features. IgG, which comprises between 10% and 20% of all serum proteins, is involved in a variety

of functions, including the immune exclusion to neutralize pathogens and toxins, opsonization of microbes and infected cells for lysis or phagocytosis, and modulation of inflammatory responses [170, 312, 313]. While functions such as immune exclusion are receptor-independent, others are mediated or facilitated by the interaction of IgG with cellular receptors. Through their Fc portion, IgG molecules interact with FcγRs on the membrane of target (immune) cells. Each FcγR is encoded by polymorphic genes, resulting in receptors with phenotypically distinct binding to various IgG forms and subclasses [314-316]. As for subclasses, IgG1 and IgG3 are generally considered to bind better to FcγRs than IgG2 and IgG4 [176]. *In vivo*, the high-affinity FcγRI (CD64) receptor is mostly occupied by monomeric serum-derived IgG. However, monomeric IgG has a very low affinity for FcγRII (CD32) and FcγRIII (CD16). Interactions with low-affinity FcγRs are only possible in the presence of immune complexes (ICs) containing IgG and multivalent antigens capable of cross-linking multiple receptors [179]. As for CD64, low-affinity receptors like CD32 have been implicated in critical processes such as Antibody-Dependent Cellular Phagocytosis (ADCP) and the clearance of pathogens and infected cells [179, 313, 317]. Immune complex binding to CD32 and internalization can induce proinflammatory cytokine release by engaging TLR signaling [204-207]. This process was also found to be important for the maturation of the DCs and subsequent efficient MHC class II-restricted presentation of the exogenous peptides [318].

In **Chapter 4**, we demonstrated that CD16 is present on the membranes of mDC subpopulations and a small proportion of monocytes, but not on the membranes of all other immune cells studied. It is known from the literature that NK cells highly express CD16 which is involved in Antibody-Dependent Cellular Cytotoxicity (ADCC) for eliminating (viral) infected cells [319, 320]. CD32 expression, however, was detected on monocytes, B lymphocytes, and mDCs. IgG ICs deliver an activating signal after binding to CD32a and CD32c, whereas binding to CD32b induces an inhibitory effect. Both activator and inhibitory CD32 are present on monocytes, whereas B cells only express CD32b, and binding of IgG ICs to this receptor regulates B cell activity and survival, as well as playing a role in maintaining peripheral tolerance for B cells [173, 321, 322]. We also observed that CD64 is present on monocytes and mDCs, whereas its expression varies considerably among DC subsets. Although we did not further characterize the mDC subsets, it is known that CD64 is present on some $CD11b^+$ and $Ly\text{-}6C^+$ mDCs [323].

The cross-species activity of antibodies has been demonstrated, and the immune-supportive impact of bovine colostrum as an IgG-rich food has been confirmed [96, 103, 107, 187, 218, 324-327]. Similar to human IgG, orally ingested bovine milk IgG (bIgG) may play a role in pathogen immune exclusion, as well as enhancing ADCC and ADCP in local tissues. Interaction with FcγRs on immune cells is one proposed mechanism for bIgG to induce immune-supportive effects. bIgG showed the ability to bind to human pathogens

and at the same time interact with the FcγRs expressed on human immune cells [102, 111]. We previously mentioned that CD64 is already filled with autologous monomeric IgG *in vivo,* leaving only low-affinity FcγRs for interaction with bIgG. ICs comprised of bIgG and antigens engage neighboring low-affinity FcγRs like CD32 to activate downstream signaling, and this engagement facilitates IC internalization [198]. Large ICs would form only when the antibody (bIgG): antigen ratios are optimal (equimolar content) and neither IgG nor the antigen arm is in excess (Fig. 2). Ingested bIgG may form ICs with respiratory pathogens from the nasal cavity after swallowing. The ICs may then interact with FcγRs on the tissue-resident macrophages and specialized mucosal DCs in the tonsillar crypts in Waldeyer's ring [10].

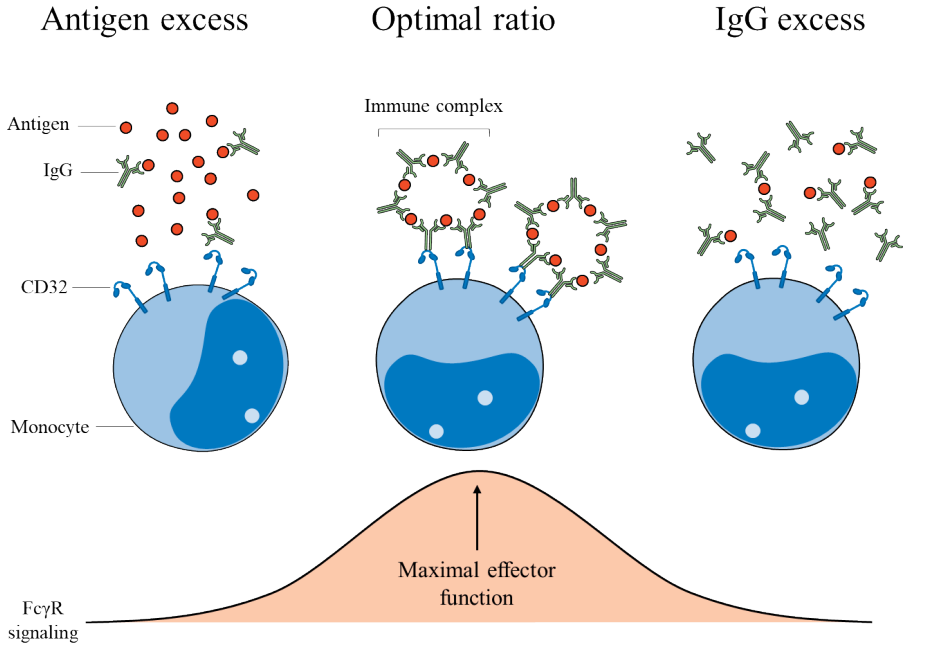

Figure 20. Stoichiometry of antibody (IgG) and antigens immune complex binding to CD32: Monomeric IgG does not bind to the low-affinity CD32. Antibody: antigen ratio is a major determinant of the formation of ICs. When either bIgG or antigen is in excess, the binding to CD32 is feeble, and hence the receptor signaling and effector function is weak. When the optimal ratio of IgG and antigen is present, immune complexes are formed and can bind to and interact with CD32. In that case, the signaling and the effector function of the ICs are maximal. (Figure is redrawn from Lu et al., Nat. Rev. Immunol. 2018)

We demonstrated direct binding of ICs formed from the optimal ratio of bIgG and RSV prefusion forms of the F protein (preF) to $CD14^{+}$ monocytes in **Chapter 4**. RSV is a major respiratory tract pathogen that is associated with a high rate of morbidity and mortality, particularly in infants and young children [328]. The F protein of the RSV is responsible for fusion and cell entry, and it has been shown that priming with this protein

activates both $CD4^+$ and $CD8^+$ T cell responses [329]. Recently, Nederend et al. reported that incubation of autologous $CD14^+$ monocytes with an optimal ratio of bIgG and RSV preF protein increases the capacity of monocytes to activate RSV-specific $CD4^+$ and $CD8^+$ *in vitro* [111]. They also found that bIgG interacts with the activator CD32a and subsequently showed that bIgG confers protection against an experimental RSV infection *in vivo* [111].

In **Chapter 4**, we established an experimental method to detect the direct binding of bIgG: RSV preF protein ICs to the $CD14^+$ monocytes, the same Antigen-Presenting Cell (APC) type used in the previous work. The optimal antibody: antigen ratio for the formation of ICs was determined by titrating the bIgG against RSV preF protein. Using the same method, we also showed the binding of ICs comprised from bIgG and mouse monoclonal anti-bIgG antibody (a-bIgG) to monocytes. Following the identification of the optimal ratio, the latter bIgG ICs generated a substantially higher binding signal than the bIgG: preF ICs, indicating the formation of larger ICs.

As previously noted, the interaction of ICs with CD32 may lead to a wide variety of effector functions. Modulation of inflammatory responses by affecting secretion or repression of pro- or anti-inflammatory cytokines was shown for ICs [206, 330-335]. As an effector function, we selected to study the putative role of bIgG-containing ICs on inducing innate immune training since bIgG previously showed the ability to train the monocytes *in vitro* [46, 47]. The training effects were observed at relatively high concentrations of bIgG (200 ug/mL) in the aforementioned studies, and the role of ICs in inducing trained immunity was not addressed. In **Chapter 4**, we showed that at low bIgG concentrations (10 ug/mL), the training effects reflected by enhanced TNF-a production are induced by bIgG-containing ICs but not the monomeric form of bIgG. The data suggest that the ICs formed from the optimal ratio of bIgG and RSV PreF protein most likely interacted with CD32 and contributed to the training effects of bIgG reported in monocytes. However, the RSV preF protein alone also increased cytokine production, albeit non-significantly. Interestingly, the preF protein was recently shown to bind to IGFR1 for cellular entry [211]. IGF, the natural ligand for this receptor, has been shown to be able to induce innate immune training [212], which can explain why the preF protein by itself has an effect in this model system as well.

Unexpectedly, incubation with the ICs composed of bIgG and a-bIgG did not affect the responsiveness of monocytes to TLR ligands. Several explanations were highlighted in **Chapter 4** for the absence of discernible training effects despite the substantial direct binding of ICs to $CD14^+$ monocytes. We speculate that the lack of antigen in the IC is the most probable explanation for no effects. Binding an epitope to the Fab portion of the IgG modifies the glycosylation of the Fc portion, which is essential for IgG functionality [199,

213, 336]. Therefore, binding an antigen such as RSV preF protein may lead to a different glycosylation pattern than anti-bIgG, which will influence the effector function of the IgG and the quality of the FcγR signaling. Besides, upon internalization of ICs, the antigen can stimulate PPRs to release inflammatory mediators, something that obviously an a-bIgG antibody cannot achieve. Altogether, it appears that binding ICs to FcγRs is necessary but not sufficient for the IgG effector functions to be exerted. Other determinants like concurrent stimulation of PRRs may be involved to synergistically strengthen the signal transduced by FcγRs to activate the cells.

Recently, the effects of a dairy product that contains high amounts of bIgG was tested in a human challenge model. PBMC from the individuals in this study were used to validate the *in vitro* findings on the ability of bIgG to train innate immune cells *in vivo*. This study, executed prior to the current research, was covered in **Chapter 5** and will be discussed in further detail in the following section of this discussion.

Evaluating the immunomodulatory effects of dietary components using human infection challenge models

Controlled human pathogenic infection models provide an alternative to large and costly field trials to study the protective effects of (nutritional) components. In **Chapter 5**, the immunomodulatory effects of Whey Protein Concentrate (WPC) consumption were evaluated using a human infection challenge model. Two-week ingestion of WPC did not change the gene expression pattern of PBMCs or induce *in vivo* trained immunity in healthy young participants. Differential Gene Expression (DGE) analysis revealed no significant variation in PBMCs gene expression patterns between the control and WPC-consuming groups. Besides, the two-week dietary intervention with WPC did not affect monocytes and mDCs *ex vivo* response to TLR agonists reflected by IL-6 and TNF-a production. Previously, the same WPC was tested in an *in vitro* trained immunity model, which was shown to be capable of modulating monocyte responses to TLR stimulation [47]. TNF-a production in monocytes treated with WPC *in vitro* increased significantly a week after training in response to TLR1/2, TLR4, and TLR7/8 stimulation. WPC is a dairy product with a high concentration of immunoglobulin G (bIgG) and MFGM proteins and phospholipids [47]. Purified bIgG also demonstrated *in vitro* monocyte training capacity [46, 47]. In **Chapter 5**, we speculated that the study target population could be one reason for the lack of clinical and immunological effect of the dietary intervention (described in (Ulfman et al, appendix 1)). For ethical reasons, the study was conducted on a healthy young population who did not have any health complications. In healthy humans, gene expression is closely regulated to maintain a continuous state of homeostasis, and a properly functioning immune system is normally capable of protecting against threats. Thus, it seems that there is no requirement or possibility for dietary components to modulate the gene expression and response of (innate) immune cells. Most

probably due to the same principle, administration of killed BCG vaccine had no effect on the response of innate immune cells in healthy volunteers [221], and healthy participants did not benefit from β-glucan supplementation [223], despite promising *in vitro* findings. These findings, however, do not rule out the possibility of local effects in the intestine that you do not pick up in peripheral blood.

Nevertheless, the impact of dietary intervention may be more significant in more vulnerable populations, including newborns, the elderly, and those with impaired immune systems. Consumption of raw bovine milk reduced the prevalence of respiratory tract infection in children [97]. We speculate that trained immunity may be a viable mechanism for supporting the immune system in youngsters whose immune systems are still developing. In infants, the TLR signaling is impaired, rendering them more susceptible to viral infections caused by e.g., RSV and influenza virus [65]. However, after training with WPC [47] and bIgG [46, 47], the TLR7/8-mediated response in monocytes was enhanced, which may influence antiviral immunity. In people susceptible to infections, such as the elderly, the immune cells are relatively in higher demand for extrinsic immune support, and perhaps they can benefit more from immunomodulation [337, 338]. With this line of reasoning, influenza vaccination have been recommended and prioritized for the elderly [339, 340] as well as in people with transplants, malignancies, and those with autoimmune conditions [341, 342].

In vitro findings, on the other hand, may not always translate into *in vivo* effects. Despite proven *in vitro* training capacity, WPC consumption did not result in *in vivo* training, as previously stated. Similar to our observations, ingestion of β-glucan, a well-characterized component with demonstrated *in vitro* innate immune training abilities, did not result in an enhanced innate immune response in healthy human volunteers [223]. In established *in vitro* trained immunity models, isolated immune cells, mostly monocytes, are exposed directly to the candidate training agent for an extended period of time (24 hours or more) [134]. These conditions may not be entirely physiologically relevant since the (dietary) components may not reach the circulation, and even if they do, their interaction with immune cells might not be comparable to the *in vitro* model. In the case of dietary components, the GI tract mucosal surfaces are the primary site of interaction between these compounds and immune cells. Ingested components interact with innate immune cells like DCs in the crypt of the Waldeyer's ring and in the intestine as long as the compounds survive the GI tract hydrolysis. Thus, the quality and duration of exposure may differ between *in vitro* and *in vivo* models, while the influence of other physiological parameters such as microbiota and their metabolites should not be disregarded. It is worth mentioning that, while many studies have demonstrated the occurrence of *in vivo* innate immune training via, e.g., vaccination [34, 250], *in vivo* studies on food components have not always been conclusive.

Nevertheless, this discrepancy does not depreciate the research conducted on *in vitro* trained immunity models. The model was critical not only for conceptualization but also for studying the trainability of distinct innate immune cells. *In vitro* studies demonstrated that certain compounds can modulate monocytes/macrophages [34, 38] and NK cells [35, 36, 252] to respond differently to secondary exposure to the same and similar stimulant. Other innate immune cells, such as neutrophils, are currently being investigated in this regard [343]. More importantly, *in vitro* models have made it possible to study the underlying mechanisms of innate immune training. The receptors and pathways involved in training induction may be identified using well-defined *in vitro* models, and the epigenetic alterations and metabolic reprogramming associated with each component can be addressed. In this context, it was demonstrated that β-glucan interacts with the Dectin-1 receptor to activate the noncanonical Raf-1 pathway [38], whereas receptor blocking studies confirmed the critical role of the NOD2 receptor in recognizing BCG and inducing trained immunity in response to the BCG vaccine [34, 221]. In *in vitro* β-glucan-trained monocytes, histone methylation and acetylation (H3K4me1 and H3K27Ac) occurred [39], and an increase in glucose intake and lactate generation, as well as a rise in NAD^+ to NADH ratio, indicated a metabolic shift from oxidative phosphorylation to glycolysis in the trained cells[49].

The *in vitro* trained immunity model has also proven to be useful for screening compounds that might have this potential and further proof-of-concept studies. The training potential of candidate compounds can be evaluated before applying them in large, expensive, and time-consuming studies in animals or humans. Despite the fact discussed earlier, in vitro screening of a large pool of compounds can help pre-select the potential candidates. Promising *in vitro* findings for chitin from *Saccharomyces cerevisiae* prompted testing *in vivo* [44], and laboratory data formed the basis for catecholamines administration to induce trained immunity in patients with pheochromocytoma/paraganglioma (PHEO) [222]. In addition, based on *in vitro* evidence, selected compounds are being used to promote innate immune training in farm animals with the goal of improving the animals' health and productivity (Reviewed in [344]).

The human challenge model applied in **Chapter 5** is not merely meant to evaluate the ability of the selected dietary compounds in training innate immune cells. This model is primarily used to determine whether (dietary) components have a beneficial clinical effect in preventing or alleviating the symptoms of experimental infection and can modify the course of a natural infection. Using the same challenge model, dietary calcium [80] and MFGM [216] were shown to be protective against diarrheagenic *E. coli* infection. After two weeks of consuming the product (WPC), subjects were orally challenged with diarrheagenic *E. coli* (strain E1392/75-2A), and the progression of diarrhea was followed for an additional two weeks. All study participants' diarrhea score and stool frequency

increased the day after infection but reverted to baseline within around two days. Yet, the variations between the control and WPC groups were not significant, and there was no evidence of an apparent protective effect of WPC consumption against diarrhea symptoms (See appendix 1. Ulfman et al., Nutrients. 2022).

