Immunomodulation by raw bovine milk and its ingredients

Effects in nutritional intervention, oral vaccination and trained immunity

Marloes van Splunter

Propositions

1. Lactoferrin supplementation in elderly women can improve immune responsiveness of pDCs in response to TLR7/8 stimulation.
   (this thesis)

2. Incidental consumption of raw bovine milk is not sufficient to induce consistent detectable immunomodulatory effects in oral cholera vaccination.
   (this thesis)

3. Healthy ageing starts *in utero.*

4. Urbanisation of land previously used for intensive agriculture, leads to a higher diversity of bees, especially if maintenance free gardens are discouraged.

5. The duration of paternity leave should be at least 4 weeks.

6. If there would be as much science in politics as politics in science, better decisions would be taken by the government.

Propositions belonging to the thesis, entitled:
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Effects in nutritional intervention, oral vaccination and trained immunity
M.E. van Splunter
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M.E. van Splunter

Thesis

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Contents

List of Abbreviations

BCG:	Bacille Camette-Guérin vaccine
bIgG:	bovine immunoglobulin G
bLF:	bovine Lactoferrin
CLA:	cutaneous lymphocyte antigen
COMP:	cartilage oligomeric matrix protein (COMP)
CRP:	C-reactive protein
CTB:	cholera toxin B
FEV:	forced expiratory volume
FVC:	forced vital capacity
GOS:	galactooligosaccharides
HLA-DR:	human leukocyte antigen- antigen D related
ICAM:	intercellular adhesion molecule
IFN:	interferon
IL:	interleukin
LP:	lamina propria
LPS:	lipopolysaccharide
MAdCAM:	mucosal addressin cell adhesion molecule
mDC:	myeloid dendritic cell
MLN:	mesenteric lymph node
NK cells:	natural killer cells
OA:	osteoarthritis
OR:	odds ratio
OVA:	ovalbumin
Pam:	PAM3CSK4 or (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4-OH
pDC:	plasmacytoid dendritic cell
PP:	Peyer's Patch
RM:	raw milk
RM-(M)ANOVA:	repeated measures (M)ANOVA
RSV:	respiratory syncytial virus
RTI:	respiratory tract infections
SCFA:	short chain fatty acid
TGF:	transforming growth factor
TLR:	Toll-like receptors
TNF:	tumor necrosis factor
TT:	tetanus toxoid
UHT:	ultra-heat treated
VCAM:	vascular cell adhesion molecule

Voor Jeroen en Waldemar

Chapter 1

General introduction

Immunity in different age groups: young and old people are susceptible to infection

Already during embryonic development the immune system of the foetus starts to develop. The placenta does not only provide nutrients for the foetus, but also transfers IgG antibodies[1] and hosts a placental microbiome, which is hypothesized to have a function in the induction of tolerance to commensal bacteria by the foetus.[2] Furthermore, there seems to be an immune suppressing environment in the uterus, so that the foetus is prevented to exert pro-inflammatory immune responses.[3] This would be beneficial to prevent excessive inflammatory responses upon colonisation by commensal bacteria and to prevent preterm abortion. At birth many immune cells are present, but their responsiveness to pathogens is less than the level of responsiveness observed in adults.[4] IgA heavy chain transcript levels are at 60 weeks of age at 25% of the adult IgA transcript levels.[5] Toll-like receptor (TLR)-induced anti-viral responses of plasmacytoid dendritic cells (pDCs) are reduced at birth but develop to fully responsive pDCs within weeks after birth.[6] In addition, it takes 3-4 weeks after birth before B and T cell areas are formed in the bronchus associated lymphoid tissues (BALT).[7,8] These data indicate that infants are more vulnerable to infections and need additional protection, which is provided by human milk upon breastfeeding. The immune supporting effects of human milk are described below in 'immunomodulation by human milk'. When comparing the infection rate of seasonal influenza A infections between different age classes, the infection rates are highest for young children and elderly (>70 years old).[9] When comparing death rates by influenza and of the inter-pandemic years (1911-1917), the highest mortality rate is observed for children <1 year old and the mortality rate starts increasing after 65 years old.[10] High Influenza infection and mortality seems to correlate perfectly with the observed curves over the ages of Th1, B cells and innate immune responses, which are needed for good anti-viral immune responses and are decreased in infants and elderly, see Figure 1.[4,11] Besides, infants and elderly have an increased anti-inflammatory status (e.g. levels of IL-10), which dampen immune responses.[4] Elderly are more vulnerable for infections, because of reduced numbers of immune cells and reduced TLR-signalling[12–14], which are examples of immunosenescence, described in more detail in 'ageing of the immune system'.

Mucosal immune system

The main function of the mucosal immune system is to protect against colonization and invasion of air- or foodborne pathogens by immune exclusion and prevent uptake of foreign antigens. When these antigens enter the body, the function of the mucosal immune system is to protect the body against harmful immune responses to these antigens in order to prevent tissue damage and immunological exhaustion.[15] The mucosal immune system consists of many different cells. First there are the epithelial cells (with a mucus layer) that form a barrier between 'the outside-world' and the body. In addition there are innate immune cells, such as monocytes, macrophages, dendritic cells and neutrophils that can phagocytose pathogens and present their antigens to lymphocytes.[16] Lymphocytes form the adaptive immune system, which consists of B cells, T cell and natural killer cells.[16] Together, these immune cells protect our body against foreign antigens and pathogens.

The mucosal immune system consist of all mucous membranes covering the aerodigestive tract (nasal cavity, oral cavity, airways and gastro-intestinal tract) as well the urogenital tract, eye conjunctiva, inner ear and ducts of all exocrine glands.[15] The mucosa-associated lymphoid tissues (MALT) is the largest mammalian lymphoid organ system[15] and approximately 80% of all immune cells reside in the MALT. The MALT encompasses different lymphoid compartments where immune responses are initiated such as the Peyers patches, mesenteric lymph node, solitary follicles in the intestine and the tonsils.[17,18] Each mucosal tissue has its own associated lymphoid tissue resulting in NALT (nasal cavity), BALT (bronchus/lower airways), GENALT (urogenital tract) and GALT (gastro-intestinal tract). One of the important molecules of the mucosal immune system is secreted polymeric IgA (sIgA), which is obvious from the fact that 80% of the plasma cells are located in the gut and of these plasma cells 80-90% produce sIgA.[19] sIgA is translocated to the intestine for immune exclusion of pathogens and their antigens in high amounts, up to 40 mg/kg body weight in adults per day, which results in several grams sIgA per day.[20] In human two types of IgA exist, IgA1, which is the dominant isotype systemically and in many mucosal secretions and tissues, except in colon where IgA2 is predominant, because IgA2 is largely resistant to bacterial proteases.[19]

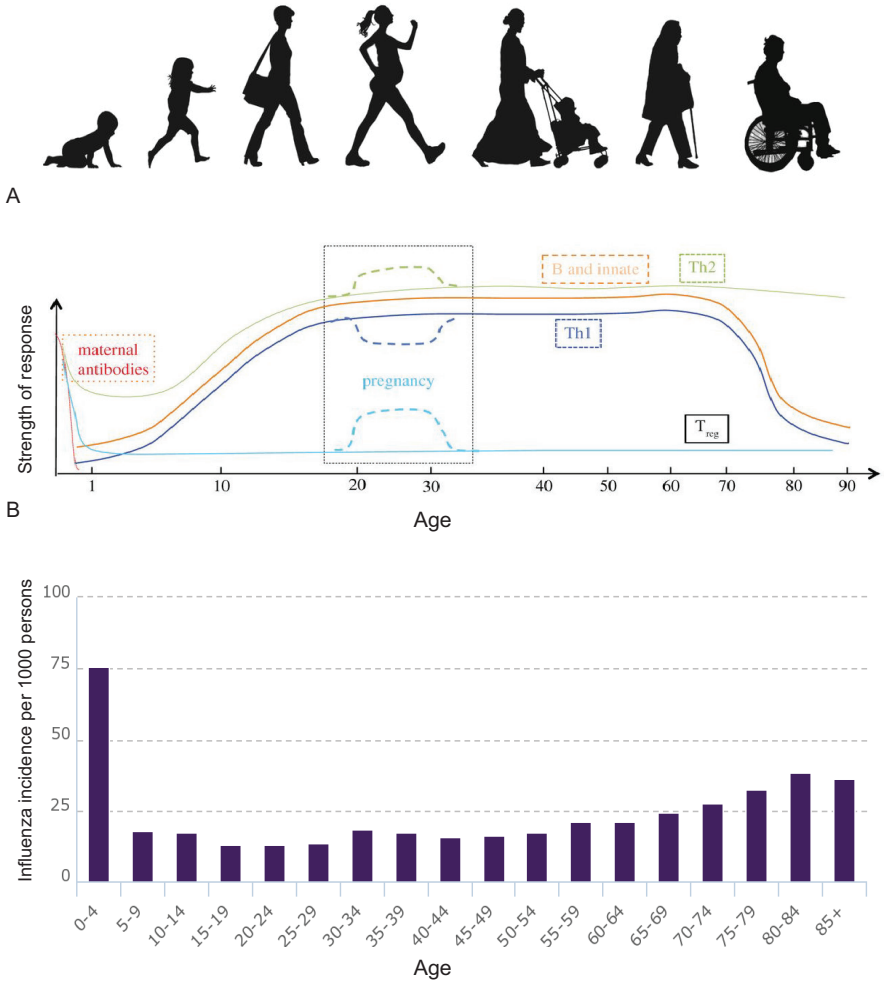

Figure 1: *Strength of immune responses and vulnerability to influenza infection over the lifetime of an individual. A) Schematic graph of the different arms of the immune response to influenza over the lifetime of an individual (adapted from Simon et al., 2015)*[11]*. B) Influenza incidence per 1000 persons per age category in 2016/2017 in the Netherlands as reported by general physicians (source: volksgezondheidenzorg.info)*

Of special interest is Waldeyer's ring, as the oropharynx (throat) is the place were intact food components food components and pathogens can interact, before they get (partially) digested in the stomach and intestine. The palatine tonsils in the oropharynx are part of Waldeyer's ring, which includes the nasopharyngeal tonsil (adenoid), paired tubal tonsils and lingual tonsil

as well.[21] The crypts of the palatine tonsils have a surface of approximately 300 cm^2, which is 6 times larger than the oropharyngeal surface and makes it especially suited for surveillance against airborne and food-derived pathogens and antigens. In the crypt epithelium lymphocytes can be found, as well as dendritic cells and macrophages.[21] Epithelial cells of the crypts of adenoids can synthesize the polymeric Ig receptor (pIgR) needed to transport IgA towards the nasal cavity. Next to this, IgA and IgG can transfer paracellular from palatine tonsils towards the oral cavity.[22]

Microfold or M-like cells present in the microcrypts (smalll pores) can serve as entrance for antigens and as passage for immune cells.[23,24] Antigens are better taken up by antigen-presenting cells when they are forming a complex with previous secreted immunoglobulins.[25–27] When pathogens/antigens are taken up by antigen-presenting cells such as dendritic cells in the tonsils or Peyers patches they are presented to naive T helper and B cells in the lymph nodes. Only the T and B cells specific for that pathogen/antigen will be activated and will start proliferating, differentiate to effector cells and migrate through the blood to the original site of activation, by a process which is called homing.[15,28] Upon stimulation, naive B cells can differentiate into short-lived plasmablasts[29] or into long-lived plasma cells[30] that both secrete specific antibodies. Plasmablasts are supposed to home towards the infected tissues where they produce antibodies locally, while plasma cells home to the bone marrow and produced antibodies systemically. Once the pathogen is cleared most B (and T cells) die by apoptosis, although some of them turn into memory cells that stay in the tissue as well as in the lymph nodes.[28] Upon a secondary encounter with the same pathogen the immune response is therefore both faster and the number of activated T and B cells will be higher leading to a faster clearance of the pathogen.[31,32] This concept is especially relevant for vaccination.

Mucosal tolerance

A typical feature of the mucosal immune system is its capacity to distinguish between inducing an immune effector response when needed, while at the same time developing a state of oral tolerance to harmless (commensal and dietary) antigens. Typically for the gut immune system, there is an intense

interaction between host cells, commensal and pathogenic bacteria, all of which can also have an impact on systemic immune responses. The local presence of defined DC subsets, together with the vitamin A metabolite RA, and the presence of Tregs are crucial in regulating intestinal tolerance and homeostasis.[33] Gut microbiota generate signals that direct intestinal responses with effector T-cells against pathogens or, in the case of commensals, induce a state of tolerance via modulation of Tregs and release of immunosuppressive cytokines like IL-10 and TGF-ß.[34] Mucosal DC are present in gut-associated tissues, including Peyer's patches (PP), mesenteric lymph nodes (MLN) and the lamina propria (LP) of the villous mucosa. Some of these DC subpopulations can selectively induce the differentiation of Treg. Normally, intestinal DC are quiescent and this state is linked to mucosal tolerance. However, these intestinal DC are responsive to inflammatory stimuli and thus able to present antigens and induce T-cell priming, homing and protective immunity.[34]

Immunomodulation by human milk

Human milk does not only supply nutritional compounds such as proteins, sugar and fat to newborns, but also stimulates and support the infant's immune system. The composition of milk is different in the first days (called colostrum) (e.g. whey:casein ratio 80:20, 2.0g/L sIgA) compared to mature milk (e.g. whey:casein ratio 60:40, 0.5 g/L sIgA) and composition varies during the day.[35,36] Because of the overall breastmilk composition, and because it's composition is adapted to the needs of the growing infant, breastfeeding is considered the golden standard to feed newborns. Breastfeeding protects against infections by the transfer of antibodies, such as secretory IgA and anti-microbial compounds such as lysozyme and lactoferrin[37,38]. This passive immunity supplied by breastfeeding is needed because the infant receives maternal antibodies in the uterus via the placenta during development, but does not produce enough specific-antibodies by themselves yet, when they are born.[39,40] Human milk contains hormones, growth factors that can influence the development of the gastrointestinal tract of newborns.[41] Next to this, many cytokines can be detected in human milk that affect and support the neonatal immune system, such as IL-1β, IL-6, IL-8, IL-10, TNF-α and IFN-γ[36,42]. In addition to proteins and fat, human milk contains high concentrations

oligosaccharides (HMOs), which makes human milk unique.[43] Human milk contains 10-15 g/L HMOs, which is 100x higher than bovine milk, that is used for infant formula.[44] There are more than 100 HMOs known, which function as prebiotic, as gut microbes use them as substrate for growth and production of metabolites.

To mimic the prebiotic function of HMOs, prebiotics have been developed for use in infant nutrition. Prebiotics are non-digestible oligosaccharides that reach the large intestine intact and allow specific changes in composition or activity in the gastrointestinal microbiota, that confer host well-being and health.[45] The best known function of prebiotics is the selective outgrowth of bifidobacteria, which can produce short-chain fatty acids as reviewed in chapter 2.[33] Next to this, HMOs can function as soluble decoy receptors that block binding of virusses (e.g. rotavirus and norovirus) and bacteria (*Campylobacter jejuni* and *E.coli*), as reviewed by Bode.[44] Approximately 1 % of the HMOs is absorbed by infants and the rest is simply excreted in feces.[44] Other functions of oligosaccharides and especially of sialylated oligosaccharides are discussed in Chapter 2.[33] The infant intestinal epithelium is immature and hence the permeability is high enough to absorb intact food- and bacterial components and immune cells present in human milk.[46,47] Furthermore, breast milk has been shown to contain bacteria (10^3-10^6 bacteria/ml milk), and has been proposed that it can thus also be considered a probiotic fluid.[48–51] There is an interplay between immune cells (macrophages, neutrophils and lymphocytes), bacterial communities, HMOs and macronutrients in human milk, although the exact mechanisms by which they are interacting remains largely elusive.[48] To summarize, human milk can support the mucosal and systemic immune system of neonates.

Differences between human and bovine milk

Consumption of raw bovine milk is associated with a reduction of the prevalence of asthma, allergies and respiratory tract infections in infants, and can thus exert a protective effect, which is described in more detail below.[52–54] Bovine milk is quite similar to human milk when comparing the composition, however the concentrations of some of the components are

different.[55] Bovine milks contains β-lactoglobulin and α_{s2}-casein, which are absent in human milk and which could contribute to their allergenic effects.[55] Lactoferrin and IgA concentrations are higher in human milk compared to bovine milk, while bovine milk contains higher IgG, TNF-α and TGF-β1 and β2 concentrations. IgM, vitamin A and D and other cytokines concentrations are comparable.[55,56] The main differences between human and bovine milk is the presence and concentration of oligosaccharides. Human milk contains a more diverse profile and higher concentrations of oligosaccharides.[57] Fucose-containing oligosaccharides are missing in bovine milk[58], but several sialylated oligosaccharides are present[59]. In bovine colostrum the levels of IgG are especially high as cow's don't have placental transfer of IgG antibodies. Hence, calves depend on the active absorption of IgG from colostrum into the blood for neonatal immunity.[60]

Box 1. The hygiene hypothesis in relation to the effect of the farming environment on allergies

In the last decades, the number of serious infectious diseases has declined rapidly in the Western world, while the occurrence of chronic inflammatory diseases, such as asthma and allergies is strongly increased.[61] Based on this inverse correlation, the hygiene hypothesis was formulated in 1989 by Strachan.[62] The hygiene hypothesis proposed that infections by bacteria and viruses are needed to induce a T-helper (Th) 1 response, thus protecting against Th2-mediated diseases, such as asthma and allergies. However, today it is known that the development of allergies and asthma does not only involve the balance between Th1 and Th2 responses, but is much more complicated, so the hygiene hypothesis has been revised recently. [63–65] Today other factors known to be involved in the development of allergies and asthma are Western lifestyle affecting gut microbiota, Tregs, Innate lymphoid cells and lung epithelial barrier.[65]

Epidemiologically, correlations between prevalence of atopy and asthma and parental social class and household size have been made, as reviewed by Strachan.[66] Next to this, the farming environment, especially in early childhood, is an important factor that has been linked to a reduced prevalence of atopy and asthma.[54,66–74] This farm effect is dependent on the type of farm.[72,74] For example, whereas adjusted odd ratios (aOR) for asthma and atopic sensitisation are lower for agricultural farms, dairy farms, and pig farms, aORs were higher for hares/rabbit farms and sheep farms.[74] So far these results have mainly been confirmed for dairy farms[72]. Several factors in the farming environment are associated with reduced asthma and atopy, such as exposure to animals,[69,70,72] endotoxin load,[68] exposure to stables and barns,[71,72,74] exposure to animal feed[72] and consumption of (raw) farm milk.[52,54,71,72,74,75]

Immunomodulation by raw bovine milk

Epidemiological studies have demonstrated an association of consumption of raw bovine milk and decreased incidence of asthma, allergy and even respiratory tract infections.[52–54] This may in part be mediated by the increased numbers of regulatory T cells in peripheral blood.[75] In the study of Perkin and Strachan, several environmental factors were tested for having a correlation with atopy (positive skin prick test) and only unpasteurized milk was associated with a reduced incidence of atopy.[54] Furthermore, also non-farming children had reduced allergies when consuming unpasteurized milk.[54,76] Next to this, raw milk consumption early in life (<6 years) is known to result in increased pulmonary function (FEV and FVC).[77] Altogether, these studies show that consuming unpasteurized farm milk is one of the main farm factors that exert a protective effect against asthma, allergy and atopy.

Besides, raw milk seems inversely associated with respiratory tract infections (RTI)[53]. One mechanism of this reduced respiratory tract infections could be the ingestion of bovine IgG (bIgG) present in raw milk[55], which is known to bind to human respiratory pathogens[78] allergens and intestinal pathogens[79,80]. Interestingly, bIgGs can also bind to FcγRII (CD32) expressed on human antigen presenting cells and neutrophils.[78] Bovine IgG can end up in the crypts of the palatine tonsils when swallowed. Hence, bovine IgG bound to respiratory pathogens may therefore result in more efficient pathogen uptake, antigen processing and activation of specific T cells towards that specific pathogen. In addition, the concept of the existence of a gut-lung axis is getting established in which changes in gut immunity affect airway immunity as well. This could play a role in the protective effect of raw bovine milk on airway immunity. In short, the gut-lung axis encompasses the influence of Toll like receptor (TLR) signalling by gut microbiota on airway immunity, the production of short chain fatty acid (SCFA) by gut microbiota after fermentation of fiber and oligosaccharides and immunomodulation of homing of immune cells by dietary components such as vitamin A and D, see chapter 2. Furthermore, bovine milk components can promote epithelial barrier function by upregulation of tight junctions and induce local regular T cell differentiation, as described in chapter 2. Another mechanism that might be responsible for reduced respiratory tract

infections is trained immunity, which is described in general below and more specifically for raw bovine milk in chapter 3.

Trained immunity

In humans, evidence for the existence of trained immunity first emerged from epidemiological studies on vaccination responses that indicated that vaccination induces protection not only against the target disease, but also induced cross-protection against other pathogens.[81] This was known to occur for the adaptive immune response, by the formation of cross-reactive antibodies after e.g. Salmonella vaccination[82,83], but it was not known for the innate immune system. The best known example of cross-protection mediated by the innate immune system is seen after Bacille Camette-Guérin (BCG) vaccination against *Mycobacterium tuberculosis,* which was shown to protect against all-cause mortality by reducing neonatal sepsis, respiratory infection and fever.[84] Other vaccines known to have non-specific beneficial effects are measles vaccination[85] and smallpox vaccine *Vaccinia,* as reviewed by Benn et al.[81] Furthermore, it was shown that BCG vaccination in humans induced trained immunity in both monocytes and NK cells three months after vaccination, which was mediated by increased H3K4 trimethylation in monocytes.[86,87] Next to this Influenza vaccination was shown to induce trained immunity in human NK cells.[88]

Until recently, only the adaptive immune system was supposed to build long-lasting immune memory upon infection and vaccination. However, recent observations have revealed that the innate immune system can also adapt to previous infections and can develop non-specific memory, a process termed trained innate immunity.[89,90] The concept of trained immunity is based on the observation that after a primary infection, an enhanced innate immune response is induced in response to the same secondary, as well as unrelated infections or stimulations, see Figure 2. In contrast to adaptive immune memory, this enhanced secondary response of trained innate immune cells is not only specific for the antigen that induced the primary response, but is rather a non-specific enhanced response to heterologous stimuli.[89] Trained immunity or innate immune memory is the opposite of immune tolerance,

which can be induced during sepsis.[91] Trained immunity results in an enhanced basal level of innate immune response, instead of returning to base line during homeostasis as is the case during short term immune priming.[91] This enhanced state involves chromatin modifications of latent enhancers, see below. To date it remains unclear which factor or pathway determines if an infection or stimulation leads to training or tolerance.

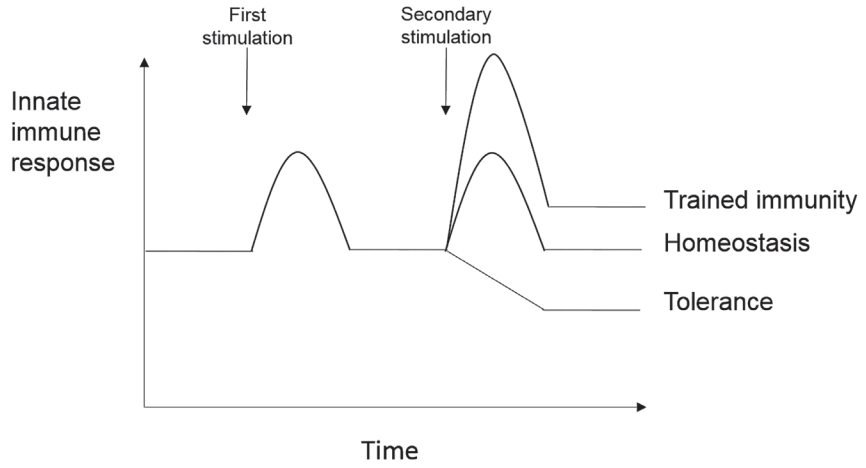

Figure 2: The concept of trained Immunity. Under homeostatic conditions a comparable innate immune response is observed after a secondary stimulation compared to the first stimulation (no innate immune memory). When innate immune cells are stimulated with TLR agonists tolerance can be induced, leading to a lower innate immune response after a secondary stimulation. When the first stimulation is able to induce trained immunity, a higher innate immune response is induced after a secondary infection (innate immune memory).

T and B cells have highly specific antigen receptors, which can distinguish pathogens at a species level. Innate immune cells cannot distinguish single species but can distinguish different pathogens via a group of pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs), Nod-like receptors (NLR) and C-type lectins (e.g. dectin-1).[92–94] These receptors can recognize pathogen associated molecular patterns (PAMPs) or endogenous danger signals from apoptotic or dead cells (danger-associated molecular patterns) (DAMPs) and can subsequently lead to trained immunity or tolerance in some cases, but not all.[95] Adaptive immune memory is very specific for one

type of antigen or pathogen and involves V-D-J gene recombination of TCR and BCR gene segments, whereas innate immune memory is not species-specific and does not involve gene recombination. In trained immunity histone methylation and acetylation of H3K4me1 or H3K27Ac of latent enhancers of pro-inflammatory cytokine genes takes place during initial activation, resulting in the enhance epigenetic status of immune cells.[96,97] These latent enhancers are regions of the genome that lack enhancers characteristics (e.g. binding of transcription factors and methylation or acetylation), but can acquire these characteristics upon stimulation leading to a fast secondary response, leading to long-term memory.[98] Trained immunity is confirmed both *in vitro* and *ex vivo* for monocyte/macraphages[86,99–101] and NK cells[87,88,102,103].

As a results of Western diet, low-density lipoprotein (oxLDL) particles are produced, which can induce trained immunity in human cells *in vitro*.[104] In mice a Western diet induced trained immunity in vivo.[105] It can thus be concluded that not only infections by pathogens or vaccination can induce trained immunity, but dietary compounds can induce trained immunity as well.

Dairy consumption in ageing adults

Both human and bovine milk are composed to provide newborn with both nutrients and protection. Biologically, this gives an advantage to the offspring of mammals, but humans uniquely evolved the possibility to digest lactose from bovine milk into adulthood.[106] This means that bovine milk can not only support the immune system of infants, but could potentially be used to modulate and support the immune system of elderly. Elderly often suffer from chronic diseases (described in more detail below) of which the onset can be delayed if the nutritional status of elderly is good.[107] However, improving nutritional status can be challenging as elderly can suffer from malabsorption, impaired sensory perception and higher micronutrient requirements[108,109]. Only a few foods that are consumed regularly by elderly provide enough proteins and micronutriens of which lean meat and dairy consumption are the best examples.[110,111] Dairy is the most important source for calcium in Europe, USA and UK and can further provide zinc, magnesium, and vitamin A, D and B-vitamins. Some studies have suggested that diary consumption is correlated with lower risk of hypertension and thereby

of developing cardiovascular diseases (CVD), improved bone health and reduced metabolic syndrome.[112–118] In the study of Laird et al. increased milk consumption in elderly was positively correlated with vitamin B2 status, but negatively correlated with vitamin B6 concentrations, while yogurt was positively correlated with both vitamin B2 and B6.[107] In a 12 year follow-up study in middle aged mixed population (±55 years old) increased total dairy intake was associated with a lower risk of incidence of prediabetes and type 2 diabetes, when comparing the lowest dairy with the highest dairy intake.[119] Furthermore, in a systematic review, dairy intake was shown in some studies to be associated with reduced anti-inflammatory markers in healthy people and in people suffering from metabolic disorders. Although some studies with a pro-inflammatory effect of dairy were found as well for healthy people, it was not found for people suffering from a metabolic syndrome.[114] In conclusion, although mixed results about the health benefits of dairy products are obtained, evidence of a possible link of dairy consumption to decreasing inflammation associated markers and diseases is emerging.

Box 2: The evolution of lactose persistence

Humans are the only species that are able to break down lactose present in cow's milk throughout life. Approximately one third of humans expresses lactase, the enzyme needed to break down lactose, although it varies per geographical region whether the lactase persistence is 5% (Southern part of Africa) or up to 100% (Northern part of Europe, West-Africa and Middle East).[106] The consumption of cow's milk in Europe dates from the late Neolithic period (approximately 3000-2300 BC).[209] However, there is evidence that milk was fermented from 6500 BC onwards in the region of Anatolia and in Europe from 6000 BC[210]. From this point onwards there is a strong natural selection on lactose persistence, with a selection coefficient of 0.04-0.05.[211,212] Therefore it seems likely that lactose persistence induces a selective advantage, compared to lactose non-persistent people. This has led to the culture-historical hypothesis that a population with higher levels of milk-dependence (e.g. nomadic herders) should be more often lactose persistent, as milk consumption provides sugar, fat and proteins. However, when measuring lactose persistent frequencies in long-term herder societies from Asia such as Mongols (12%)[213], and Kazakhs (24-30%)[213,214], as well as Sami reindeer herders in Scandinavia (40-75 versus 91% in Swedish population)[215] the frequencies are low. Ségural et al. explain this phenomena by pointing to advantages for lactose intolerant people, especially when consuming fermented low-lactose dairy products, as source of protein and fat, and even converted lactose to glucose by bacterial lactase. Furthermore many lactose intolerant people have a high threshold level for lactose (one glass of milk or 15 g of lactose) before getting harmful side effects as diarrhea.[106] In addition, lactose can exert prebiotic properties in the colon by increasing short chain fatty acid production[216,217], but lactose only reaches the colon when it's not broken down by lactase in the small intestine, resulting in a beneficial effect for lactose non persistent individuals[218,219]. When the tolerance levels for lactose are high enough there is no need to adapt the lactase gene.[106] So for adults there is an advantage to consume bovine milk.

What causes ageing and age-related diseases?

The world population is growing rapidly from 2.5 billion in 1950, through 7.5 billion in 2017 to 9.7 billion in 2050[120,121]. In addition, the population is ageing quickly. It is estimated that in 2050 1.5 billion people (15.8%) are over 65 years old, compared to 0.65 billion in 2017 (8.7%)[121]. As elderly people are more susceptible to infections and frequently develop non-communicable, inflammation-related diseases[122], this will lead to an increasing burden on the global health care system, unless preventive measures are taken. According to the WHO, the number of elderly that suffer from chronic diseases could be reduced by 80% if main risk factors like smoking, lack of exercise and poor diet would be tackled.[123]

Ageing is broadly defined as a time-dependent functional decline that affects living organisms, although we do not yet understand the mechanisms causing ageing.[124–126] The general cause of ageing is the time-dependent accumulation of cellular damage, caused by different hallmarks of ageing.[127] Nine hallmarks of ageing were defined by López Otín et al. being: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. These hallmarks can be subdivided three categories. Primary hallmarks that are causes of damage including genomic instability (e.g. DNA damage of chromosomal DNA or mitchondrial DNA), telomere shortening, epigenetic alteration and loss of proteostasis, which leads to incorrectly folded proteins. Secondary hallmarks are processes that have at a low level beneficial effects, but at increased level have detrimental effects. These hallmarks include deregulated nutrient sensing, mitochondrial dysfunction including the formation of reactive oxygen species (ROS) and cellular senescence. Cellular senescence is a process that permanently arrest proliferation in cells responding to stressors, which is important in prevention against the development of cancer, but in increased form leads to increased number of infections.[127] Short telomeres or telomere exhaustion causes replicative senescence or Hayflick limit of *in vitro*-cultured cells[128,129] and increased mortality risks in humans.[130] However, there are other non-telomeric age-related stimuli that induce cellular senescence such as DNA damage.[131] Senescent cells are known to secrete

more inflammatory proteins leading to the "senescence-associated secretory phenotype" (SASP)[132–134] and results in reduced autophagy.[135] Autophagy is an important mechanism to degrade incorrect folded proteins and is known to reduce upon ageing[136], while increased autophagy increases lifespan in yeast, nematodes and flies[136,137]. Age-related diseases such as Alzheimer's disease, and Parkinson's disease are the results of chronic unfolded, misfolded and aggregated proteins, underlining the importance of good proteostatis, including autophagy.[138] Proper autophagy is also important for other age-related diseases like arteriosclerosis, and type 2 diabetes.[139]

Activated macrophages and neutrophils can produce reactive oxygen species (ROS) that can destroy pathogens.[16] ROS production is beneficial for proliferation and survival, but in high levels can cause DNA damage as well, next to other causes such as reduced DNA repair and exogenous DNA damage.[140,141] In the 60's the "free radical theory of aging" was postulated, which proposed that there is a negative feedback loop between mitochondrial dysfunction that occurs upon aging and increased ROS production leading to increased mitochondrial dysfunction and global cellular damage.[142] The free radical theory is partly revised as ROS was found to increase lifespan and did not accelerate ageing in mice, resulting in a more balanced theory that ROS has homeostatic purposes, but when ROS are increased upon ageing leads to enhanced age-associated damage.[143]

The last category of ageing hall marks includes integrative hallmarks being stem cell exhaustion and altered intercellular communication, such as inflammaging, which is ultimately responsible for the deleterious effects of ageing. When organisms have a good turn-over system it clears senescent cells and re-establish cell numbers by activation progenitor or stem cells. Upon ageing, the number of senescent cells are increasing, which ultimately can lead to exhausted stem cells and increased senescent cells in tissues.[127] All these hallmarks are related to metabolic alterations as well.[144]

Different pathways that sense and respond to nutrient fluctuations are dysregulated upon ageing, of which the "insulin and IGF1 signaling" (IIS) pathway is the best known and evolutionary most conserved pathway involved in glucose sensing.[145,146] Dietary restriction is known to extend

lifespan or health span in many species and this relies on suppressing the IIS pathway.[146–148] Although during physiological ageing IIS pathway is decreased as well, probably to minimize cell growth and metabolism in case of systemic damage.[149] Next to the IIS pathway, three interconnected metabolic pathways play a role in ageing. These pathway are involved in sensing of high amino acid concentration (mTOR), in sensing high AMP levels (AMPK) or high NAD^+ levels (sirtuins) corresponding to low-energy state.[150] Altogether, it is hypothesized that anabolic signaling (IIS pathway) accelerates ageing, while decreased nutrient sensing (mTOR, AMPK and sirtuin pathways) extends longevity.[146]

Western lifestyle is characterized by hypercaloric food intake, with insufficient healthy food components (vegetables, fruit, fibres), too much fat and proteins in combination with a sedentary life style. This has a negative impact on health as it leads to increased blood pressure, high plasma glucose levels, high serum triglycerides and low high-density lipoproteins, as well as obesity. All these markers increase upon ageing, and obesity even enhances ageing, as reviewed by López-Otín et al.[144]

To counteract the negative effect of Western diet, multiple metabolic interventions can have positive outcomes. Caloric restriction or starvation for 24h can have anti-inflammatory effects.[151–154] An isocaloric diet, with the depletion of methionine and/or tryptophan can extend longevity in many model organisms.[155,156] Furthermore "fasting-mimicking diet" (FMD), which was taken 5 days a month for three months, included 44% ± 10% of average caloric intake and only 10% of the proteins resulted in a human trial to reduced baseline glucose, IGF1 and C-reactive protein (CRP).[157] Also the Mediterranean diet, which is high in fibres and carbohydrates may result in reduced ageing.[158] Next to this exercise is beneficial to prevent age-related decline, as it reduces morbidity and mortality in humans.[159]

Ageing of the immune system

Ageing is also associated with changes in the immune system. History of infections, microbiota changes, diet, physical activity and stress all contribute to decreased immune function in elderly people.[160] Immune deficiency from ageing occurs at two levels: irreversible primary immune deficiency and reversable secondary immune deficiency of which low nutritional status is an example.[161] Immunosenescence can be seen as an example of primary immune deficiency, in which both adaptive immune responses by B and T cells are reduced, as well as responses of the innate immune system. In addition to this, first line immune defenses such as fragile skin and antibody production by the mucosal immune system, are decreased in elderly.[162]

Much is known about the effect of ageing on the adaptive immune system, as reviewed by Ventura et al.[163] and Sansoni et al.[164] Numbers of naïve T cells, due to thymic involution, and effector memory T cells are declining, except for people infected with Cytomegalovirus (CMV). CMV infection was also related to lower responses to Influenza vaccination and accelerated ageing. Besides, $CD8^+$ T cells and regulatory T cells numbers are increased. Increasing Treg numbers correlate to increasing incidence of cancers, neurodegenerative diseases, decreased responses to vaccination, and decreased capability to cope with infections.[165] Furthermore, an accumulation of dysfunctional senescent cells occur due to reduced sensitivity of damage-induced apoptosis.[163] In contrast, fewer mature B cells are found upon ageing due to declining numbers of progenitors. Serum levels of IgM and IgD are reduced, while IgG and IgA levels are increasing upon ageing.

Immunosenescence seems to involve, among others, changes in the number and function of lymphocytes and innate immune cells, as well as altered expression of Toll-like receptors (TLRs).[12–14] The reduction in age-related responses to TLR triggering is best described for myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs).[166–170] mDCs and pDCs are two types of human blood DCs that derive from different progenitors and have different functions.[171] Whereas pDCs are the main producers of type I interferons (IFN-α, IFN-β), mDCs regulate pro-inflammatory responses via inducing T-helper 1 and cytotoxic T lymphocyte responses upon bacterial and

viral stimulation[166]. Interestingly, in relation to anti-viral immune responses, elderly have reduced numbers of pDCs and their pDCs have been shown to produce lower concentrations of antiviral IFN-α and pro-inflammatory cytokines upon TLR7 and TLR9 stimulation, resulting in lowered antiviral immunity.[15,16,173] MDCs are not reduced upon ageing[135]. During ageing the number of monocytes[174] and neutrophils [175] seems unchanged, although for neutrophils the effector response induced by Fc gamma receptor signalling is reduced[175]. The number of macrophages pre-cursors are decreased [174]. Altogether, these compromised innate and adaptive immune responses result in reduced ability of elderly people to respond to infection and vaccination.[13,14,176,177] Furthermore, many age-related health issues, such as osteoarthritis, metabolic diseases, cognitive decline, onset of frailty, and cardiovascular diseases are associated with inflammation, often referred to as inflammaging[4,122,166,176,178]. Inflammaging is associated with increased serum concentrations of pro-inflammatory cytokines[14], acute-phase proteins and soluble adhesion markers.[160] Inflammaging is found both in diseased elderly as well as healthy centenarians, although high levels of IL-6 are correlated to increased morbidity and mortality.[179] It is hypothesized that after a lifetime of inflammatory immune responses, the immune system in elderly fails to downregulate these responses, resulting in a low-grade chronic inflammation.[180] A possible explanation could be the decreased Treg function towards auto-immune inflammation, while Treg function towards infections and cancer remains intact, as reviewed by Jagger.[165] Other causes of inflammaging are accumulation of pro-inflammatory tissue damage (by ROS), loss of capability to effectively clear pathogens and accumulation of senescent cells with their senescence-associated pro-inflammatory secretory phenotype.[127] It should be noted however, that inflammaging by itself is a conserved evolutionary concept present from invertebrates to vertebrates and does not only exist in humans.[179,180] This makes sense in an evolutionary perspective, as life expectancy increased far beyond our reproductive age and the immune system did not adapt to this elongation of inflammatory exposures.[180]

Based on all these changing markers upon ageing a phenotype of Immune Risk Profile (IRP) was defined, which correlates for 57% of the cases with mortality and for 97% with survival in the next 2 years.[181] Characteristic markers included in IRP are inversion of the CD4/CD8 ratio and CMV-positivity.[181]

Next to this IL-6 is associated to increased mortality, just as reduced number of B cells.[163] Therefore it would be beneficial to support the immune system in elderly to promote healthy ageing.

Mucosal vaccination

The aim of vaccination is to protect an organism against infection by a specific pathogen. Parenteral vaccination (e.g. intramuscular or subcutaneous injection) is the most common vaccination strategy for many pathogens. Parenteral vaccination predominantly elicits systemic immune responses to the vaccine, whereas mucosal vaccination via the oral or intranasal route induces adaptive immune responses targeted to the mucosal tissues and intestinal lumen to inhibit colonization and invasion by pathogens.[182–184]

Mucosal administration of vaccine antigens may induce T- and B-cell tolerance rather than immunity, particularly without the use of an adjuvant. Classical tolerance is dependent on the dose and the timing of antigen delivery with 'low-zone' tolerance referring to low antigen doses over prolonged periods of time and 'high-zone' tolerance dealing with high doses of antigen overwhelming the immune system. In both cases antigen is specifically recognized and induces central or peripheral deletion of reactive T- and B-cells, while at the same time generating antigen-specific Tregs. Slow release of antigen with a low dose, but also rapid delivery of a high antigen dose at a mucosal surface are thus more likely to induce tolerance and thereby lose the benefit of using adjuvants. Thus, optimal antigen release kinetics must be controlled particularly when designing mucosal vaccines while using suitable adjuvants.[34]

Commonly used oral vaccines include Vibrio Cholerae, Salmonella Typhi, and Rotavirus vaccination[185] for prevention of diarrheal diseases. The only intranasal vaccine licensed for use in humans is against Influenza A and B (Flumist®)[186] and other licensed vaccines for respiratory infections are administered parenterally. To obtain prevention against mucosal infections induction of the proper amount of memory cells specific for an antigen of a pathogen is needed. The final outcome will be that once there are enough memory B and CD4+ helper T cells specific for the antigen a fast secondary immune response can develop leading to high levels of antigen-specific antibodies. In general it is considered that after a first infection the peak of

antibodies in serum is at day 7 for IgM and between 14-21 days for IgG[187,188], while after a secondary infection the peak of antibody production is after 3 days already.[16] This means that the activated effector B and T cells are migrating through the blood mostly before these time points. Antibodies can bind to the pathogen leading to either neutralization or immune exclusion by binding to the pathogen-binding site or viral fusion protein so that infection of host cells is impossible (sIgA or sIgM in MALT).[189–191] Besides, opsonisation by antibodies leading to fixation of the complement system and subsequently death of the pathogen[192,193] or the formed antibody-antigen complexes results in increased uptake by antigen presenting cells expressing FcR and complement receptors [194]. Oral antigens primarily interact with the immune system through the tonsils of Waldeyer's ring in the oral cavity[21] or via the Peyer's patches in the small intestine[17]. Therefore it is hypothesized that oral vaccination follows the same route of uptake. In addition, other structures in the gut associated lymphoid tissue (GALT) and isolated lymphoid follicles in the colon can also play a role in immune responses to oral vaccines. Oral vaccination is known to induce vaccine-specific sIgA not only in the small intestine and colon, but also in upper respiratory tract (e.g. in saliva and nasal secretions) and to a lesser extend in the tonsils and lower respiratory tract.[15,195–197] These studies were all performed with cholera toxin B (CTB) or the cholera vaccine Dukoral®. Conversely, CTB vaccination in the rectum and female genital tract mainly induces local vaccine-specific IgA production.[197–199] Part of this compartmentalization of the mucosal immune system may be explained by tissue-specific homing properties of B and T cells. Tissue-specific homing of lymphocytes is dependent on their expression pattern of homing receptors that bind addressins expressed on the endothelium of the target tissue.[200] Examples are binding of Integrin α4β1 to vascular cell adhesion molecule (VCAM)-1 and Integrin α4β7 to mucosal addressin cell adhesion molecule (MAdCAM)-1.[19] Furthermore, locally produced chemokines (e.g. CCL25, CCL28) attract lymphocytes via their chemokine receptors (CCR9[201,202] and CCR10[19], respectively) and direct them into the tissue.[203,204] It is not known to what extent an oral cholera vaccine can induce a local mucosal immune response by modulating the homing profile of peripheral lymphocytes. Another factor that can determine lymphocyte trafficking and tissue homing is the location of the initial immune activation, as was recently described for plasmablasts.[205,206] In addition, homing is influenced by external factors, such

as stromal factors in the local microenvironment and food components, such as retinoic acid.[207,208]

Oral vaccination, in contrast to parenteral vaccination, follows the same route of uptake as food. Therefore it might be possible that food components modulate the homing capacity of effector B and T cells from the gut towards the airways. In this respect, there is much interest in the potential for oral vaccination to elicit a more protective immune responses in the upper respiratory tract. Therefore, oral vaccination can serve as a model to test immunomodulation by raw milk or other dietary components.

Aims and outline of the thesis

Both infants and elderly people have compromised immune systems, as discussed in the general introduction. For infants human milk provides support for the immune system. Infants need immunological support, as their immune system needs time to develop. Furthermore, infants are faced with microbial colonisation and changed nutrition from breast milk towards solid food. In addition to commensal bacteria, infants are exposed to food- and airborne pathogens as well, which can cause infections in the intestine and airways. As especially infants suffer from respiratory virus infections, it would be beneficial to improve airway immunity by dietary modulation. Next to this, the epithelial barrier of infants is not fully closed, especially just after birth. As a result, intact food an microbial components can cross the epithelium into the mucosa. The infant should not develop allergies or anti-inflammatory immune responses towards these macronutrients and is supposed to induce oral tolerance against both food components as well as to commensal bacteria.

Elderly people often suffer from non-communicable inflammation-related inflammatory diseases as a result of immunosenescence and inflammaging. Next to this, elderly respond less to vaccination and are more vulnerable to infections. The ageing population is growing worldwide and therefore the burden of age-related disease on the global health care system is increasing as well. Hence, it would be beneficial when people become older without developing these diseases, which is termed healthy ageing. The **main objective**

of this project was to study the immunomodulatory effect of raw bovine milk and its ingredients in nutritional intervention, oral cholera vaccination and trained immunity and its possible relevance for different stages in life.

Raw bovine milk consumption in early life is known to be associated with reduced incidence of asthma, allergies and respiratory tract infections, all associated with immunity in the airways. The **aim** of this thesis was to investigate to which extent raw bovine milk can modulate immune responses to improve airway immunity. To this end the route of bovine milk uptake and the local effect of milk components on barrier functions, dendritic cells, microbiota and potentially on the gut-lung axis is described in **chapter 2**. Furthermore, the homology between human milk and bovine milk is described and the epidemiological studies that correlates raw bovine milk consumption with respiratory health are reviewed, which form the basis of this thesis.

In **chapter 3**, we investigated the potential of raw bovine milk, milk fractions, lactoferrin and bovine IgG to induce trained immunity as a mechanistic concept for protection against infection. Freshly isolated monocytes were stimulated with raw bovine milk or milk components for 24h. After a resting period of five days the cells were stimulated with TLR ligands. Using raw bovine milk and its components we studied its capacity to induce trained immunity in monocytes.

One of the mechanisms by which raw milk can enhance airway immunity is by increasing homing of B cells from the gut towards the airways leading to higher local antibody production. Oral vaccination follows the same route of uptake as nutrition and is known to induce a vaccine-specific immune response in the gut as well as systemically and in the upper airways. Therefore we hypothesized that oral vaccination could serve as model to study immunomodulation by food, and raw milk in particular. In **chapter 4,** we used oral cholera vaccination as model to study the tissue homing potential of memory B cells. To this end, we developed flow cytometry panels that could distinguish different homing profiles for airway, small intestine, colon, skin and peripheral homing. Next to this, we measured the kinetics of vaccine and CTB-specific antibodies in serum. In **chapter 5**, the aim was to study the modulation of the oral cholera vaccination by raw milk, pasteurized and ultra-heat treated milk. To this end,

serum, nasal wash, saliva and fecal samples were collected to determine the amount of vaccine-specific antibodies and compare those levels between the control vaccinated group and the group vaccinated in the presence of (raw) milk. In addition, airway homing potential of memory B cells was investigated.

Upon ageing the immune system becomes dysregulated, leading to reduced cell numbers, reduced TLR expression by immune cells and reduced TLR responses, reflecting a reduced response against pathogen exposure. The aim of **chapter 6** was to compare innate immune function towards TLR-mediated responses between elderly and young adult women. To this end, elderly women and young female adults were compared to study the effect of ageing on the frequency and reactivity to TLR-mediated responses of plasmacytoid and myeloid dendritic cells (pDC, mDC). Next to this, TLR expression and generation of pro-inflammatory markers in serum were investigated. In **chapter 7** we performed a double-blind placebo-controlled nutritional intervention study, to investigate the potential of bovine lactoferrin, galacto-oligosaccharides (GOS) and vitamin D to restore TLR responsiveness of pDCs and mDCs and to reduce inflammatory cytokines in serum in elderly women.

In the last chapter, **chapter 8**, the results of the previous chapters are discussed and integrated with respect to recent scientific findings. I place the results of immunomodulation by raw bovine milk and its components in the light of trained immunity and discuss the implications of our research for the rational design of intervention strategies with raw bovine milk.

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Chapter 2

Cow's milk and immune function in the respiratory tract: potential mechanisms

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Abstract

During the last decades, the world has witnessed a dramatic increase in allergy prevalence. Epidemiological evidence shows that growing up on a farm is a protective factor, which is partly explained by the consumption of raw cow's milk. Indeed, recent studies show inverse associations between raw cow's milk consumption in early life and asthma, hay fever, and rhinitis. A similar association of raw cow's milk consumption with respiratory tract infections is recently found. In line with these findings, controlled studies in infants with milk components such as lactoferrin, milk fat globule membrane, and colostrum IgG have shown to reduce respiratory infections. However, for ethical reasons, it is not possible to conduct controlled studies with raw cow's milk in infants, so formal proof is lacking to date. Because viral respiratory tract infections and aeroallergen exposure in children may be causally linked to the development of asthma, it is of interest to investigate whether cow's milk components can modulate human immune function in the respiratory tract and *via* which mechanisms. Inhaled allergens and viruses trigger local immune responses in the upper airways in both nasal and oral lymphoid tissue. The components present in raw cow's milk are able to promote a local microenvironment in which mucosal immune responses are modified and the epithelial barrier is enforced. In addition, such responses may also be triggered in the gut after exposure to allergens and viruses in the nasal cavity that become available in the GI tract after swallowing. However, these immune cells that come into contact with cow's milk components in the gut must recirculate into the blood and home to the (upper and lower) respiratory tract to regulate immune responses locally. Expression of the tissue homing-associated markers α4β7 and CCR9 or CCR10 on lymphocytes can be influenced by vitamin A and vitamin D3, respectively. Since both vitamins are present in milk, we speculate that raw milk may influence homing of lymphocytes to the upper respiratory tract. This review focuses on potential mechanisms *via* which cow's milk or its components can influence immune function in the intestine and the upper respiratory tract. Unraveling these complex mechanisms may contribute to the development of novel dietary approaches in allergy and asthma prevention.

Introduction

In the Western world, the prevalence of chronic inflammatory diseases, including allergies, has increased dramatically in the last few decades, while the number of serious infectious diseases has declined rapidly[1]. An inverse correlation, indicating a "protective effect" of infectious diseases against chronic inflammatory diseases (e.g. allergy and asthma), was postulated in 1989 by Strachan, who formulated the hygiene hypothesis[2]. The hygiene hypothesis suggests that the exposure to viruses and bacteria is essential to induce a T-helper (Th)1 response, which balances the immune system and protects against Th2-mediated diseases. With the discovery of additional T cell subsets such as Th17 cells and regulatory T cells (Tregs), this paradigm had to be revised. For example, it was demonstrated that suppressive dendritic cells (DCs) induced by helminths restored the disturbed Th1/Th2 balance by induction of Tregs[3]. The immune education of DCs was suggested to be an important step toward understanding the complex relation between infectious diseases and allergies[4]. Th2 responses are now known to be enhanced by the production of type 2 cytokines (e.g. TSLP, IL-25, IL-33) secreted by group 2 innate lymphoid cells and epithelial cells[5,6]. Thus, different cell types are responsible for Th2-mediated diseases such as allergies. Allergy is initiated as an aberrant immune response towards a harmless antigen (allergen). *Via* activation of Th2 cells, the allergen triggers the production of allergen-specific IgE by B cells that binds to high-affinity FcεR1 on effector cells like mast cells and basophils. Effector cells release soluble factors (e.g. histamine) upon secondary exposure to the allergen that cause immediate type I allergic symptoms. The term "atopic march" refers to the sequence of IgE responses and clinical symptoms initiated in early life[7].

In the first year of life, the mucosal immune system is shaped by microbial colonization and dietary components, which contributes to health later in life[8]. Viral infections during this critical period also impact health later in life. For example, respiratory syncytial virus (RSV) infection in early life was shown to increase the risk of wheezing up to 11 years of age[9] and allergic sensitization and development of asthma into adulthood[10–12]. The exact mechanism by which allergy and viral infection in the upper airways results in the development of asthma is not yet elucidated. However, Holt and Sly[13] proposed a mechanism in which viral infection can trigger excessive type I

interferon production that can result in upregulation of FcεR1 expression on airway resident DCs. FcεR1-mediated signaling in DCs has been suggested to contribute to allergic airway inflammation depending on the environmental stimuli[14]. In mice, cross-linking of virus-specific IgE on these airway DCs results in the production of Th2 cytokines and the chemoattractant CCL28, recruiting effector Th2 cells to the airways[15,16]. However, recent evidence shows that not all asthma patients have this typical Th2 profile in early life[17]. Nevertheless, atopy and viral infections in early life are risk factors for asthma development. Therefore, preventive strategies for asthma, such as dietary interventions, should be targeted at early life to suppress allergen- or viral-induced airway inflammation.

In Europe, rapid evolutionary changes are found in the lactase persistance gene suggesting health benefits of cow's milk consumption to humans (Box 1). The existing epidemiological evidence shows that consumption of cow's milk in early life is associated with a lower prevalence of allergies, respiratory tract infections, and asthma. This suggests that milk components (e.g. proteins, sialylated oligosaccharides, and vitamins) may contribute to the protection against the development of allergies[18] and respiratory viral infections[19]. Since raw cow's milk may contain pathogenic bacteria, intervention studies in infants are impossible due to safety risks. Nevertheless, a recent mouse study showed a causal relation between raw milk consumption and the protection against house dust mite (HDM)-induced asthma, which was not seen in mice receiving heated milk[20]. The mechanisms underlying this protective effect of raw cow's milk remains speculative. Therefore, in this review we discuss potential mechanisms by which dietary components, using cow's milk as an example, can protect against airway inflammation.

Box 1.

The consumption of cow's milk in Europe and the Middle East already dates back to the Neolithic cultural period. Milk fatty acids were traced by carbon isotope analysis on Middle Eastern pottery, showing that cow's milk was already processed since 6500 BC[21]. This introduction of processed ruminant milk might explain why it was adopted so quickly, despite lactose intolerance. Lactase persistence (i.e., the capacity to digest lactose into adulthood) seems to have arisen around 5500 BC in the European population due to a specific mutation in the gene encoding the lactase enzyme[22]. Its rapid expansion in the ancestral population suggests high selective pressure[23]. This makes it appealing to speculate that this mutation confers health benefits to the host by consuming cow's milk.

Homology between cow's milk and breast milk

By comparing the immunomodulatory components in breast milk with those in cow's milk, conserved mechanisms could be identified, which contribute to immune homeostasis in early life. Overall bovine and human milk contain similar components. However, the concentration or presence of several specific components (e.g. β-lactoglobulin specific for cow's milk) may differ. For a complete overview comparing breast milk and cow's milk, we refer to a review by van Neerven et al. who compared the composition of breast milk to cow's milk[18]. We briefly describe several immunomodulatory components in cow's milk that are used in this review to illustrate potential mechanisms by which cow's milk may affect respiratory health.