This type of single-tier challenge model aims to study the ability of the compounds to passively support the immune system. Neutralizing the microbe via immune exclusion and increasing the phagocytic capability of neutrophils and macrophages are among the postulated mechanisms for supporting the immune system. In our single-tier challenge model, we investigated the possibility of inducing innate immune training as another supportive mechanism. Generally, in these types of challenge models, the primary goal is not to induce protection by increasing T- or B cell responses. Due to the short duration of the study and possibly low immunogenicity of the dietary ingredients, activation and expansion of antigen-specific T and B cells are not likely. In contrast, 2-tier challenge models allow researchers to investigate the active impacts of the components on the adaptive immune system as well as potential protective mechanisms targeting the innate immune system. Unlike single-tier infection models, when an initial challenge is followed by a secondary challenge a few weeks later, it is possible to explore adaptive immunity via cellular and humoral responses. In fact, serum IgG/IgA, fecal IgG/IgA, Antibody in Lymphocyte Supernatant (ALS), and memory B and T cells are all primary immunological readout parameters that have been recommended for evaluation in these types of controlled human *E. coli* infection models [84].

Generally, in challenge models as used for vaccine efficacy and to study (viral) infection mechanisms, the most relevant immunological readouts are cellular and humoral immunity parameters reflected as serum and mucosal IgG and IgA and the development of antigen-specific memory B and T cells [84, 237]. In our study, serum anti-CFAII IgG (the *E. coli* strain-specific antibody) increased dose-dependently following the primary infectious challenge and reached the highest levels 14 days post-infection. These findings indicate the expansion of B cells and the production of *E. coli*-specific antibodies induced by primary infection. However, on the following time points, the anti-CFAII IgG levels remained constant throughout the study period and did not further increase upon reinfection. Similar to our observations, in a study that used a two-tier model, a higher initial dose (5E10 CFU), serum *E. coli*-specific IgG levels increased only following the first challenge [236]. The serum IgG levels seem to have reached their maximal values after the primary challenge with doses as low as 1E6 CFU and remained at the peak even after the second challenge. The former study team also reported the increase in fecal *E. coli*-specific (CFAII) IgA after the primary infection [236]. Despite the increase in fecal IgA and serum IgG specific against the *E. coli*, association analysis could not establish a correlation between antibody response and the protection after secondary challenge [236].

This feature was previously reported for other *E. coli* challenge studies in which there was no direct association between antibody levels and protection [240, 246]. In fact, a single immune response may not be sufficient for protection against *E. coli* infection, and it requires the involvement of different components of the immune system [345, 346].

In **Chapter 6**, we conducted a pilot human challenge study to optimize a currently available diarrheagenic *E. coli* challenge model for dietary intervention studies. Previously, oral inoculation of volunteers with high concentrations (1E10 or 5E10 CFU) of *E. coli* strain E1392/75-2A led to mild and transient diarrheal clinical symptoms but was protective against a three week later second challenge with the same pathogen [236]. Although the initial challenge resulted in mild diarrhea, clinical symptoms of diarrhea and gastrointestinal discomfort did not significantly increase upon rechallenge, indicating optimal protection. Full protection against reinfection is not ideal for the challenge model. Since this model was primarily designed to assess the supportive effect of (food) ingredients at reinfection, the full protection conferred by the bacteria itself may obscure the effects of the tested compound. We hypothesized that by reducing the initial inoculation dose, we could partially eliminate the protective effects of *E. coli* allowing us to evaluate the influence of the tested dietary component. However, as described in **Chapter 6**, our observations were relatively comparable to those of the earlier study that only used a high-dose primary infection [236]. Although the primary inoculum concentrations were lowered, we observed the same protective effects of primary *E. coli* strain E1392/75-2A infection. Despite using a wide range of primary doses (1E6, 1E7, 1E8, 1E9, and 1E10 CFU), protection against reinfection with a high dose (1E10 CFU) of the bacteria was achieved. Few clinical parameters, namely % of fecal wet weight and stool frequency were significantly lower in the group challenged primarily with 1E6 CFU indicative suboptimal protective effects. These clinical parameters can be used in future studies to evaluate the effectiveness of dietary intervention in reducing *E. coli*-induced diarrhea. However, we think the model can benefit from further optimization to identify other biological biomarkers or clinical symptoms to complement the aforementioned parameters, thereby making it a more reliable model.

In **Chapter 6**, we further explored the contribution of myeloid innate immune cells in the protection against reinfection for the first time in a two-tier model. 3 weeks after the primary infectious challenge with *E. coli*, monocytes and mDCs were considerably more responsive to TLR ligand stimulation *ex vivo* compared to before infection. Blood samples were collected just before the primary infection and prior to the rechallenge. Three weeks after the first infection, when monocytes and mDCs were stimulated *ex vivo* with the same *E. coli* strain, a larger percentage of monocytes and mDCs produced IL-6 and TNF-a. Given the 21 days difference between the first and second infection, priming effects of the primary stimulation seem improbable, especially since the heightened response was also towards individual TLR ligands like LPS and flagellin from other bacterial species.

A likely explanation for the observations could be the occurrence of trained immunity *in vivo*, although this was not formally demonstrated.

Infectious agents from fungi (*Candida albicans*) [38], bacteria (BCG) [34], to even parasites (*Plasmodium falciparum*) [347, 348] have previously been demonstrated to induce innate immune training, and the list continues to grow [349]. Higher immunogenicity of microbial compounds (compared to dietary components tested in e.g., **Chapter 5**) and engaging multiple PRRs by *E. coli* may initiate the response in innate immune cells that could lead to trained immunity comparable to vaccination with BCG. The primary exposure to the *E. coli* bacteria in our model could have stimulated monocytes and DCs to respond differently to the second stimulant. The response of pDCs to *E. coli* was unchanged, which is consistent with the fact that these cells lack TLR4 and TLR5 and therefore have a limited response to bacterial ligands [151, 152]. Although the precise mechanism needs to be unraveled, we propose that the interaction between the bacteria and the immune cells occurred during the transient colonization of the gut by *E. coli*. For future research, it would be relevant to study the alteration in the epigenetic markers or metabolic pathways of the monocytes and mDCs to confirm the state of trained immunity. Such data could substantiate our conclusion in **Chapter 6** on the involvement of both adaptive and innate immunity in conferring protection against the secondary *E. coli* challenge.

In the future, an alternate research line could be linked to the *E. coli* strain used in **Chapter 5** and **Chapter 6** challenge models. Although it is beyond the scope of this thesis, we believe that the *E. coli* strain E1392/75-2A used in these challenge models is a promising candidate for the development of vaccines against more virulent enterotoxigenic *E. coli* (ETEC). This antibiotic-susceptible *E. coli* strain is a mutant of LT/ST toxin-producing ETEC strain O6:H16 with the deletion of genes for enterotoxins. Due to this deletion, challenges with this strain cause mild, transient and self-limiting diarrhea, making it acceptable for challenge models [147, 148, 151, 161]. Despite the induction of mild symptoms, the protection against this attenuated strain was shown to be 75% protective against the wild-type strain scored as reduction of diarrhea incidence [237, 241]. Here we discussed that oral inoculation doses as low as 1E6 CFU to high concentrations as high as 5E10 CFU effectively protected against reinfection without causing severe diarrhea or clinical gastrointestinal symptoms. Moreover, despite the lack of a definite association, increased serum and fecal IgG and IgA levels, as well as enhanced monocyte and mDC responses, may explain the mechanism of protection. The efficacy and safety profile of *E. coli* strain E1392/75-2A, in our opinion, makes it an interesting candidate for the development of (mucosal) vaccines against ETEC, and future research may be used to adjust the dose and optimize the readouts.

Concluding remarks and future perspectives

Nutritional intervention could be an approach to support the immune system of vulnerable individuals and contribute to the protection against infections and allergies. Bovine milk can be used not only because of its nutritional values but also because it contains numerous bioactive components with the ability to modulate the humans' immune system. Bovine IgG and metabolites induced by milk oligosaccharides (SCFAs) display immunomodulatory properties and putative immune-supportive mechanisms. In this thesis, we focused on the immunomodulatory activity of nutritional interventions to support the immune system of vulnerable individuals and contribute to the protection against infections.

Major milk components are known to contribute to the protection against allergies and asthma during childhood by potentially influencing the human epigenome to exert their immune-supportive effects. Innate immune training was introduced in this chapter as a method of epigenetic modification, and throughout this thesis, the ability of components to induce trained immunity was investigated next to other specific readouts.

Direct exposure to acetate, propionate, and butyrate suppressed inflammatory responses in innate and adaptive immune cells, but the latter two SCFAs were more efficient inhibitors. We speculated how differential SCFA receptor and transporter expression might contribute to these effects, but additional specific research into this feature of SCFAs may help identify novel prophylactic or therapeutic applications for human diseases.

Direct binding of bIgG-containing ICs to human $CD14^+$ monocytes was demonstrated. While ICs composed of bIgG and RSV preF protein induced trained immunity, ICs formed from bIgG and monoclonal anti-bIgG antibodies did not. The binding of ICs to FcγRs appears to be necessary but not sufficient for effector function to be induced, and additional factors may be critical. Possibly, these findings can be extrapolated to generate ICs containing human IgG and RSV antigens to study their role in training the innate immune system.

A two-week nutritional intervention with bIgG-rich WPC neither induced trained immunity nor significantly changed the gene expression pattern in PBMCs of the volunteers in a human challenge model. This could be due to the healthy study population or an unsuccessful intervention.

Primary infection with *E. coli* protected participants in a human challenge model from reinfection, which was accompanied by a rise in serum CFAII-specific IgG and the elevated response of myeloid innate immune cells. Both adaptive and innate immunity seems to be involved in this protection, and *in vivo,* innate immune training induced by the *E. coli* bacteria could be a significant contributing factor. Future research can substantiate the effects of infection on epigenetic markers and a possible switch in the metabolic pathways for generating energy.

Summary

In this thesis, we aimed to evaluate the immunomodulatory effects of (selected) bovine milk components (and metabolites induced by them) on the immune function of humans. **Chapter 1** gives an introduction to the topic and provides an outline of fundamental aspects of the immune system that are referred to in later chapters.

In **Chapter 2**, we addressed the primary components of bovine milk that have the potential to induce epigenetic changes to exert their immune-supportive effects during childhood. We reviewed the proposed mechanisms, including innate immune training that induces epigenetic modification. Through these mechanisms, the components may exert an effect on the immune system with implications for allergies and asthma. Living in a farm environment and raw bovine milk bioactive components were addressed as contributing factors that may reduce allergies in infancy and beyond.

HMOs and bovine milk oligosaccharides (mainly 3'-Sialyllactose) serve as substrates for the SCFA-producing microbiota. SCFAs are potent immune modulators and have significant roles in maintaining homeostasis and steering the response of the immune cells to the environment. In **Chapter 3**, we showed that butyrate and propionate had inhibitory effects on the activation of myeloid cells and lymphocytes, whereas acetate had a more selective impact on the immune cells. Production of inflammatory cytokines were suppressed in monocytes, mDCs, and pDCs as well T lymphocytes. SCFAs could not train the monocytes for enhanced response to secondary TLR stimulation *in vitro* but instead induced a tolerance-like phenotype. We attempted to explain the observed effects according to the differential expression of relevant SCFA receptors and transporters.

Bovine milk IgG (bIgG) binds to human pathogens such as RSV and, via the Fc portion, interacts with the FcγRs on human immune cells. The relevance of bIgG-containing immune complexes (ICs) on activation of CD32 was studied in **Chapter 4**, where we could establish a method to show the direct binding of bIgG ICs to immune cells. It was demonstrated that ICs containing bIgG are directly bound to human $CD14^{+}$. Subsequently, we could show the role of bIgG ICs on induction of trained immunity after binding to monocytes while contrary to previous reports, (monomeric) bIgG alone did not have similar effects suggestive of the presence of other contributing factors.

Human infection challenge models are used as an alternative to field trials to study the immune-supportive effects of dietary components. In **Chapter 5**, we found that ingestion of a dairy product (WPC) in a human challenge model did not influence the responsiveness of myeloid cells from healthy volunteers to *ex vivo* stimulation with TLR ligands. It also did not change the gene expression pattern of the PBMCs isolated from the same donors.

Although the model was utilized successfully before, the study product did not exert beneficial effects. We speculated that the study population might be a critical factor for no apparent impact.

In **Chapter 6**, we focused on optimizing an *E. coli* infection challenge model in humans to study the protective effects of dietary components and the correlates of protection at rechallenge. Primary infection with even low doses (1E6 CFU) of *E. coli* protected the subjects against reinfection with a high dose (1E10 CFU) of the same pathogen. Following the primary infection, serum anti-CFAII IgG levels were raised, and monocytes and mDCs responded more strongly to *ex vivo* stimulation. The latter may indicate the occurrence of trained immunity *in vivo*.

Throughout **Chapter 7**, we reviewed and discussed the most important findings of our research and placed the findings in a broader context by relating them to the most recent published literature. In addition, we identified subjects for future study perspectives to follow the work done in this project.

Immunomodulation by nutrition or supplementation of the food with potent immunomodulatory components can provide immune support for the immune system in individuals with an immature or impaired immune system. To substantiate the dietary components' beneficial effects and define the supporting mechanisms involved, we studied bovine IgG and metabolites induced by milk oligosaccharides to substantiate their health-promoting and immune-mediated effects.

References

1. Brodin, P., et al., *Variation in the Human Immune System Is Largely Driven by Non-Heritable Influences.* Cell, 2015. **160**(1): p. 37-47.
2. Brodin, P. and M.M. Davis, *Human immune system variation.* Nature Reviews Immunology, 2017. **17**(1): p. 21-29.
3. Martino, D.J. and S.L. Prescott, *Silent mysteries: epigenetic paradigms could hold the key to conquering the epidemic of allergy and immune disease.* Allergy, 2010. **65**(1): p. 7-15.
4. Suarez-Alvarez, B., et al., *DNA methylation: a promising landscape for immune system-related diseases.* Trends in Genetics, 2012. **28**(10): p. 506-514.
5. Kim, Lark K., et al., *Oct-1 Regulates IL-17 Expression by Directing Interchromosomal Associations in Conjunction with CTCF in T Cells.* Molecular Cell, 2014. **54**(1): p. 56-66.
6. Kuo, M.-H. and C.D. Allis, *Roles of histone acetyltransferases and deacetylases in gene regulation.* BioEssays, 1998. **20**(8): p. 615-626.
7. Gregory, P.D., K. Wagner, and W. Hörz, *Histone Acetylation and Chromatin Remodeling.* Experimental Cell Research, 2001. **265**(2): p. 195-202.
8. Bannister, A.J. and T. Kouzarides, *Regulation of chromatin by histone modifications.* Cell research, 2011. **21**(3): p. 381-395.
9. Onodera, A., K. Kokubo, and T. Nakayama, *Epigenetic and Transcriptional Regulation in the Induction, Maintenance, Heterogeneity, and Recall-Response of Effector and Memory Th2 Cells.* Frontiers in Immunology, 2018. **9**.
10. Bégin, P. and K.C. Nadeau, *Epigenetic regulation of asthma and allergic disease.* Allergy, Asthma & Clinical Immunology, 2014. **10**(1): p. 27.
11. Baskara-Yhuellou, I. and J. Tost, *Chapter Six - The impact of microRNAs on alterations of gene regulatory networks in allergic diseases*, in *Advances in Protein Chemistry and Structural Biology*, R. Donev, Editor. 2020, Academic Press. p. 237-312.
12. Melnik, B.C., et al., *Milk: a postnatal imprinting system stabilizing FoxP3 expression and regulatory T cell differentiation.* Clinical and Translational Allergy, 2016. **6**(1): p. 18.
13. Georgountzou, A. and N.G. Papadopoulos, *Postnatal Innate Immune Development: From Birth to Adulthood.* Frontiers in Immunology, 2017. **8**.
14. Torow, N., et al., *Neonatal mucosal immunology.* Mucosal Immunology, 2017. **10**(1): p. 5-17.
15. McDade, T.W., *Early environments and the ecology of inflammation.* Proceedings of the National Academy of Sciences, 2012. **109**(Supplement 2): p. 17281-17288.
16. West, C.E., N. D'Vaz, and S.L. Prescott, *Dietary Immunomodulatory Factors in the Development of Immune Tolerance.* Current Allergy and Asthma Reports, 2011. **11**(4): p. 325-333.
17. Aagaard, K., et al., *The Placenta Harbors a Unique Microbiome.* Science Translational Medicine, 2014. **6**(237): p. 237ra65-237ra65.
18. Gil, A., et al., *Is there evidence for bacterial transfer via the placenta and any role in the colonization of the infant gut? – a systematic review.* Critical Reviews in Microbiology, 2020. **46**(5): p. 493-507.
19. Dunstan, J.A., et al., *Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune*

responses and clinical outcomes in infants at high risk of atopy: A randomized, controlled trial. Journal of Allergy and Clinical Immunology, 2003. **112**(6): p. 1178-1184.

20. Gunaratne, A.W., M. Makrides, and C.T. Collins, *Maternal prenatal and/or postnatal n-3 long chain polyunsaturated fatty acids (LCPUFA) supplementation for preventing allergies in early childhood.* The Cochrane database of systematic reviews, 2015. **2015**(7): p. CD010085-CD010085.
21. Schaub, B., et al., *Maternal farm exposure modulates neonatal immune mechanisms through regulatory T cells.* Journal of Allergy and Clinical Immunology, 2009. **123**(4): p. 774-782.e5.
22. Fragkou, P.C., et al., *Impact of Early Life Nutrition on Children's Immune System and Noncommunicable Diseases Through Its Effects on the Bacterial Microbiome, Virome and Mycobiome.* Frontiers in Immunology, 2021. **12**.
23. Azagra-Boronat, I., et al., *Lactobacillus fermentum CECT5716 supplementation in rats during pregnancy and lactation affects mammary milk composition.* Journal of Dairy Science, 2020. **103**(4): p. 2982-2992.
24. Hogenkamp, A., et al., *Supplementation of Mice with Specific Nondigestible Oligosaccharides during Pregnancy or Lactation Leads to Diminished Sensitization and Allergy in the Female Offspring.* The Journal of Nutrition, 2015. **145**(5): p. 996-1002.
25. Hogenkamp, A., et al., *Supplementing Pregnant Mice with a Specific Mixture of Nondigestible Oligosaccharides Reduces Symptoms of Allergic Asthma in Male Offspring.* The Journal of Nutrition, 2014. **145**(3): p. 640-646.
26. van Vlies, N., et al., *Effects of short-chain galacto- and long-chain fructo-oligosaccharides on systemic and local immune status during pregnancy.* Journal of Reproductive Immunology, 2012. **94**(2): p. 161-168.
27. Eberharter, A. and P.B. Becker, *Histone acetylation: a switch between repressive and permissive chromatin.* EMBO reports, 2002. **3**(3): p. 224-229.
28. Loss, G., et al., *The protective effect of farm milk consumption on childhood asthma and atopy: The GABRIELA study.* Journal of Allergy and Clinical Immunology, 2011. **128**(4): p. 766-773.e4.
29. Riedler, J., et al., *Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey.* The Lancet, 2001. **358**(9288): p. 1129-1133.
30. Brick, T., et al., *The Beneficial Effect of Farm Milk Consumption on Asthma, Allergies, and Infections: From Meta-Analysis of Evidence to Clinical Trial.* The Journal of Allergy and Clinical Immunology: In Practice, 2020. **8**(3): p. 878-889.e3.
31. von Mutius, E. and D. Vercelli, *Farm living: effects on childhood asthma and allergy.* Nature Reviews Immunology, 2010. **10**(12): p. 861-868.
32. Sozańska, B., et al., *Consumption of unpasteurized milk and its effects on atopy and asthma in children and adult inhabitants in rural Poland.* Allergy, 2013. **68**(5): p. 644-650.
33. Ege, M.J., et al., *Not all farming environments protect against the development of asthma and wheeze in children.* Journal of Allergy and Clinical Immunology, 2007. **119**(5): p. 1140-1147.
34. Waser, M., et al., *Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe.* Clinical & Experimental Allergy, 2007. **37**(5): p. 661-670.
35. Perkin, M.R. and D.P. Strachan, *Which aspects of the farming lifestyle explain the inverse association*

with childhood allergy? Journal of Allergy and Clinical Immunology, 2006. **117**(6): p. 1374-1381.
36. Schuijs, M.J., et al., *Farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells.* Science, 2015. **349**(6252): p. 1106-1110.
37. LEYNAERT, B., et al., *Does Living on a Farm during Childhood Protect against Asthma, Allergic Rhinitis, and Atopy in Adulthood?* American Journal of Respiratory and Critical Care Medicine, 2001. **164**(10): p. 1829-1834.
38. Kilpeläinen, M., et al., *Childhood farm environment and asthma and sensitization in young adulthood.* Allergy, 2002. **57**(12): p. 1130-1135.
39. Wlasiuk, G. and D. Vercelli, *The farm effect, or: when, what and how a farming environment protects from asthma and allergic disease.* Current Opinion in Allergy and Clinical Immunology, 2012. **12**(5): p. 461-466.
40. van Neerven, R.J.J., et al., *Which factors in raw cow's milk contribute to protection against allergies?* Journal of Allergy and Clinical Immunology, 2012. **130**(4): p. 853-858.
41. Perdijk, O., et al., *Cow's Milk and Immune Function in the Respiratory Tract: Potential Mechanisms.* Frontiers in Immunology, 2018. **9**(143).
42. Abbring, S., et al., *Raw cow's milk consumption and allergic diseases – The potential role of bioactive whey proteins.* European Journal of Pharmacology, 2019. **843**: p. 55-65.
43. Roduit, C., et al., *High levels of butyrate and propionate in early life are associated with protection against atopy.* Allergy, 2019. **74**(4): p. 799-809.
44. Arrieta, M.-C., et al., *Early infancy microbial and metabolic alterations affect risk of childhood asthma.* Science Translational Medicine, 2015. **7**(307): p. 307ra152-307ra152.
45. Khaleva, E., et al., *Transforming growth factor beta in human milk and allergic outcomes in children: A systematic review.* Clinical & Experimental Allergy, 2019. **49**(9): p. 1201-1213.
46. Manzoni, P., et al., *Bovine Lactoferrin Supplementation for Prevention of Late-Onset Sepsis in Very Low-Birth-Weight Neonates: A Randomized Trial.* JAMA, 2009. **302**(13): p. 1421-1428.
47. Manzoni, P., et al., *Bovine Lactoferrin Prevents Invasive Fungal Infections in Very Low Birth Weight Infants: A Randomized Controlled Trial.* Pediatrics, 2012. **129**(1): p. 116-123.
48. King, J.C.J., et al., *A Double-Blind, Placebo-Controlled, Pilot Study of Bovine Lactoferrin Supplementation in Bottle-fed Infants.* Journal of Pediatric Gastroenterology and Nutrition, 2007. **44**(2): p. 245-251.
49. Chen, K., et al., *Effect of bovine lactoferrin from iron-fortified formulas on diarrhea and respiratory tract infections of weaned infants in a randomized controlled trial.* Nutrition, 2016. **32**(2): p. 222-227.
50. Lönnerdal, B., et al., *Growth, Nutrition, and Cytokine Response of Breast-fed Infants and Infants Fed Formula With Added Bovine Osteopontin.* Journal of Pediatric Gastroenterology and Nutrition, 2016. **62**(4): p. 650-657.
51. West, C.E., et al., *Effects of osteopontin-enriched formula on lymphocyte subsets in the first 6 months of life: a randomized controlled trial.* Pediatric Research, 2017. **82**(1): p. 63-71.
52. Timby, N., et al., *Infections in Infants Fed Formula Supplemented With Bovine Milk Fat Globule Membranes.* Journal of Pediatric Gastroenterology and Nutrition, 2015. **60**(3): p. 384-389.
53. Collins, A.M., et al., *Bovine milk, including pasteurised milk, contains antibodies directed against allergens of clinical importance to man.* Int Arch Allergy Appl Immunol, 1991. **96**(4): p. 362-7.

54. den Hartog, G., et al., *Specificity and Effector Functions of Human RSV-Specific IgG from Bovine Milk.* PLOS ONE, 2014. **9**(11): p. e112047.
55. Nederend, M., et al., *Bovine IgG Prevents Experimental Infection With RSV and Facilitates Human T Cell Responses to RSV.* Frontiers in immunology, 2020. **11**: p. 1701-1701.
56. van Splunter, M., et al., *Induction of Trained Innate Immunity in Human Monocytes by Bovine Milk and Milk-Derived Immunoglobulin G.* Nutrients, 2018. **10**(10): p. 1378.
57. Hellinga, A.H., et al., *In Vitro Induction of Trained Innate Immunity by bIgG and Whey Protein Extracts.* International Journal of Molecular Sciences, 2020. **21**(23): p. 9077.
58. Zivkovic, A.M. and D. Barile, *Bovine Milk as a Source of Functional Oligosaccharides for Improving Human Health.* Advances in Nutrition, 2011. **2**(3): p. 284-289.
59. Koh, A., et al., *From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites.* Cell, 2016. **165**(6): p. 1332-1345.
60. Tan, J., et al., *Chapter Three - The Role of Short-Chain Fatty Acids in Health and Disease*, in *Advances in Immunology*, F.W. Alt, Editor. 2014, Academic Press. p. 91-119.
61. Blaak, E.E., et al., *Short chain fatty acids in human gut and metabolic health.* Benef Microbes, 2020. **11**(5): p. 411-455.
62. van der Hee, B. and J.M. Wells, *Microbial Regulation of Host Physiology by Short-chain Fatty Acids.* Trends in Microbiology, 2021.
63. Liu, P., et al., *The role of short-chain fatty acids in intestinal barrier function, inflammation, oxidative stress, and colonic carcinogenesis.* Pharmacological Research, 2021. **165**: p. 105420.
64. Bezkorovainy, A., *Probiotics: determinants of survival and growth in the gut.* The American Journal of Clinical Nutrition, 2001. **73**(2): p. 399s-405s.
65. Corrêa-Oliveira, R., et al., *Regulation of immune cell function by short-chain fatty acids.* Clinical & translational immunology, 2016. **5**(4): p. e73-e73.
66. Krautkramer, K.A., et al., *Diet-Microbiota Interactions Mediate Global Epigenetic Programming in Multiple Host Tissues.* Molecular Cell, 2016. **64**(5): p. 982-992.
67. Li, M., et al., *Pro- and anti-inflammatory effects of short chain fatty acids on immune and endothelial cells.* European Journal of Pharmacology, 2018. **831**: p. 52-59.
68. Segain, J.-P., et al., *Butyrate inhibits inflammatory responses through NFκB inhibition: implications for Crohn's disease.* Gut, 2000. **47**(3): p. 397-403.
69. Usami, M., et al., *Butyrate and trichostatin A attenuate nuclear factor κB activation and tumor necrosis factor α secretion and increase prostaglandin E2 secretion in human peripheral blood mononuclear cells.* Nutrition Research, 2008. **28**(5): p. 321-328.
70. Ohira, H., et al., *Butyrate Attenuates Inflammation and Lipolysis Generated by the Interaction of Adipocytes and Macrophages.* Journal of Atherosclerosis and Thrombosis, 2013. **20**(5): p. 425-442.
71. Le Poul, E., et al., *Functional Characterization of Human Receptors for Short Chain Fatty Acids and Their Role in Polymorphonuclear Cell Activation.* Journal of Biological Chemistry, 2003. **278**(28): p. 25481-25489.
72. Tazoe, H., et al., *Roles of short-chain fatty acids receptors, GPR41 and GPR43 on colonic functions.* Journal of physiology and pharmacology : an official journal of the Polish Physiological Society,

2008. **59 Suppl 2**: p. 251-62.

73. Thangaraju, M., et al., *GPR109A is a G-protein-coupled receptor for the bacterial fermentation product butyrate and functions as a tumor suppressor in colon.* Cancer research, 2009. **69**(7): p. 2826-2832.
74. Wu, J., et al., *Butyrate-induced GPR41 Activation Inhibits Histone Acetylation and Cell Growth.* Journal of Genetics and Genomics, 2012. **39**(8): p. 375-384.
75. Masui, R., et al., *G protein-coupled receptor 43 moderates gut inflammation through cytokine regulation from mononuclear cells.* Inflammatory Bowel Diseases, 2013. **19**(13): p. 2848-2856.
76. Halnes, I., et al., *Soluble Fibre Meal Challenge Reduces Airway Inflammation and Expression of GPR43 and GPR41 in Asthma.* Nutrients, 2017. **9**(1): p. 57.
77. Vinolo, M.A.R., et al., *Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils.* The Journal of Nutritional Biochemistry, 2011. **22**(9): p. 849-855.
78. Donohoe, Dallas R., et al., *The Warburg Effect Dictates the Mechanism of Butyrate-Mediated Histone Acetylation and Cell Proliferation.* Molecular Cell, 2012. **48**(4): p. 612-626.
79. Asarat, M., et al., *Short-Chain Fatty Acids Regulate Cytokines and Th17/Treg Cells in Human Peripheral Blood Mononuclear Cells in vitro.* Immunol Invest, 2016. **45**(3): p. 205-22.
80. Fontenelle, B. and K.M. Gilbert, *n-Butyrate Anergized Effector CD4+ T Cells Independent of Regulatory T cell Generation or Activity.* Scandinavian Journal of Immunology, 2012. **76**(5): p. 457-463.
81. Cavaglieri, C.R., et al., *Differential effects of short-chain fatty acids on proliferation and production of pro- and anti-inflammatory cytokines by cultured lymphocytes.* Life Sciences, 2003. **73**(13): p. 1683-1690.
82. L. MILLARD, A., et al., *Butyrate affects differentiation, maturation and function of human monocyte-derived dendritic cells and macrophages.* Clinical & Experimental Immunology, 2002. **130**(2): p. 245-255.
83. Liu, L., et al., *Butyrate interferes with the differentiation and function of human monocyte-derived dendritic cells.* Cellular Immunology, 2012. **277**(1): p. 66-73.
84. Newman, J.D., et al., *Induction of the insulin receptor and other differentiation markers by sodium butyrate in the Burkitt lymphoma cell, Raji.* Biochemical and Biophysical Research Communications, 1989. **161**(1): p. 101-106.
85. Yamamoto, I., et al., *Interleukin-2-dependent augmentation of the anti-tnp antibody production by sodium butyrate in cultured murine splenic b cells.* International Journal of Immunopharmacology, 1997. **19**(6): p. 347-354.
86. Takeuchi, H., et al., *Efficient Induction of CCR9 on T Cells Requires Coactivation of Retinoic Acid Receptors and Retinoid X Receptors (RXRs): Exaggerated T Cell Homing to the Intestine by RXR Activation with Organotins.* The Journal of Immunology, 2010. **185**(9): p. 5289.
87. Bohmig, G.A., et al., *n-butyrate downregulates the stimulatory function of peripheral blood-derived antigen-presenting cells: a potential mechanism for modulating T-cell responses by short-chain fatty acids.* Immunology, 1997. **92**(2): p. 234-43.
88. Zapolska-Downar, D. and M. Naruszewicz, *Propionate reduces the cytokine-induced VCAM-1 and ICAM-1 expression by inhibiting nuclear factor-κ B (NF-κB) activation.* Journal of Physiology and Pharmacology, 2009. **60**(2): p. 123-131.

89. Nastasi, C., et al., *The effect of short-chain fatty acids on human monocyte-derived dendritic cells.* Scientific Reports, 2015. **5**(1): p. 16148.
90. Vinolo, M.A.R., et al., *Regulation of inflammation by short chain fatty acids.* Nutrients, 2011. **3**(10): p. 858-876.
91. Reichert, N., M.-A. Choukrallah, and P. Matthias, *Multiple roles of class I HDACs in proliferation, differentiation, and development.* Cellular and Molecular Life Sciences, 2012. **69**(13): p. 2173-2187.
92. Divangahi, M., et al., *Trained immunity, tolerance, priming and differentiation: distinct immunological processes.* Nature Immunology, 2021. **22**(1): p. 2-6.
93. Foster, S.L., D.C. Hargreaves, and R. Medzhitov, *Gene-specific control of inflammation by TLR-induced chromatin modifications.* Nature, 2007. **447**(7147): p. 972-978.
94. Schroeder, H.W. and L. Cavacini, *Structure and Function of Immunoglobulins.* The Journal of allergy and clinical immunology, 2010. **125**(2 0 2): p. S41-S52.
95. Forthal, D.N., *Functions of Antibodies.* Microbiology spectrum, 2014. **2**(4): p. 1-17.
96. Lu, L.L., et al., *Beyond binding: antibody effector functions in infectious diseases.* Nature Reviews Immunology, 2017. **18**: p. 46.
97. Clark, M.R., et al., *Molecular basis for a polymorphism involving Fc receptor II on human monocytes.* The Journal of Immunology, 1989. **143**(5): p. 1731.
98. Ory, P.A., et al., *Characterization of polymorphic forms of Fc receptor III on human neutrophils.* The Journal of clinical investigation, 1989. **83**(5): p. 1676-1681.
99. Warmerdam, P.A., et al., *A single amino acid in the second Ig-like domain of the human Fc gamma receptor II is critical for human IgG2 binding.* The Journal of Immunology, 1991. **147**(4): p. 1338.
100. Bruhns, P., et al., *Specificity and affinity of human Fcγ receptors and their polymorphic variants for human IgG subclasses.* Blood, 2009. **113**(16): p. 3716-3725.
101. Anania, J.C., et al., *The Human FcγRII (CD32) Family of Leukocyte FcR in Health and Disease.* Frontiers in Immunology, 2019. **10**(464).
102. Bournazos, S., T.T. Wang, and J.V. Ravetch, *The Role and Function of Fcγ Receptors on Myeloid Cells.* Microbiology spectrum, 2016. **4**(6): p. 10.1128/microbiolspec.MCHD-0045-2016.
103. Boulé, M.W., et al., *Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes.* The Journal of experimental medicine, 2004. **199**(12): p. 1631-1640.
104. Means, T.K., et al., *Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9.* The Journal of clinical investigation, 2005. **115**(2): p. 407-417.
105. Parcina, M., et al., *Staphylococcus aureus-Induced Plasmacytoid Dendritic Cell Activation Is Based on an IgG-Mediated Memory Response.* The Journal of Immunology, 2008. **181**(6): p. 3823-3833.
106. Bunk, S., et al., *Internalization and Coreceptor Expression Are Critical for TLR2-Mediated Recognition of Lipoteichoic Acid in Human Peripheral Blood.* The Journal of Immunology, 2010. **185**(6): p. 3708-3717.
107. Ravetch, J.V., *Fc receptors: Rubor redux.* Cell, 1994. **78**(4): p. 553-560.
108. Shore, S.L., et al., *Detection of cell-dependent cytotoxic antibody to cells infected with herpes simplex*