Systematic reviews conclude that TGFβ consumption in early life protects against allergies in humans and animal models[24,25]. Strikingly, the active forms of TGFβ1 and TGFβ2 are identical between cow and human[26]. Although articles report different concentrations of TGFβ in breast and cow's milk, there is a consensus that TGFβ2 is several fold more abundant compared to TGFβ1[24,27]. Remarkably, the concentrations of TGFβ1 and TGFβ2 are approximately fivefold more abundant in cow's milk compared to breast milk[18]. The concentration of TGFβ1 in cow's milk decline significantly after processing and are non-detectable in processed milk[28].

Bovine lactoferrin has 77% homology to human lactoferrin on mRNA level and 69% on protein level[29]. Nevertheless, bovine lactoferrin is taken up by the human lactoferrin receptor and exerts similar bioactivities as human lactoferrin on human colon epithelial cells such as induction of proliferation, differentiation, and TGFβ expression[30]. Similarly, bovine IL-10 is 76.8% homologous and affects human cells[31]. Cow's milk shows lower IgA and higher IgG levels compared to human milk[18].

While the quantities of proteins in human milk are quite similar to cow's milk, the oligosaccharide composition is completely different. In contrast to cow's milk, human milk is unique among mammals in its high and diverse levels of complex oligosaccharides[32]. Cow's milk contains only small amounts and a non-diverse profile of oligosaccharides, which is dominated by sialylated

oligosaccharides. Therefore, this review will only address the effect of sialylated oligosaccharides present in cow's milk. In colostrum, the concentrations of sialylated oligosaccharides range between 0.23–1.5 and 1–3.3g/L in cows and humans, respectively[33]. The concentrations of sialylated oligosaccharides in mature bovine milk are approximately 10-fold lower compared to colostrum[33]. Most sialylated oligosaccharides in human- and cow's milk are monomeric [e.g. 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL)] and are present in very low concentrations in infant formulas[34].

Vitamin A and D, which are not specific for cow's milk, are essential for the development of the mucosal immune system. Vitamin A can be obtained from different dietary sources and can be converted to its active metabolite retinoic acid (RA) by epithelial cells and DCs in the gut[35]. Breast milk contains, depending on the vitamin D status of the mother, low levels of vitamin D3, and additional vitamin D3 supplementation is recommended for infants[36]. Cow's milk contains similar concentrations of vitamin A, vitamin D3, and 1,25-hydroxyvitamin D3 (active form of vitamin D3) compared to breast milk[18,37].

Epidemiological evidence for the immune modulatory role of cow's milk on respiratory health

It is now well established that children growing up on a farm less often develop allergies and asthma.[38] Of the different environmental factors investigated in these epidemiological studies, contact with farm animals, endotoxin levels in house dust, and the consumption of farm milk (i.e., cow's milk with an unknown heating status) showed the strongest association with the protection of childhood asthma and allergy[38,39]. The consumption of farm milk was associated with higher Treg numbers in blood, which were negatively associated with asthma and serum IgE levels[40]. Moreover, increased demethylation of the *FOXP3* gene and increased FoxP3$^+$ T cell numbers were detected in PBMC cultures of children who were exposed to farm milk, suggesting that farm milk consumption induces an immunoregulatory phenotype.

Raw cow's milk consumption in the first year of life showed an inverse correlation with the prevalence of atopy and doctors-diagnosed asthma in farmers and non-farmers[41]. This study showed that raw cow's milk consumption in the first year of life is inversely associated with atopic sensitization and asthma independently of the farming environment. Children who consumed raw cow's milk produced higher IFN-γ levels upon whole blood stimulation[42]. Since IFN-γ production is associated with a Th1 profile, this finding—even though counterbalancing Th2 responses—is in contrast to studies showing a regulatory phenotype induced by farm milk consumption in the first year of life[40]. Nevertheless, both studies show that either raw cow's milk or farm milk is associated with lower total serum IgE levels and allergic diseases[40,42]. Other epidemiological studies have specifically addressed the question whether heating of farm milk influences its effect on allergic diseases.

Loss et al. showed that the protective effect of cow's milk on asthma and hay fever incidence was only noted in children who consumed raw milk and not in children who consumed high heat-treated shop milk (>85°C). Indeed, the thermosensitive whey proteins BSA, α-lactalbumin, and β-lactoglobulin were associated with the protective effects. Similar trends were found for lactoferrin and total IgG. No associations were found between microbiological communities or cell counts in the milk, showing that the protective effect was not primarily caused by bacteria in the raw cow's milk[43]. A follow-up study investigated the association between raw, boiled, or commercially available cow's milk consumption and the occurrence of common infections in infants (2–12 months of age). In comparison to ultra-heat-treated milk, raw milk consumption in the first year of life was inversely associated with the occurrence of rhinitis, otitis, and respiratory tract infections at 12 months of age. In addition, soluble CRP levels were lower in the infants that received raw cow's milk. Interestingly, respiratory tract infections and fever were also reduced in infants receiving boiled cow's milk[19]. It was suggested that the milk fat globule membrane contributes to this negative association between boiled milk consumption and respiratory tract infections[19]. Indeed, non-heat-sensitive cow's milk components may also contribute to the induction of a regulatory phenotype[40]. Nevertheless, these studies show that the thermosensitive fraction of the milk (i.e., proteins, most likely whey fraction) is an important driver of the protection against not only allergies and asthma but also viral infections, fever, and inflammatory conditions in the upper airways.

These epidemiological findings cannot be confirmed in controlled intervention studies in infants due to safety risks. However, controlled trails with infants fed experimental infant formulas rich in immune-related bovine milk components have shown effects on respiratory tract infections. Infants fed with a bovine milk fat globule membrane preparation rich in IgG and lactoferrin showed a reduced prevalence of acute otitis media and showed lower pneumococcal-specific IgG levels in serum[44]. Similarly, infants of 4–6 months of age receiving infant formula supplemented with lactoferrin showed fewer respiratory illness[45,46]. A reduction in respiratory tract infections was also observed in an intervention study with children of 1–6 years of age receiving bovine colostrum that is extremely rich in IgG[47]. These findings indicate that bovine milk components may prevent respiratory tract infections in early life.

Passage through the gastrointestinal tract

After swallowing milk components, allergens, or pathogens, they pass through the GI tract and are exposed to different pH levels and proteases, varying from pancreatic, gastric, or peptidases on the enterocytic brush border. In adults, there is little evidence that intact dietary proteins can reach the circulation in homeostasis[48]. In early life, however, the digestion of proteins is lower compared to adults, which has several causes. First, the gastric acid production in infants only reaches the levels of adults after 6 months of age. Infants therefore have a higher pH in the stomach compared to adults. Lower gastric acid levels impair the activity of pepsins. Second, concentrations of other proteases (e.g. chymotrypsin and enterokinase) are significantly lower in the small intestine of neonates (10–60% of that of adults) compared to adults[49]. Thus, in infants, a significant fraction of milk proteins reaches the small intestine intact and may interact with intestinal immune cells (e.g. epithelial cells and sampling DC). For instance, 10% of the orally fed bovine IgG (bIgG) can be found in stool of infants, compared to <0.1% in adults[50]. In addition, the infants gut is in a "leaky state"[51], which may promote sensitization to allergens and bacteria- or virus-induced inflammation. On the other hand, it is a window in which (milk-derived) components have an opportunity to induce tolerance.

Some milk proteins are less sensitive to the low pH and proteases and pass the GI tract intact or can even be activated by an acidic environment or protease activity. For instance, TGFβ, which is present in milk in its latent form first needs to be activated before exerting any effector function. The activation of this exogenous latent TGFβ can be triggered by multiple factors such as macrophages membrane-bound receptor TSP-1, αvβ-3/5/6 and αvβ8 integrins, ROS, low pH during passage of the stomach, and proteases [52,53]. Thus, TGFβ can be activated by binding integrins in the upper airways or by activation in the stomach and small intestine. Significant amounts of the abundant milk protein lactoferrin reach the small intestine intact and retain their functional activity in both adults and infants[29].

In contrast to proteins, milk oligosaccharides escape enzymatic hydrolysis in the small intestine and low pH of the stomach and are fermented in the colon[54]. By escaping degradation in the small intestine, they function as a carbon source for the microbiota in the colon and can be converted into metabolites such as short-chain fatty acids (SCFAs). In breastfed infants, the genus *Bifidobacterium* is commonly present, which comprises mainly *Bifidobacterium bifidum, Bifidobacterium longum* subsp. *infantis*, and *Bifidobacterium breve*. Of the three, *B. longum infantis* has the right machinery to ferment sialylated oligosaccharides directly[55,56] and is unique in its ability to import and degrade low-molecular-weight oligosaccharides[57]. Indeed, several *B. longum* strains were capable of converting 3'SL and 6'SL, which are abundantly present in bovine milk, into SCFA *in vitro*[58]. Nevertheless, it is unknown whether the concentrations of sialyllactose present in cow's milk alters the microbiota *in vivo*. *B. longum* is abundantly present in breast-fed neonates and is thought to confer various health benefits (e.g. enhanced barrier functioning and anti-inflammatory effects) to the host[59]. Although most of these sialylated oligosaccharides are fermented by these *Bifidobacteria*, a small fraction of oligosaccharides reaches the circulation intact[60,61]. Therefore, sialylated oligosaccharides might impact immunity directly. Interestingly, it has been shown that the microbial community in the upper respiratory tract can be differentially modulated by breast milk compared to formula-fed children[62]. Interestingly, breastfed infants showed a higher prevalence of *Dolosigranulum* that was negatively associated with respiratory tract infections[62]. Children with asthma show a lower nasal microbiota composition

and higher abundance of *Moraxella*[63]. *Moraxella* was not associated with asthma in children who were exposed to a farming environment, which was in contrast to children who were not exposed to a farming environment. This indicates that the farming environment might protect the children from the detrimental effects of *Moraxella*. To date, it is unknown whether raw cow's milk alters the nasopharyngeal microbiota composition and if this influences susceptibility toward upper respiratory tract infections or allergies.

Binding of bovine IgG to respiratory pathogens

One mechanism by which food components could modulate immunity in the (upper) respiratory tract is by preventing contact between pathogens or allergens and the host immune system. In early life, maternal antibodies are essential for passive protection of the infant against viral infections. Interestingly, maternal RSV-specific antibodies in amniotic fluid were recently shown to protect mouse pups from RSV infection for at least 1 week after birth[64]. Human antibodies are found against conserved parts of the pre-fusion F protein of human RSV and metapneumovirus (PMV) that cross-neutralize bovine RSV[65]. This cross-reactivity could also work *vice versa* if bovine IgG could recognize conserved patterns on human RSV. Cross-reactive antibodies to other human pathogens have also been demonstrated in bovine milk and colostrum. Indeed, as reviewed by van Neerven, feeding colostrum of cows vaccinated against specific human pathogens protected children from subsequent infections[66]. Interestingly, bovine IgG was shown to bind human RSV and to induce phagocytosis *via* FcγRII receptors on macrophages, neutrophils, and monocytes[67]. The binding of bovine IgG to RSV also directly neutralizes RSV, as shown by protection of Hep2 cells from infection with RSV *in vitro*[67]. Bovine IgG isolated from cow's milk does not only bind to human viruses but was also found to bind to inhaled allergens (e.g. HDM)[68]. In addition, bovine IgG inhibits translocation of Pam3CSK4 over the epithelial barrier, thereby suppressing the production of pro-inflammatory cytokines *in vitro*[69]. Thus, bovine IgG can neutralize RSV infection *in vitro* and might also play a role in preventing sensitization by binding allergens and supporting barrier functioning by preventing binding of TLR ligands to the epithelium.

Next to bovine IgG, milk oligosaccharides have also been shown to prevent binding of viruses to host cells[32]. Viruses use lectin-like structures to adhere and infect host cells. It was hypothesized that breast-fed infants developed less otitis caused by viral infections (e.g. RSV and influenza) due to the decoy receptor activity of milk oligosaccharides[32]. However, as stated in the review by ten Bruggencate et al., it is to date uncertain which sialylated oligosaccharides can serve as a decoy receptor for human respiratory infecting viruses[33]. Thus, IgG and sialylated oligosaccharides present in cow's milk might shield allergens or virus pathogens from inducing infection and inflammation (Figure 1).

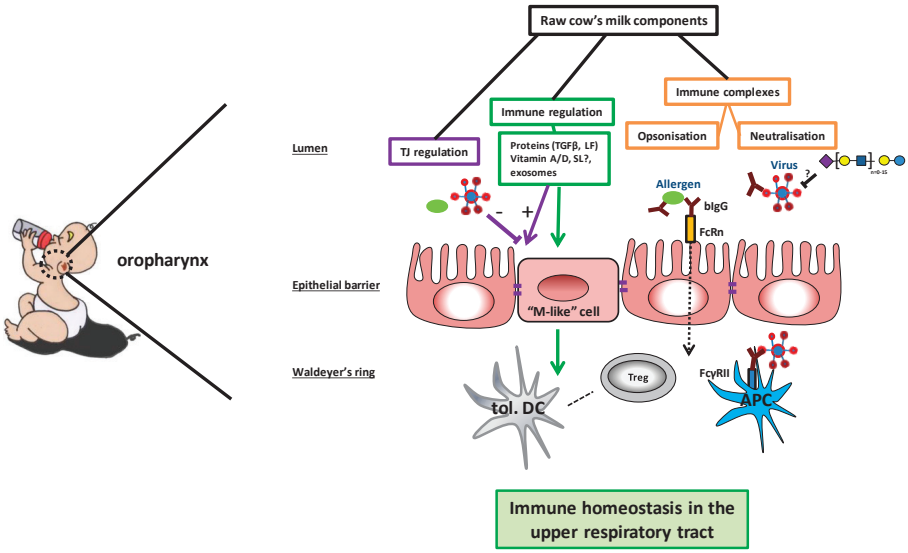

Figure 1: Potential mechanisms of cow's milk-induced immune homeostasis in the upper respiratory tract. In the oropharynx, raw cow's milk components can contribute to immune homeostasis via different mechanisms. First, bovine IgG can bind and possibly even neutralize bacteria, viruses, or allergens. Immune complexes are transported over the epithelial barrier by neonatal Fc receptor (FcRn) or transported via M-like cells to reach the mucosal tissue in the Waldeyer's ring. The immune complexes can bind to FcγRII on antigen-presenting cells (APCs), leading to phagocytosis and clearance of the pathogens—as well as antigen presentation to (regulatory) T cells. Second, sialylated oligosaccharides may function as decoy receptors for viruses in the lumen of the oropharynx, preventing viral adhesion. Further, the expression of tight junction proteins can be enhanced by several milk components, thus strengthening the mucosal barrier against breaching by allergens and pathogens. Finally, several milk components contribute to immune regulation by inducing the differentiation into tolerogenic dendritic cells (tol. DC) and immunoregulatory T cells (Tregs). In this way, raw cow's milk can promote a local microenvironment that contributes to immune homeostasis in the upper airways.

The first host barrier: the epithelium

When the allergen or virus is not neutralized, it will encounter the epithelial cell layer of mucosal tissues. This epithelial cell layer is the first line of defense in mucosal tissues. Epithelial cells are covered by a thick layer of mucus that keeps harmful compounds from entering the body. Epithelial cells act as the first physical barrier and the first responders of the innate immune system. The epithelial cells protect against inflammation and sensitization by preventing bacteria and virus entry and leakage of allergens into the mucosal tissue. The integrity of the epithelial barrier in the upper airways[70] and intestine[71] is regulated by tight junctions (TJs). Homeostasis is maintained by hyporesponsiveness of epithelial cells toward bacterial constituents, because inflammation disrupts barrier functioning[72]. Therefore, the barrier functioning of the upper airways is under constant threat of environmental factors, including viral infections (e.g. RSV) and allergens[73]. Breaching of the barrier can results in tissue modifications in the upper airways as seen in patients suffering from allergic rhinitis or sinusitis[74].

Tight junctions

The integrity of the epithelial barrier is maintained by structural elements including adherens junctions, desmosomes, and TJs. TJs consists of a "ziplock-like" structure of multiple protein strands that are connected to the cytoskeleton, allowing selective transport across the barrier[75]. Cytosolic scavenger proteins (e.g. ZO-1) link the actin cytoskeleton to paracellular located proteins: the claudins and occludins. The claudin family consists of transmembrane proteins, which *via* the interaction of claudin strands, are connected to each other by extracellular loops[76]. Claudins can be subdivided into pore-forming (e.g. claudin-2) and sealing claudins (e.g. claudin-4) that increase and decrease permeability, respectively[76]. Notably, pro-inflammatory cytokines (e.g. IL-6 and TNF) increase the expression of the pore-forming claudin-2 and thereby reduce the epithelial barrier function[77]. The function and distribution of occludins is highly influenced by its phosphorylation status that is regulated by protein kinase C[78]. TJ proteins are key in maintaining epithelial barrier function and are shown to regulate proliferation on gene expression level[79]. For further reading about TJ proteins, we would like to refer to other excellent reviews specifically about occludins[78], claudins[76], or the function and morphology of TJs in general[75,80].

Many allergens have protease activity that could breach barrier functioning[81]. For instance, the protease activity of one of the major HDM allergens, Der p 1, was shown to disrupt the cleavage site in the extracellular loops of claudins and occludins[82]. In contrast, RSV disrupts barrier functioning by remodeling the actin cytoskeleton and interfering with cytosolic scavenger proteins[83]. Disruption of barrier functioning in the upper airways results in increased exposure of allergens and viral particles to the underlying immune system (Figure 1), which could result in chronic inflammatory diseases such as asthma and allergies[84]. In early life, the epithelial barrier is more permeable compared to adults. Closure of the barrier occurs only after a few weeks in humans, while in mice, this is a more gradual process that develops during weaning[51,85].

Effect of milk components on barrier functioning

Breast milk contains many growth factors that facilitate gut maturation. Neonates receiving infant formulas were shown to have a higher gut permeability compared to breast-fed neonates. This stresses the importance of identifying functional milk components that promote barrier functioning[51]. Currently, there is no *in vivo* evidence on effects of cow's milk on epithelial barrier functioning. Nevertheless, at least two recent studies investigated the effect of cow's milk on epithelial cells *in vitro*. To study barrier functioning of dietary components *in vitro*, most studies use colon carcinoma cell lines (Caco-2 or HT-29). It is to date impossible to study the effect of dietary components on barrier functioning in the upper airways since no human oropharyngeal epithelial cell lines are available that form TJs. Caco-2 cells express enzymes that are expressed in the fetal intestine and are biochemically and morphologically similar to ileal enterocytes[86]. These *in vitro* models are thus one of the few limited models available to study the effect of dietary components on TJ regulation. The anti-inflammatory properties of milk components on the epithelium are reviewed by Chatterton et al.[26]. In this review, the role of dairy components on epithelial barrier function in terms of epithelial proliferation, differentiation, and TJ regulation is addressed.

The first study that looked at the effect of cow's milk *in vitro* showed that cow's milk induces the expression of the pore-forming TJ protein claudin-2 in Caco-2 cells[87]. However, no differences were observed in permeability, which was proposed to be counteracted by the milk-induced increase of endogenous

TGFβ expression. A second study stimulated HT-29 cells with raw milk versus pasteurized cow's milk preparations. The authors showed with microarray analysis that raw milk induced the expression of genes related to immunity compared to the pasteurized cow's milk or medium control[88]. This study showed that the thermosensitive milk fraction (i.e., proteins) induced the expression of immune-related pathways and thereby indirectly barrier functioning.

One of the proteins in cow's milk that is important for epithelial barrier functioning is TGFβ. Apart from these exogenous sources of TGFβ, TGFβ is endogenously produced. In the gut, TGFβ is most prominently expressed in epithelial cells compared to its expression in the underlying lamina propria[89]. TGFβ1 is capable of promoting barrier functioning by regulating TJ expression and proliferation. TGFβ1 induces the expression of claudin-4 and protein kinase C expression *in vitro*, both strengthening the barrier[90,91]. On the other hand, TGFβ inhibits the proliferation of epithelial cells[89,92]. Interestingly, the production of endogenous TGFβ1 by epithelial cells is regulated through a positive feedback loop by other milk proteins like lactoferrin that triggers an intracellular cascade that results in the production of TGFβ1. Bovine and human lactoferrin were shown to have similar effects on barrier functioning. Moreover, in low concentrations, lactoferrin induces differentiation of epithelial cells, whereas lactoferrin stimulates proliferation in higher concentrations[29].

In high concentrations, sialylated milk oligosaccharides affect the cell cycle and induce differentiation of intestinal epithelial cells[93]. In the colon, these oligosaccharides are fermented by the microbiota. These microbes produce SCFAs that also impact barrier functioning. As reviewed by Tan et al., SCFAs reduce paracellular permeability and induces the expression of TJ genes and MUC2 expression, thus strengthening the epithelial barrier[94] that may subsequently protect the host against infections[95].
Another milk ingredient shown to have immunomodulatory effects is vitamin D. More specifically, the inactive and circulating form of vitamin D3 (25(OH)2D3) is converted to the active form (1,25(OH)2D3) by the enzyme 1α-hydroxylase, which is highly expressed in the kidney and lowly expressed in epithelial cells[96]. Epithelial cells transport the inactive form of vitamin D3 over the membrane, which can be subsequently systemically metabolized[97]. The conversion locally by epithelial cells of dietary inactive vitamin D3 into

the active form can create a microenvironment containing active vitamin D3. Stimulation of Caco-2 cells with 1,25(OH)2D3 was shown to result in the induction of E-cadherin, which indirectly promotes the transcription of ZO-1 and induces differentiation[98]. In support of this, blocking vitamin D receptor transcription resulted in a decreased transepithelial electrical resistance and expression of ZO-1 and E-cadherin and claudin 1, 2, and 5 but not occludin[99]. Thus, it is evident that vitamin D3 contributes to epithelial barrier function by regulating TJ protein expression. Less is known about the effect of vitamin A on barrier functioning. RA was shown to enhance differentiation of epithelial cells, as indicated by the increase in alkaline phosphatase expression. In contrast, RA also decreased the expression of claudin-2, resulting in a decrease in permeability of the Caco-2 model[100]. Thus, several components present in cow's milk promote epithelial barrier functioning (Figure 1).

Do milk components promote immune homeostasis?

The nasal mucus is cleared to the back of the throat every 10–15 minutes by the movement of cillia. Thus, it is likely that allergens and viruses are trapped in this thick layer of mucus and are subsequently swallowed. The oropharynx (throat) is the place where milk components, bacteria, viruses, and allergens may interact before they are digested. Lymphoid tissues in the upper airways are the lingual tonsils, tubal tonsils, palatine tonsils, and adenoids, together forming the Waldeyer's ring[101]. Uptake of antigens by the tonsils occurs *via* M-like cells in specialized induction sites, which are composed of follicles containing both myeloid and lymphoid cells[102]. Similarly, in the GI tract, antigens can be taken up by columnar epithelial cells (transcellular), M cells, neonatal Fc receptor-mediated uptake[103], or direct uptake by specific sampling subsets of DCs[104].

The mucosal immune system is capable of distinguishing between harmful and harmless compounds resulting in inflammation or tolerance, respectively. Food components are important non-self-antigens to which an immune response constantly needs to be suppressed. This type of tolerance induction is known as oral tolerance. Food does not only trigger local tolerance but also systemic tolerance, and thus food makes the systemic and mucosal immune

systems relatively unresponsive to these food antigens. Breast milk contains many components that dampen immune responses. It is suggested that this regulatory milieu induced to breast milk components favors tolerance inductions towards other harmless antigens such as allergens[105]. This suppression of immune responses is antigen specific and long lasting.

The consumption of farm milk is associated with higher regulatory FoxP3$^+$ T cell numbers, which were negatively associated with doctors-diagnosed asthma and IgE levels[40]. We here address several potential cow's milk components that might promote these regulatory responses. Literature supports that raw cow's milk contains a multitude of components, including proteins and vitamins, that promote the development of human "tolerogenic" or regulatory monocyte-derived DCs (moDCs) *in vitro*.

Cow's milk and colostrum contain several immunoregulatory cytokines such as TGFβ and IL-10. Interestingly, a population of tolerogenic IL-10 producing DCs (IL-10 DCs) with similar characteristics to *in vitro* monocyte-derived DCs, differentiated in the presence of IL-10, were identified in human blood[106,107]. Not only human IL-10 but also bovine IL-10, which has 70% homology to human IL-10, was shown to induce a dose-dependent reduction of CD80/CD86 expression and IL-12 and TNF production[28]. DCs with low CD86/CD80 expression in the presence of TGFβ or IL-10 are known to polarize naive T cells into FoxP3$^+$ T cells[108]. IL-10 DCs also express PD-L1 which is critical for the induction of T cell anergy. Similarly, moDC differentiated in the presence of bovine lactoferrin showed inhibited cytokine responses and surface marker expression upon stimulation with TLR ligands[109].

TGFβ is an unique pleiotropic cytokine that is produced by leukocytes and epithelial cells[110]. The dual role of TGFβ was shown in a recent review, which showed that TGFβ-induced SMAD proteins are key in balancing immunity[111]. DCs from the lamina propria are essential for inducing FoxP3 expression in naive T cells, which requires an exogenous source of TGFβ[112]. Interestingly, pups of mice exposed to airborne allergens developed oral tolerance towards the allergen that was dependent on milk-derived TGFβ[113]. In addition, TGFβ and IL-10 inhibit type I interferon production by pDCs[114]. Immunosuppressive cytokines such as IL-10 and TGFβ in milk are important in maintaining immune

homeostasis and the suppression of type I interferon production. These immunosuppressive cytokines in cow's milk could be essential for inducing a regulatory milieu, which subsequently may result in tolerance towards allergens.

Antigen-specific IgG in breast milk was shown to protect against OVA-induced asthma in a mouse model by inducing regulatory responses. Moreover, pups of mothers that were exposed to antigen aerosols during lactation resulted in a regulatory immune response that protected them from developing asthma[115]. The proposed mechanism involves binding of IgG to neonatal Fc receptor (FcRn), which resulted in the expansion of antigen-specific Tregs. Bovine IgG shows some affinity for human FcRn[116] and is specific for human allergens[68], and it is therefore possible that the uptake of bovine IgG–allergen complexes induces FoxP3$^+$ T cells (Figure 1). These functional properties of milk proteins are lost upon heating. Another heat-sensitive fraction of bovine milk that has been suggested to induce immune regulation is exosomal microRNA[117].

The role of milk oligosaccharides in the induction of oral tolerance remains inconclusive. 6'SL was shown to alleviate OVA-induced food allergic symptoms by promoting IL-10-producing T cells[118]. In contrast, pups fed milk that contained 3'SL had more severe induced colitis compared to pups fed milk devoid of 3'SL. *Ex vivo* cultures of mesenteric lymph node (MLN) DC showed direct TLR4 activation by 3'SL[119]. However, 3'SL did not induce TLR4-mediated activation of human immune cells *ex vivo*[120]. In addition, sialylated milk oligosaccharides were shown to alter the microbiota composition and growth in infants[121]. These changes in microbiota composition in turn impact the production of SCFAs that were shown to be essential, together with vitamin A, in oral tolerance induction[122]. In summary, the direct immunomodulatory effect of sialylated oligosaccharides remains inconclusive. Rather than having direct effect on the immune system, sialylated oligosaccharides may promote immune homeostasis indirectly by promoting the outgrowth of SCFA-producing bacteria.

In the gut, a subset of migratory DC expressing the integrin CD103 are known to convert vitamin A into RA. In mouse models, RA induces the differentiation of naive T cells into Tregs *in vivo*[123]. These findings were confirmed in vitro

in humans by differentiating moDC in the presence of RA. These RA DCs expressed CD103 and were capable of polarizing naive T cells into Tregs[124] or FoxP3$^-$ IL-10-producing T cells[125]. Similarly, under steady-state conditions, lung macrophages produce RA and TGFβ toward harmless airborne antigens and induce antigen-specific Tregs[126]. Thus, dietary vitamin A triggers endogenous RA production that is essential to induce Tregs in the gut and the lung. However, it is unknown whether dietary vitamin A contributes in the upper airways to induce antigen-specific Tregs. The active form of vitamin D3, 1,25(OH)2D3, halts the differentiation of monocytes into moDC *in vitro* and does not affect pDCs[127]. These vitamin D3 DCs are less sensitive to TLR ligands and develop a semi-mature phenotype upon stimulation. These authors show that this semi-mature phenotype is instrumental for priming naive T cells to become Tregs and to induce T cell anergy[128]. In summary, cow's milk contains a variety of components that are known to promote immune homeostasis and induce regulatory responses by human immune cells *in vitro* (Figure 1). Therefore, we hypothesize that these immune regulatory effects aid in tolerance induction toward allergens or suppress immune responses in the upper airways that could aid in the protection against asthma exacerbation.

Systemic responses; the gut-lung axis

Several recent studies have shown that immune responses triggered in the GI tract can influence immunity in the respiratory tract. Evidence for the existence of this so-called gut–lung axis is increasing, although the exact mechanisms involved are not yet completely understood[128–131]. The importance of TLR signaling by commensal microbiota in relation to airway immunity was demonstrated in multiple studies[132–134]. Mice treated with antibiotics before influenza infection showed a higher viral load in the lungs, reduced CD4$^+$ T cells responses and reduced influenza specific antibody titers compared to control mice. Intrarectal administration of TLR agonists could restore immune responses to influenza infection in this model. To clear the influenza infection, commensal bacteria or TLR agonists were needed to induce inflammasome-dependent cytokine release (IL-1β and IL-18). These cytokines allowed lung DCs to migrate to the mediastinal lymph node where they activate specific T cells[132]. Another study in mice showed that oral administration of a bacterial

extract (OM-85) reduced the viral load in the respiratory tract after influenza infection. The bacterial extract also boosted specific polyclonal antibodies against *Klebsiella pneumoniae* and *Streptococcus pneumoniae*, which protected the mice against these airway pathogens[133]. Furthermore, germ-free mice showed increased susceptibility to pulmonary infection with *K. pneumoniae*, which could be restored by i.p. injection of LPS[131]. These studies indicate that there is cross-talk between the commensal microbiota and immunity in the respiratory tract *via* TLR signaling (Figure 2).

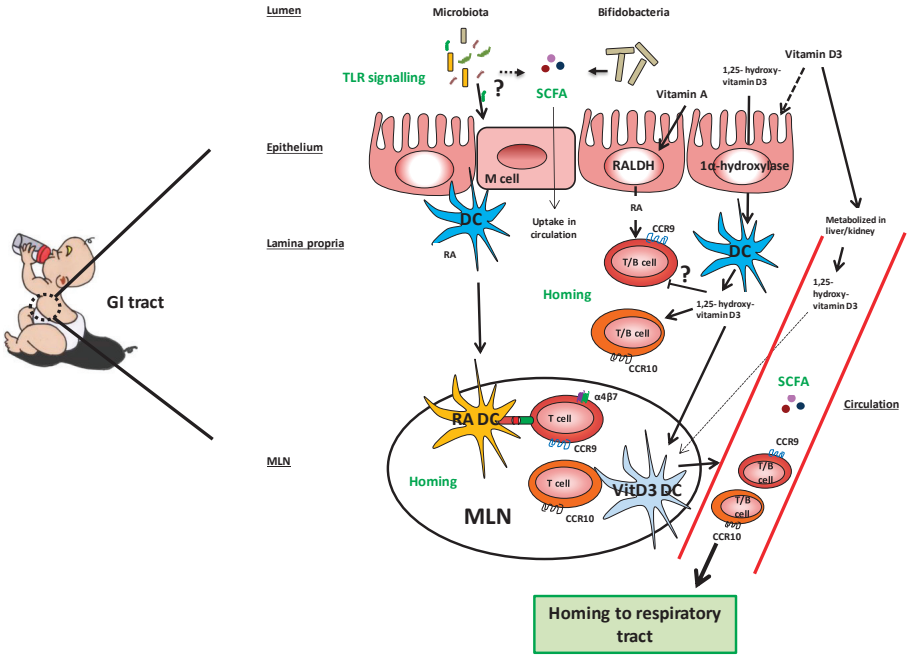

Figure 2: Mechanisms involved in the gut-lung axis linked to milk components. TLR signaling by microbiota in the gut results in improved airway immunity although the exact mechanism in this gut–lung axis is unknown. Microbiota, specifically Bifidobacteria, may ferment sialylated oligosaccharides present in cow's milk into short-chain fatty acids (SCFAs), which are taken up into the circulation. Cow's milk contains vitamin A, vitamin D3, and 1,25-hydroxyvitamin D3. Vitamin A and vitamin D3 can be taken up by epithelial and dendritic cells (DCs) and converted by the enzymes RALDH and 1α-hydroxylase into retinoic acid (RA) and 1,25-hydroxyvitamin D3, respectively. However, the majority of vitamin D3 is taken up into the system and converted into its active form in the kidneys. In the lamina propria, RA may induce expression of gut homing markers CCR9 and α4β7 on T cells and CCR9 on B cells directly or prime DC to induce the expression of these markers in the mesenteric lymph node (MLN). 1,25-hydroxyvitamin D3 may induce the expression of homing marker CCR10 on T and B cells directly or indirectly via vitamin D3-primed DC (VitD3 DC). In addition, 1,25-hydroxyvitamin D3 downregulates the expression of CCR9 on lymphocytes in a direct way. Lymphocytes expressing CCR9 have a homing capacity toward the small intestine, while lymphocytes expressing CCR10 have a homing potential toward the colon and respiratory tract, providing a potential mechanism of the gut–lung axis.

Another link between airway immunity and the gut microbiota is the release of SCFAs. SCFAs are metabolites produced by bacteria in the gut from dietary non-digestible fibers. One type of non-digestible fibers are sialylated oligosaccharides present in cow's milk. As mentioned earlier, milk oligosaccharides are fermented in the colon into SCFAs[54]. B. longum infantis has been shown to ferment sialylated oligosaccharides directly[55,56]. After release into the colon, SCFAs such as acetate and to a lesser extent propionate are taken up into the circulation in mice[135] and humans[136]. SCFAs bind to metabolite-sensing G protein-coupled receptors, and signaling influences gene expression via induction of histone deacetylases[136]. Both acetate and propionate bind via GPR[41], which is expressed on various tissues and cells including enteroendocrine cells and PBMCs[137]. Several studies indicate that SCFAs play an important role in the gut–lung axis by regulating immune activation in the lung[135,136] (Figure 2). A high-fiber diet was prevented against allergic airway disease (AAD) in mice[135,136]. This protective effect was shown to be mediated by acetate produced by the microbiota. Direct oral administration of acetate resulted in higher Treg numbers in the lung and protection against HDM-induced AAD[136]. In another study, oral administered propionate did not affect Treg numbers in the lung, but resulted in hematopoiesis in the bone marrow of DCs that were found in the lungs. These DCs had a more immature phenotype (lower levels of MHCII and CD40) and therefore a reduced capacity of activating Th2 cells[135]. These studies demonstrate that microbial metabolites produced in the intestines can have an effect on immune function in the airways. In addition, other microbial components such as TLR ligands may be taken up in the circulation and impact immunity in the respiratory tract. TLR stimulation in the gut could activate DCs leading to the activation of lymphocytes in the mediastinal lymph node. Upon activation, these lymphocytes can migrate to the lung and potentially to the gut. Besides, microbiota can have an indirect effect via SCFA production, as SCFAs in the circulation can affect DCs and Tregs in the respiratory tract. Currently, direct effects of SCFAs on the induction of homing markers on DCs or lymphocytes are not known.

After activation, lymphocytes can migrate (i.e. home) to tissues depending on their homing marker (e.g. selectins, integrins, and chemokine receptors) expression. These receptors can bind to tissue-specific ligands (e.g. addressins and chemokines) expressed by the endothelium. In humans, mucosal vaccination was used as a model to show that the site of induction of a mucosal

immune response resulted in IgA production in restricted mucosal tissues. Holmgren and Czerkinsky showed that specific IgA antibodies are produced in the upper respiratory tract and gut in cholera toxin B (CTB) vaccinated individuals. In contrast, intranasal vaccination with CTB resulted in specific IgA production in both upper and lower respiratory tract and genital tract, but not in the gut. Furthermore, rectally vaccinated individuals only produced specific IgA locally in the rectum[138]. The fact that orally administered antigens result in effector cells being present both in the gut and the upper respiratory tract indicates that homing markers might overlap. Well-studied homing marker interactions in humans are among others, CCR9 binding to locally produced CCL25 in the small intestine[139,140] and CCR10 binding to CCL28 produced in the airways and colon[141]. For B cells, there is no clear homing marker that differentiates between upper and lower respiratory tract homing as CCR10 expressed on B cells binds CCL28 produced locally in lower and upper respiratory tracts. In contrast, T cells express CCR10 to bind CCL28 produced in the lower respiratory tract and salivary glands[16,142], while T cells express CCR3 to bind CCL28 in the nasal mucosal[143]. Another homing marker that could be important in migration between gut and lung is CCR6 as its ligand CCL20, which are expressed in both tissues[144].

Thus, the homing potential of immune cells is affected by the site of induction and is dependent on local production of tissue-specific stromal factors. Recent evidence suggests that it can also be modified by dietary components. Of all dietary components, the effect on homing is best studied for RA and 1,25-dihydroxyvitamin D3 (Figure 2). Dietary vitamin A as a source of RA is essential for efficient homing of T cells to the GALT[145,146]. In mice, RA production by mucosal $CD103^+$DCs[146,147] or stromal cells in the MLN[148] is essential for efficient differentiation of naive T cells into $FoxP3^+$ Tregs that express the gut homing markers α4β7 and CCR9 in the MLN. Interestingly, human RA-primed $CD103^+$ DCs were also shown to induce differentiation of naive T cells into IL-10-producing T cells expressing gut homing markers *in vitro*[125]. RA is also a factor that regulates B cell proliferation, differentiation, and class switching[149]. Moreover, RA and TGFβ1 induce IgA class switching[150]. Similarly to the effects observed on T cells, RA derived from GALT-DCs alone was shown to induce gut homing markers on B cells[151]. Interestingly, vitamin D3 blocks the upregulation of RA-induced gut homing marker expression on

T cells[152,153] although this was not observed by Baeke et al.[154]. The majority of dietary vitamin D3 is taken up along the GI tract and converted into its active form 1,25(OH)2D3 in the kidney and becomes systemically available[97]. In addition, vitamin D3 is shown to be converted in its active metabolite by DCs and epithelial cells[96,152]. Dietary supplementation of vitamin D3 to HIV-infected patients was shown to induce CCR10 expression on Tregs[155]. This finding is in line with *in vitro* studies showing that vitamin D3 induces CCR10 expression on human B and T cells[152,154,156]. Interestingly, RA also induces CCR10 expression in human B cells and acts even synergistically with 1,25-dihydroxyvitamin D3[156]. The balance of vitamin A and vitamin D3 may thereby regulate homing of lymphocytes to gut or respiratory tract, respectively (Figure 2). However, it should be noted that the concentrations of vitamin A and vitamin D3 are relatively low in cow's and breast milk. In addition, the active metabolites of these vitamins can be endogenously produced (e.g. by stromal cells) in the secondary lymphoid tissues. In summary, we are only beginning to unravel the complex interplay between gut and lung. Hence, we can only speculate about the mechanisms by which cow's milk through sialylated oligosaccharides and vitamin A and D could affect microbiota composition or homing of lymphocytes, respectively.

Concluding remarks

The existing epidemiological evidence suggests that the consumption of raw cow's milk contributes to protection against allergies and asthma and respiratory tract infections. In this review, we discussed potential mechanisms by which cow's milk and its components may exert these immunological effects. Bovine IgG can bind to bacterial and viral pathogens, enhance phagocytosis, and may neutralize pathogens. Other milk components like TGFβ promote epithelial barrier functioning by upregulation of TJ genes and might favor the differentiation of Tregs that can reduce inflammation locally. Finally, recent evidence show an interplay between gut and lung. We speculate about the effect of milk components on trafficking of lymphocytes from the intestine to the upper airways through modulation of homing receptors and microbiota. Further unraveling the impact of milk components on local responses in the respiratory tract, microbiota and immune trafficking are necessary to fully understand their effects on allergy, infection, and asthma.

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Chapter 3

Induction of trained innate immunity in human monocytes by bovine milk and milk-derived IgG

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Abstract

Innate immune memory, also termed trained immunity in vertebrates, has been recently described in a large variety of plants and animals. In most cases trained innate immunity is induced by pathogens or pathogen-associated molecular patterns (PAMPs), and is associated with long-term epigenetic, metabolic and functional reprogramming. Interestingly, recent findings indicate that food components can mimic PAMPs effects and induce trained immunity. The aim of this study was to investigate whether bovine milk or its components can induce trained immunity in human monocytes. To this aim monocytes were exposed for 24 h to β-glucan, TLR-ligands, bovine milk, milk fractions, bovine lactoferrin (bLF), and bovine Immunoglobulin G (bIgG). After washing away the stimulus and a resting period of 5 days, the cells were re-stimulated with TLR ligands and TNF-α and IL-6 production was measured. Training with β-glucan resulted in higher cytokine production after TLR1/2, TLR4 and TLR7/8 stimulation. When monocytes trained with raw milk were re-stimulated with TLR1/2 ligand Pam3CSK4, trained cells produced more IL-6 compared to non-trained cells. Training with bIgG resulted in higher cytokine production after TLR4 and TLR7/8 stimulation. These results show that bovine milk and bIgG can induce trained immunity in human monocytes. This confirms the hypothesis that diet components can influence the long-term responsiveness of the innate immune system.

Introduction

The immune system is divided in two arms, the innate immune system and the adaptive immune system, of which only the latter is known to build long-lasting immune memory in T and B cells. However, recent observations have revealed that the innate immune system can also adapt to previous insults and develop a non-specific memory after infections, a process termed trained innate immunity.[1,2] The concept of trained immunity is based on the observation that after a primary infection, an enhanced innate immune response is induced in response to secondary infection or stimulation. In contrast to adaptive immune memory, this enhanced secondary response of trained innate immune cells is not only specific for the antigen that induced the primary response, but is rather a non-specific enhanced response to heterologous stimuli.[1] It is known that invertebrates respond better towards secondary infections, both to the same pathogen as well as towards other unrelated infections. It was shown in plants and invertebrates, processes termed systemic acquired resistance and immune priming occur widely in organisms that possess only an innate immune system.[3–9] Among vertebrates, interesting from an evolutionary perspective, there are indications that trained immunity occurs in teleost fish, which are the first vertebrates having a functioning adaptive and innate immune system, as reviewed by Petit and Wiegertjes.[10]

In humans, evidence for the existence of trained immunity first emerged from epidemiological studies on vaccination responses that indicated that vaccination induces protection not only against the target disease, but also induced cross-protection against other pathogens.[11] The best known example of this cross-protection is seen after Bacillus Camette-Guérin (BCG) vaccination against *Mycobacterium tuberculosis,* which was shown to protect against all-cause mortality by reducing neonatal sepsis, respiratory infection and fever.[12] Furthermore, it was shown that BCG vaccination in humans induced trained immunity in both monocytes and NK cells three months after vaccination, which was mediated by increased H3K4 trimethylation in monocytes.[13,14]

The mechanism of trained immunity was first described by Quintin et al., who showed that mice survived when treated first with a sublethal dose of *Candida*

albicans followed by a secondary lethal *C. albicans* infection.[15] This outcome was found both in wild-type mice and in T/B-cell defective (Rag 1-deficient) mice, indicating that the adaptive immune system was not involved in the induction of trained immunity. Subsequently, it was shown that β-glucan derived from *C. albicans* could induce trained immunity in purified human monocytes.[15] Some types of β-glucans are present in the cell wall of *C. albicans*, while other types are also present in food as mushrooms, baker's and brewer's yeast and cell walls of plants including wheat and oat, as reviewed by Meena et al.[16] These dietary β-glucans may induce trained immunity as well, although this remains to be demonstrated.

Low-density lipoprotein (oxLDL) particles induced in blood as a result of Western diets, are known to induce trained immunity in human cells *in vitro*.[17] A Western diet resulted in transient systemic inflammatory responses in mice, yet at the same time induce long-lived epigenetic and transcriptomic reprogramming of granulocyte-monocyte progenitor cells, leading to trained immunity by monocytes.[18] It can thus be concluded that not only infections by pathogens or vaccination can induce trained immunity, but also dietary components.

Epidemiological studies have revealed that children growing up on a farm and consuming (raw) farm milk have a reduced incidence of asthma, atopy, hay fever, respiratory tract infections (RTI) and otitis media compared to children that consumed heat treated milk.[19–21] The components that may cause the reduction of allergy and infections in children are thus milk processing sensitive, and are therefore thought to be heat sensitive milk proteins. These findings indicate that raw milk or its components can modify immune responses *in vivo*.

More than 400 components have been identified in bovine milk and these can be subdivided in multiple fractions.[22] Bovine milk is composed of water (87%), lactose (4-5%), protein (whey and casein) (3%), lipids and fat (3-4%), minerals (0.8%) and vitamins (0.1%).[23–26] Whey proteins make up 20% of protein concentration of milk, whereas caseins represent 80% of milk proteins.[27] The most abundant whey proteins are β-lactoglobulin, α-lactalbumin, immunoglobulins, such as bovine IgG (bIgG), serum albumin

and lactoferrin (bLF).[27] Immunologically the best studied whey proteins are bovine IgG and lactoferrin.[28–31]

The aim of this study was to study whether raw bovine milk, milk fractions, or milk proteins such as lactoferrin and IgG can induce trained immunity in human monocytes.

Materials and Methods

Blood samples and monocyte purification

Buffy coats were collected from healthy blood donors at the Sanquin Blood Supply in Nijmegen, the Netherlands. Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats, using Ficoll plaque plus (17-1440-02, GE Healthcare Life Sciences, Uppsala, Sweden). Monocytes were enriched from PBMCs using negative selection with Easysep human mono-enrichment kit according to manufacturer's protocol (19359, Stemcell Technologies). Purity was tested by flow cytometry staining isolated cells with α-CD14 (555397, BD Pharmingen), α-CD3 (555334, BD Pharmingen) α-CD19 (562947 BD Horizon), α-CD56 (555516, BD Pharmingen) and fixable viability dye eFluor 450 (65-0863-14, eBioscience). Stained cells were measured on FACS CANTO II. Monocytes isolated from subjects used for analysis all had a purity of >70% $CD14^+$ monocytes and <4% $CD3^+$ T cells.

Trained immunity model in human monocytes

The trained immunity model in human monocytes was performed as previously described[32,33] with some adjustments and is depicted in Figure 1. Isolated monocytes were transferred into a 96-wells plate ($1x10^5$ monocytes/well) (Costar3596) and the training stimulation or culture medium RPMI 1640 Dutch modifications from Sigma-Aldrich, supplemented with 1% gentamicin, 1% L-glutamine, and 1% pyruvate (Life Technologies, Nieuwerkerk, the Netherlands) was added in a total volume of 200 µl for 24h at 37˚C. For the different training stimuli and their concentrations see section 'reagents'. After 24h plates were washed twice with warm PBS and RPMI medium + 10% human pooled serum was added for 5 days and refreshed after 2-3 days. In these 5 days monocytes differentiated towards macrophages and at day

6 macrophages were stimulated in the presence or absence of TLR-ligands Pam3CSK4 10µg/ml (L2000; EMC microcollections) Ultra-pure LPS 0.1 µg/ml (3pelps, Invivogen) and R848 10 µg/ml (TLRL-R848-5, Invivogen). After 24h of secondary stimulation, supernatant was collected and stored at -80 ˚C.

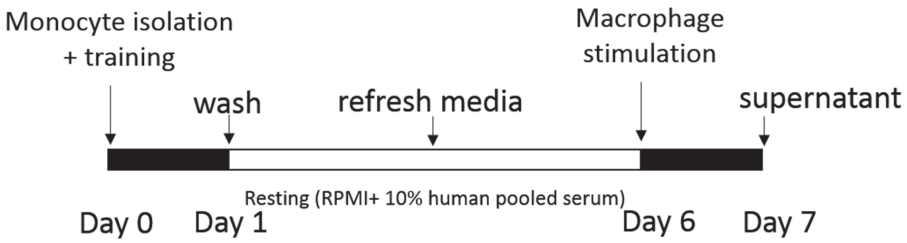

Figure 1: Trained immunity model in human monocytes. At day 0 PBMCs were isolated from buffy coats and monocytes were isolated by negative selection. Monocytes are stimulated (trained) for 24 hours, the stimulus is washed away and monocytes differentiated towards macrophages during a 5 day resting period. At day 6 macrophages were re-stimulated with TLR-ligands and after 24 hours supernatant was collected and cytokines were measured.

Reagents

Candida albicans β-glucan (1µg/ml) was a kind gift of Prof. David Williams (East Tennessee State University), and was isolated and purified as previously described[34]. Raw bovine milk (1:100), bovine lactoferrin (100 µg/ml), bovine IgG (200 µg/ml), lactose (463 µg/ml), whey protein (53.41 µg/ml), casein (264 µg/ml), cream (3.6 µg/ml), and milk fat (459.9 µg/ml) were obtained from FrieslandCampina, and TGF-β1 (5 ng/ml) & TGF-β2 (18 ng/ml) were obtained from Peprotech. As bLF and bIgG contained some endotoxin, they were treated with Triton-X114 to remove LPS as described by Teodorowicz et al.[35] After LPS removal, bIgG and bLF contained less than 10 pg/ml LPS at the dilutions used for all experiments. Endotoxin levels were measured using Endozyme recombinatant Factor C assay (Hyglos).

Cytokine measurement

In supernatant the production of IL-6 (558276, BD Pharmingen) and TNF-α (560112, BD Pharmingen) was measured using cytometric bead array on the FACS CANTO II, according to manufacturer's protocol

Statistical analysis

IBM SPSS Statistics software, version 23, was used to perform statistical analysis. All experiments with β-glucan, raw bovine milk, bovine lactoferrin and bovine IgG were performed at least five times, with monocytes isolated from PBMC of in total a minimum of eleven volunteers. In order to assess the training effect of a specific ligand upon a secondary stimulation, non-trained cells stimulated with Pam3CSK4, LPS or R848 were compared with trained cells re-stimulated with Pam3CSK4, LPS or R848. Differences between the groups were analyzed using the Wilcoxon signed-rank test and were considered statistically significant at a P value of <0.05. No statistical analysis was performed on the screening of the training stimuli (Figure 2 and 3).

Results

Screening of bovine milk, milk fractions and isolated milk proteins for induction trained immunity

To determine if milk and its components can induce trained immunity we screened the effects of raw milk, whey proteins, casein, milk fat, cream as well as a number of purified milk proteins on monocytes using the experimental set-up as depicted in Figure 1 (based on [32,33]).

Figure 2 shows that monocytes stimulated with β-glucans as positive control, and re-stimulated with R848, produced higher IL-6 and TNF-α levels compared to control cells (non-trained cells in culture medium). The same induction of IL-6 and TNF-α was also seen when cells trained with β-glucans were re-stimulated with Pam3CSK4 (Pam) and LPS (data not shown). In contrast, training with TLR-ligands Pam, LPS and R848 induced tolerance to re-stimulation as published elsewhere[32,33] and was not due to cell death.

When raw milk and milk components were tested for their ability to induce trained immunity, raw milk induced variable production of IL-6 and TNF-α for different secondary stimulations while inducing higher levels of IL-6 upon Pam, LPS (both not shown) and R848 stimulation compared to non-trained cells (Figure 3A). Whey proteins induced trained immunity when re-stimulated with R848 (Figure 3A, C). This was also seen after stimulation with Pam and LPS (data not shown). The only other milk fraction that might induce some

trained immunity was cream. As whey proteins induced trained immunity comparable to β-glucan, the effect of three prominent immune-related whey proteins was investigated (Figure 3B, D). LPS-free, isolated bovine lactoferrin (bLF) and bovine IgG (bIgG), but not TGF-β, induced trained immunity (Figure 3B, D). Bovine milk proteins are thus able to induce trained immunity.

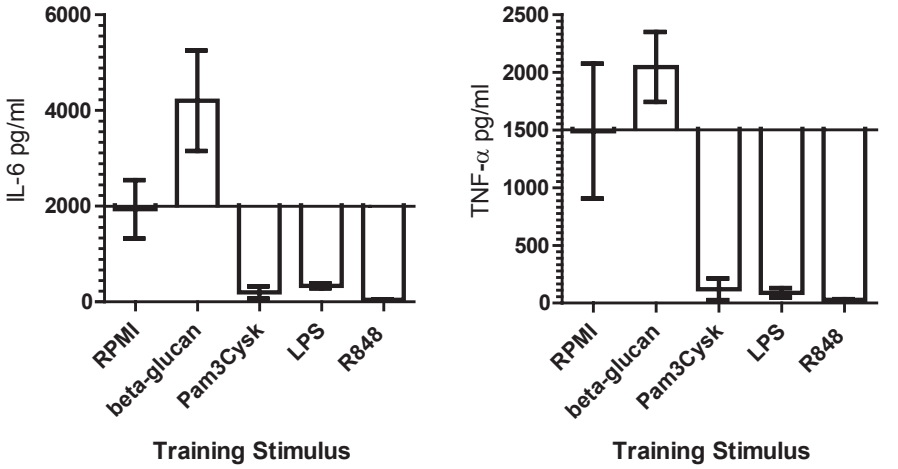

Figure 2: Induction of trained immunity or tolerance is dependent on the training of monocytes. Monocytes were stimulated 24h in the presence or absence of β-glucan (1μg/ml); Pam3CSK4 (10 μg/ml), LPS (0.1 μg/ml) or R848 (10 μg/ml), after 5 days of rest the differentiated macrophages were stimulated for 24h with R848 (10 μg/ml). In supernatant the produced IL-6 and TNF-α (pg/ml) was measured. Data shown as mean ± SEM, with the IL-6 and TNF-α production of non-trained cells (RPMI) as x-axis. RPMI and β-glucan n=5; TLR stimuli n=3.

Induction of trained immunity by raw milk and bIgG

To extend our observations, we studied the induction of trained immunity by raw milk, bIgG, and bLF in a number of additional individuals. β-glucan was included as positive control. Stimulation of isolated monocytes with β-glucans consistently resulted in higher IL-6 production compared to non-trained monocytes upon re-stimulation with TLR-ligands Pam, LPS and R848, see Figure 4. Also, a higher TNF-α production by β-glucan trained monocytes was observed for LPS and R848 re-stimulated macrophages, but not for Pam re-stimulated macrophages.