virus. Nature, 1974. **251**(5473): p. 350-352.
109. Jegerlehner, A., et al., *Influenza A Vaccine Based on the Extracellular Domain of M2: Weak Protection Mediated via Antibody-Dependent NK Cell Activity.* The Journal of Immunology, 2004. **172**(9): p. 5598.
110. Pearse, R.N., et al., *SHIP Recruitment Attenuates FcγRIIB-Induced B Cell Apoptosis.* Immunity, 1999. **10**(6): p. 753-760.
111. Ravetch, J.V. and L.L. Lanier, *Immune Inhibitory Receptors.* Science, 2000. **290**(5489): p. 84-89.
112. Nimmerjahn, F. and J.V. Ravetch, *Fcγ receptors as regulators of immune responses.* Nature Reviews Immunology, 2008. **8**: p. 34.
113. Langlet, C., et al., *CD64 Expression Distinguishes Monocyte-Derived and Conventional Dendritic Cells and Reveals Their Distinct Role during Intramuscular Immunization.* The Journal of Immunology, 2012. **188**(4): p. 1751-1760.
114. Ebina, T., et al., *PREVENTION OF ROTAVIRUS INFECTION BY COW COLOSTRUM CONTAINING ANTIBODY AGAINST HUMAN ROTAVIRUS.* The Lancet, 1983. **322**(8357): p. 1029-1030.
115. Rump, J.A., et al., *Treatment of diarrhoea in human immunodeficiency virus-infected patients with immunoglobulins from bovine colostrum.* The clinical investigator, 1992. **70**(7): p. 588-594.
116. AK, M., et al., *Hyperimmune cow colostrum reduces diarrhoea due to rotavirus: a double-blind, controlled clinical trial.* Acta Paediatrica, 1995. **84**(9): p. 996-1001.
117. SARKER, S.A., et al., *Successful treatment of rotavirus diarrhea in children with immunoglobulin from immunized bovine colostrum.* The Pediatric Infectious Disease Journal, 1998. **17**(12): p. 1149-1154.
118. Brooks, H.J., et al., *Potential prophylactic value of bovine colostrum in necrotizing enterocolitis in neonates: an in vitro study on bacterial attachment, antibody levels and cytokine production.* FEMS Immunology & Medical Microbiology, 2006. **48**(3): p. 347-354.
119. Marit, K., et al., *Anti-HIV-1 antibody-dependent cellular cytotoxicity mediated by hyperimmune bovine colostrum IgG.* European Journal of Immunology, 2012. **42**(10): p. 2771-2781.
120. Xu, M.L., et al., *The effect of dietary bovine colostrum on respiratory syncytial virus infection and immune responses following the infection in the mouse.* Journal of Microbiology, 2015. **53**(9): p. 661-666.
121. Savarino, S.J., et al., *Prophylactic Efficacy of Hyperimmune Bovine Colostral Antiadhesin Antibodies Against Enterotoxigenic Escherichia coli Diarrhea: A Randomized, Double-Blind, Placebo-Controlled, Phase 1 Trial.* The Journal of Infectious Diseases, 2017. **216**(1): p. 7-13.
122. Kelly, G.S., *Bovine colostrums: a review of clinical uses.* Altern Med Rev, 2003. **8**(4): p. 378-94.
123. Jaumouillé, V. and S. Grinstein, *Receptor mobility, the cytoskeleton, and particle binding during phagocytosis.* Current Opinion in Cell Biology, 2011. **23**(1): p. 22-29.
124. Govers, C., et al., *Ingestion, Immunity, and Infection: Nutrition and Viral Respiratory Tract Infections.* Frontiers in Immunology, 2022. **13**.
125. Borchers, A.T., et al., *Respiratory syncytial virus--a comprehensive review.* Clinical reviews in allergy & immunology, 2013. **45**(3): p. 331-379.
126. Srikiatkhachorn, A. and T.J. Braciale, *Virus-specific CD8+ T lymphocytes downregulate T helper cell type 2 cytokine secretion and pulmonary eosinophilia during experimental murine respiratory syncytial virus*

infection. The Journal of experimental medicine, 1997. **186**(3): p. 421-432.
127. Fernández, N., M. Renedo, and M. Sánchez Crespo, *FcγR receptors activate MAP kinase and up-regulate the cyclooxygenase pathway without increasing arachidonic acid release in monocytic cells.* European Journal of Immunology, 2002. **32**(2): p. 383-392.
128. Fernández, N., et al., *Activation of Monocytic Cells Through Fcγ Receptors Induces the Expression of Macrophage-Inflammatory Protein (MIP)-1α, MIP-1β, and RANTES.* The Journal of Immunology, 2002. **169**(6): p. 3321-3328.
129. Jancar, S. and M.S. Crespo, *Human diseases associated with tissue immune complex deposition.* Trends in Immunology, 2005. **26**(1): p. 48-55.
130. Tsuboi, N., et al., *Human neutrophil Fcgamma receptors initiate and play specialized nonredundant roles in antibody-mediated inflammatory diseases.* Immunity, 2008. **28**(6): p. 833-846.
131. Gallo, P., R. Gonçalves, and D.M. Mosser, *The influence of IgG density and macrophage Fc (gamma) receptor cross-linking on phagocytosis and IL-10 production.* Immunology letters, 2010. **133**(2): p. 70-77.
132. Zhang, Y., et al., *Ligation of Fc gamma receptor IIB enhances levels of antiviral cytokine in response to PRRSV infection in vitro.* Veterinary Microbiology, 2012. **160**(3): p. 473-480.
133. Griffiths, C.D., et al., *IGF1R is an entry receptor for respiratory syncytial virus.* Nature, 2020. **583**(7817): p. 615-619.
134. Bekkering, S., et al., *Metabolic Induction of Trained Immunity through the Mevalonate Pathway.* Cell, 2018. **172**(1): p. 135-146.e9.
135. Lux, A. and F. Nimmerjahn. *Impact of Differential Glycosylation on IgG Activity*. 2011. New York, NY: Springer New York.
136. Lux, A., et al., *Impact of Immune Complex Size and Glycosylation on IgG Binding to Human FcγRs.* The Journal of Immunology, 2013. **190**(8): p. 4315.
137. Hayes, J.M., et al., *Glycosylation and Fc Receptors*, in *Fc Receptors*, M. Daeron and F. Nimmerjahn, Editors. 2014, Springer International Publishing: Cham. p. 165-199.
138. Arts, R.J.W., et al., *Long-term in vitro and in vivo effects of γ-irradiated BCG on innate and adaptive immunity.* Journal of Leukocyte Biology, 2015. **98**(6): p. 995-1001.
139. Leentjens, J., et al., *The Effects of Orally Administered Beta-Glucan on Innate Immune Responses in Humans, a Randomized Open-Label Intervention Pilot-Study.* PLOS ONE, 2014. **9**(9): p. e108794.
140. Loss, G., et al., *Consumption of unprocessed cow's milk protects infants from common respiratory infections.* Journal of Allergy and Clinical Immunology, 2015. **135**(1): p. 56-62.e2.
141. Kollmann, T.R., et al., *Innate immune function by Toll-like receptors: distinct responses in newborns and the elderly.* Immunity, 2012. **37**(5): p. 771-783.
142. Dorshkind, K., E. Montecino-Rodriguez, and R.A.J. Signer, *The ageing immune system: is it ever too old to become young again?* Nature Reviews Immunology, 2009. **9**(1): p. 57-62.
143. van Splunter, M., et al., *Bovine Lactoferrin Enhances TLR7-Mediated Responses in Plasmacytoid Dendritic Cells in Elderly Women: Results From a Nutritional Intervention Study With Bovine Lactoferrin, GOS and Vitamin D.* Frontiers in immunology, 2018. **9**: p. 2677-2677.
144. Ciabattini, A., et al., *Vaccination in the elderly: The challenge of immune changes with aging.* Seminars

in Immunology, 2018. **40**: p. 83-94.
145. Weinberger, B., *Vaccines for the elderly: current use and future challenges.* Immunity & ageing : I & A, 2018. **15**: p. 3-3.
146. Zbinden, D. and O. Manuel, *Influenza vaccination in immunocompromised patients: efficacy and safety.* Immunotherapy, 2014. **6**(2): p. 131-9.
147. Bosaeed, M. and D. Kumar, *Seasonal influenza vaccine in immunocompromised persons.* Human vaccines & immunotherapeutics, 2018. **14**(6): p. 1311-1322.
148. Domínguez-Andrés, J., et al., *In vitro induction of trained immunity in adherent human monocytes.* STAR protocols, 2021. **2**(1): p. 100365-100365.
149. Giamarellos-Bourboulis, E.J., et al., *Activate: Randomized Clinical Trial of BCG Vaccination against Infection in the Elderly.* Cell, 2020. **183**(2): p. 315-323.e9.
150. Kleinnijenhuis, J., et al., *Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes.* Proc Natl Acad Sci U S A, 2012. **109**(43): p. 17537-42.
151. Quintin, J., et al., *Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes.* Cell host & microbe, 2012. **12**(2): p. 223-232.
152. Sun, J.C., *Re-educating natural killer cells.* The Journal of experimental medicine, 2010. **207**(10): p. 2049-2052.
153. Sun, J.C., J.N. Beilke, and L.L. Lanier, *Immune memory redefined: characterizing the longevity of natural killer cells.* Immunological reviews, 2010. **236**: p. 83-94.
154. Kleinnijenhuis, J., et al., *BCG-induced trained immunity in NK cells: Role for non-specific protection to infection.* Clinical Immunology, 2014. **155**(2): p. 213-219.
155. Kalafati, L., et al., *Innate Immune Training of Granulopoiesis Promotes Anti-tumor Activity.* Cell, 2020. **183**(3): p. 771-785.e12.
156. Saeed, S., et al., *Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity.* Science (New York, N.Y.), 2014. **345**(6204): p. 1251086-1251086.
157. Cheng, S.-C., et al., *mTOR- and HIF-1α-mediated aerobic glycolysis as metabolic basis for trained immunity.* Science (New York, N.Y.), 2014. **345**(6204): p. 1250684-1250684.
158. Rizzetto, L., et al., *Fungal Chitin Induces Trained Immunity in Human Monocytes during Cross-talk of the Host with Saccharomyces cerevisiae*.* Journal of Biological Chemistry, 2016. **291**(15): p. 7961-7972.
159. Heijden, C.D.C.C.v.d., et al., *Catecholamines Induce Trained Immunity in Monocytes In Vitro and In Vivo.* Circulation Research, 2020. **127**(2): p. 269-283.
160. Byrne, K.A., C.L. Loving, and J.L. McGill, *Innate Immunomodulation in Food Animals: Evidence for Trained Immunity?* Frontiers in immunology, 2020. **11**: p. 1099-1099.
161. Bovee-Oudenhoven, I.M.J., et al., *Diarrhea caused by enterotoxigenic Escherichia coli infection of humans is inhibited by dietary calcium.* Gastroenterology, 2003. **125**(2): p. 469-476.
162. Ten Bruggencate, S.J., et al., *Dietary Milk-Fat-Globule Membrane Affects Resistance to Diarrheagenic Escherichia coli in Healthy Adults in a Randomized, Placebo-Controlled, Double-Blind Study.* The Journal of Nutrition, 2015. **146**(2): p. 249-255.

163. Hanevik, K., et al., *The way forward for ETEC controlled human infection models (CHIMs).* Vaccine, 2019. **37**(34): p. 4794-4799.
164. Levine, M.M., E.M. Barry, and W.H. Chen, *A roadmap for enterotoxigenic Escherichia coli vaccine development based on volunteer challenge studies.* Human Vaccines & Immunotherapeutics, 2019. **15**(6): p. 1357-1378.
165. van Hoffen, E., et al., *Characterization of the pathophysiological determinants of diarrheagenic Escherichia coli infection using a challenge model in healthy adults.* Sci Rep, 2021. **11**(1): p. 6060.
166. Porter, C.K., et al., *A systematic review of experimental infections with enterotoxigenic Escherichia coli (ETEC).* Vaccine, 2011. **29**(35): p. 5869-85.
167. Zhang, W. and D.A. Sack, *Progress and hurdles in the development of vaccines against enterotoxigenic Escherichia coli in humans.* Expert Review of Vaccines, 2012. **11**(6): p. 677-694.
168. Rao, M.R., et al., *Serologic Correlates of Protection against Enterotoxigenic Escherichia coli Diarrhea.* The Journal of Infectious Diseases, 2005. **191**(4): p. 562-570.
169. Tobias, J., et al., *Preexisting antibodies to homologous colonization factors and heat-labile toxin in serum, and the risk to develop enterotoxigenic Escherichia coli-associated diarrhea.* Diagnostic Microbiology and Infectious Disease, 2008. **60**(2): p. 229-231.
170. McCall, M.B.B., et al., *Plasmodium falciparum Infection Causes Proinflammatory Priming of Human TLR Responses.* The Journal of Immunology, 2007. **179**(1): p. 162-171.
171. Rodrigues, J., et al., *Hemocyte differentiation mediates innate immune memory in Anopheles gambiae mosquitoes.* Science (New York, N.Y.), 2010. **329**(5997): p. 1353-1355.
172. Rusek, P., et al., *Infectious Agents as Stimuli of Trained Innate Immunity.* International Journal of Molecular Sciences, 2018. **19**(2): p. 456.
173. Hornung, V., et al., *Quantitative Expression of Toll-Like Receptor 1–10 mRNA in Cellular Subsets of Human Peripheral Blood Mononuclear Cells and Sensitivity to CpG Oligodeoxynucleotides.* The Journal of Immunology, 2002. **168**(9): p. 4531-4537.
174. Reizis, B., *Plasmacytoid Dendritic Cells: Development, Regulation, and Function.* Immunity, 2019. **50**(1): p. 37-50.
175. Tacket, C. and M. Levine, *Vaccines against enterotoxigenic Escherichia coli infections.* New generation vaccines, 2nd ed. Marcel Dekker, Inc., New York, NY, 1997: p. 875-883.

Summary

In this thesis, we aimed to evaluate the immunomodulatory effects of (selected) bovine milk components (and metabolites induced by them) on the immune function of humans. **Chapter 1** gives an introduction to the topic and provides an outline of fundamental aspects of the immune system that are referred to in later chapters.

In **Chapter 2**, we summarized and addressed the primary components of bovine milk that have the potential to induce epigenetic changes to exert their immune-supportive effects during childhood. We reviewed the proposed mechanisms, including innate immune training that induces epigenetic modification. Through these mechanisms, the components may exert an effect on the immune system with implications for allergies and asthma. Living in a farm environment and raw bovine milk bioactive components were addressed as contributing factors that may reduce allergies in infancy and beyond.

HMOs and bovine milk oligosaccharides (mainly 3'-Sialyllactose) serve as substrates for the SCFA-producing microbiota. SCFAs are potent immune modulators and have significant roles in maintaining homeostasis and steering the response of the immune cells to the environment. In **Chapter 3**, we showed that butyrate and propionate had inhibitory effects on the activation of myeloid cells and lymphocytes, whereas acetate had a more selective impact on the immune cells. Production of inflammatory cytokines were suppressed in monocytes, mDCs, and pDCs as well T lymphocytes. SCFAs could not train the monocytes for enhanced response to secondary TLR stimulation *in vitro* but instead induced a tolerance-like phenotype. We attempted to explain the observed effects according to the differential expression of relevant SCFA receptors and transporters.

Bovine milk IgG (bIgG) binds to human pathogens such as RSV and, via the Fc portion, interacts with the FcγRs on human immune cells. The relevance of bIgG-containing immune complexes (ICs) on activation of CD32 was studied in **Chapter 4**, where we could establish a method to show the direct binding of bIgG ICs to immune cells. It was demonstrated that ICs containing bIgG are directly bound to human $CD14^+$. Subsequently, we could show the role of bIgG ICs on induction of trained immunity after binding to monocytes while contrary to previous reports, (monomeric) bIgG alone did not have similar effects suggestive of the presence of other contributing factors.

Human infection challenge models are used as an alternative to field trials to study the immune-supportive effects of dietary components. In **Chapter 5**, we found that ingestion of a dairy product (WPC) in a human challenge model did not influence the responsiveness of myeloid cells from healthy volunteers to *ex vivo* stimulation with TLR ligands. It also did not change the gene expression pattern of the PBMCs isolated from the same donors. Although the model was utilized successfully before, the study product did not exert beneficial effects. We speculated that the study population might be a critical factor for no

apparent impact.

In **Chapter 6**, we focused on optimizing an *E. coli* infection challenge model in humans to study the protective effects of dietary components and the correlates of protection at rechallenge. Primary infection with even low doses (1E6 CFU) of *E. coli* protected the subjects against reinfection with a high dose (1E10 CFU) of the same pathogen. Following the primary infection, serum anti-CFAII IgG levels were raised, and monocytes and mDCs responded more strongly to *ex vivo* stimulation. The latter may indicate the occurrence of trained immunity *in vivo*.

Throughout **Chapter 7**, we reviewed and discussed the most important findings of our research and placed the findings in a broader context by relating them to the most recent published literature. In addition, we identified subjects for future study perspectives to follow the work done in this project.

Immunomodulation by nutrition or supplementation of the food with potent immunomodulatory components can provide immune support for the immune system in individuals with an immature or impaired immune system. To substantiate the dietary components' beneficial effects and define the supporting mechanisms involved, we studied bovine IgG and metabolites induced by milk oligosaccharides to substantiate their health-promoting and immune-mediated effects.

APPENDIX

I

A Double Blind Randomized Intervention Study on the Effect of a Whey Protein Concentrate on *E. coli*-Induced Diarrhea in a Human Infection Model

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Abstract

Infectious diseases are a main cause of morbidity and mortality worldwide. Nutritional interventions may enhance resistance to infectious diseases or help to reduce clinical symptoms. Here, we investigated whether a whey protein concentrate (WPC) could decrease diarrheagenic Escherichia coli-induced changes in reported stool frequency and gastrointestinal complaints in a double-blind parallel 4-week intervention study. Subjects were randomly assigned to a whey hydrolysate placebo group, a low dose WPC group or a high dose WPC group. After 2 weeks of consumption, subjects (n = 121) were orally infected with a high dose of live, but attenuated, diarrheagenic *E. coli* (strain E1392/75-2A; 1E10 Colony Forming Units). Subjects recorded information on stool consistency, frequency and severity of symptoms through an online diary. Primary outcome parameters were a change in stool frequency (stools per day) and a change in GSRS (Gastrointestinal Symptom Rating Score) diarrhea score between first and second day after infection. Dietary treatment with neither dose of the whey protein concentrate affected the *E. coli*-induced increase in stool frequency or GSRS diarrhea score, compared to placebo treatment. The composition of the microbiota shifted between start of the study and after two weeks of consumption of the products, but no differences between the intervention groups were observed, possibly due to dietary guidelines subjects had to adhere to during the study. In conclusion, consumption of -the whey protein concentrate by healthy adults did not reduce diarrhea scores in an *E. coli* infection model compared to a whey hydrolysate placebo control.

Introduction

Diarrheal disease is a common cause of illness and death worldwide, especially under 5 years of age (1). Pathogenic *E. coli* strains are among the most common causes of diarrheal diseases in Asia and Africa (2). Access to safe water, sanitation, vaccination programs and nutrition are important factors in the prevention of diarrheal disease (3,4).

Dairy ingredients have been shown to affect infectious diseases in preclinical studies as well as in *in vivo* intervention trials (5,6). Both the native whey components, such as bovine immunoglobulins (reviewed in (5)), lactoferrin (reviewed in (7)), and milk fat globule membrane (MFGM) components (reviewed in (6,8)) such as phospholipids and MFGM proteins may contribute to these effects. With respect to MFGM type of products, some (9–12) but not all (13,14) studies show reduced incidence of infectious outcomes of interventions for infants (9–11,14) and children (13). Three (9,11,12) of the four studies that found an effect of the intervention should be interpreted with caution since infection was not defined as the primary outcome of the study. One study was hampered by low incidence of diarrheal disease (14). Another study did find an effect on decreased febrile episodes, suggesting an immune modulating effect (13).

Randomized double blind intervention field trials are the gold standard to study the effects of nutritional interventions on the prevention of diarrheal disease. However, these studies are very time-consuming and costly and therefore not always the first choice to test a new food ingredient. Human pathogenic infection challenge models are therefore a very relevant alternative as a first step to test whether a nutritional intervention can affect the course of a natural infection.

The human infection challenge model used was a diarrheagenic *E. coli* strain challenge developed at NIZO (The Netherlands) (15). The *E. coli* used is a well-characterized, antibiotic susceptible organism that has been associated with mild diarrhea and gastrointestinal symptoms (severity and duration). Indeed, previous intervention studies using the diarrheagenic *E. coli* strain showed transient induction of symptoms including increased stool frequency, mild diarrhea, mild abdominal pain and bloating (16–18). The diarrheagenic *E. coli* strain E1392/75-2A is a spontaneous mutant with deletion of the genes encoding the heat-labile (LT) and heat-stable (ST) toxins, and therefore cannot produce any toxins. However, it expresses colonization factor antigen II (CFA/II) and provides 75% protection against challenge with an LT, ST, CFA/II strain (19,20). This model has been successfully used to test the effect of nutritional intervention, including a phospholipid rich stream (21).

As described above, whey proteins and MFGM components such as specific proteins and

phospholipids have been associated with anti-infection and immune related outcomes. The whey protein concentrate tested in this study contained these components. The main objective of the current study was to test the effect of two concentrations of a whey protein concentrate on *E. coli*-induced diarrheal disease in otherwise healthy adults as measured by the number of stools per day and diarrhea score between day 2 to 3 after infection as compared to the control group.

Materials and Methods

Trial Design

The trial had a parallel design with 3 treatment arms and a 4 weeks intervention duration of which 2 weeks before the *E. coli* challenge and 2 weeks after the *E. coli* challenge (October 22nd, 2020- December 4th 2020). The set up was comparable to a previous study in which a phospholipid rich dairy product was tested for its reducing effect of *E. coli* -induced stool frequency and gastrointestinal complaints in adults (21). Participants visited the study site (NIZO, Ede, the Netherlands) 3 times, once for information on the trial, once for baseline measurements and once for *E. coli* challenge. All other interaction with participants was via online tools and coerier services for pick-up of samples/materials.. The study schedule is available in the supplementary Table S1.

Participants, Ethics and Trial Registration

This human intervention study was approved by the Medical Ethics Committee (METC) of Brabant, Tilburg, the Netherlands (July 2019) and registered as Protocol NL66645.028.18. In addition, the study was registered at the Netherlands Trial Register as NTR7613 NTR (trialregister.nl). Healthy male subjects between 18 and 55 years with a BMI between 18.5 and 30 kg/m2 were included in the study. For a complete in- and exclusion criteria list see Supplementary data S2. Subjects who were willing to participate were asked to sign an informed consent and were checked for eligibility by the Principal and Medical Investigator. Participants (149) signed informed consent, of which 121 were eligible for the study and of which 116 completed the study. For details on screening failures (n = 28) and dropouts (n = 5) see subject flow diagram Figure 1.

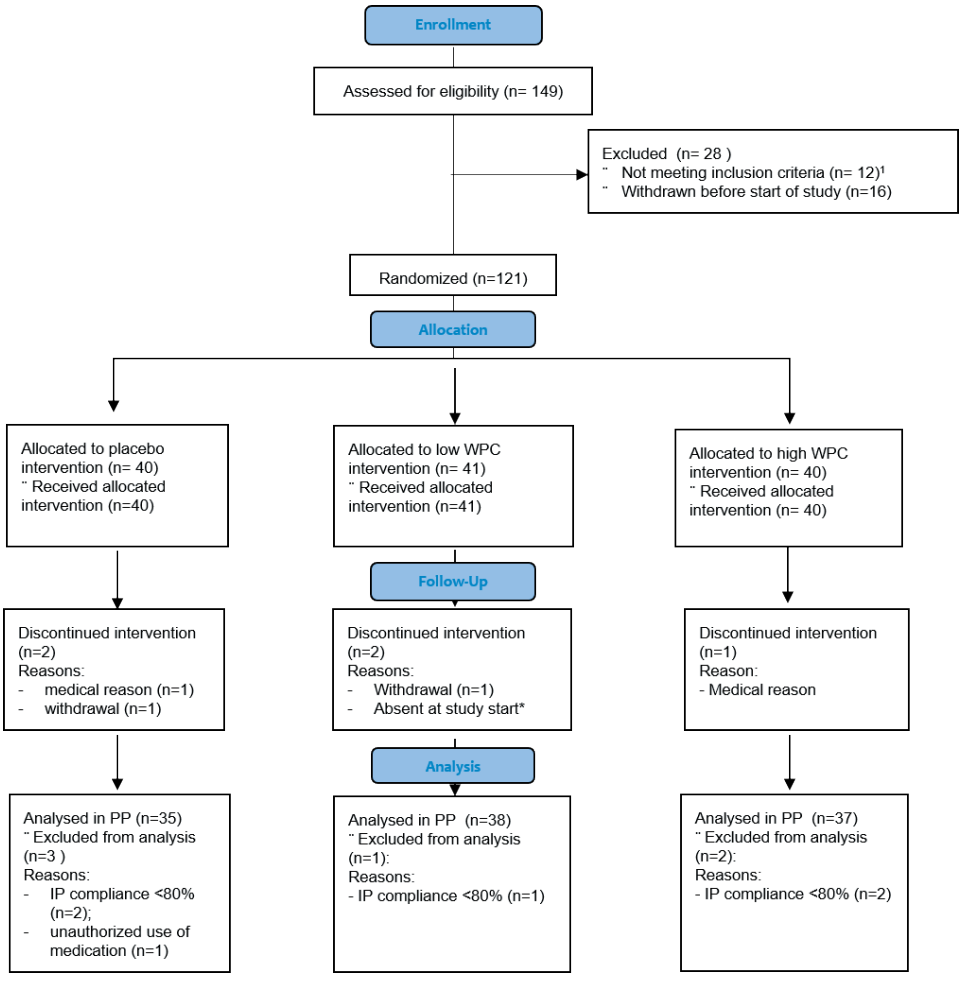


Figure 1. Flow diagram of the GIGA study. 1 Reasons for not meeting inclusion criteria; participation other study (n = 1), frequency defecation (n = 3), travel diarrhea (n = 4), participation ETEC study before (n = 1), BMI (n = 1), diagnoses IBS (n=1), planned operation during infection week (n = 1). * This person was replaced by another person in cohort 2 that started a week later. Discontinued intervention in total were n = 5 (dropouts).

Randomisation and stratification

Subjects were stratified by an independent NIZO scientist according to age and BMI and randomly assigned to one of three treatment groups; placebo, WPC low dose, WPC high dose. Stratified randomization of subjects to treatment group was performed using the Research Manager software (Deventer, The Netherlands).

Study products were supplied by FrieslandCampina and each of the 3 products received 3 unique codes. Blinding and labeling of the investigational product (IP) was performed

manually by the independent NIZO scientist by placing a label with the right subject code on the assigned study product, which covered the unique code.

All researchers of the NIZO project team, with the exception of the independent NIZO scientist, were kept blind to the assignment of treatment, and so were the study subjects. The code of the randomization was kept by the independent scientist. The investigators and participating subjects were blinded until after the blind data review. After sending the approved data master file to the statistician, the key to the treatment allocation was sent to the statistician by the independent NIZO scientist.

The randomization code of the study was broken after (i) all laboratory reports related to primary and secondary outcomes were authorized by the Principle Investigator, (ii) the Data Master File was documented as meeting the cleaning and approval requirements of the Principle Investigator, (iii) the finalization and approval of the statistical analysis plan by the Principal Investigator.

Medical guidelines

Use of medication known to affect GI functionality that contain non-steroidal anti-inflammatory drugs, acid suppression medication and antimotility agents were prohibited three days before, during and four days after the diarrheagenic *E. coli* challenge (Day 11 until Day 18). Antibiotics were prohibited from the run-in period and during the whole study. Paracetamol up to 2 g/day was allowed as escape medication during these days. Subjects received a list of prohibited and permissible medication. However, if medication was prescribed by the general practitioner or study physician, subjects were allowed to take these medications but needed to register intake (dose and frequency).

Dietary guidelines and Calcium restriction

Subjects were instructed to maintain their habitual diet, except for their dairy intake. Dairy has a high calcium content and contributes significantly to total daily calcium intake in the Dutch diet (22). From previous studies, it is known that calcium can significantly reduce the gastro-intestinal symptoms induced by the *E. coli* strain (17). Therefore, subjects were asked to omit dairy products. It was estimated that this restriction in dairy intake would lower the subject's daily calcium intake without the treatment to 400-500mg. Including the additional calcium intake through the intervention products (430-460 mg/day), overall calcium intake was in the range of the recommended daily calcium intake of 1000 mg in The Netherlands.

Probiotics and prebiotics/fibers

Subjects were instructed to avoid probiotics; non-dairy yoghurts with active cultures of probiotics; probiotic based supplements; products with added prebiotics/fibers; prebiotic/

fiber supplements, starting from the run-in period and during the whole study due to potential effects on duration of gut infection-induced diarrhea.

Dietary intervention products

Nutritional values

The investigational products (IP) were a partial hydrolysed whey protein (as placebo) and a whey protein concentrate (WPC) as the test product. All products were produced by FrieslandCampina, The Netherlands. All products were mixed with 18% maltodextrin to increase the solubility of the powder. The high dose of the WPC was pure WPC, and the low dose WPC was a mixture of WPC and the placebo product at a ratio of 0.9:1. All products were manufactured under the scope of a certified COKZ Z0949 approved Food Safety Management system. Table 1 shows the nutritional composition of the three IPs in % weight. Since calcium is a potential confounder in the study, the calcium levels were equalized in all 3 products. Subjects were asked to dissolve the content of a sachet (28 gram) slowly in 180 mL of stirring water using a vortex provided to them. Consumption was twice a day, during breakfast and during an evening meal. The control product had a bitter taste which was masked with instant coffee or syrup. To prevent deblinding, all participants were asked to use either syrup or instant coffee powder with or without sweetener. Usages of these taste options were equally distributed over the 3 groups. Questions from subjects about the study product were asked to an independent staff member to keep study team blind at all stages.

Table 1. Nutritional composition of Placebo, WPC low and WPC high Investigational Products indicated as percentage by weight of the powder except for calcium which is depicted as mg/kg.

Description	Unit	Placebo	WPC low	WPC high
Fat	w/w%	5	10	16
of which Phospholipids	w/w%	0	4,4	7,0
Protein	w/w%	60	58	57
of which HA300	w/w%	60	31	0
Lactose	w/w%	1,7	1,0	1,0
Maltodextrin	w/w%	18	18	18
Moisture	w/w%	4,4	4,7	4,0
Ash 525°C	w/w%	7,7	5,3	4,0
Calcium content	mg/kg	7700	7967	8200

Compliance with dietary guidelines and supplement intake

Participants were requested to daily record IP intake and indicate whether they complied with the dietary guidelines in their online diary. IP compliance was cross-checked by the returned sachets of IP.

Diarrheagenic E. coli challenge

For logistic reasons, the subjects were randomly split into 2 cohorts, which followed an identical study schedule, with a 1-week delay for the second cohort.

After a run-in period of 13 days, on day 13 a standardized meal was provided to the subjects. On study day 14, after an overnight fast, a blood sample was drawn. Under the supervision of the project team, subjects drank a NaHCO3 solution (100 mL 2% NaHCO3) to neutralize the gastric acid. After 5 minutes, they drank a fruit syrup drink (100 mL) containing the attenuated diarrheagenic *E. coli* strain E1392/75-2A (1E10 CFU). Subjects went home but were not allowed to drink or eat for 1 hour, after which they consumed the breakfast dose of the investigational product.

Reported stool consistency, stool frequency and gastrointestinal symptoms

Subjects had to report information on stool frequency, stool consistency by using the Bristol Stool Scale (BSS) (23), and registration of medication intake. Also, subjects had to record the frequency and severity of symptoms related to Reflux, Abdominal pain, Indigestion, Diarrhea and Constipation by the validated Gastro-intestinal Symptom Rating Scale (GSRS) (24). The GSRS is a disease-specific instrument of 15 items combined into five symptom clusters. The GSRS has a seven-point graded Likert-type scale where 1 represents the absence of troublesome symptoms and 7 represents very troublesome symptoms. Online data management system 'De Research Manager' was used to record all subject information.

Fecal sample collection

Fecal samples were collected one day before inoculation (day 12 or 13), at day 14 (inoculation day), during 4 days after the inoculation day (day 15 until day 18), and on the last study day (day 27 or 28). Subjects were asked to collect all 24h stool samples in collection bags and freeze them on-site using a provided mini freezer. The frozen samples were transported to NIZO, where they were sorted, homogenized, aliquoted, and stored at -20°C until later analysis

Microbiota and SCFA were analysed from those subjects that completed the entire study, had >90% compliance of study product intake, had 100% compliance of study product intake at day 11, 12, and 13 and collected a stool sample at baseline and on day 12/13. This resulted in n=33 samples from the placebo group, n=30 from WPC low and n=27 from WPC high group. Of these 90 subjects, the fecal samples at the start (before consumption of product, t=1) and at day 12, 13 (before inoculation, t=2) were analyzed on microbiota and SCFA to investigate the effect of 2 weeks consumption of IP.