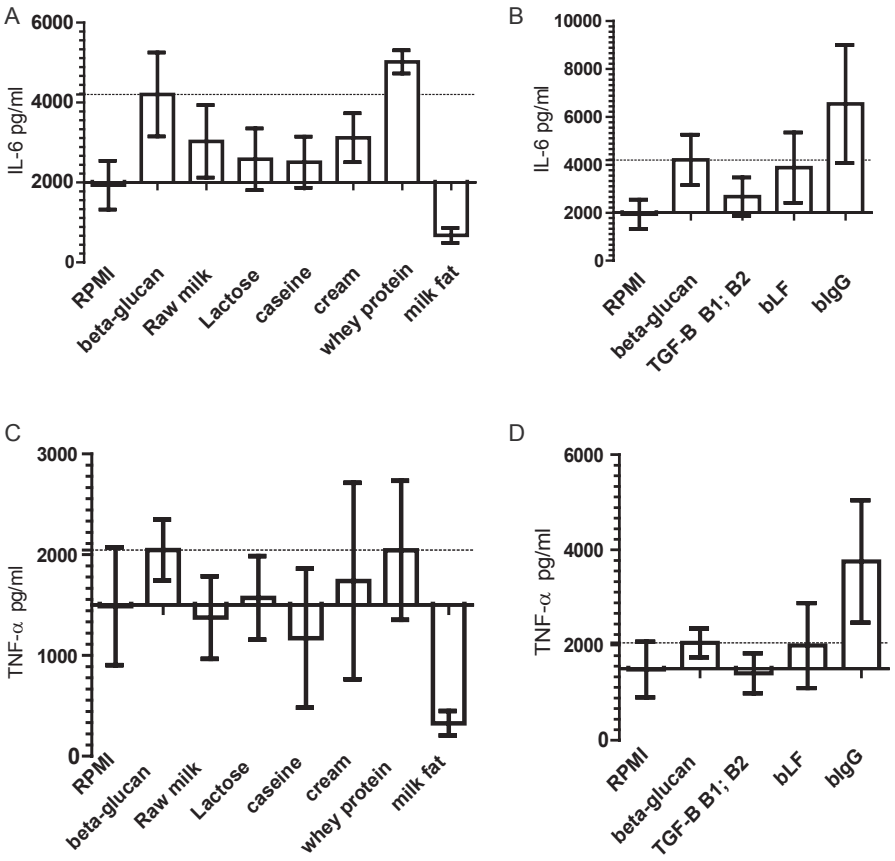

Figure 3: Selection of bovine milk fractions (A,C) and purified bovine milk ingredients (B,D) that can induce trained immunity. Monocytes were stimulated 24h in the presence or absence of β-glucan (1µg/ml); raw milk (1:100); lactose (463 µg/ml); casein (264 µg/ml); cream (3.6 µg/ml); whey protein (53.41 µg/ml); milk fat (458.9 µg/m)l; TGF-β1 (5ng/ml) β2 (18ng/ml); bovine lactoferrin (bLF) (100 µg/ml); bIgG (200 µg/ml). After 5 days of rest the differentiated macrophages were stimulated for 24h with R848 (10 µg/ml). In supernatant the produced IL-6 and TNF-α (pg/ml) was measured. Data shown as mean ± SEM, with the IL-6 and TNF-α production of non-trained cells (RPMI) as x-axis and the cytokine production by β-glucan trained cells (positive control) as dotted lines. RPMI, Beta-glucan, raw milk, lactose, TGF-β, bIgG n=5; bLF n=4, all other stimuli n=3.

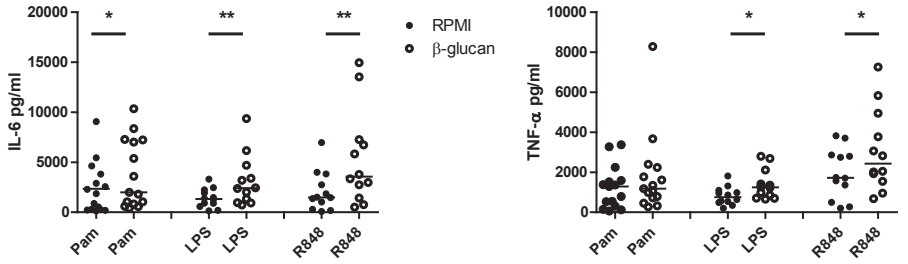

Figure 4: Induction of trained immunity by β-glucan. Monocytes were stimulated 24h in the presence or absence of β-glucan (1µg/ml), after 5 days of rest the differentiated macrophages were re-stimulated for 24h with Pam3CSK4 (Pam) (10 µg/ml), LPS (0.1 µg/ml) or R848 (10 µg/ml). In supernatant the produced IL-6 and TNF-α (pg/ml) was measured. N=15 (Pam) or n=12 (LPS, R848) in 5-6 independent experiments. Data shown as dot plot with median. Statistics was done by performing a Wilcoxon signed rank test between ß-glucan and RPMI for every secondary stimulation (Pam, LPS, R848). * p<0.05; ** p<0.01

Figure 5 shows that only upon re-stimulation with Pam, training with raw bovine milk is able to induce higher production of IL-6 compared to non-trained (RPMI) monocytes. This effect is not observed for IL-6 production after re-stimulation with other TLR-ligands and not for TNF-α production after stimulation with any TLR-ligand, see also Supplementary Figure 1B.

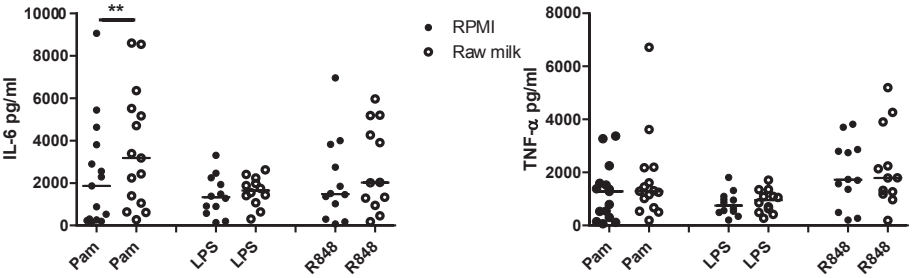

Figure 5: Induction of trained immunity by raw bovine milk. Monocytes were stimulated 24h in the presence or absence of raw bovine milk (1:100), after 5 days of rest the differentiated macrophages were re-stimulated for 24h with Pam3CSK4 (Pam) (10 µg/ml), LPS (0.1 µg/ml) or R848 (10 µg/ml). In supernatant the produced IL-6 and TNF-α (pg/ml) was measured. N=15 (Pam) or n=12 (LPS, R848) in 5-6 independent experiments. Data shown as dot plot with median. Statistics was done by performing a Wilcoxon signed rank test between raw bovine milk and RPMI for every secondary stimulation (Pam, LPS, R848). ** p<0.01

In contrast to the previous results (Figure 3B and 3D), bLF did not consistently induce trained immunity when more subjects were tested, see Figure 6. Supplementary Figure 1C and Supplementary Table 1 show that donors respond

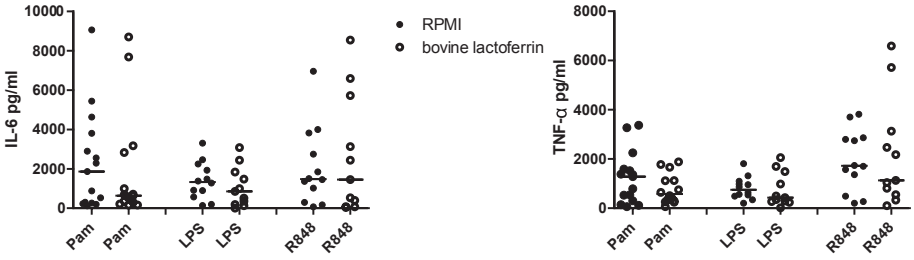

Figure 6: Bovine lactoferrin does not induce trained immunity. Monocytes were stimulated 24h in the presence or absence of bovine lactoferrin (bLF) (100 µg/ml), after 5 days of rest the differentiated macrophages were re-stimulated for 24h with Pam3Cysk4(10 µg/ml), LPS (0.1 µg/ml) or R848 (10 µg/ml). In supernatant the produced IL-6 and TNF-α (pg/ml) was measured. N=15 (Pam) or n=11 (LPS, R848) in 5-6 independent experiments. Data shown as dot plot with median. Statistics was done by performing a Wilcoxon signed rank test between bLF and RPMI for every secondary stimulation (Pam, LPS, R848). No significant differences were observed.

to bLF training in a very heterogeneous manner, in which only some donors show increased training by bLF, resulting in no statistically significant effect overall.

In line with the selection experiment and raw bovine milk, bovine IgG was able to consistently induce trained immunity (Figure 7). When monocytes were stimulated with bIgG and re-stimulated with R848 the cells produced more IL-6 and TNF-α and more TNF-α upon LPS stimulation compared to non-trained cells (RPMI). Upon re-stimulation with Pam, bIgG tended to increase the production of IL-6 and TNF-α, albeit not significantly (Supplementary Figure 1D).

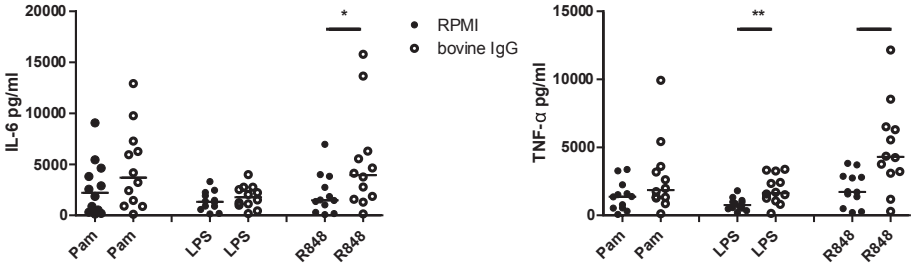

Figure 7: Induction of trained immunity by bovine IgG. Monocytes were stimulated 24h in the presence or absence of bovine IgG (200 µg/ml), after 5 days of rest the differentiated macrophages were re-stimulated for 24h with Pam3CSK4 (Pam)(10 µg/ml), LPS (0.1 µg/ml) or R848 (10 µg/ml). In supernatant the produced IL-6 and TNF-α (pg/ml) was measured. N=12 in 5 independent experiments. Data shown as dot plot with median. Statistics was done by performing a Wilcoxon signed rank test between bovine IgG and RPMI for every secondary stimulation (Pam, LPS, R848). * $p<0.05$

Discussion

Here we show that bIgG and raw bovine milk can induce trained immunity in human monocytes. These findings confirm the hypothesis that dietary components can modulate the responsiveness of the innate immune system to pathogen-related stimuli.

One of the first components that was shown to induce trained immunity was β-1,3-(D)-glucan derived from *C. albicans,* that exerted this effect via epigenetic changes in trimethylation of H3K4, induced after binding to Dectin-1.[15] Epigenomic profile analysis revealed changes in H3K4m1, H3K4me3 and H3K27ac when comparing β-glucan trained compared to non-trained macrophages.[33] Based on these studies β-1,3-(D)-glucan derived from *C. albicans* has become a model compound to study the mechanisms of trained immunity.

Cell metabolism is important in monocyte to macrophage differentiation, and also for M1 vs M2 function.[36] Resting and tolerant macrophages use oxidative phosphorylation to generate ATP, while activated (trained) macrophages shift to aerobic glycolysis (Warburg effect) via the dectin-1-Akt-mTOR and HIF-1α pathway.[33,37] Resting macrophages have a functional tricarboxylic acid (TCA) cycle that together with glycolysis enhances membrane synthesis and induce TLR-mediated activation in dendritic cells.[33] Two other metabolic pathways that are important *in vitro* and *in vivo* for trained immunity induction are glutaminolysis and the cholesterol synthesis pathway.[38,39] β-glucan induced trained immunity via the dectin-1 pathway also effects the TCA cycle as glycolysis inhibitors (rapamycin) also inhibit β-glucan mediated trained immunity.[38] This links cell metabolism, metabolites and epigenetic mechanisms, suggesting that β-glucan induces trained immunity by modifying cell metabolism.

In our hands β-glucan induced higher levels of IL-6 compared to non-trained cells when trained macrophages were re-stimulated with ligands for TLR1/2 (Pam), TLR4 (LPS) and TLR7/8 (R848). TNF-α production was increased after LPS and R848 re-stimulation. Unexpectedly, TNF-α production was not increased after Pam re-stimulation, which is in contrast to Ifrim et al., albeit using the same concentration and same supplier of Pam3CSK4.[32]

Until now, it is not clear if dietary components can also induce immune training in humans after nutritional intervention. In a pilot study in humans where baker's yeast (*S. cerevisiae)* – derived β-glucans (1000 mg/day) were used as food supplement for 7 days, no trained immunity effects were observed.[40] In contrast, it was recently shown that diets high in fat can also induce trained immunity *in vivo* in mice, and oxLDL can induce immune training in human monocytes *in vitro*[17,18], suggesting that nutrition may indeed directly affect innate immune function.

As breast milk and bovine milk contain many immune-modulating components[22], we set out to study whether (raw) bovine milk or milk components can induce trained immunity in human monocytes as well. A trained immunity effect was observed for IL-6 production by raw bovine milk when stimulated with TLR1/2 ligand Pam3CSK4, but not for stimulation through TLR4 or TLR7/8 (Figure 5). In contrast, treatment with bIgG induced trained immunity after stimulation of TLR4 (LPS) and TLR7/8 (R848), but not after TLR1/2 (Pam) stimulation. This indicates that the training effect of raw milk (for TLR1/2) is not mediated via bLF or bovine IgG, as no effect was observed for training with bLF and bIgG after TLR1/2 stimulation. Further, as undiluted and low dilutions of milk compromised viability of the monocytes, raw milk was tested at a 100-fold dilution, whereas bIgG was tested at the levels present in raw milk. This can explain why raw milk does not induce trained immunity via TLR7/8.

It is not clear which component in raw milk is responsible for this training effect. One possibility is that bovine miRNAs in extracellular vesicles (exosomes) may do this. These vesicles are described to induced higher production of IL-6, but not higher TNF-α production, in LPS stimulated RAW264.7 cells.[41]

In our initial screen of raw milk and the major milk components, the clearest training effect after TLR7/8 stimulation was seen for whey proteins as well as for the isolated whey proteins bLF and bIgG (Figure 3). As whey proteins contain bIgG and bLF this suggests that bIgG and maybe bLF are responsible for the induction of trained immunity. Next to whey proteins, lipid-rich cream but not milk fat (triglycerides) seemed to induce a small training effect. This indicates that either the lipids or the intact milk fat globular membranes

present in cream serum, but not the triglycerides, may induce trained immunity to some extent.

For bIgG a consistent trained immunity effect was observed for re-stimulation with TLR7/8. However, this was not seen for bLF. bLF could only induce trained immunity in some donors (Supplementary Table 1, Supplementary Figure 1C), but the overall response was not significant (Figure 6). The lack of training induced by bLF in most donors, was not due to cell death. Besides, in supplementary table 1 it can be observed that subjects in which no training for bLF is seen, trained immunity induction by other training-stimuli is possible. It can be thus concluded that the variability of trained immunity responses depends on the training stimulus, and the mechanisms responsible for this effect remain to be elucidated in future studies. Furthermore, we have recently observed that a 3-week dietary intervention with bLF could enhance the response of pDC of elderly women to TLR7/8 (van Splunter, unpublished observations), and future studies should determine the involvement of trained immunity in this effect.

BLF can be taken up by human cells via three different receptors: intelectin[42], low-density-lipoprotein (CD91)[43] and CXCR4[43]. Most likely bovine LF can also bind to soluble CD14 and TLR4, as human LF is 69% homologous to bovine LF and can bind sCD14[44] and TLR4[45], which are expressed by monocytes.[46] In a human study in which subjects received 200 mg bLF and 100 mg Ig-rich whey proteins twice per day a significant reduction in rhinovirus induced common cold was observed.[47] This protective effect of bLF and bIgG on rhinovirus infections that are recognized by the immune system via TLR2 and TLR7/8 might thus be linked to trained immunity.[48]

In addition to the effect shown after TLR7/8 re-stimulation, bIgG was also able to induce trained immunity when re-stimulated with TLR4, but not with TLR1/2. bIgG is known to bind to human monocytes, macrophages and monocyte-derived dendritic cells via FcyRII (CD32 receptor).[49] Den Hartog et al. showed that bIgG could bind to human airway pathogens, such as respiratory syncytial virus (RSV), *haemophilus influenzae* type b (Hib) and influenza virus.[49] These pathogens can activate TLR2 and TLR4 (Hib and RSV), TLR7 (RSV and influenza) and TLR8 (RSV).[49–51]

We propose that the mechanism for the trained immunity induction by bIgG occurs via binding to FcγRII (CD32). In murine macrophages crosslinking of FcγRII/III leads to activation of MAP kinase family members p38 and JNK.[52] In human monocytes, inhibiting p38 and JNK abolished trained immunity induced by flagellin (TLR5) after re-stimulating through TLR4.[32] TLR induced signaling via MyD88 or TRIF leads to pro-inflammatory cytokine production and is partly regulated by p38MAPK/MK2 pathway.[53,54] Furthermore, in trained immunity epigenetic changes (e.g. H3K4 trimethylation) on the promotor of pro-inflammatory cytokines genes (IL-6, TNF-α) are induced by training stimuli, resulting in increased cytokine production.[15] Therefore, altogether, we hypothesize that bIgG can exert a trained immunity effect in macrophages by activating MAP kinase pathway via FcyRII, thereby inducing epigenetic changes on the promotors of IL-6 and TNF-α genes, leading to enhanced TLR-mediated responses.

In summary, our data show that raw bovine milk and bIgG isolated from raw colostrum can induce trained immunity in human monocytes. This strengthens the hypothesis that diet can influence the responsiveness of the innate immune system. Importantly to underline however is that the trained immunity program induced by milk and milk components is very likely different from that induced by other dietary components, such as Western-type diet. This can be concluded by the deleterious effects of Western-diet induced trained immunity on atherosclerosis, whereas no such effects have been reported for milk. Future whole-genome transcriptome and epigenome studies should describe the trained immunity activation program induced by milk, fully describing the impact of dairy milk on long-term reprogramming of innate immunity.

Acknowledgements

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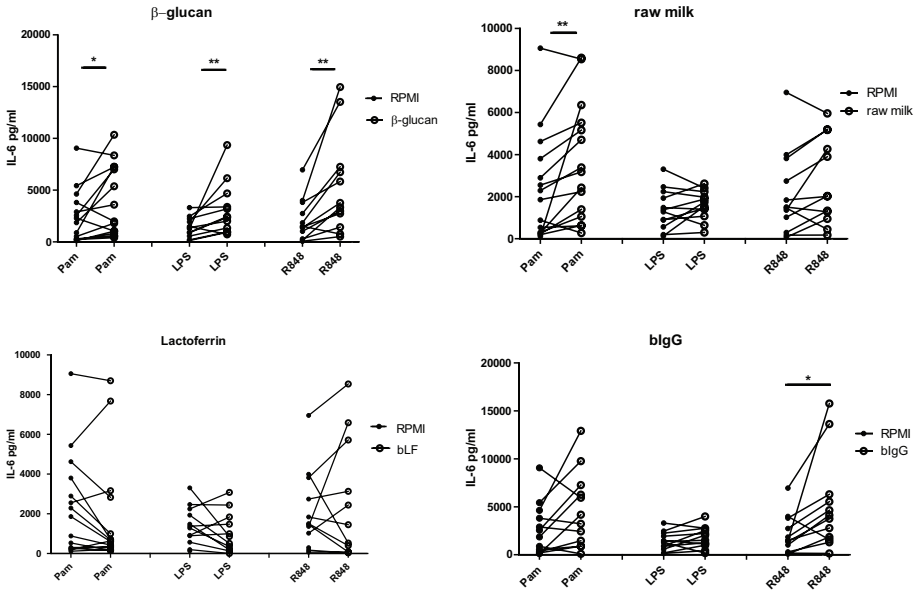
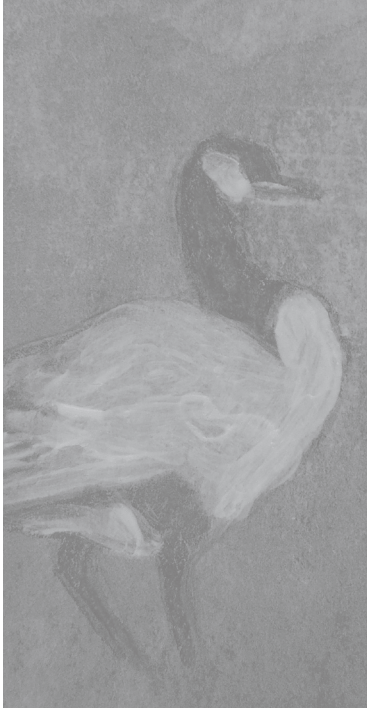
Supplementary Table 1: Trained immunity induced per training stimulus compared to non-trained cells after TLR7/8 (R848) stimulation (IL-6 production).

donor	β-glucan	Raw milk	Bovine lactoferrin	Bovine IgG
1	+	+	+	+
2	+	+	+	+
3	+	-	+	+
4	+	+	0	+
5	+	+	-	+
6	-	0	-	-
7	+	0	0	+
8	+	+	-	+
9	+	+		+
10	+	-	-	-
11	+	+	-	+
12	0	-	-	-
Training	10/12	7/12	3/11	9/12

+: >10% higher IL-6 production of training stimulus compared to non-trained cells: trained immunity;
-: <-10% lower IL-6 production of training stimulus compared to non-trained cells: tolerance;
0: comparable (-10 < % < 10%) IL-6 production of training stimulus compared to non-trained cells



Supplementary Figure 1: Paired analysis plots of monocytes trained with β-glucan, raw milk, bLF and bIgG and re-stimulated with Pam, LPS or R848 at day 6. In supernatant of day 7 IL-6 (pg/ml) was measured.



Chapter 4

Oral cholera vaccination promotes homing of IgA+ memory B cells to the large intestine and the respiratory tract

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Abstract

Oral cholera vaccination is used to induce immune responses in the intestines to protect against cholera infection. However, oral vaccination may also affect immune responses in other mucosal tissues. To study this, tissue-specific homing potential and kinetics of B cell responses were characterized after oral cholera vaccination.

Healthy adult volunteers received two doses of Dukoral® and blood, saliva, nasal wash and fecal samples were collected over time to detect vaccine-specific antibodies. Additionally, homing potential of lymphocytes to small intestine, colon, airways, skin and periphery was measured by expression of Integrin β1 and β7, CCR9, CCR10, CCR7 and CLA.

After vaccination, antibody responses to cholera toxin B (CTB) and Dukoral® were detected in serum and nasal wash. CTB-specific memory B cells in peripheral blood and tissue homing profiles of memory B cells peaked at day 18. IgA^+ memory B cells expressed markers that enable homing to the airways and colon, while IgA^- memory B cells primarily expressed small intestine homing markers. These data show that oral cholera vaccination has a differential effect on immune responses in various mucosal sites, including the respiratory tract.

Introduction

Mucosal surfaces are frequently exposed to potentially harmful microorganisms. As a result, mucosal tissues can be infected by many different pathogens, such as Influenza A, rhinovirus, Respiratory syncytial virus (RSV), and *Streptococcus pneumoniae* in the respiratory tract and *Vibrio cholerae*, Rotavirus, Norovirus, *Salmonella typhi*, *Escherichia coli* and *Campylobacter jejuni* in the gastrointestinal tract.

Parenteral vaccination predominantly elicits systemic immune responses to the vaccine, whereas mucosal vaccination via the oral or intranasal route induces adaptive immune responses targeted to the mucosal tissues and lumen to inhibit colonisation and invasion by pathogens[1–3]. Oral vaccines used for prevention of diarrheal diseases caused by *Vibrio cholerae*, *Salmonella typhi* and Rotavirus[4] are commonly used for populations at risk. The only intranasal vaccine licensed for use in humans is against Influenza A and B[5] and other licensed vaccines for respiratory infections are administered parenterally. In this respect, there is much interest in the potential for enteral vaccination to elicit a more protective immune responses in the upper respiratory tract than parenteral vaccines.

Oral vaccines primarily interact with the immune system through the tonsils of Waldeyer's ring in the oral cavity or via the Peyer's patches in the small intestine. In addition, other structures in the GALT and isolated lymphoid follicles in the colon can also play a role in immune responses to oral vaccines. Oral vaccination is known to induce larger amounts of vaccine specific-IgA in the small intestine, colon, mammary glands, salivary glands and nasal cavity, than in the genital tract, tonsils and the lower respiratory tract[6–9]. Conversely, vaccination in the rectum and female genital tract mainly induces local vaccine specific-IgA production[10–12]. Part of this compartmentalization of the mucosal immune system may be explained by tissue-specific homing properties of B and T cells. Tissue-specific homing of lymphocytes is dependent on their expression pattern of homing receptors which bind addressins expressed on the endothelium of the target tissue[13]. Examples are binding of Integrin α4β1 to vascular cell adhesion molecule (VCAM)-1 and Integrin α4β7 to mucosal addressin cell adhesion molecule (MAdCAM)-1.[14] Furthermore, locally produced chemokines

(e.g. CCL25, CCL28) attract lymphocytes via their chemokine receptors (CCR9[15,16] and CCR10[14], respectively) and direct them into the tissue[17,18]. It is not known to what extent an oral cholera vaccine can induce a local mucosal immune response by modulating the homing profile of peripheral lymphocytes. Another factor that can determine lymphocyte trafficking and tissue homing is the location of the initial immune activation, as was recently described for plasmablasts[19,20]. In addition, homing is influenced by external factors, such as stromal factors and food components, such as retinoic acid[21,22].

The aim of this study was to evaluate whether oral cholera vaccination is able to induce potent IgA and IgG responses in serum, as well as at mucosal surfaces. In addition, the kinetics of these antibody responses and the tissue homing marker expression profiles of memory B cells (in contrast to plasmablasts as described recently)[19] toward airways, small intestine, colon, periphery and skin (Supplementary Figure S1) was investigated. This is to our knowledge the first study which investigated the kinetics of extensive homing profiles of memory B cells upon oral vaccination.

Material and Methods

Volunteers, specimens and sample collection

Healthy adult volunteers (total n=14, range 20-46 years, 25.07 ±6.53 years, 10 females, 4 males) gave informed consent to participate in this study. The study was approved by the Research Ethics Committee of Wageningen University and registered at www.clinicaltrials.gov with identification-number: NCT02238548. Volunteers, who had not previously been vaccinated with *Vibrio Cholera* or *E.coli*, were vaccinated with the oral cholera vaccine Dukoral® (EU/1/03/263/002, Crucell, Leiden, The Netherlands). The vaccine consisted of a total of 1.25×10^{11} formalin- and heat-killed *Vibrio cholerae* bacteria (strains O1 Inaba and O1 Ogawa) and 1 mg recombinant cholera toxin B-subunit (rCTB). Each individual received Dukoral® in the sodium carbonate buffer supplied with the vaccine.

Blood, nasal washes, saliva and fecal samples were collected at baseline on study day 0, and at day 7, day 14, day 18 and day 28 after the first vaccination

dose. On the vaccination days (days 0 and 14) biological samples were collected before the vaccine was given. Blood was collected in K_2-EDTA tubes (367525 BD, Franklin Lakes, NJ, USA) for peripheral blood mononuclear cell (PBMC) isolation and in serum tubes (BD 367895, Plymouth, United Kingdom) for serum collection. Serum was stored at -80˚C. PBMCs were isolated within 24 hours using 50 ml Leucosep tubes (227290, Greiner Bio-One, Alphen a/d Rijn, The Netherlands) filled with Ficoll plaque plus (17-1440-02, GE Healthcare Life Sciences, Uppsala, Sweden) according to manufacturer's protocol. Remaining PBMCs were cryopreserved and stored in liquid nitrogen.

For collection of nasal wash, volunteers were instructed not to use any nasal sprays from the evening before until the collection. For the nasal wash 3 ml pre-warmed saline (32˚C) was injected in each nostril and collected into a container. Containers were put on ice and processed within 2 hours after collection. Nasal wash samples were centrifuged at 3000xg for 10 minutes at 4˚C. Supernatant was stored at -80˚C.

For saliva collection volunteers were not allowed to ingest anything else than water for 1h preceding sampling. Ten minutes after rinsing the mouth with water, saliva was collected by passive drooling for 6 minutes. Saliva was collected, vortexed and stored at -80˚C.