Microbiota composition profiling by 16S rRNA gene sequencing

DNA isolation, including vigorous bead-beating steps, was performed as described previously (25). Barcoded amplicons from the V3–V4 region of 16S rRNA genes were generated using a 2-step PCR and according to previously described methods (25). For the second PCR in combination with sample-specific barcoded primers, purified PCR products were shipped to BaseClear BV (Leiden, The Netherlands). PCR products were checked on a Bioanalyzer (Agilent) and quantified. This was followed by multiplexing, clustering and sequencing on an Illumina MiSeq with the paired-end (2×) 300 bp protocol and indexing. FASTQ read sequence files were generated using bcl2fastq2 version 2.18. Initial quality assessment was based on data passing the Illumina Chastity filtering. From the raw sequencing data, the sequence reads of too low quality (only "passing filter" reads were selected) were discarded and reads containing adaptor sequences or PhiX control were removed with an in-house filtering protocol. On the remaining reads, quality assessment was performed using the FASTQC tool version 0.11.5.

Sequences of the 16S rRNA gene were analyzed using a workflow based on Qiime 1.8 (26). On average, 45,862 (range 13,179 – 57,858) 16S rRNA gene sequences per sample were analyzed. We performed operational taxonomic unit (OTU) clustering (open reference), taxonomic assignment and reference alignment with the pick_open_reference_otus.py workflow script of Qiime, using uclust as clustering method (97% identity) and GreenGenes v13.8 as reference database for taxonomic assignment. Reference-based chimera removal was done with Uchime (27). The RDP classifier version 2.2 was performed for taxonomic classification (28).

Fecal SCFA analysis

For organic acids (lactate, acetate, propionate, butyrate, isobutyrate, valerate, isovalerate), fecal samples were prepared according to a modified and previously described method (see also the Supplementary Materials and methods) (29).

Data and statistical analysis

Sample size calculation

Sample size calculation was based upon 2-sided statistical testing, unpaired analysis, α=0.05 (chance of type I error) and ß=0.20 (chance on type II error) and took the 3 study arms into account with an expected dose-response of the 2 WPC arms. Based on reported stool frequency (number of stools per day) and diarrhea complaints (Gastro-intestinal Symptom Rating Scale, domain diarrhea) in an earlier study (21) and taking into account potential dropouts, pre-exposure of subjects to *E. coli* (1-2 per study based on experience in the past) and a possible effect whether the subject was in the first or the second logistic group, the calculated sample size was 40 subjects per arm. In total this resulted in n=120 subjects for the study, of which 121 were realized (see below).

Statistical analysis of primary outcomes

Intention-to-treat (ITT) and per protocol (PP) analysis was performed for all outcomes and for all randomized subjects who received the challenge and for whom results for the primary outcomes were available. ITT analysis included all consented and randomized subjects, having (reportedly) consumed the study product at least once, PP analysis excluded subjects with protocol violations or major protocol deviations. Criteria for subjects to be excluded from the PP analysis were as follows:

- Subjects who did not fulfill the in- and exclusion criteria;
- Subjects who reported protocol violations or major protocol deviations;
- Subjects for whom IP compliance was <80%;
- Subjects for whom >50% of the data points were missing.

After inclusion, 5 subjects dropped out of the study. One subject (193/131) had to be excluded from the study before the 2nd cohort started with the trial. This subject was replaced in the 2nd cohort. The other 4 subjects dropped out before the infection day. Therefore, 116 healthy males completed the study (see also Figure 1).

Outcome analysis

Primary outcomes were a change in the number of stools per day, d15-16, and a change in GSRS score, domain diarrhea, d15-16. These are continuous variables and were analyzed using mixed model analysis with study treatment and baseline variable on day 15 as fixed effects, and age, BMI and logistical groups as covariates. Correction for multiple testing was done by correcting the threshold using the Hochberg procedure because 2 primary outcomes were defined (30). If both primary parameters had a p-value below the threshold of 0.05 both null hypotheses were rejected. If one p-value was above 0.05, the other one must be lower than 0.025 to reject the null hypothesis and thus accept a significant effect of the intervention.

Secondary outcomes were a) change in stool consistency measured by BSS score (1-7) between day 15 and 16 using a chi-square test, b) percentage change in stool frequency d16 vs. d15 using a mixed model with study dose, baseline variable on day 15 as fixed effects and age, BMI, and logistical groups as covariates c) Stool consistency as measured as mean BSS score per day and maximum BSS score per day. Change and percentage change for both parameters were analysed using the same mixed model as for the primary parameters, d) Kinetics of Gastro-intestinal symptoms (GSRS), measured as sum total GSRS score, sum domain diarrhea score, and sum domain abdominal pain score. Changes and percentage changes for all the parameters of days 14 to 18 were analysed using mixed model repeated measures (MMRM) with dose and timepoints as the fixed factors and age, BMI and logistical group as covariates, e) Maximum GSRS score, measured by sum total

GSRS score, sum domain diarrhea score, and sum domain abdominal pain score over days 14 to 18, analysed using ANCOVA with dose as factor and age, BMI and logistical group as covariates.

SCFAs as exploratory outcome were analysed using the same mixed model as for the primary parameters.Analyses were performed according to a statistical analysis plan and all analyses were performed using Stata, version 12.

Microbiota analysis

Between-treatment group differences in alpha-diversity (Faith's phylogenetic diversity, Shannon index and Richness were assessed by the non-parametric Kruskal–Wallis test with Dunn's post hoc test. Alpha-diversity differences between t1 and t2 of the combined treatment groups were assessed by the Wilcoxon signed rank test. In the bivariate explorative analysis of all taxa, the Mann–Whitney U test with FDR (Benjamini-Hochberg) correction for multiple testing was applied to assess differences between two treatment groups. Taxon relative abundance differences between t1 and t2 of the combined treatment groups were assessed by the Wilcoxon signed rank test, followed by FDR correction. For longitudinal analysis, the change of taxon relative abundance over time, 2log ratios were calculated, in which the relative abundance of a taxon at endpoint was divided by the relative abundance of the same taxon at baseline. Ratios were compared between groups by Mann–Whitney U tests with FDR correction for multiple testing.

Redundancy analyses (RDAs) on the gut microbiota composition as assessed by 16S rRNA gene sequencing was performed in Canoco version 5.11 using default settings of the analysis type "Constrained" (31). Relative abundance values of OTUs were used as response data and metadata as the explanatory variable. For visualization purposes, genera (and not OTUs) were plotted as supplementary variables. Longitudinal effects of the intervention were assessed by calculating 2log ratios in which the relative abundance of an OTU or genus at endpoint was divided by the relative abundance of the same OTU or genus at baseline. These ratios were used as response variables in RDAs and were weighted based on the average relative abundance of each OTU in all subjects. RDA calculates p-values by permutating (Monte Carlo) the sample status. Partial RDA was employed to account for covariance attributable to age and BMI; these were first fitted in the regression modeling and then partialled out (removed) from the ordination as described in the Canoco 5 manual (31).

Results

Baseline Characteristics and compliance with diet and investigational products

In total, 121 healthy male human subjects who fulfilled all the inclusion criteria and none of the exclusion criteria were included in the study (see Figure 1 flow diagram of study participants). Five subjects dropped out before the infection day, resulting in 116 subjects completing the study.

Based on online diaries and check on returned empty sachets at the study location, in total 5 subjects had compliance lower than 80% and were therefore excluded from the PP population. All subjects had a compliance of more than 80% for following the calcium restrictions. One subject used medication in the restricted period and was also excluded from the PP population. Baseline characteristics of the PP population (n=110) are depicted in Table 2. The results presented here are for the PP population.

Table 2. Baseline characteristics of subjects. No significant differences were observed between the groups with respect to age and BMI.

Variable		Placebo	Ingredient Low Dose	Ingredient High Dose
Gender	N	35	38	37
	Male	100%	100%	100%
Age	Mean (SD)	36.29 (11.6)	34.16 (11.93)	33.7 (9.98)
BMI (kg/m2)	Mean (SD)	24.43 (2.24)	23.85 (2.93)	24.02 (2.81)

Primary outcomes

The main objective of the current study was to test the effect of a whey protein concentrate on *E. coli*-induced diarrheal disease in otherwise healthy adults as measured by a faster decline in stool frequency and diarrhea symptoms between day 15 (one day after infection) and day 16 (two days after infection) compared to placebo. Figure 2 shows the stool frequency (Figure 2a) and diarrhea score (Figure 2b) of the three treatment groups during the complete trial. On day 14 the *E. coli* challenge was performed which resulted in an increase in symptoms on day 15 in all groups. On day 16 the symptoms declined as expected.

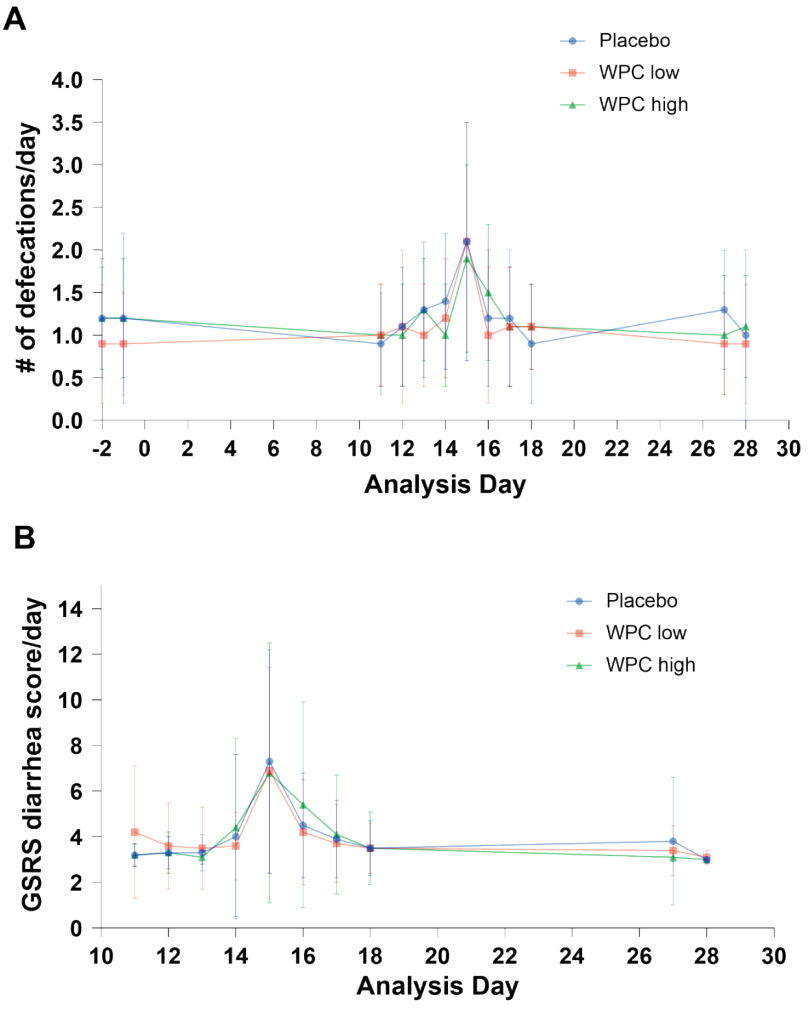


Figure 2. Response curves for stool frequency (mean # of defecations per day +/- SD) (A) and GSRS (mean domain score diarrhea per day +/- SD) (B) for the three treatment groups for the per protocol population. No statistically significant differences were found between placebo (circles, blue),WPC low (squares, red) and WPC high (triangles, green) using mixed model statistical analysis.

There was an overall significant effect of treatment dose on change in stool frequency ($p<0.01$), see also Table 3. Indeed, the difference between the high dose and placebo group suggested a somewhat delayed recovery in the high dose group, but this difference was not significant ($p>0.025$) after correction for multiple testing (30). Post-hoc analysis on primary outcomes taking only the high responders (> 1 stool on day 15) into account also did not show significant differences between the groups, excluding a diluting effect of the non-responders on the primary outcomes (data not shown).

Table 3. Statistical study outcomes in relation to stool frequency.

Parameter	Variable	Placebo	WPC low	WPC high	p-value trend
Change in stool frequency (# defecations/day) d16 vs. d15 P	N (Nmiss)	35 (0)	38 (0)	37 (0)	P 0.01
	Mean (SD)	-0.9 (1.5)	-1.2 (1.6)	-0.4 (1.4)	
Percentage change in stool frequency d16 vs. d15 S	N (Nmiss)	33 (2)	37 (1)	35 (2)	P 0.053
	Mean (SD)	-36.2 (45.2)	- 38.8 (67.3)	-3.2 (80.8)	
Stool frequency d15 PH	N (Nmiss)	35 (0)	38 (0)	37 (0)	P 0.931
	Mean (SD)	2.1 (1.4)	2.1 (1.4)	1.9 (1.1)	
Stool frequency d16S	N (Nmiss)	35 (0)	38 (0)	37 (0)	P 0.034
	Mean (SD)	1.2 (0.8)	1.0 (0.8)	1.5 (0.8)	
AUC for stool frequency d11-d18 PH	N (Nmiss)	35 (0)	38 (0)	37 (0)	P 0.707
	Mean (SD)	9.2 (3.9)	8.5 (2.8)	8.9 (2.8)	

P Primary, S Secondary and PH post-hoc analysis. PP analysis is shown. The significant trend between the 3 groups was observed for primary outcome change in stool frequency d16 vs. d15 disappeared after correction for multiple testing (p>0.025) according to the Hochberg procedure.

Secondary outcome

Secondary study outcomes were tested in three areas of E.coli-induced infection effects: stool frequency (Table 3), gut comfort complaints (Table 4) and stool consistency (Table 5). For none of these secondary outcomes, a significant effect was found in the PP analysis. In the ITT analysis, the % change in stool frequency between day 15 and 16 was statistically significant (p=0.035) between the intervention groups as was the percentage change in GSRS diarrhea score (p=0.035). However, a sensitivity analysis of these parameters did not confirm these results (p=0.112 and p=0.249 respectively).

Table 4. Statistical study outcomes in relation to diarrhea.

Parameter	Variable	Placebo	WPC low	WPC high	Statistics
Change in GSRS diarrhea d16 vs. d15 P	N (Nmiss)	35 (0)	38 (0)	37 (0)	P 0.13
	Mean (SD)	-2.8 (4.7)	-2.7 (4.6)	-1.4 (4.7)	
Percentage change in GSRS diarrhea d16 vs. d15 S	N (Nmiss)	35 (0)	38 (0)	37 (0)	P 0.09
	Mean (SD)	-20.1 (37.9)	-21.3 (46.6)	-0.7 (61.0)	
GSRS max diarrhea score day 14 – day 18 S	N (Nmiss)	35 (0)	38 (0)	37 (0)	P 0.84
	Mean (SD)	8.3 (4.9)	7.4 (4.4)	7.9 (5.9)	
GSRS diarrhea d15 PH	N (Nmiss)	35 (0)	38 (0)	37 (0)	P 0.48
	Mean (SD)	7.3 (4.9)	6.9 (4.5)	6.8 (5.7)	
AUC GSRS diarrhea d11-d18 PH	N (Nmiss)	35 (0)	38 (0)	37 (0)	P 0.54
	Mean (SD)	29.7 (8.8)	29.2 (8.1)	30.4 (14.7)	

P Primary, S Secondary and PH post-hoc analysis.

Post-hoc analysis

Post-hoc analyses were performed in 2 areas of E.coli-induced infection effects: stool frequency and GSRS diarrhea. Stool frequency as well as GSRS diarrhea scores were compared between the groups at day 15 (the peak of the infection). Furthermore, the area under the curve for both stool frequency as well as GSRS diarrhea scores were compared between the groups (Table 3 and 4, indicated with PH). Again, no significant differences were found. For the other sub-domain of the GSRS, abdominal pain, also no statistically significant differences were found (data not shown).

Table 5. Stool consistency as measured by Bristol Stool Scale .

Parameter	Variable	Placebo	WPC low	WPC high	Statistics
Change in BSS max d16 vs. d15	N (Nmiss)	28 (7)	26 (12)	34 (3)	P 0.79
	Mean (SD)	-0.82 (2.06)	-1.00 (1.7)	-0.38 (1.54)	
Percentage change in BSS max d16 vs. d15	N (Nmiss)	28 (7)	26 (12)	34 (3)	P 0.58
	Mean (SD)	-7.53 (43.01)	-14.88 (27.51)	-1.51 (36.11)	
BSS max d15	N (Nmiss)	33 (2)	37 (1)	35 (2)	P 0.07
	Mean (SD)	5.61 (1.60)	5.57 (1.48)	4.83 (1.64)	

BSS scores were calculated as absolute and percentage change in BSS at day 16 compared to day 15, and as maximum BSS score on d15. Parameters were all secondary outcomes as defined in the statistical analysis plan.