Fecal samples were collected using a fecotainer (AT Medical BV, Enschede, The Netherlands) and stored at home at -20˚C and transported on dry ice to the research facility at the end of the study. Fecal extracts were prepared by adding 3.6 ml of PBS and 40µl Protease inhibitor (Sigma-Aldrich, Zwijndrecht, The Netherlands P8340) to 0,4 gram of feces, vortexing at least 30 seconds, and centrifuged at 3000x g for 10 minutes at 4˚C. Fecal extracts were stored at -80˚C until measurement.

T cell proliferation

To assess antigen-specific T cell proliferation (day 0, day 14, day 28), PBMCs were freshly isolated from blood, plated in a 24 wells culture plate (Costar 3526, Sigma-Aldrich, Zwijndrecht, The Netherlands; $1x10^6$ PBMCs/well) and stimulated with different stimuli for 7 days in IMDM medium (Gibco 31980-022, Grand Island, New York USA) supplemented with 5% Human

AB serum, 1% Pen/Strep, 0,2% Normocin (#ant-nr-1 Invivogen, San Diego, California USA). The stimuli were CTB 10 µg/ml (Sigma-Aldrich, C9903, Zwijndrecht, The Netherlands), Tetanus toxoid (TT) 5 lf/ml (Statens Serum Institut, Copenhagen, Denmark, kindly provided by W. van de Veen, SIAF Switzerland), Ovalbumin (OVA) 10 µg/ml (Invivogen 9006-59-1, Toulouse, France) and Dukoral® vaccine 10^8 bacteria/ml and 0,8 µg CTB/ml. After 7 days, supernatants were collected and stored at -80˚C for cytokine analysis using BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Kit, (BD Bioscience 560484, Franklin Lakes, New Jersey, USA). Cells were collected and analyzed on FACS CantoII for proliferation by staining cells for the following markers: anti-CD3-Pe-Cy5 (BD Pharmingen 555334, Franklin Lakes, New Jersey, USA), anti-CD19 BV510 (BD Horizon 562947, Franklin Lakes, New Jersey, USA), anti-Ki67-PE or Ki67 isotype control (BD Pharmingen 556027, Franklin Lakes, New Jersey, USA) and fixable viability dye V450 (Ebioscience 65-0863-18, Waltham, Massachusetts, USA).

Flow cytometric analysis of peripheral B cells

Freshly isolated PBMCs were stained with antibody panels and/or fluorescently labelled CTB to detect CTB-specific memory B cells, and analyzed by using multi-color flow cytometry. The selection of tissue specific homing marker profiles was done based on literature research and three antibody panels were composed, see Supplementary Table 1. For every panel, $3x10^6$ freshly isolated PBMCs were stained in a 96 well plate (NUNC PP Sigma-Aldrich 7116, Zwijndrecht, The Netherlands) for 30 minutes at 4˚C, washed with cold FACS buffer (PBS, 0,5% BSA, 3% FCS, 2mM EDTA, 0,05% NaN3) and centrifuged at 400xg for 3 minutes at 4˚C. Cells were analyzed at medium flow for 180s using a FACS Canto II. Due to technical issues, the FACS measurement had to be repeated for all day 0 samples, using defrosted PBMCs that had been stored in liquid nitrogen. The effect of freeze/thawing was examined on 5 donors at time point day 14 and most markers are slightly increased or equal after thawing (Integrin Beta 7, Integrin Beta 1, CCR10 and CLA), while only CCR9 is slightly lower expressed (data not shown). However, this did not compromise the results as most of these markers at day 0 resulted in lower number of cells with these homing profile compared to other time points and for CCR9 even higher numbers at day 0 compared to day 14. To exclude dead cells in these samples (day 0), specific B cells were stained with fixable viability dye V450

in PBS for 30 minutes. For the gut, airways and skin homing panel 3, DRAQ 7 (70250, Biostatus, Leicestershire, United Kingdom) was added as live/dead marker to exclude dead cells (day 0 only). The viability of cells was measured for specific B cells (panel 1) at all time points using fixable viability dye V450 and resulted in 100% alive cells as expected for fresh cells. This in combination with strict gating on preferably mutually exclusive markers as CD3 and CD19 should result in reliable data for all panels at all time points. For all panels fluorescent minus one (FMO) controls and isotype controls were included. This was done for: CTB (only FMO), CCR7, CLA, CCR10 and CCR9.

Flow cytometry data analysis was performed by using FlowJo software (version 10 TreeStar, Inc.) and gating was performed as is shown in Supplementary Figure S1. Data were exported as % of memory B cells and subsequently converted to 'number of homing memory B cells/million of memory B cells'.

ELISA

CTB- and Dukoral®- specific IgA and IgG antibodies in serum, saliva, nasal wash and fecal extract were determined by enzyme-linked immunosorbent assay (ELISA) in high binding 96 well plates (Greiner Bio one 655061, Monroe, North Carolina, USA) and performed as described previously[23]. In short, plates were coated with either 0.5 µg/ml CTB or $1x10^8$ bacteria + 0,8 µg/ml CTB from Dukoral®/ml PBS (Lonza Be17-516F, Basel, Switzerland) and incubated overnight at 4˚C. After washing with wash buffer (PBS + 0,05% Tween-20), plates were blocked with ELISA blocking buffer (Roche 11112589001, Mannheim, Germany) for 1h. For specific-IgA and IgG measurements serum was diluted in ELISA blocking buffer. Before diluting the samples nasal wash and saliva were centrifuged for 15 minutes at 1500xg and fecal extracts for 2 minutes at 1000xg. Nasal wash, saliva and fecal extracts were incubated for 15 minutes in High Performance Elisa (HPE) buffer (Sanquin M1940, Amsterdam, The Netherlands). Samples were added to the plate in duplicate and incubated for 2h. A pooled serum sample, from all vaccinated donors at day 18, was used to make a reference calibration line in all ELISAs and is expressed in arbitrary units/ml (AU/ml). After sample incubation, detection antibodies goat-anti-human IgA or IgG-horseradish peroxidase (HRP) (Southern Biotech 2050-05, 2040-05, Birmingham, Alabama, USA) diluted in Elisa blocking buffer (serum) or HPE buffer (saliva, nasal wash and fecal extracts samples) were incubated

for 1h. For development of all ELISAs TMB (SDT, Baesweiler, Germany) was added and the reaction was stopped by adding 2% HCL solution. Plates were read in a Filtermax at 450 nm minus 620 nm as reference value.

Statistics

Data were 10log transformed (ELISA) or logistically transformed (FACS) to obtain normally distributed data. A one-way repeated measures ANOVA was conducted using transformed data with IBM SPSS Statistics 23. Differences were considered significantly different when $p<0.05$. First, the assumption of sphericity was tested, using Mauchly's Test of Sphericity. If $p<0.05$, sphericity could not be assumed and hence, a Greenhouse-Geisser correction was applied to test within-subject effects (time). Only when a significant within subject effect was observed (time), a pairwise comparison between timepoints with a Bonferroni correction was made.

Results

Oral vaccination with Dukoral® induces vaccine-specific IgA and IgG antibodies

To measure the humoral response to oral cholera vaccination, vaccine-specific IgA and IgG, so Dukoral® and cholera toxin B (CTB)- specific antibodies, were measured in serum and mucosal fluids. The values are depicted as dot-plot in Figure 1. Vaccine-specific antibody levels in serum increased over time, with a maximum at day 28 post vaccination. Levels of both Dukoral® -specific IgA (Figure 1a) and cholera toxin B (CTB)-specific IgA (AU/ml) (Figure 1b) were significantly increased at day 18 and 28. Vaccine-specific IgG (Figure 1c and d) in serum was increased significantly at day 28. Dukoral®-specific IgA in fecal extracts and vaccine-specific IgA in nasal wash were just above the detection limit (0.12 AU/ml) and did not increase over time. Dukoral®-specific IgG was not detectable in fecal extracts. The concentration of vaccine specific IgG in nasal wash was significantly increased at day 28. Saliva samples were not available for all donors at all time points. Low levels of CTB-specific IgA were detected in saliva and showed no significant vaccination response. CTB-specific IgG in saliva was below the detection limit. In conclusion, vaccine-specific antibodies were induced in serum (IgA and IgG) and nasal wash (IgG) following oral vaccination

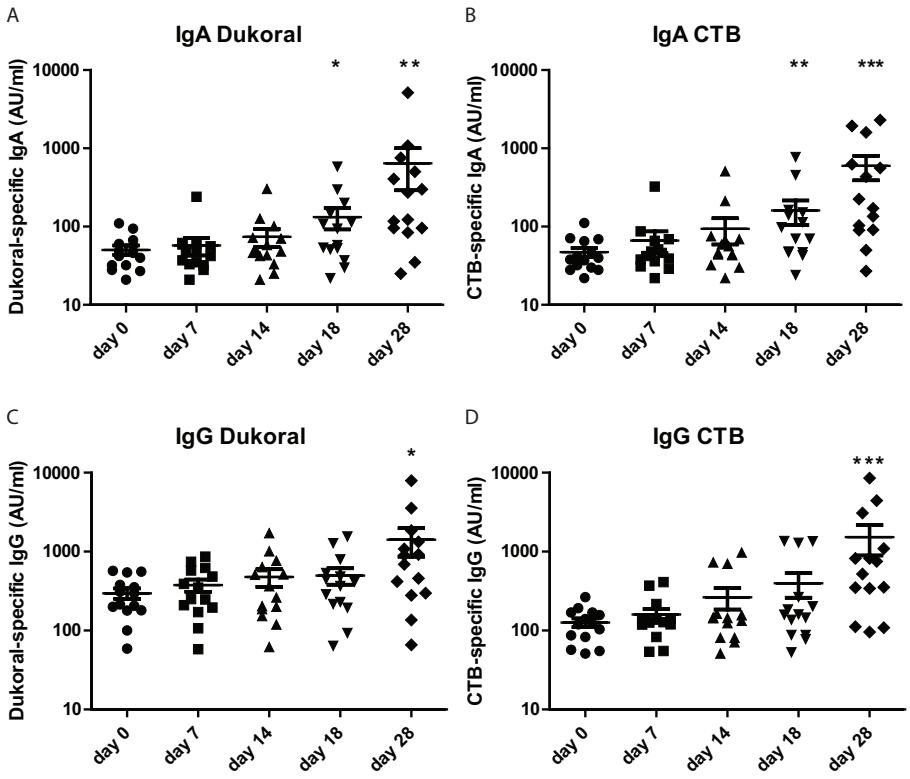

Figure 1: Serum of healthy volunteers (n=14) was measured on day 0, 7, 14, 18 and day 28. Dukoral-specific IgA (A), CTB-specific IgA (B), Dukoral-specific IgG (C) and CTB-specific IgG (D) was measured over time. Data are shown as dot plots with mean ± SEM in AU/ml. Serum IgA was diluted 75x, serum IgG 300x. Repeated measures ANOVA was performed after 10log transformation of the data. Stars indicate significant differences at time points compared to day 0 * = $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Cholera toxin B-specific memory B cells in blood increase after oral vaccination

In addition to measuring the levels of specific antibodies in serum, the induction of CTB-specific B cells in peripheral blood was investigated. The presence of IgA^+ and IgG^+ CTB-specific B cells in peripheral blood mononuclear cells (PBMCs) was analyzed by flow cytometry (Figure 2a). The frequency of CTB-specific IgA^+ as well as IgG^+ memory B cells significantly increased after oral vaccination and peaked at day 18 (Figures 2b and 2c).

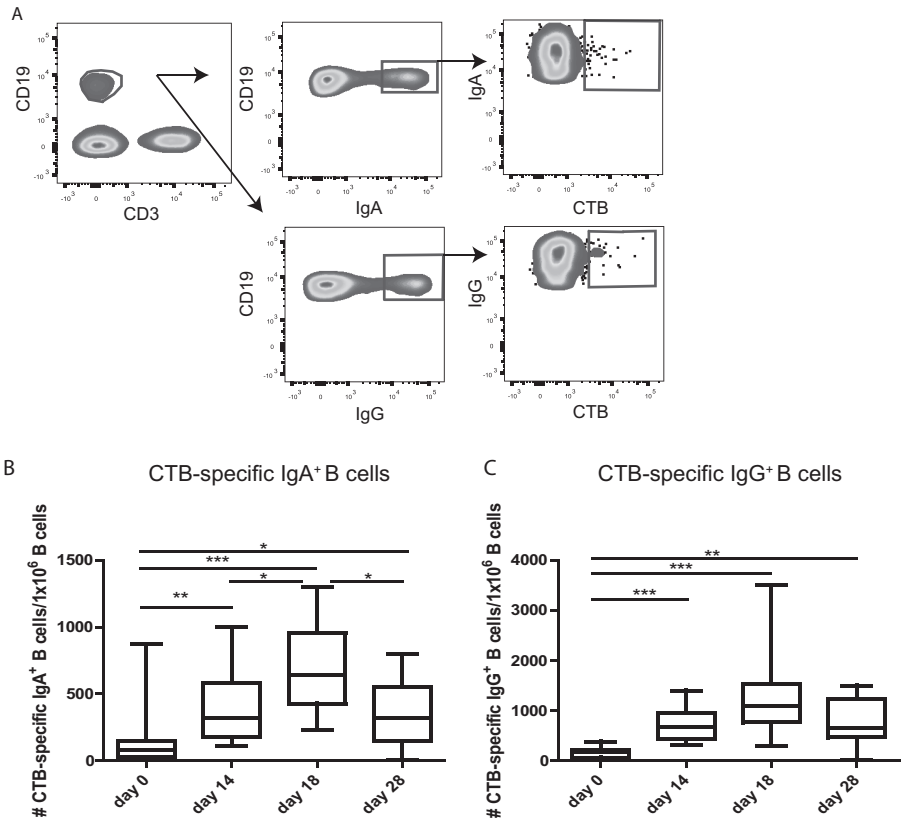

Figure 2: Kinetics of cholera toxin B (CTB)-specific memory B cells in peripheral blood. PBMCs of healthy volunteers (n=14) were stained and measured on day 0, 14, 18 and 28. CTB-specific IgA$^+$ and IgG$^+$ memory B-cells were analysed by flow cytometry (A) and are shown as #CTB-specific IgA$^+$ memory B cells/ 1x10^6 memory B cells at day 0, 14, 18 and 28 (B,C). Data are shown in Whisker-plots with a median, 25% and 75 % quartile and 5-95% confidence interval. Repeated measures ANOVA was performed * = p<0.05; ** p<0.01; *** p<0.001.

The B cells identified using this gating strategy are isotype-switched CD19$^+$ memory B cells. To compare the effect of oral vaccination on memory B cells with the effect on plasmablasts, we devised an alternative gating strategy to study plasmablasts (Supplementary Figure S2). Using this strategy the kinetics of IgG$^+$ as well as IgA$^+$ and IgG$^-$IgA$^-$ (IgM) plasmablasts were analysed. In contrast to the results shown for IgA$^+$ and IgG$^+$ memory B cells, the numbers of IgA$^+$ and IgG$^+$ plasmablasts in peripheral blood did not increase significantly after vaccination on the days studied. The number of IgA$^-$IgG$^-$ plasmablasts seemed to decrease after vaccination, but this decrease was also not significant.

Based on the fact that we only see a clear vaccination response in the memory B cells all further analyses were performed on the memory B cells.

Oral vaccination induces markers associated with homing to the colon on IgA$^+$ memory B cells and to the small intestine on IgA$^-$ memory B cells

In addition to the presence of CTB-specific memory B cells, the kinetics of memory B cells with homing marker profiles consistent for homing to different organs was analyzed with panels of antibodies for relevant homing markers (Supplementary Figure S1). In Figure 3, the overall gut homing potential of IgA$^+$ and IgA$^-$ memory B cells (CD19$^+$CLA$^-$ Integrin β7$^+$), based on Integrin β7$^+$ expression alone, was compared with chemokine receptor expression of CCR9 alone(CD19$^+$CLA$^-$CCR9$^+$) and CCR10 alone (CD19$^+$CLA$^-$CCR10$^+$). Furthermore, homing potential toward the colon (CD19$^+$CLA$^-$Integrinβ7$^+$CCR10$^+$CCR9$^-$) and small intestine (CD19$^+$CLA$^-$Integrinβ7$^+$CCR10$^-$CCR9$^+$) was compared by combining expression of integrin β7 and chemokine receptors. Upon vaccination, the percentage of IgA$^+$ memory B cells with overall homing potential to the gut, colon and small intestine, and also CCR9 or CCR10 expression alone, increased. Overall, the gut homing potential of IgA$^+$ memory B cells, based on Integrin β7$^+$ expression alone, was significantly increased, most profoundly at day 14. The clearest vaccination response for IgA$^+$ memory B cells was observed, when combining Integrin β7$^+$ expression with CCR10 as markers for colon homing potential, with a peak at day 18 ($p<0.001$, Figure 3b), which corresponded with a peak in the number of CTB-specific IgA$^+$ memory B cells. The same kinetics were observed when IgA$^+$ memory B cells were only gated for the expression of CCR10. When combining Integrin β7$^+$ expression of IgA$^+$ memory B cells with CCR9, a significant increase in homing potential to the small intestine was observed at day 14, while this increase was smaller for CCR9 expression alone. For IgA$^-$ memory B cells, the kinetics and tissue selectivity of the vaccination-induced homing response were different. Homing potential of IgA$^-$ memory B cells toward the colon was significantly increased at day 18 and a similar trend was observed for single CCR10$^+$ IgA$^-$ memory B cells. Homing potential toward the small intestine was much more profound for IgA$^-$ memory B cells as compared to IgA$^+$ memory B cells, with a peak at day 18, a trend also observed for single CCR9$^+$ IgA$^-$ memory B cells.

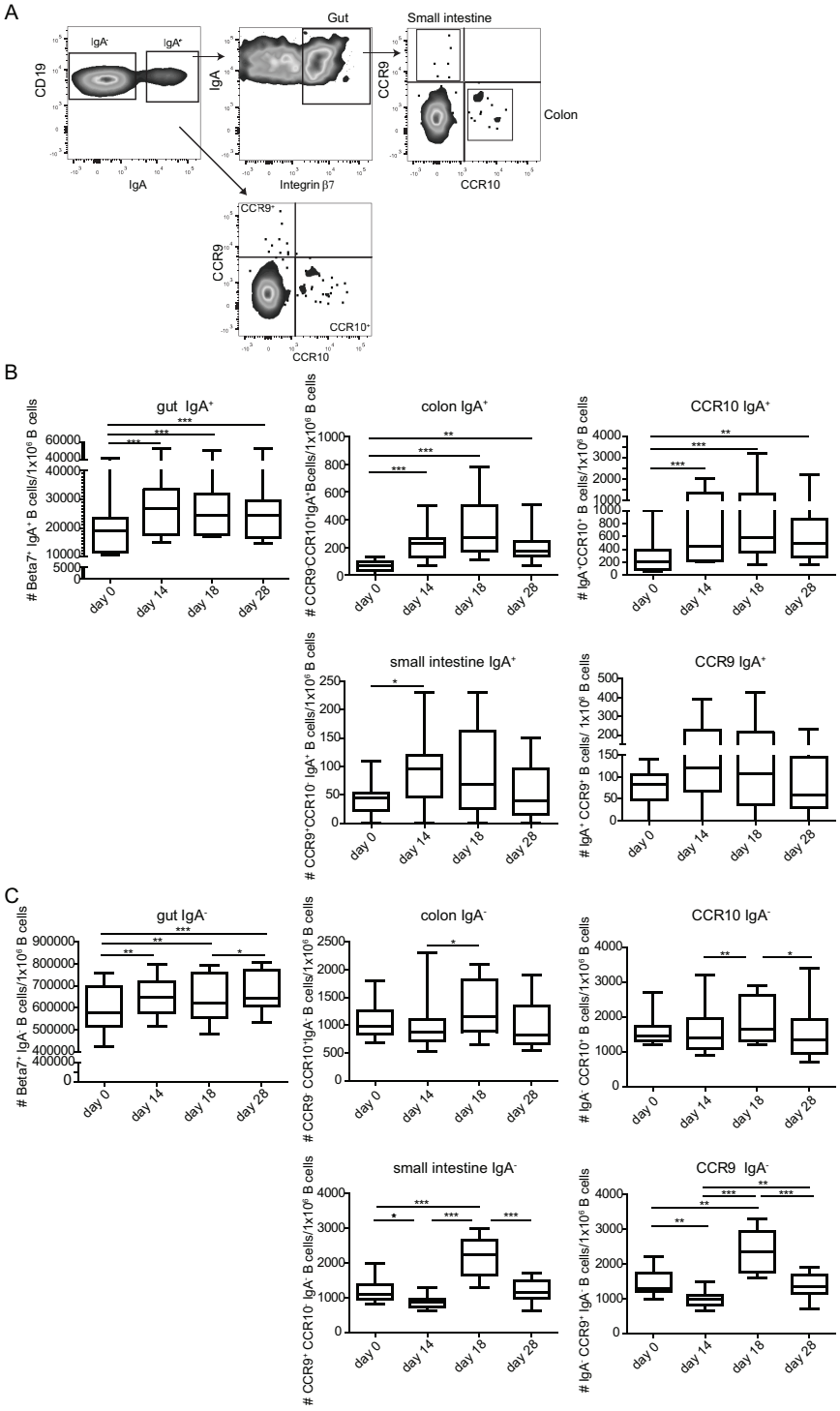
A
IgA-
IgA+
CD19
IgA
Gut
IgA
Integrin β7
Small intestine
CCR9
CCR10
Colon
CCR9+
CCR10+
B
gut IgA+
Beta7+ IgA+ B cells/1x10^6 B cells
colon IgA+
CCR9+CCR10-IgA+B cells/1x10^6 B cells
CCR10 IgA+
IgA+CCR10+ B cells/1x10^6 B cells
small intestine IgA+
CCR9+CCR10- IgA+ B cells/1x10^6 B cells
CCR9 IgA+
IgA+ CCR9+ B cells/ 1x10^6 B cells
day 0
day 14
day 18
day 28
C
gut IgA-
Beta7+ IgA- B cells/1x10^6 B cells
colon IgA-
CCR9 CCR10+IgA- B cells/1x10^6 B cells
CCR10 IgA-
IgA- CCR10+ B cells/1x10^6 B cells
small intestine IgA-
CCR9+ CCR10- IgA- B cells/1x10^6 B cells
CCR9 IgA-
IgA- CCR9+ IgA- B cells/1x10^6 B cells

Figure 3: Kinetics of IgA^+ and IgA^- memory B-cells with a gut, colon and small intestine homing potential or expression of CCR9 alone and CCR10 alone. PBMCs of healthy volunteers (n=14) were analysed by flow cytometry on day 0, 14, 18 and 28. A) Gating strategy used to differentiate between IgA^+ (B) and IgA^- (C) memory B cells with a gut ($CD19^+CLA$-Integrin$\beta7$+), colon ($CD19^+CLA$-Integrin$\beta7$+CCR10+CCR9-), small intestine ($CD19^+CLA$-Integrin$\beta7$+CCR10-CCR9+), $CCR9^+$ alone ($CD19^+CLA$-CCR9+) and CCR10+ alone($CD19^+CLA$-CCR10+) homing profile. Data are shown in Whisker-plots with a median, 25% and 75 % quartile and 5-95% confidence interval. Repeated measures ANOVA was performed * = $p<0.05$; ** $p<0.01$; *** $p<0.001$.

The total number of IgA^+ and IgA^- memory B cells with distinct homing profiles on day 18 was higher compared to the number of CTB-specific memory B cells. This can be explained by the fact that the response to CTB represents only part of the total response to the vaccine that also contains formalin- and heat-killed *Vibrio cholerae* bacteria.

In conclusion, the homing potential of memory B cells toward the colon and small intestine increased upon oral vaccination with Dukoral®, with a distinct preferential increase of IgA^+ memory B cells expressing a colon homing profile and of IgA^- memory B cells expressing a small intestine homing profile.

Oral vaccination induces homing to airways with different kinetics for IgA+ and IgA- memory B cells

As oral vaccines are in contact with the oral mucosa and tonsils in the Waldeyer's ring, it was evaluated whether oral cholera vaccination also induced homing of IgA^+ and IgA^- memory B cells to the airway mucosa. Oral vaccination induced an increased airway homing potential of peripheral IgA^+ memory B cells, ($CD19^+CLA^-$Integrin$\beta1^+\beta7^-CCR10^+$) already at day 14, with a peak at day 18 (Figure 4). In IgA^- memory B cells, the strongest increase of cells with airway homing potential was observed on day 14.

Distribution of homing potential of lymphocytes at day 0 and day 18

Additionally, we analyzed the effect of the cholera vaccine on distribution of homing potential to the periphery (lymph nodes (LN) and spleen, CLA^- IgA^+/IgG^+CCR7^+) (Supplementary Figure S3) and skin ($CLA^+IgA^{+/-}$Integrin $\beta1^+$ integrin $\beta7^-CCR10^+$) (Supplementary Figure S4). Homing potential of memory B cells to the LN and spleen was significantly increased at day 18 for IgA^+ memory B cells and at day 28 for both IgA^+ and IgG^+ memory B cells. The number of IgA^- memory B cells with skin homing potential did not significantly

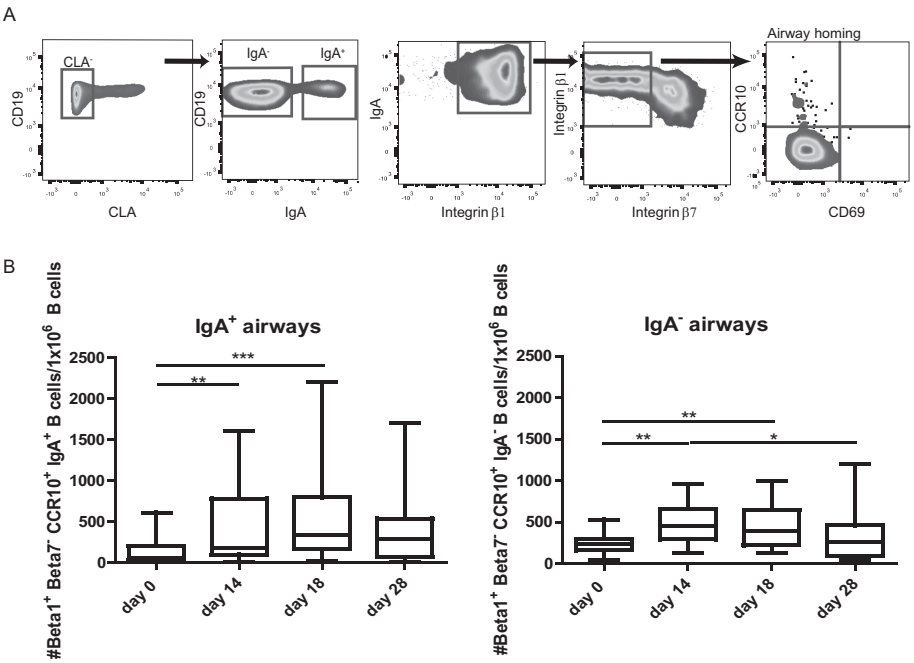

Figure 4: Kinetics of IgA$^+$ and IgA$^-$ memory B cells with an airways homing potential. PBMCs of healthy volunteers (n=14) were analysed on day 0, 14, 18 and day 28, using flow cytometry. Gating strategy (A) and kinetics (B) of IgA$^+$ and IgA$^-$ memory B cells with homing potential to airways (CD19$^+$CLA$^-$ Integrinβ1$^+$β7$^-$CCR10$^+$). Data are shown in Whisker-plots with a median, 25% and 75 % quartile and 5-95% confidence interval. Repeated measures ANOVA was performed * = p<0.05; ** p<0.01; *** p<0.001.

change over time (Supplementary Figure S4). This was in contrast to IgA$^+$ memory B cells, which had a significantly increased skin homing potential from day 14 onwards.