Microbiota results

Exploratory analysis on microbiota composition was performed to test the hypothesis that a whey protein concentrate modulates the microbiota differently compared to the whey hydrolysate placebo. Microbiota composition at the start of the intervention was compared with microbiota obtained after 2 weeks (day 12/13 of the study) of consuming the intervention products, but before the *E. coli* infection. In line with the primary outcomes, microbiota diversity (Faith's phylogenetic diversity, Shannon index, Richness) was not different between the intervention groups at either time point. From baseline to day 12/13, Shannon diversity significantly increased (p=0.0176) in the study population (combined treatment groups), but Faith's phylogenetic diversity and Richness were not different (Supplementary Figure S1). Cross-sectional analysis by Redundancy analysis (RDA) showed no difference between the microbiota composition of the WPC low, WPC high, and placebo groups at d12/13 (variation explained by treatment was 0.1%, p=0.36). However, there was a significant effect of time across all groups (variation explained by time point was 2.5%, p=0.002), with a similar effect in all groups (Figure 3). Baseline (t1) was associated with higher relative abundances of e.g. Streptococcus and Lactococcus, while d12/13 (t2) was associated with e.g. Parabacteroides and Odoribacter. Bivariate analysis of all taxa across all groups, between baseline and d12/13, showed e.g. lower Streptococcus and Lactococcus (p=0.0015 and p=0.0009, respectively) and e.g. higher Odoribacter and Bacteroides (p=0.0005 and p=0.0144, respectively) at d12/13 (Supplementary Table S2) Yet, RDA on change over time (ratios) confirmed that microbiota change was not different

between treatment groups (variation explained 0.4%, p=0.21). Also, no association of microbiota composition with complaint scores was found (data not shown). In line with the lack of difference in microbiota composition between the intervention groups, also no difference in fecal SCFA composition was observed (Supplementary Figure S3).

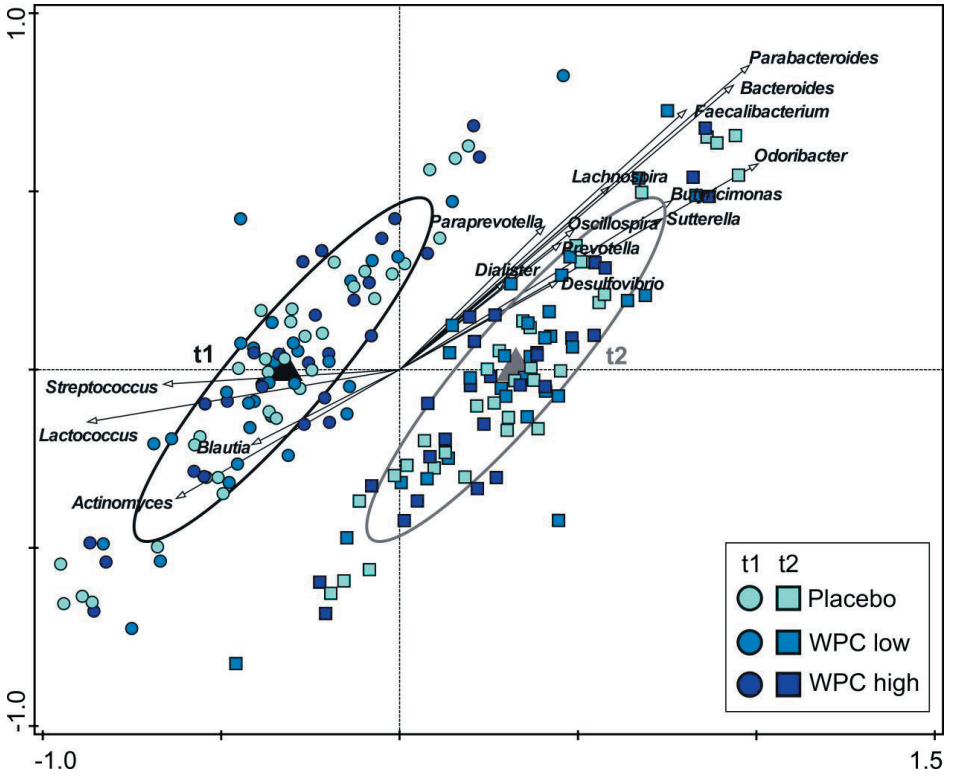


Figure 3. Redundancy analysis (RDA) on the OTU level. OTUs were used as response data and time point was explanatory data, the bacterial genera that contributed most were plotted. The covariance attributed to subject was first fitted by regression and then "partialled out" (removed) from the ordination. Variation explained by time point was 2.5%, p=0.002. Ellipses cover 66% of the observations associated with each time point. Circles are t=1 samples, squares are t=2 samples. Samples were separated based on time point on the first (horizontal) constrained axis, the second (vertical) unconstrained axis captures a fraction of variation explained by factors other than time point.

Adverse Events

Overall, study execution was safe and adverse events did not deviate from expectations. During the study, 124 adverse events (AE) were reported of which 49 were probably related to the *E. coli* inoculation, 22 were probably related to the investigational products and 53 were probably not related to the study. No serious adverse events have been reported during the study.

Discussion

Dairy streams and ingredents are of interest as a source of nutrition and can also be a source of immune active components (5,8,32,33). Indeed, many bovine proteins and complex lipids are highly similar to their human counterparts (34,35) and show functional responses *in vitro* (5,36–39) and *in vivo* (5–10,13,40,41).

Whey protein concentrates have been studied by multiple research groups in clinical intervention trials, (9,10,12,13,42) especially in infants because of the relatively high demand for immune support at that vulnerable age. Most of these whey protein concentrates contain proteins and lipids associated with the milk fat globule membrane (43). Here, we used an adult infection model using E.coli to study the effect of a whey protein concentrate.

In the present study, dietary treatment with WPC did not lead to a faster recovery from *E. coli*-induced diarrhea compared to a control hydrolyzed whey containing product, as concluded from the primary outcomes (ITT and PP). Although a significant effect was observed in secondary outcomes, % change of stool frequency and % change GSRS diarrhea in the ITT population, suggesting a slower recovery, this was not robust as concluded from the sensitivity analyses. Also, for the PP population in which all subjects were compliant with study product intake, no significant effects were found. The primary outcomes were selected based on the results obtained in an earlier study with a phospholipid rich dairy product (21). In that study, a significant effect was found on the secondary outcomes stool frequency and gastrointestinal symptoms in the days immediately after the infection.

The reason for not finding an effect in the present study is not known but may in part be explained by several factors. First, the type of protein the products contain. In the current study, the WPC contained >95% whey proteins as did the placebo, although hydrolyzed in the latter. The protein fraction of MFGM has been shown to have anti-*E. coli* effects *in vitro* (38), but no effect of the whey hydrolysate on the study outcomes was expected. However, it is possible that some of the peptides or amino acids present in the hydrolysate could have an antimicrobial or immune modulating activity (44). It can therefore not be fully excluded that the whey hydrolysate had some effect on the outcomes thereby potentially masking an effect of WPC. However, the recently published study using the same dose of *E. coli* showed a very comparable height of *E. coli* induced diarrhea responses in a non-treated population (15), making it less likely that the placebo in the current study suppressed diarrhea responses.

The phospholipid component, which has been shown to contain antibacterial effect, reviewed in (45), was highly comparable in composition (data not shown) and dose (WPC

high) between the current study and previously published data (21), and thus cannot explain the observed differences.

Intake of protein in the current study was 30 g. A hypothesis is that the 30 gram of high quality nutritious whey protein supports the immune system well in the WPC high, the WPC low (which is a mixture of WPC and hydrolysate) as well as in the hydrolysate group. Indeed, an increased intake in daily protein consumption to meet the demand during acute infection has been estimated (46,47), and immune function is dependent on available amino acids (48). Therefore, it is possible that both sources of high quality whey proteins used in our study were sufficient in recovering fast from the infection challenge in healthy adults. Arguments against this are that this population is a well-nourished population already and furthermore, the severity of the E.coli-induced diarrhea in the current study was comparable to a previous study (15). A limitation of the study is that only one dose was tested. Whether a higher dose would have had an effect remains to be established.

A potential confounder in the current study using E1392/75-2A *E. coli* challenge model is dairy-derived calcium-phosphate, that was previously shown to confer protection against gastrointestinal infection in this model (17). Comparison of a dose of 1100 mg versus 60 mg resulting in ~1500 mg vs. ~400 mg daily calcium intake between the two groups resulted in a reduction in *E. coli*-induced infection in this model. In the current study, 430-460 mg calcium per day was obtained from the study products and an additional ~400 mg from the calcium restriction diet. As a result, all participants consumed around 900 mg of calcium daily. Since it is not known from a dose response study at what concentration the protection by calcium is apparent, it cannot be excluded that calcium in the current study had a dampening effect on the diarrhea symptoms in all subjects. Again, a recent study using the same model in which no investigational product was used but where subjects did comply with a low calcium diet (<500 mg calcium in feces/day) (15) showed that *E. coli* induced a very similar symptom pattern compared to the current study arguing against a suppressed diarrhea effect because of higher total calcium. Indeed, post hoc analysis showed that even in the subgroup analysis of the high responders, no treatment effect was found.

Consumption of WPC had no differential effect on fecal microbiota composition and diversity between the low dose, high dose and placebo groups after two weeks of intervention. This indicates that the whey proteins and phospholipids in the WPC do not modulate gut microbiota composition differently when compared to the control, hydrolyzed whey. Although effects on the primary readouts and on the fecal microbiota may be independent, the comparable gut microbiota composition between the intervention groups fits with not finding an effect on the primary readouts. Most striking was the change in microbiota composition and diversity/evenness (Shannon index) over time, that was

observed for all intervention groups. The most likely explanation for this change over time is the change in diet by the dietary guidelines, as all subjects were instructed to adhere to a calcium-restricted diet, specifically omitting dairy products. However, the specific dietary drivers for the microbial compositional change could not be identified. Calcium intake may affect the gut microbiota, but we did not specifically determine the actual calcium intake of the subjects. The restriction of dairy products and supplementation of the intervention products may have influenced the casein/whey ratio, but this ratio was previously shown not likely to be a driver of microbiota changes (49). Interestingly, a high dairy diet has previously been found to increase Streptococcus, Lactococcus and Leuconostoc, while Faecalibacterium decreased, but Shannon diversity was not different (50). In the current study Streptococcus and Lactococcus were associated with baseline (before dairy restriction), while Faecalibacterium was one of the taxa associated with the two weeks time point (after dairy restriction) and therefore the change in microbiota may be the result of dairy restriction.

In this study, we used the *E. coli* infection challenge model to study the effects of intervention on infection and diarrhea as a model to evaluate whether the WPC might offer protection against infection in infants. A limitation of this study is that this type of inoculation can only be performed in healthy adults with a well-functioning immune system (51). This population does not represent a vulnerable infant population with a high disease burden.

Field trials in infants have shown that whey protein concentrates, which contain milk fat globule membrane components, may have a beneficial effect on infection outcomes (10,13). Also, multiple studies have shown beneficial effects of polar lipids and specific proteins present in the MFGM fraction on diverse infectious microbes in preclinical (40,52) and *in vitro* studies (37–39,53–56). In the current study, no effect of a short nutritional intervention with a WPC on acute gastrointestinal infection readouts in adults was found. Whether the current WPC offers protection on infection readouts in an infant population remains to be established.

Conclusions

Short term consumption of a whey protein concentrate by healthy adults a) did not reduce diarrhea scores in an *E. coli* infection model, and b) did not modify the composition of the intestinal microbiota. More research is needed to exploit the potential of whey protein concentrates on infection readouts *in vivo*.

Abbreviations

AE	Adverse Events
AUC	Area Under the Curve
BSS	Bristol Stool Scale
CFA	Colonisation Factor Antigen
CFU	Colony Forming Unit
ETEC	Entero Toxigenic Escherichia Coli
GI	Gastro-intestional
GIGA	Gastrointestinal benefit of dairy Ingredients to prevent symptoms of Gut infection in healthy Adult subjects
GSRS	Gastrointestinal Symptom Rating Score
IP	Investigational Product
ITT	Intention to treat
LT	Heat labile
METC	Medisch Etisch Toetsings Commissie
MFGM	Milk Fat Globular Membrane
MMRM	Mixed model repeated measures
NTR	Netherlands Trial Register
OTU	operational taxonomic unit
PCR	Polymeric Chain Reaction
PP	Per Protocol
RDA	Redundancy analysis
(S)AE	Severe Adverse Events
SCFA	Short Chain Fatty Acids
ST	Heat stable
WPC	Whey Protein Concentrate

References

1. Troeger C, Forouzanfar M, Rao PC, Khalil I, Brown A, Reiner RC, et al. Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015. Lancet Infect Dis. 2017;17(9):909–48.
2. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet (London, England). 2013 Jul;382(9888):209–22.
3. Brennhofer S, Reifsnider E, Bruening M. Malnutrition coupled with diarrheal and respiratory infections among children in Asia: A systematic review. Public Health Nurs. 2017 Jul;34(4):401–9.
4. Mokomane M, Kasvosve I, de Melo E, Pernica JM, Goldfarb DM. The global problem of childhood diarrhoeal diseases: emerging strategies in prevention and management. Ther Adv Infect Dis. 2018 Jan;5(1):29–43.
5. Ulfman LH, Leusen JHW, Savelkoul HFJ, Warner JO, van Neerven RJJ. Effects of Bovine Immunoglobulins on Immune Function, Allergy, and Infection. Front Nutr. 2018 Jun 22;5:52.
6. Hernell O, Timby N, Domellöf M, Lönnerdal B. Clinical Benefits of Milk Fat Globule Membranes for Infants and Children. J Pediatr. 2016 Jun;173:S60–5.
7. Drago-Serrano M, Campos-Rodríguez R, Carrero J, de la Garza M. Lactoferrin: Balancing Ups and Downs of Inflammation Due to Microbial Infections. Int J Mol Sci. 2017 Mar 1;18(3):501.
8. Brink LR, Lönnerdal B. Milk fat globule membrane: the role of its various components in infant health and development. J Nutr Biochem. 2020 Nov;85:108465.
9. Timby N, Hernell O, Vaarala O, Melin M, Lönnerdal B, Domellöf M. Infections in infants fed formula supplemented with bovine milk fat globule membranes. J Pediatr Gastroenterol Nutr. 2015 Mar;60(3):384–9.
10. Zavaleta N, Kvistgaard AS, Graverholt G, Respicio G, Guija H, Valencia N, et al. Efficacy of an MFGM-enriched complementary food in diarrhea, anemia, and micronutrient status in infants. J Pediatr Gastroenterol Nutr. 2011 Nov;53(5):561–8.
11. Li X, Peng Y, Li Z, Christensen B, Heckmann AB, Stenlund H, et al. Feeding Infants Formula With Probiotics or Milk Fat Globule Membrane: A Double-Blind, Randomized Controlled Trial. Front Pediatr. 2019;7:347.
12. Li F, Wu SS, Berseth CL, Harris CL, Richards JD, Wampler JL, et al. Improved Neurodevelopmental Outcomes Associated with Bovine Milk Fat Globule Membrane and Lactoferrin in Infant Formula: A Randomized, Controlled Trial. J Pediatr. 2019 Dec;215:24-31.e8.
13. Veereman-Wauters G, Staelens S, Rombaut R, Dewettinck K, Deboutte D, Brummer R-J, et al. Milk fat globule membrane (INPULSE) enriched formula milk decreases febrile episodes and may improve behavioral regulation in young children. Nutrition. 2012 Jul;28(7–8):749–52.
14. Poppitt SD, McGregor RA, Wiessing KR, Goyal VK, Chitkara AJ, Gupta S, et al. Bovine complex milk lipid containing gangliosides for prevention of rotavirus infection and diarrhoea in northern Indian infants. J Pediatr Gastroenterol Nutr. 2014 Aug;59(2):167–71.

15. van Hoffen E, Mercenier A, Vidal K, Benyacoub J, Schloesser J, Kardinaal A, et al. Characterization of the pathophysiological determinants of diarrheagenic Escherichia coli infection using a challenge model in healthy adults. Sci Rep. 2021;11(1):1–14.
16. Ten Bruggencate SJM, Girard SA, Floris-Vollenbroek EGM, Bhardwaj R, Tompkins TA. The effect of a multi-strain probiotic on the resistance toward Escherichia coli challenge in a randomized, placebo-controlled, double-blind intervention study. Eur J Clin Nutr. 2015 Mar;69(3):385–91.
17. Bovee-Oudenhoven IMJ, Lettink-Wissink MLG, Van Doesburg W, Witteman BJM, Van Der Meer R. Diarrhea caused by enterotoxigenic Escherichia coli infection of humans is inhibited by dietary calcium. Gastroenterology. 2003 Aug;125(2):469–76.
18. Ouwehand AC, ten Bruggencate SJM, Schonewille AJ, Alhoniemi E, Forssten SD, Bovee-Oudenhoven IMJ. Lactobacillus acidophilus supplementation in human subjects and their resistance to enterotoxigenic Escherichia coli infection. Br J Nutr. 2014 Feb;111(3):465–73.
19. Levine MM, Tacket CO. Vaccines against Enterotoxigenic Escherichia Coli Infections. Part Ii: Live Oral Vaccines and Subunit (Purified Fimbriae and Toxin Subunit) Vaccines. In: New generation Vaccins. Marcel Dekker, Inc; 1997. p. 875–83.
20. Levine MM, Barry EM, Chen WH. A roadmap for enterotoxigenic Escherichia coli vaccine development based on volunteer challenge studies. Hum Vaccines Immunother [Internet]. 2019;15(6):1357–78. Available from: https://doi.org/10.1080/21645515.2019.1578922
21. Ten Bruggencate SJ, Frederiksen PD, Pedersen SM, Floris-Vollenbroek EG, De Bos EL van, Van Hoffen E, et al. Dietary milk-fat-globule membrane affects resistance to diarrheagenic escherichia coli in healthy adults in a randomized, placebo-controlled, double-blind study. J Nutr. 2016 Feb 1;146(2):249–55.
22. Rossum, C.T.M. van; E.J.M. Buurma-Rethans; Dinnissen CS, Beukers, M.H.; Brants, H.A.M.;Dekkers, A.L.M.;Ocké MC. The diet of the Dutch. Results of the Dutch National Food Consumption Survey 2012-2016. 2016.
23. Heaton KW, Ghosh S, Braddon FEM. How bad are the symptoms and bowel dysfunction of patients with the irritable bowel syndrome? A prospective, controlled study with emphasis on stool form. Gut. 1991;32(1):73–9.
24. Svedlund J, Sjödin I, Dotevall G. GSRS--a clinical rating scale for gastrointestinal symptoms in patients with irritable bowel syndrome and peptic ulcer disease. Dig Dis Sci. 1988 Feb;33(2):129–34.
25. Rahman S, Kortman GAM, Boekhorst J, Lee P, Khan MR, Ahmed F. Effect of low-iron micronutrient powder (MNP) on the composition of gut microbiota of Bangladeshi children in a high-iron groundwater setting: a randomized controlled trial. Eur J Nutr. 2021 Sep;60(6):3423–36.
26. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Vol. 7, Nature methods. 2010. p. 335–6.
27. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011 Aug 15;27(16):2194–200.
28. Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, et al. The Ribosomal Database Project:

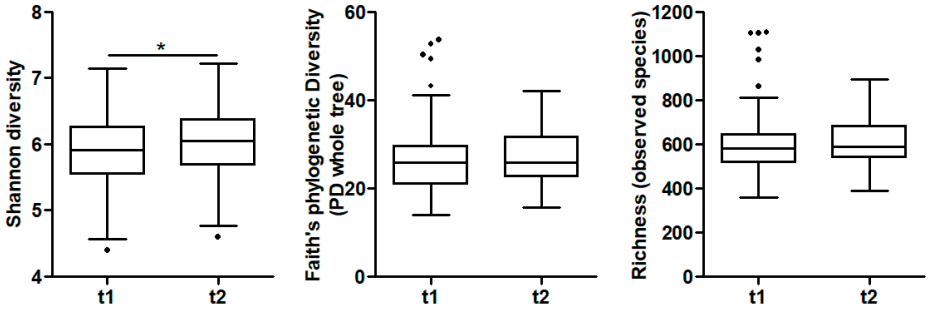
improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 2009 Jan;37(Database issue):D141-5.

29. Gommers LMM, Ederveen THA, van der Wijst J, Overmars-Bos C, Kortman GAM, Boekhorst J, et al. Low gut microbiota diversity and dietary magnesium intake are associated with the development of PPI-induced hypomagnesemia. FASEB J. 2019 Oct;33(10):11235–46.
30. Hochberg Y. A sharper Bonferroni procedure for multiple tests of significance. Biometrika. 1988 Dec 1;75(4):800–2.
31. ter Braak CJF, Smilauer P. Canoco reference manual and user's guide: software for ordination, version 5.0. Ithaca USA: Microcomputer Power; 2012.
32. van Neerven J. The effects of milk and colostrum on allergy and infection: Mechanisms and implications. Vol. 4, Animal Frontiers. 2014. 16–22 p.
33. Hammarström L, Weiner CK. Targeted antibodies in dairy-based products. Adv Exp Med Biol. 2008;606:321–43.
34. Hageman JHJ, Danielsen M, Nieuwenhuizen AG, Feitsma AL, Dalsgaard TK. Comparison of bovine milk fat and vegetable fat for infant formula: Implications for infant health. Int Dairy J. 2019;92:37–49.
35. Pierce A, Colavizza D, Benaissa M, Maes P, Tartar A, Montreuil J, et al. Molecular cloning and sequence analysis of bovine lactotransferrin. Eur J Biochem. 1991;196(1):177–84.
36. den Hartog G, Jacobino S, Bont L, Cox L, Ulfman LH, Leusen JHW, et al. Specificity and Effector Functions of Human RSV-Specific IgG from Bovine Milk. PLoS One. 2014 Jan;9(11):e112047.
37. Fuller KL, Kuhlenschmidt TB, Kuhlenschmidt MS, Jiménez-Flores R, Donovan SM. Milk fat globule membrane isolated from buttermilk or whey cream and their lipid components inhibit infectivity of rotavirus *in vitro*. J Dairy Sci. 2013 Jun;96(6):3488–97.
38. Tellez A, Corredig M, Guri A, Zanabria R, Griffiths MW, Delcenserie V. Bovine milk fat globule membrane affects virulence expression in Escherichia coli O157:H7. J Dairy Sci. 2012 Nov;95(11):6313–9.
39. Guri A, Griffiths M, Khursigara CM, Corredig M. The effect of milk fat globules on adherence and internalization of Salmonella Enteritidis to HT-29 cells. J Dairy Sci. 2012 Dec;95(12):6937–45.
40. Berding K, Wang M, Monaco MH, Alexander LS, Mudd AT, Chichlowski M, et al. Prebiotics and bioactive milk fractions affect gut development, microbiota, and neurotransmitter expression in piglets. J Pediatr Gastroenterol Nutr. 2016;63(6):688–97.
41. Lee H, Padhi E, Hasegawa Y, Larke J, Parenti M, Wang A, et al. Compositional Dynamics of the Milk Fat Globule and Its Role in Infant Development. Front Pediatr. 2018;6:313.
42. Lee H, Zavaleta N, Chen S-Y, Lönnerdal B, Slupsky C. Effect of bovine milk fat globule membranes as a complementary food on the serum metabolome and immune markers of 6-11-month-old Peruvian infants. NPJ Sci food. 2018;2:6.
43. Brink LR, Herren AW, McMillen S, Fraser K, Agnew M, Roy N, et al. Omics analysis reveals variations among commercial sources of bovine milk fat globule membrane. J Dairy Sci. 2020;103(4):3002–16.
44. Kiewiet MBG, Faas MM, de Vos P. Immunomodulatory protein hydrolysates and their application.

Nutrients. 2018;10(7):1–22.

45. Fontecha J, Brink L, Wu S, Pouliot Y, Visioli F, Jiménez-Flores R. Sources , Production , and Clinical Treatments of Milk. Nutrients. 2020;12:1–1607.
46. Kurpad A V. The requirements of protein & amino acid during acute & chronic infections. Indian J Med Res. 2006 Aug;124(2):129–48.
47. Blackburn GL. Nutritional assessment and support during infection. Am J Clin Nutr. 1977 Sep;30(9):1493–7.
48. Li P, Yin Y-L, Li D, Kim SW, Wu G. Amino acids and immune function. Br J Nutr. 2007 Aug;98(2):237–52.
49. Aslam H, Marx W, Rocks T, Loughman A, Chandrasekaran V, Ruusunen A, et al. The effects of dairy and dairy derivatives on the gut microbiota: a systematic literature review. Gut Microbes. 2020 Nov;12(1):1799533.
50. Swarte JC, Eelderink C, Douwes RM, Said MY, Hu S, Post A, et al. Effect of high versus low dairy consumption on the gut microbiome: Results of a randomized, cross-over study. Nutrients. 2020;12(7):1–13.
51. Porter C, Talaat K, Isidean S, Kardinaal A, Chakraborty S, Gutiérrez R, et al. The Controlled Human Infection Model for Enterotoxigenic Escherichia coli. Curr Top Microbiol Immunol. 2021 Oct 21;
52. Sprong RC, Hulstein MFE, Lambers TT, van der Meer R. Sweet buttermilk intake reduces colonisation and translocation of Listeria monocytogenes in rats by inhibiting mucosal pathogen adherence. Br J Nutr. 2012 Dec;108(11):2026–33.
53. Martín M-J, Martín-Sosa S, Alonso JM, Hueso P. Enterotoxigenic Escherichia coli strains bind bovine milk gangliosides in a ceramide-dependent process. Lipids. 2003 Jul;38(7):761–8.
54. Idota T, Kawakami H. Inhibitory effects of milk gangliosides on the adhesion of Escherichia coli to human intestinal carcinoma cells. Biosci Biotechnol Biochem. 1995 Jan;59(1):69–72.
55. Otnaess AB, Laegreid A, Ertresvåg K. Inhibition of enterotoxin from Escherichia coli and Vibrio cholerae by gangliosides from human milk. Infect Immun. 1983 May;40(2):563–9.
56. Sprong RC, Hulstein MF, Van der Meer R. Bactericidal activities of milk lipids. Antimicrob Agents Chemother. 2001 Apr;45(4):1298–301.

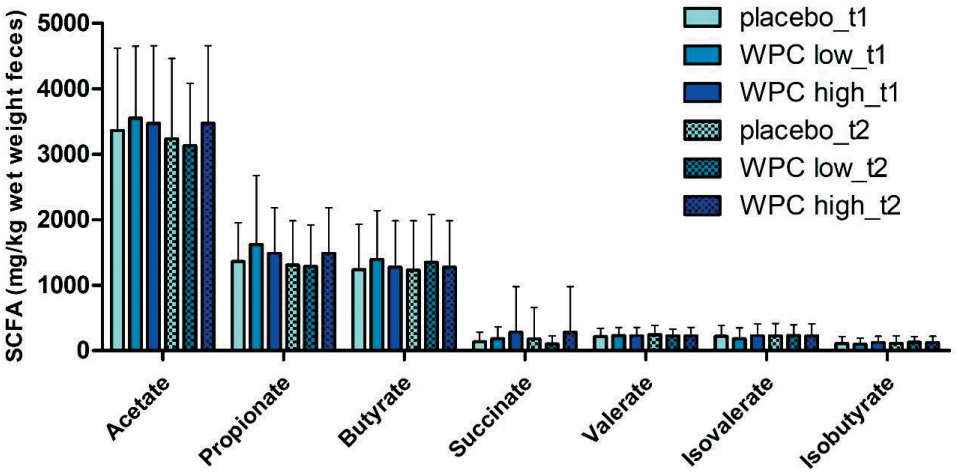
Supplementary materials



Supplementary Figure S1 Shannon diversity index (left) was slightly higher at t2 compared to t1 (p=0.0176). Faith's phylogenetic diversity (middle) and Richness (right) were not different. Boxplots are displayed as Tukey whiskers.

Supplementary Table S2 Taxa that were significantly different between t1 and t2 in bivariate analysis of each taxon, after correction for multiple testing by FDR. The mean 2log ratio column is conditionally formatted, in which an increase is indicated by positive (red) ratios, and a decrease by negative (blue) ratios.

Phylogenetic level	Taxon	t1 mean relative abundance (%)	t2 mean relative abundance (%)	Mean 2log ratio	p-value	p-value FDR
family	Clostridiaceae	5.44	3.72	-0.55	1.67E-06	4.03E-04
family	Ruminococcaceae	17.90	20.48	0.19	7.74E-04	1.56E-02
genus	Coprococcus	7.38	8.09	0.13	2.07E-03	3.26E-02
class	Bacilli	2.34	1.34	-0.80	2.25E-05	1.46E-03
order	Lactobacillales	2.11	1.19	-0.82	5.58E-05	2.75E-03
family	Streptococcaceae	1.74	0.88	-0.99	7.74E-06	7.62E-04
genus	Streptococcus	1.69	0.87	-0.96	2.59E-05	1.46E-03
genus	Lactococcus	0.06	0.01	-2.47	1.15E-05	9.05E-04
phylum	Bacteroidetes	3.08	4.12	0.42	8.70E-04	1.56E-02
class	Bacteroidia	3.08	4.12	0.42	8.70E-04	1.56E-02
order	Bacteroidales	3.08	4.12	0.42	8.70E-04	1.56E-02
family	Bacteroidaceae	1.38	1.88	0.45	6.21E-04	1.44E-02
genus	Bacteroides	1.38	1.88	0.45	6.21E-04	1.44E-02
family	Rikenellaceae	0.32	0.45	0.50	2.78E-04	1.20E-02
family	[Odoribacteraceae]	0.05	0.08	0.81	2.05E-06	4.03E-04
genus	Butyricimonas	0.02	0.03	0.77	5.03E-04	1.32E-02
genus	Odoribacter	0.03	0.05	0.85	3.44E-06	4.51E-04
family	Alcaligenaceae	0.02	0.04	0.64	3.90E-04	1.20E-02
genus	Sutterella	0.02	0.03	0.62	3.96E-04	1.20E-02
class	Deltaproteobacteria	0.02	0.03	0.85	3.35E-03	4.98E-02
order	Desulfovibrionales	0.02	0.03	0.85	3.54E-03	4.98E-02
family	Desulfovibrionaceae	0.02	0.03	0.85	3.54E-03	4.98E-02
order	Actinomycetales	0.09	0.06	-0.42	3.84E-04	1.20E-02
family	Actinomycetaceae	0.07	0.05	-0.31	3.56E-04	1.20E-02
genus	Actinomyces	0.06	0.05	-0.31	6.89E-04	1.51E-02



Supplementary Figure S3 Fecal SCFA (mg/kg wet weight feces) as determined by HPLC. There were no statistically significant differences between the treatment groups.

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When I started my PhD, I was extremely enthusiastic about learning science. However, my experience was far more extensive than that. During the course of the project, I learned a great deal more than just science. I also met a number of remarkable individuals and formed friendships that I hope will last for long. Now that I am proudly presenting my PhD dissertation, I would like to express my gratitude to those who assisted me along the way and emphasize that without their assistance, a successful completion of the PhD trajectory would not have been possible.

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During my PhD research, we collaborated with Friesland Campina and the NIZO food research institute to conduct two extensive human studies. I would like to extend my sincere appreciation to all colleagues who participated in these studies; **Laurien**, **Talitha**, **Els**, **Alwine**, **Maartje**, **Joyce**, **Anita**, and others who assisted me in collecting and analyzing data for this thesis. In addition, numerous data were generated from experiments conducted on blood samples collected at the Wageningen University blood collection center. Thank you to **Henriëtte** and her colleagues for the blood collection, as well as the generous blood donors who contributed.

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About the author

Mojtaba Porbahaie was born on May 20, 1979 in Shiraz, Iran. He has always been fascinated by nature, animals, and how various things function. He spent many hours exploring various plants and animals in the surrounding natural environment. Moreover, even before he could read, flipping the pages and scanning the books was one of his main interests. Through the encouragement of his mother and the influence of his older siblings, particularly his brother, he developed a passion for and interest in science. After graduating from high school in his hometown, he pursued a veterinary medicine degree at Kazeroun Azad University, where he earned his DVM degree in 2005. After working as a professional in science-related fields for several years, he decided to return to school and engage in self-education to satisfy his thirst for knowledge.

In 2015, he enrolled in the Animal Sciences MSc program at Wageningen University and Research (WUR) and chose a specialization to dive into human and veterinary immunology topics. For his MSc thesis, he joined the Cell Biology and Immunology group under the supervision of Gosia Teodorowicz. During his MSc research, he investigated the interaction between dietary advanced glycation end products (AGEs) and antigen-presenting cell receptors (APCs). In October 2017, following his MSc graduation, he began a PhD project in the same group under the supervision of Joost van Neerven and Huub Savelkoul. The aim of the PhD research was to investigate the direct and indirect effects of bovine milk components, including milk immunoglobulins, on the human immune system. During his doctoral studies, he collaborated with Friesland Campina B.V. and the NIZO food research institute. In this thesis, the findings of the research conducted for this project are presented and discussed.

During his PhD, Mojtaba participated in a variety of extracurricular activities, such as coordinating the animal health and immunology discussion group (AHIDG) and organizing the 2019 WIAS annual conference. He also developed expertise in flowcytometry analysis and spent the last six months of his PhD as a part-time analyst and provided help for other projects and studies. Mojtaba's journey at Wageningen University is not yet complete, as he will continue his scientific career as a researcher in the Cell Biology and Immunology group after receiving his PhD.

Overview of completed training activities

The Basic Package	***1.8 ECTS***
WIAS Introduction Day	2018
Course on philosophy of science and/or ethics	2018
Disciplinary Competences	***9.3 ECTS***
Research proposal	2018
Advanced course immunology, UMC Utrecht	2019
Basic statistics for PhDs	2019
Flowcytometry course, U of Antwerp	2019
Professional Competences	***11.2 ECTS***
The Essentials of Scientific Writing and Presenting	2019
Scientific Writing	2021
WIAS Science Day	2019
Project and Time Management	2019
Scientific publishing	2021
Animal Health and Immunology Discussion Group	2018-2020
Career perspectives	2021
Effective and efficient verbal communication in academia and beyond	2021
Presentation Skills	***2 ECTS***
WIAS Annual Conference, Lunteren - Oral Presentation,	2020
World Immune Regulation Meeting, Davos - Oral presentation	2020
Teaching competences	***6 ECTS***
Cell Biology (CBI-10306)	2018-2020
Supervision of MSc theses	2018-2020

Completion of the training activities is in fulfilment of the requirements for the education certificate of the Graduate School of the Wageningen Institute of Animal Sciences (WIAS). This overview is based on the European Credit Transfer System (ECTS). One ECTS equals a study load of 28 hours.

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