Both the number of CTB-specific memory B cells and specific homing profiles peaked at day 18. For each homing profile, the number of memory B cells was plotted at day 0 and day 18 as relative percentage (r%) to compare the relative contribution per homing profile (Figure 5). The total percentage (t%) of IgA$^+$ memory B cells with a distinct homing profile at day 0 was 1.77 t%, while at day 18 this increased to 2.17 t%, resulting in a 1.22-fold increase (Figure 5a). IgA$^-$ memory B cells had a lower percentage of cells with a homing potential at day 0 (1.53 t%) while this was 2-fold increased at day 18 to 3.06 t%. The majority of the memory B cells had a homing potential toward the periphery at both day 0 and day 18 (84-99 r%) (Figure 5a-b). In the mucosal tissues (small intestine, colon and airways), the relative number of IgA$^+$ memory B cells with homing

potential toward these organs were increased 3.8-fold (Figure 5c). The largest relative increase of IgA$^+$ memory B cells was found for homing potential toward airways (from 36 to 50 r%), followed by colon (from 38 to 40 r%), whereas small intestine showed a decrease (from 26 to 10 r%). As the total frequency of IgA$^+$ memory B cells was increased, the number of IgA$^+$ memory B cells with a homing potential toward small intestine, colon and airways per million of memory B cells was calculated for day 0 and day 18. Indeed, Figure 5e shows that there was a large increase in number of IgA$^+$ memory B cells with homing potential toward airways (5.3-fold), followed by colon (4.0-fold) and skin (3.5-fold) whereas a small increase was observed for small intestine homing potential (1.5-fold) and peripheral homing (1.19-fold).

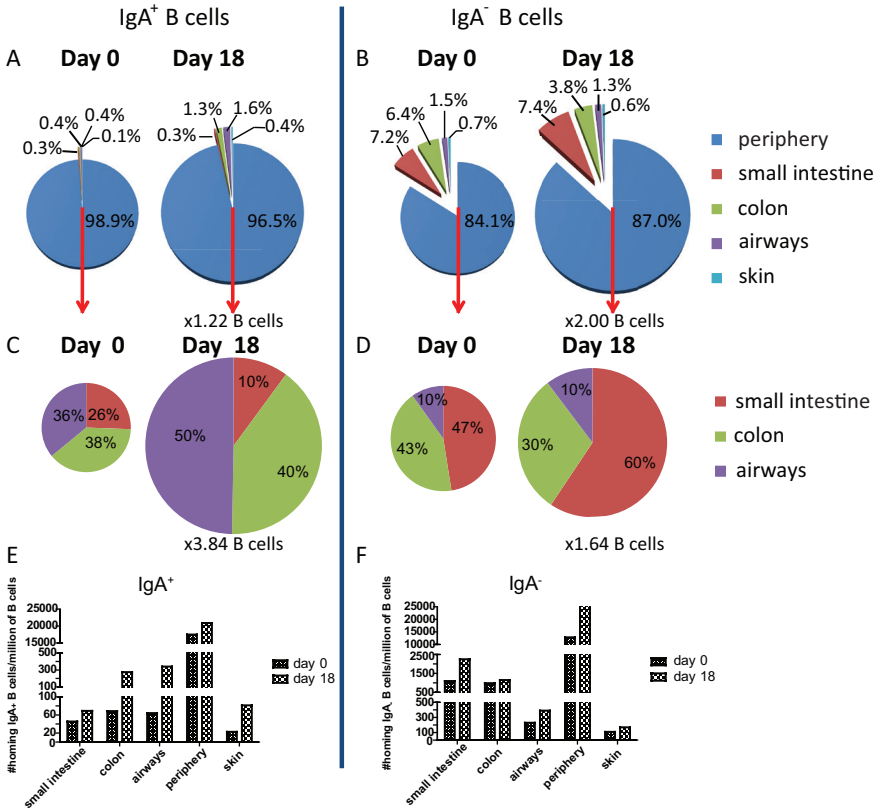

Figure 5: Distribution of homing potential of IgA$^+$ and IgA$^-$ memory B cells at day 0 and day 18. In the piecharts (A-D) relative percentages (r%) of homing potential of periphery, small intestine, colon, airways and skin (together 100%) are depicted for IgA$^+$ memory B cells (A) and IgA$^-$ memory B cells (B). Figures C and D show the distribution in relative percentage (r%) of homing potential toward small intestine, colon and airways only (together 100%) at day 0 versus day 18. The increase in total number of cells at day 18 is shown as x1.22-3.84 B cells under the piechart. In Figures E and F, the number of cells with a homing potential toward small intestine, colon, airways, periphery and skin is given (#homing potential cells/10^6 memory B cells) at day 0 and day 18.

Remarkably, IgA^- memory B cells showed a completely different profile. Only 10 r% of the IgA^- memory B cells had an airway homing potential both at day 0 and day 18 (Figure 5d). Colon homing potential decreased from 43 r% to 30 r%. Small intestine homing potential strongly increased to 60 r% at day 18 compared to 47 r% at day 0. Of all IgA^- memory B cells expressing homing receptors, 90 r% had a gut homing potential on days 0 and day 18, while this was only around 60 r% for IgA^+ memory B cells. At day 18 after vaccination, the number of IgA^- memory B cells per million of memory B cells with specific homing potential for mucosal sites increased 2-fold for the small intestine , 1.7–fold for the airway, 1.6-fold for the skin and 1.2 fold for the colon (Figure 5f).

Oral Cholera vaccination does not increase vaccine-specific T cell proliferation

To investigate whether vaccine-specific T cell responses were increased in response to oral cholera vaccination PBMCs were stimulated for 7 days in the presence of the vaccine antigens CTB and Dukoral®, control antigen ovalbumin (OVA) or recall antigen tetanus toxoid (TT). A recall antigen is used as control to elicit a specific immune response in previously (Tetanus) vaccinated subjects. After 7 days, T-cell proliferation was measured by staining the cells for expression of the proliferation marker Ki67 (Figure 6). After stimulation with CTB no increased T cell proliferation was observed compared to medium. When Dukoral® and TT induced proliferation was compared to medium controls, there was a highly significant difference at all time points ($p<0,001$). This was already the case at day 0, also for Dukoral®, which is caused by cross reactivity as the patients were not vaccinated yet. When Dukoral-specific proliferation was followed over time and compared to Dukoral stimulation at day 0, no significant differences between the time points were observed, albeit day 14 seems to have increased T cell proliferation ($p= 0,2$).

Additionally no significant increase in cytokine production (IFN-y, IL-5, IL-13, IL-10 and IL-17) was measured in vaccine antigen stimulated T-cells compared to the medium and OVA controls (data not shown). Thus, we concluded that oral cholera vaccination had no vaccine–specific effects on T cell proliferation and cytokine production at the time points analysed.

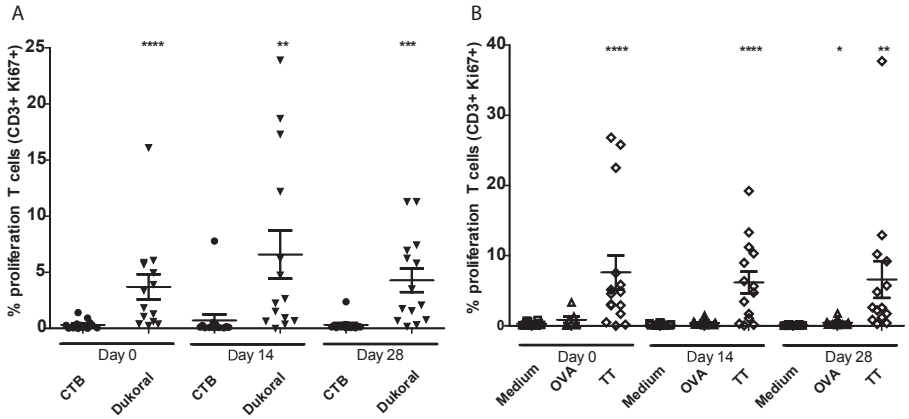

Figure 6: Antigen-specific T cell proliferation. PBMCs of healthy volunteers (n=14) were collected on day 0 and 14 before receiving Dukoral vaccination, and at day 28. PBMCs were stimulated for 7 days with A) Cholera toxin B (10 µg/ml) or Dukoral (10^{8} ® bacteria/ml) or B) IMDM medium, Ovalbumin (OVA)10 µg/ml or tetanus toxoid (TT) (5 lf/ml). Proliferating T-cells ($CD3^{+}$ $Ki67^{+}$) were analysed by flow cytometry. Data are shown as mean +/- SEM. Repeated measures ANOVA was performed per stimulation, no significant differences found. For the comparisons between stimulations and medium control per day a univariate ANOVA (GLM) was used, * = p<0.05; ** p<0.01; *** p<0.001.

Discussion

To our knowledge, this is the first study that documents the kinetics and detailed tissue homing preference of memory B cells following oral cholera vaccination. Oral vaccination induced Dukoral®- and CTB-specific IgG and IgA responses in serum, and enhanced the number of circulating memory B cells expressing marker profiles associated with homing to small intestine, colon, airways, skin and periphery. The number of circulating CTB-specific memory B cells as well as memory B cells with distinct tissue homing profiles peaked on day 18. Interestingly, IgA^{+} memory B cells preferentially expressed markers that enable homing to the airways and to the colon, while IgA^{-} (mainly IgG^{+}) memory B cells primarily expressed markers associated with homing to the small intestine.

The amounts of Dukoral- and CTB-specific serum IgA and IgG increased until day 28 post-vaccination. Other studies with this vaccine reported an earlier peak in the vaccine-specific antibody responses (day 18 to 21)[7,24,25]. This is not caused by a difference in age range or gender ratio of the study groups. [26,27]

In contrast to serum IgG and IgA levels, the number of circulating CTB-specific memory B cells in blood peaked at day 18, which is four days after the second oral vaccination, and decreased at day 28. This decrease in circulating cholera-specific memory B cells at day 28 may indicate that these cells have entered peripheral mucosal tissues, whilst still contributing to serum IgA and IgG levels by producing these antibodies locally.

Rudin et al.[7] and Leach et al.[25] observed similar kinetics of vaccine-specific antibody producing B cells in peripheral blood, but Rahman et al.[28], observed a peak at day 7 that was absent at day 21. While the first two studies were performed in Sweden, the study by Rahman et al. was performed in Bangladesh. As cholera is endemic in Bangladesh but not in Europe, the kinetics in the study by Rahman might thus reflect a memory response rather than a primary response, explaining the different kinetics seen between these studies.

In nasal washes, saliva and fecal extracts, the amount of vaccine-specific IgA antibody was low and no significant increase was detected after vaccination. The levels of vaccine-specific IgG in nasal washes were low but significantly increased at day 28. The finding that larger amounts of vaccine-specific IgG were measured in the serum and nasal washes than vaccine-specific IgA is in line with previous reports[7,24,25,29]. We speculate that this might be due to uptake of the oral vaccine in the lymphoid tissue of the palatine tonsils, resulting in activation of larger numbers of IgG plasma cells (55-72%) than IgA plasma cells (13-18%), which will preferentially home toward the respiratory tract.[30] This will lead to more vaccine specific IgG in the nasal cavity compared to vaccine-specific IgA. Besides, it is known that the nasal mucosa harbours the largest number of IgG plasma cells compared to other mucosal tissues, even though in steady state more IgA plasma cells are present locally compared to IgG plasma cells.[14]

In addition to studying the effect of oral vaccination on vaccine-specific memory B cells and antibody production, we investigated whether oral vaccination would also affect tissue homing potential of B cells. Tissue homing-related molecules such as selectins, integrins, chemokines and chemokine receptors can be expressed on several different cell types as well as in different tissues. For this reason a combination of several homing markers rather than single

markers is needed to define distinct tissue homing properties. In this study we focussed on integrin β1, integrin β7, CCR9, CCR10 and CLA to define homing to gut, airways, skin, small intestine and colon.[15–18,31–37]

CCR10 can bind to CCL28 that is expressed in the respiratory tract together with VCAM-1[35,36], but also in the colon, tonsils and lactating mammary glands.[14,16] In addition, CCR10 can also bind to CCL27 produced in the skin.[17,34,38,39] Homing to the gastrointestinal tract can be specified to colon versus small intestine on the basis of expression of CCR10 and CCR9. The ligand of CCR9, CCL25 is expressed both in the small intestine as well as in the thymus.[40] Therefore, expression of CCR10 and CCR9 cannot be used to define tissue homing preference of lymphocytes without studying additional markers. For differentiating between markers related to homing to the skin, small intestine, colon, and airways, expression of Integrin β1 and integrin β7, and CLA were also measured. Integrin β1 and integrin β7 represent the expression of the heterodimer integrin α4β1[41,42] and α4β7[20,43] leading respectively to homing potential to non-intestinal ($\alpha4\beta7^-$, $\alpha4\beta1^+$) and intestinal tissues ($\alpha4\beta7^+$). The homing profiles of B cells that can home to airways and skin are quite comparable, and are based on expression of CCR10 in combination with α4β1 without α4β7. They are differentiated by expression of the skin homing marker CLA. CLA can bind E-selectin, which is expressed in the skin, as wells as on oral mucosa and inflamed tissues.[18,32,33] However, even in the presence of CLA, homing to airways cannot be excluded. As recently shown by Seong et al., CLA^+ plasmablasts are able to migrate into the oral cavity under inflammatory conditions, when E-selectin expression is upregulated. Furthermore, patients suffering from upper respiratory tract infections show a strong increase in CLA and CD62L expression[19]. As the present study was performed in healthy donors we have made the assumption that CLA expression in this study does not reflect homing to the oral cavity.

To date, no markers are known for B cells to distinguish between homing to the lower and upper respiratory tract, even though CCR3 has been described as a marker for differential T cell homing to the nasal mucosa[37]. In addition to the markers we used, the use of additional markers like CCR3 and GPR15[19,37] in even more extensive homing panels will help to further specifying tissue homing potential of circulating lymphocytes in future studies.

The tissue homing potential of the memory B cell populations were characterized using the homing marker combinations designed for airways, small intestine, colon, periphery and skin (Supplementary Figure S1). Cholera vaccination enhanced the number of circulating memory B cells for all tissue homing marker profiles with kinetics following the kinetics as seen in the CTB-specific memory B cells, suggesting a vaccination induced expression of distinct homing profiles.

Interestingly, IgA^+ memory B cells preferentially expressed a homing marker profile associated with homing to the airways ($CLA^-Integrin\beta1^+$ $Integrin\beta7^-$ $CCR10^+$), colon (CLA^- $Integrin\beta7^+$ $CCR10^+CCR9^-$), and even to the skin (CLA^+ $Integrin\beta1^+$ $Integrin\beta7^-$ $CCR10^+$) (Figure 5). All of these homing profiles are linked to expression of CCR10. In contrast to IgA^+ memory B cells, IgA^- memory B cells showed homing marker profiles consistent with homing toward the small intestine (CLA^- Integrin $\beta7^+$ $CCR10^-CCR9^+$; Figure 5). It should be noted however, that this conclusion is based on the expression of tissue homing markers on peripheral blood lymphocytes, and do not formally demonstrate homing of these cells to these peripheral tissues. As stromal factors, such as retinoic acid[44], determine tissue homing properties of locally activated lymphocytes, it has been suggested that antigens taken up in the tonsils of Waldeyer's ring will induce the formation of especially IgG-antibody producing memory B cells with homing capacity to lungs, upper airways and peripheral blood.[30] In contrast, IgA^+ B cells induced in the Peyer's patches are thought to preferentially home to the intestine, specifically the small intestine[14,45]. This hypothesis is in line with the antibody production detected in nasal wash (mainly IgG) and fecal water (mainly IgA). However, this hypothesis does not explain the observation of IgA^+ memory B cells homing preferences to airways or colon, and of IgA^- memory B cells homing to the small intestine, respectively. Although we have no evidence on where the B cells were activated after vaccination, a possible explanation could be that the IgA^- memory B cells we detect in blood were induced in the PP and are instructed to home to the small intestine under the influence of stromal factors, and that the IgA^+ memory B cells that can home to the airways or colon were induced elsewhere, either in Waldeyer's ring or in the isolated follicles of the colon. If the vaccine induces a proinflammatory response in PP, it may induce IgG there, whereas a more regulatory-biased IgA response to the vaccine is induced in Waldeyers ring or colon.

CD19 is maintained on peripheral B-cells, including memory B-cells, but also on plasmablasts and subsets of plasma cells. Based on CD19 expression only, memory B-cells will also include a small number of $CD19^+$ plasmablasts[46]. Subsets of plasmablasts and plasma cells are further defined by the level of expression of CD38 and CD138. The CD38hi subset of peripheral B-cells comprises both newly-generated plasmablasts and plasma cells that recirculate from the bone marrow.

The B cells in the present study are primarily $CD19^+$ memory B cells, as shown in Supplementary Figure S2. The data are thus complementary to the homing potential of CD19- plasmablasts that were described recently[19]. Our data on memory B cells also differs from earlier work in relation to the kinetics of CTB-specific plasmablasts[47]. In this study CTB-specific spot forming cells were studied after oral vaccination against cholera. The number of CTB-specific cells peaked at day 7 and day 21, and were almost at background levels at day 14 and 28. This is in contrast to our results where we have a peak at a similar moment, but at day 14 and day 28 the number CTB-specific IgA and IgG memory B cells is clearly increased compared to day 0. This would imply that CTB-specific memory B cells remain in circulation for a prolonged period compared to plasma cells. Likewise, the expression of homing marker integrin β7 also seems to differ between memory B cells. After oral vaccination with *Salmonella typhi* Ty21a 100% of the IgA, IgG and IgM plasmablasts express α4β7[20]. This is very much in contrast with our findings after cholera vaccination, where only 2,5% (% of all memory B cells) of the IgA^+ memory B cells are positive for β7 expression and 65% (% of all memory B cells) of the IgA^- memory B cells expressed β7 after oral cholera vaccination. This difference is not caused by β7 expression alone, as β7 expression was compared to α4β7 expression in this study and all the differences in β7 expression was due to α4β7 expression[20]. A possible explanation is the differences in the vaccine used. *Salmonella typhi* Ty21a may be a more potent inducer of gut homing than the cholera vaccine.

In addition to the paper of Kantele et al. we measured CCR9 and CCR10 in combination of α4β7 to be able to distinguish potential homing toward the colon and small intestines for IgA^+ and IgA^- memory B cells. That the combination of the markers β7, CCR10 and CCR9 is able to distinguish

between colon and small intestine homing potential is confirmed by another study[48]. In this study both IgA^+ and IgA^- plasmablasts in healthy donors had a higher level of CCR9 expression compared to CCR10 expression. However, during severe Ulcerative Colitis CCR10 is upregulated in the $\alpha4\beta7^+$ IgA^+ and IgA^- plasmablasts. This is in contrast with our finding that upon oral cholera vaccination IgA^+ memory B cells have a higher $\alpha4\beta7$ $CCR10^+$ (colon) expression and IgA^- memory B cells have a $\alpha4\beta7$ $CCR9^+$ (small intestine) expression.

Despite consistent induction of humoral immune responses in blood and mucosal fluids, we were not able to detect significant increases in vaccine-specific T cell proliferation and cytokine production after oral vaccination as reported by others[49]. However, one study reported the induction of T cell proliferation following oral cholera vaccination[50]. One possible explanation is that the T cell proliferation takes place in the draining lymph nodes, and that the recirculation of the T cells in peripheral blood peaks at different time points than the selected study days (day 0, 14 and 28). As tissue homing of non-B cells (mainly T cells) followed comparable kinetics compared to B cells (data not shown), the peak of recirculating vaccine-specific T cells may have been at day 18 rather than at day 14 or 28.

Taken together, this study demonstrates that oral cholera vaccination not only induces humoral immunity in the gastrointestinal tract, but also in other mucosal locations, such as the respiratory tract. Therefore, oral vaccination could be considered as an option for vaccination against respiratory pathogens. This may be an obvious route of vaccination, as food components such as 1,25-dihydroxyvitamin D3 can induce expression of CCR10 in human B cells and thereby direct B cells to mucosal sites, including lungs.[39] Furthermore, retinoic acid (RA), a vitamin A metabolite, is known to induce immune cells to express CCR9, thereby promoting homing to the small intestine.[21,22] The immunomodulating properties of vitamin A and D could be part of the overall gut-lung axis framework.[51] In this 'gut-lung axis'-theory the interplay between diet, gut microbiota and improved respiratory health by migrating immune cells is linked together, although the exact mechanisms are not yet fully understood.[52–55] Oral cholera vaccination may therefore also be a useful model to study the immunomodulatory capacity of (food) components.

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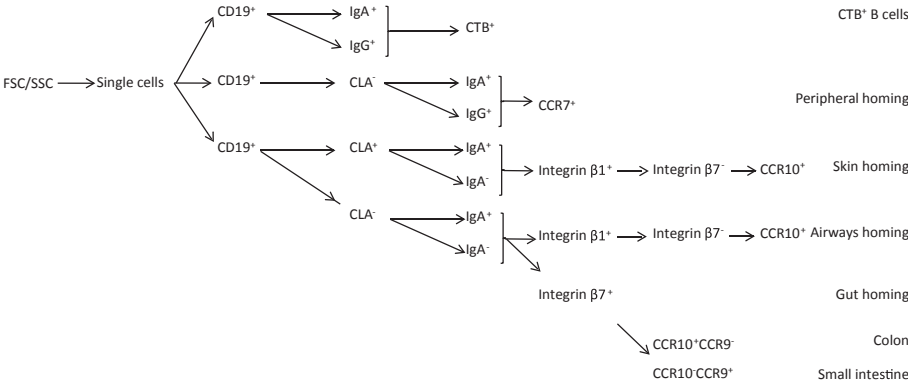
Supplementary Data

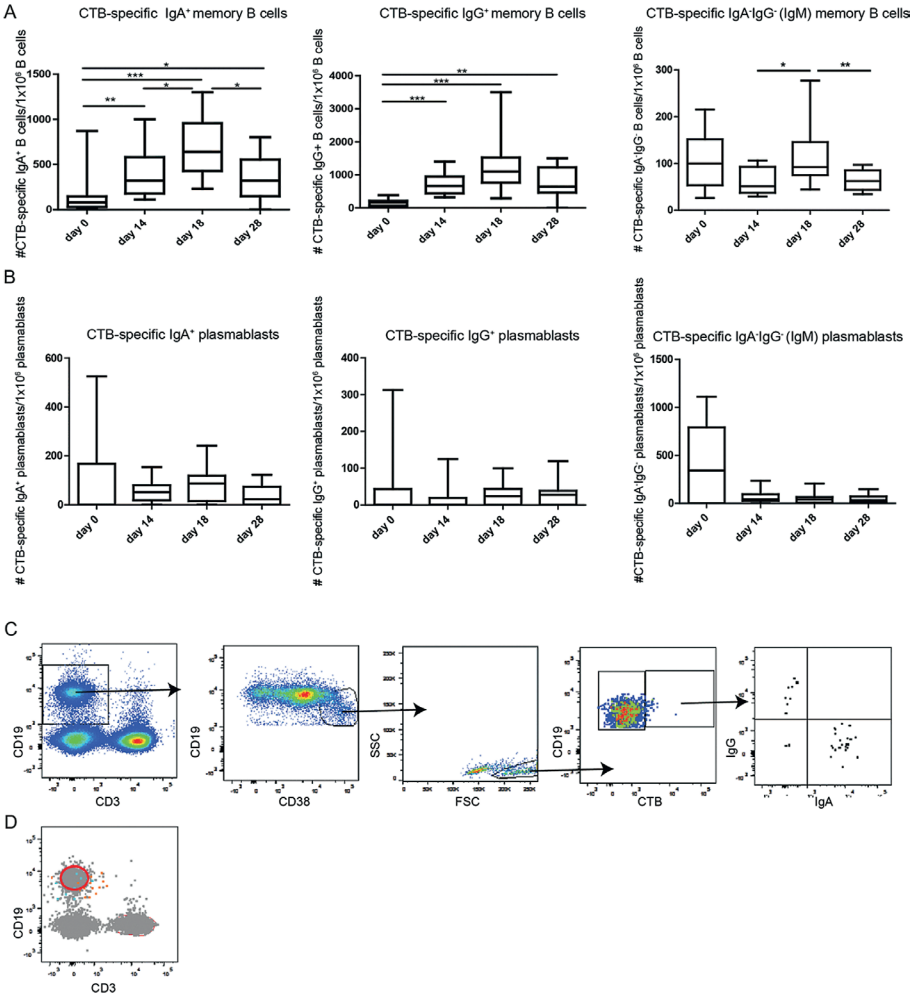
Supplementary Table 1: Antibodies used to analyze PBMCs for CTB-specific B cells (panel 1); periphery homing (panel 2) and homing to airways, gut, colon, small intestine and skin (panel 3).

Antibody	Fluor-chrome	host	isotype	light chain	clone	company	catalog number	panel
Cholera toxin B	Alexa Fluor 488					Thermo Fisher Scienctific*	C22841	1
α-CD19	BV510	mouse	IgG1	κ	SJ25C1	BD Horizon*	562947	1,2,3
α-CD3	PE-Cy5	mouse	IgG1	κ	UCHT1	BD Pharmingen*	555334	1,2
α-IgA	APC	mouse	IgG1	κ	G18-145	Miltenyi*	130-093-113	1,2,3
α-IgG	PE-cy7	mouse	IgG1	κ	G18-145	BD Pharmingen*	561298	1
α-CD38	PE	mouse	IgG1	κ	HIT2	BD Pharmingen*	555460	1
α-IgG	FITC	mouse	IgG1	κ	G18-145	BD Pharmingen*	561298	2
α-CCR7 (CD197)	PE-cy7	Rat	IgG2	κ	3D12	BD Pharmingen*	560922	2
α-CLA	BV421	Rat	IgM	κ	HECA-452	BD Horizon*	563961	2,3
α-CCR10	PE	mouse	IgG2a	κ	1B5	BD Pharmingen*	563656	2,3
α-Integrin β7	PE-cy7	Rat	IgG2a	κ	FIB504	Ebioscience*	25-5867	3
α-Integrin β1 (CD29)	PE-CY5	mouse	IgG1	κ	MAR4	BD Pharmingen*	559882	3
α-CCR9	Alexa Fluor 488	mouse	IgG2a	κ	112509	BD Pharmingen*	561608	3

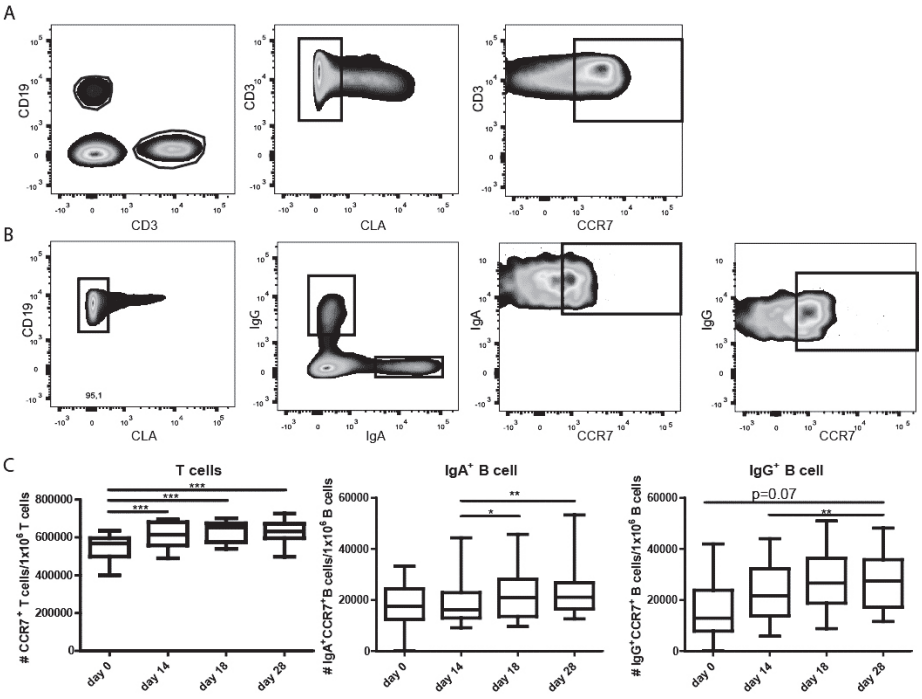
Company affiliations(*) are Thermo Fisher Scientic/Ebioscience(Waltham, Massachusetts, USA); all BD companies (Franklin Lakes, New Jersey, USA) and Milteny (Bergisch Gladbach, Germany)



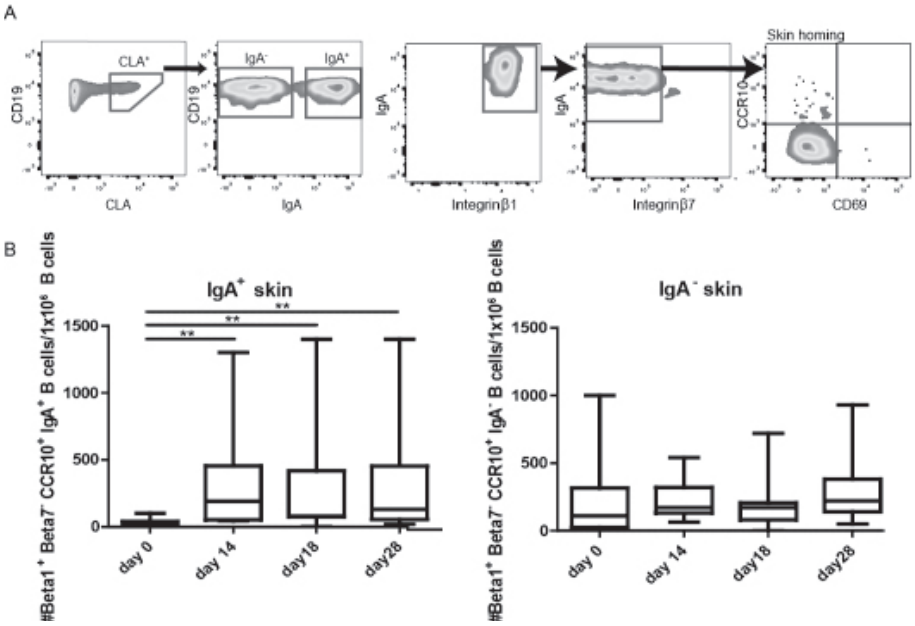
Supplementary Figure S1: Gating strategy of specific B cells and homing potential of IgA^+ and IgA^- B cells towards periphery, skin, airways, gut or colon and small intestine.



Supplementary Figure S2: Kinetics of cholera toxin B (CTB)-specific memory B cells versus plasmablasts in peripheral blood. PBMCs of healthy volunteers (n=14) were stained and measured on day 0, 14, 18 and 28. CTB-specific IgA^+, IgG^+ and IgA^-IgG^- (IgM) memory B-cells were analysed by flow cytometry (A) and are shown as # CTB-specific IgA^+ (memory) B cells/ $1x10^6$ (memory) B cells at day 0, 14, 18 and 28. CTB-specific IgA^+, IgG^+ and IgA^-IgG^- (IgM) plasmablasts were analysed by flow cytometry (B,C) and are shown as # CTB-specific IgA^+ plasmablasts/ $1x10^6$ plasmablasts at day 0, 14, 18 and 28. Figure S2d shows that IgG (blue) and IgA (orange) plasmablasts are mostly excluded in the CD19 gate used for memory B cells. Data are shown in Whisker-plots with a median, 25% and 75 % quartile and 5-95% confidence interval. Repeated measures ANOVA was performed * = $p<0.05$; ** $p<0.01$; *** $p<0.001$.



Supplementary Figure S3: Peripheral homing potential of T and B cells. Gating strategy is shown for T-cells (A) and B cells (B). Before CD3 and CD19 gating, PBMCs were gated using FSC/SSC and single cells gating. PBMCs were analysed by flow cytometry and the number of $CCR7^+$ T (or B) cells/million T (or B) cells is shown per study day (C). Data are shown in Whisker-plots with a median, 25% and 75 % quartile and 5-95% confidence interval. Repeated measures ANOVA was performed * = $p<0.05$; ** $p<0.01$; *** $p<0.001$.



Supplementary Figure S4: Kinetics of IgA$^+$ and IgA$^-$ B cells with skin homing potential. PBMCs of healthy volunteers (n=14) were analysed on day 0, 14, 18 and day 28, before receiving Dukoral vaccination at day 0 and day 14, using flow cytometry. A) Gating strategy of IgA$^+$ and IgA$^-$ B cells with skin (CD19$^+$ CLA$^+$ Integrinβ1+Integrin β7-CCR10$^+$) homing potential. Data are shown in Whisker-plots with a median, 25% and 75 % quartile and 5-95% confidence interval. Repeated measures ANOVA was performed * = p<0.05; ** p<0.01; *** p<0.001.

Chapter 5

Nutritional intervention with raw bovine milk during oral cholera vaccination

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Abstract

Consumption of raw bovine milk is associated with a reduced prevalence of asthma, allergies and airway infections, indicating that raw milk may affect the immune system in the airways. Oral vaccination follows the same route of uptake as food, and may therefore serve as a model to study the impact of dietary interventions on the immune response in the gastrointestinal tract as well as the upper airways. To this end, a pilot study (N=14) and a larger follow up study (N=27) were performed to investigate whether raw milk can specifically enhance the vaccine-specific immune response in the upper airways using oral cholera vaccination as a model.

Volunteers were vaccinated in both studies on day 0 and day 14. The effect of raw milk on vaccine-specific antibody production was measured in serum, nasal wash, saliva and fecal water, at day 0, 7, 14, 18 and day 28. As in the pilot study the vaccine-specific antibody response did not yet level off at day 28, it was decided to extend the follow up study until day 42.

In the pilot study, raw milk induced a more prominent vaccine-specific IgA response at early time points after vaccination in serum compared to the control group. However, in the follow up study this could not be confirmed. Here the only significant difference after Bonferroni correction was for the raw milk group a tendency to have lower vaccine-specific antibodies (IgG) compared to the control group in nasal wash. Surprisingly, the control group responded significantly different between the two studies.

In conclusion, opposite results were obtained for both studies. Therefore, it cannot be concluded if raw milk, when only supplied during and the day after vaccination, is able to increase or decrease the production of vaccine-specific antibodies after oral cholera vaccination, when only supplied around the vaccination days. It is recommended for potential follow up studies to focus instead on an extended nutritional intervention period with (raw) milk before, during and after the vaccination period.

Introduction

Farm milk consumption is correlated with a reduced prevalence of atopy and asthma.[1–6] In the GABRIELA study, a comparison was made between multiple types of milk consumed by children: exclusive shop milk consumption, mixed milk consumption (shop and farm milk), or only farm milk consumption (only boiled farm milk, any unboiled, *versus* daily unboiled farm milk).[6] Farm milk consumption had lower odds ratios (ORs) for asthma, hay fever and atopy, and mixed milk consumption protected against hay fever and atopy, while consumption of only boiled farm milk was not protective. In others studies, also non-farming children had reduced allergies when consuming raw milk.[3,7] These effects may in part be explained by the induction of increased numbers of regulatory T cells in peripheral blood of children consuming raw milk. Altogether, these studies show that consuming unpasteurized farm milk is one of the main farming factors that is associated with a protective effect against asthma, allergy and atopy.

Raw milk and reduced infections

Next to reduced development of asthma and atopy, raw milk seems inversely associated with rhinitis, respiratory tract infections (RTI) and otitis, although some of these effects were also seen for boiled farm milk and pasteurized milk.[8] One of the mechanism underlying this reduced prevalence of upper respiratory tract infections may be the ingestion of bovine IgG (bIgG) present in raw milk.[9] Bovine IgG is known to bind to human respiratory pathogens, including Influenza and respiratory syncytial virus.[10] Bovine IgGs can also bind to allergens and intestinal pathogens[11] and reviewed by Ulfman et al.[12] Interestingly, bIgGs can also bind to FCγRII (CD32) expressed on human antigen presenting cells and neutrophils.[10] Antigens are better taken up by antigen-presenting cells when they are forming a complex with immunoglobulins.[13–15] Bovine IgG (and raw milk) can end up in the crypts of the palatine tonsils when swallowed and before it gets digested. Hence, bovine IgG bound to respiratory pathogens may therefore result in more efficient pathogen uptake, antigen processing and activation of specific T cells towards that specific pathogen.

Raw milk and oral vaccination

Oral vaccination is known to induce protective immune responses, not only in the small intestine and colon, but also in the upper respiratory tract (e.g. in saliva and nasal secretions) and to a lesser extent in the tonsils and lower respiratory tract.[16–19] Previous vaccination studies were performed with cholera toxin B or the oral cholera vaccine Dukoral®. In a previous pilot study, we investigated the homing profiles of memory B cells after oral cholera vaccination in healthy adult subjects.[20] Oral cholera vaccination was shown to induce IgA^+ memory B cells with an airway and colon homing potential, while IgA^- (IgG) memory B cells obtained a small intestine homing profile, suggesting that oral cholera vaccination induces protective IgA-mediated immune responses in the (upper) airways. Oral vaccination, in contrast to parenteral vaccination, follows the same route of uptake as food, and may therefore serve as a model to study the impact of dietary interventions on the immune response in the upper airways. As described above, consumption of raw bovine milk is associated with a reduced prevalence of asthma, allergies and airway infections, which may indicate that it affects the airways as well.
Based on these observations, it was hypothesized in the current study that, on the one hand, immunoglobulins in milk may induce a more efficient vaccination response, mediated by the formation of immune complexes between bovine immunoglobulins and the vaccine components. On the other hand, raw milk might selectively increase IgA formation against the oral cholera vaccine because of the presence of selective switch-inducing factor TGF-ß in milk.[21]
To this aim, a pilot study and a larger follow up study were performed to investigate whether raw milk can specifically enhance the vaccine-specific immune response in the upper airways (e.g. in saliva and nasal wash) using oral cholera vaccination as a model.

Material and methods

Pilot study set-up MOSAIC I

In an initial randomized controlled single-blind pilot study, subjects, who previously had not been vaccinated with *Vibrio Cholerae* or *E. coli*, were vaccinated at day 0 and day 14 with the oral cholera vaccine Dukoral® (EU/1/03/263/002, Crucell, The Netherlands). The vaccine consisted of a

total of 1.25×10^{11} formalin- and heat-killed *Vibrio cholerae* bacteria (strains O1 Inaba and O1 Ogawa) and 1 mg recombinant cholera toxin B-subunit (rCTB). The design of the pilot study has been described before.[20] In short, one group (n=14) received the vaccine in the regular carbonate buffer. Another group (n=14) received the regular carbonate buffer, followed by the vaccine diluted in 150 ml raw milk. All subjects gave written informed consent before inclusion in the study. The demographic characteristics of the subjects are summarized in Table 1. The study was approved by the Research Ethics Committee of Wageningen University and registered at www.clinicaltrials.gov with identification-number: NCT02238548. The study was conducted from October 2014-November 2014.

Table 1: demographic characteristics of subjects in MOSAIC I and MOSAIC II study

	Study	Control group	Raw milk group	Pasteurized group	UHT group
Number of subjects	MOSAIC I	14	14	-	-
	MOSAIC II	27	27	27	27
Age: median (range)	MOSAIC I	24 (19-46)	23 (18-41)	-	-
	MOSAIC II	23 (18-49)	21 (18-47)	22 (18-49)	23 (18-49)
Gender: number M/F	MOSAIC I	4/10	4/10	-	-
	MOSAIC II	6/21	8/19	7/20	7/20
Salmonella vaccination: number Y/N/U	MOSAIC I	2/3/9	1/3/10	-	-
	MOSAIC II	1/26/0	1/26/0	1/26/0	2/25/0

M=male; F=female; Y=yes; N=no; U=unknown

Study set-up MOSAIC II

The follow-up study consisted of a 6-week, randomized controlled single-blind trial in healthy human subjects (4 groups of 27 subjects, total n=108; male and female). The study was approved by the Research Ethics Committee of Wageningen University, and registered at clinicaltrials.gov as NCT02924246. Upon providing written informed consent, subjects were selected based on age (18-50), no previous *Vibrio Cholerae* or *E.coli* vaccination, no tonsillectomy, no gastroenteritis or antibiotics use, no excessive drugs and alcohol use, being not immunocompromised, willing to consume raw milk. Subjects were stratified according to age, gender, and smoking (as determined at screening). Demographic characteristics of the subjects are summarized in Table 1. Subjects received two dosages of the oral cholera vaccine Dukoral® (Valneva

SE, France), on day 0 and day 14 of the study. The composition of Dukoral® was the same as in MOSAIC I, albeit obtained from a different supplier.

One group received the vaccine in the regular carbonate buffer. One group received the regular carbonate buffer, followed by the vaccine diluted in 150 mL raw milk. One group received the regular carbonate buffer, followed by the vaccine diluted in 150 mL full-fat pasteurized milk (Campina 'volle melk'). One group received the regular carbonate buffer followed by the vaccine diluted in 150 mL full-fat ultra-heat treated (UHT) milk (Campina 'langlekker volle melk'). The groups that received the vaccine in milk, received two additional portions of 0.5 L raw milk, 1 L of pasteurized milk, or 1 L of UHT milk, to be ingested spread over the evening and next morning. The control group received two portions of 0.5 L water instead.

For logistic reasons, the 108 subjects were divided into two groups of around 54 subjects, who were invited for each visit on one of two subsequent calendar days (Thursday/Friday). On the vaccination days, female participants were asked for current pregnancy status, and were offered a pregnancy test in case of doubt, for safety reasons in view of the raw milk and the vaccine.

During the whole study, subjects were instructed to maintain their habitual diet, with the exception of pre- and probiotics consumption. Furthermore, during the three days around each vaccination dose, subjects were not allowed to consume any dairy products. The evening before each study visit, no alcohol intake was allowed. Besides, subjects were only allowed to drink water 1 hour before the study visit. The subjects were asked to fill in an online diary during the study period to record any adverse events, and to monitor compliance with dietary guidelines. The study was conducted from October 2016-December 2016.

Raw milk selection

Raw milk selection was the same for both studies. Five farms performing excellent on microbiological safety were selected, based on FrieslandCampina's Quality programme (Foqus planet). Raw milk was collected from three days before study day 0 and day 14. Quality of milk samples were checked based on parameters used for "Vorzugsmilch", raw milk sold to consumers in Germany.

The most important parameters were total bacteria count and the absence of *Salmonella* and *Listeria monocytogenes*. The best milk was selected to use in the study, see Table 2.

Sample collection

For the MOSAIC I study, serum and nasal wash samples were collected at day 0 and day 14, before vaccination and at study days 7, 18 and 28. For the MOSAIC II study, blood, saliva, nasal wash, and fecal samples were collected directly before each vaccination at day 0 and day 14, and at study day 28 and 42.

Blood was collected for serum storage (2x10 mL tubes; cat.no. 367895 Becton Dickinson Company, Erembodegem, Belgium). Serum tubes were left at room temperature (RT) for at least 30 min before centrifugation at 2000 x g 10 min at RT. Serum was aliquoted and stored at -80°C.

Nasal wash was collected using sterile pre-warmed physiological salt solution. Study participants were asked to hold their breath and keep their head tilted backward while 3 mL salt solution was provided in one nostril. After 10 seconds, they bent their head forward and collected the nasal wash solution in a jar. This was repeated for the other nostril, and collected in the same jar. In MOSAIC I, nasal wash samples were centrifuged at 3000 x g for 10 minutes at 4°C and stored at -80°C. In the MOSAIC II study, samples were centrifuged at 3000 x g for 10 minutes at 4°C, after which 2 mL was stored in a 15 mL tube, and freeze-dried. The remaining volume was aliquoted and stored at -80°C.

Saliva samples were collected using the Salivette system for active saliva induction (cat.no. 51.1534 Sarstedt AG & Co, Nümbrecht, Germany). Ten minutes before collection, subjects flushed their mouth with tap water. At the time of collection, subjects swallowed the saliva present in the mouth, and subsequently placed a cotton swab in their mouth and chewed for 1 min. The swab was placed back into the collection tube, and put on ice until further processing. Samples were centrifuged for 2 min at 1,000 x g at 4°C. Saliva was aliquoted and stored at -80°C.

Fecal samples were collected by subjects at home, using a Fecotainer (AT Medical BV, Enschede, The Netherlands). Fecal spot samples were collected,

stored frozen at home until the end of the study, and then transported on dry ice to the research facility. Fecal water was prepared by homogenizing 400 mg of feces in 3.6 mL of sterile PBS (Lonza, BE17-516F) and 40 µL of protease inhibitor (Sigma-Aldrich, P8340). Fecal samples were vortexed until a homogeneous suspension was obtained, and centrifuged for 10 min at 3000 x g at 4°C. Supernatant was aliquoted and stored at -80°C.

ELISAs

Dukoral®- specific IgA and IgG antibodies in serum, saliva, nasal wash, and fecal extract were determined by enzyme-linked immunosorbent assay (ELISA) in high binding 96-well plates (Greiner Bio one 655061, Monroe, North Carolina, USA) and performed as described previously.[20] Plates were coated with the Dukoral® vaccine, diluted in PBS (1×10^8 bacteria + 0.8 µg CTB / 1 mL of PBS) (Lonza Be17–516F, Basel, Switzerland), and incubated overnight at 4 °C. After washing with wash buffer (PBS + 0.05% Tween-20), plates were blocked with ELISA blocking buffer (Roche 11112589001, Mannheim, Germany) for 1 h. For specific IgA, IgG and IgM measurements, serum was diluted in ELISA blocking buffer (IgA 75x diluted, IgG 300x diluted, and IgM, only MOSAICII study, 75x diluted).

Before use in the ELISA, the freeze-dried nasal wash samples from MOSAIC II were reconstituted in 650 µl HPE buffer, resulting in 3.1 times concentrated samples for ELISA (IgA, IgG). Reconstituted samples were rolled on a roller bank for three minutes at 50 RPM and stored at 4 C° overnight. Nasal wash samples (aliquots MOSAIC I or reconstituted freeze-dried sample MOSAIC II) were spun down at 1000g for 3 minutes before ELISA analysis. Before ELISA, saliva and fecal water were centrifuged for 15 min at 1500×g. Nasal wash from MOSAIC I, saliva, and fecal extracts were incubated for 15 min in High Performance Elisa (HPE) buffer (Sanquin M1940, Amsterdam, The Netherlands) before dilution. In MOSAIC I, nasal wash was diluted 2 times (IgA, IgG), whereas in MOSAIC II a 3 times concentrated sample was used. In MOSAICII the reconstituted nasal wash was used without further dilution. In both studies, saliva was diluted 10 times (IgA) and 5 times (IgG) and fecal water IgA was diluted 2 times. Samples were added to the plate in duplicate and incubated for 2 h at room temperature (RT). A pooled serum sample, from all vaccinated donors at day 18 of MOSAIC I, was used to make a

reference calibration line in all ELISAs and is expressed in arbitrary units/ml (AU/ml). After sample incubation, detection antibodies goat-anti-human IgA (1:2000), IgG (1:4000) or IgM (1:2000)-horseradish peroxidase (HRP) (Southern Biotech 2050–05, 2040–05, 2020-05 Birmingham, Alabama, USA) were diluted in Elisa blocking buffer (serum) or HPE buffer (saliva, nasal wash and fecal extracts samples) and incubated for 1 h at RT. For development of all ELISAs, TMB (SDT, Baesweiler, Germany) was added and the reaction was stopped by adding 2% HCL solution. Plates were read in a Filtermax at 450 nm minus 620 nm as reference value.

Presence of cow-derived antibodies in cow's milk, cross-reactive with Dukoral®, were determined in the same way as described above, with the exception that milk samples were diluted 3-96 times and only OD was measured. Detection antibodies were sheep-anti-cow-IgA-HRP (A10-131P-1), sheep-anti-cow-IgG-HRP (A10-118-27) and sheep-anti-cow-IgM-HRP (A10-101P-29), all from Betyl Laboratories inc.

Airway homing potential of peripheral B cells

The potential of freshly isolated B cells to home to the airways was analysed by determining the expression of a panel of cell surface markers using CD19, CLA, Integrin β1, Integrin β7, CCR9 and CCR10, as described before[20]. In short, 3×10^6 freshly isolated PBMCs were stained with CD19, CLA, Integrin β1, Integrin β7, CCR9 and CCR10. Cells were considered to have airway homing potential when expressing the following marker: $CD19^+CLA^-Integrin\beta1^+\beta7^-CCR10^+$. Data were exported as % of memory B cells and subsequently converted to 'number of homing memory B cells/million of memory B cells'.

Statistical analysis

Data were 10 log-transformed (ELISA) to obtain normally distributed data. When normalization was not achieved, data were subsequently rank transformed. No outliers were removed. Repeated measures ANOVA was conducted using transformed data with IBM SPSS Statistics 23. First, the assumption of sphericity was tested, using Mauchly's Test of Sphericity. If $p<0.05$, sphericity could not be assumed and hence, a Greenhouse–Geisser (epsilon <0.75) or a Huynh-Feldt (epsilon >0.75) correction was applied to test within-subject effects (time x treatment). When a within-subject

effect was observed (time x treatment), either significant or at least with $p<0.2$, a contrast analysis (difference; comparing study days with day 0) was performed. Using contrast analysis, differences over time compared to day 0 between the raw milk vaccination group and the control vaccination group were tested. No Bonferroni correction was applied during the initial Repeated measures ANOVA analysis. To correct for the number of comparisons during the statistical analysis by contrasts, a Bonferroni correction was applied manually. Differences were considered significantly different when $p<0.05$.
To compare results from MOSAIC I and MOSAIC II studies, an independent t-test was performed on normal distributed delta 10log (AU/ml) data on day 14 and day 28 compared to day 0 for serum IgA and IgG. Delta 10log values were tested for normal distribution. If normalization was not achieved, a rank transformation was performed before the statistical analysis.

Results

Safety and tolerability of raw milk

The raw milk used for both oral cholera vaccination studies were considered safe. Microbial safety was determined based on the parameters used for 'Vorzugsmilch', which defines microbial safety for the sale of raw milk in Germany, see Table 2. In the MOSAIC I study, only mild adverse events were reported, and these were as much reported in the control group (n=7) as in the raw milk group (n=6). Also in the follow-up MOSAIC II study, mild adverse events possibly related to the study product were reported for the raw milk group (n=4) and control group (n=1). Symptoms consisted of abdominal pain (bloating), cramp, nausea and mild diarrhoea. These side effect have been described as potential side effects of the oral cholera vaccination. Therefore, a direct link with the raw milk intake was considered unlikely. No moderately severe adverse events related to the study were reported. Furthermore, one non-study related serious adverse event was reported.

Table 2: Quality criteria of the raw milk used for the MOSAIC study I and II

		MOSAIC I batch 1	MOSAIC I batch 2	MOSAIC II batch 1	MOSAIC II batch 2
Total bacteria	CFU/ml	11.000	<4000	<1000	<1000
Coliform	CFU/ml	610*	<40	<40	<10
E.coli		0	0	0	0
Staphylococcus aureus		-	+	0	0
Streptococcus agalactiae		0	0	0	0
Somatic cells	/ml	103.000	115.000	112.000	86.000
Salmonella	CFU/ 25 ml	0	0	0	0
Listeria monocytogenes	CFU/ 25 ml	0	0	0	0

CFU colony forming units; * test sample was obtained directly at start of milk collection without having milk flow through the outlet of the tank in advance; - negative + present, in range

Dukoral®-specific antibodies in serum: comparing the MOSAIC I and MOSAIC II study

To assess the systemic immune response to oral vaccination, serum levels of vaccine-specific IgG and IgA were measured. In Figure 1, Dukoral®-specific IgA (Figure1A) and IgG (Figure1C) measured in serum at day 0, 7, 14, 18 and 28 of the MOSAIC I study are shown. The serum levels of vaccine-specific IgG were higher than vaccine-specific IgA, but not significantly different between the raw milk group and the control group. In contrast, Dukoral®-specific IgA in the raw milk group is significantly elevated at day 7 and day 14. However, upon Bonferroni correction, no significant differences are observed for MOSAIC I serum analyses. For both vaccine-specific antibodies, the response had not yet levelled off at day 28. Therefore, it was decided in MOSAIC II study to extend the study until day 42. Based on the serum data of MOSAIC I, a higher number of subjects was recruited for MOSAIC II (n=27 per group). In Figure 1, the Dukoral®-specific IgA (Figure 1B), IgG (Figure 1D) and IgM (Figure 1E) measured in serum is shown for the raw milk and control group of the MOSAIC II study. The figures show that the peak for most antibodies was reached at day 28, and that at day 42 vaccine-specific antibody levels are slightly declining or stable compared to day 28. In contrast to MOSAIC I, raw milk did not induce increased vaccine-specific IgA levels at day 14 (Figure 1B), but rather resulted in lower vaccine-specific IgA at day 28 compared to the control group. Before Bonferroni correction this result was significant, after correction is was a trend of p=0.06. Also vaccine-specific IgG was reduced at day 28 and day 42 in the raw milk group compared to

the control group, although p>0.1 after Bonferroni correction. Furthermore, vaccine-specific IgM levels were lower (after Bonferroni correction p>0.1) in the raw milk group at day 14 and day 28 in the MOSAIC II study. So, in the MOSAIC I study raw milk induces higher levels of vaccine-specific antibodies, while in MOSAIC II raw milk induces lower levels of vaccine-specific antibodies compared to the control. In Supplementary Figure 1, the overall vaccination response at day 14 and day 28 compared to day 0 between MOSAIC I and MOSAIC II is shown. Supplementary Figure 1 shows that, in the control group, vaccination induced significantly higher levels of vaccine-specific IgA and IgG in MOSAIC II compared to MOSAIC I, while in the raw milk group significantly lower vaccine-specific IgA levels were induced in MOSAIC II compared to MOSAIC I. Therefore, it is not possible to draw conclusions as not only the raw milk responses are different in the two studies, but the control group reacts significantly different as well.

Dukoral®-specific antibodies in nasal wash: comparing the MOSAIC I and MOSAIC II study

As a measure of immune responsiveness to oral cholera vaccination in the upper respiratory tract, vaccine-specific IgG and IgA antibody levels were determined in nasal wash. In Figure 2, vaccine-specific IgA and IgG antibody levels in nasal wash in MOSAIC I (Figure 2A, C) and MOSAIC II (Figure 2B, D) are shown. In MOSAIC I, no Dukoral®-specific IgA and IgG levels in nasal wash were detected. Therefore, in MOSAIC II nasal wash samples were freeze-dried and then reconstituted, concentrating the samples ±3 times. Figure 2 shows that no significant differences between the raw milk and control group were observed for vaccine-specific IgA in nasal wash of MOSAIC II. However, Dukoral®-specific IgG was higher in the control group compared to the raw milk group at day 28 and day 42. Even after Bonferroni correction, IgG levels at day 42 remain significantly higher (p=0.015) in the control group compared to the raw milk group. So, the control vaccination group has higher vaccine-specific IgG levels in nasal wash compared to the raw milk group, while no significant differences in vaccine-specific IgA are observed.

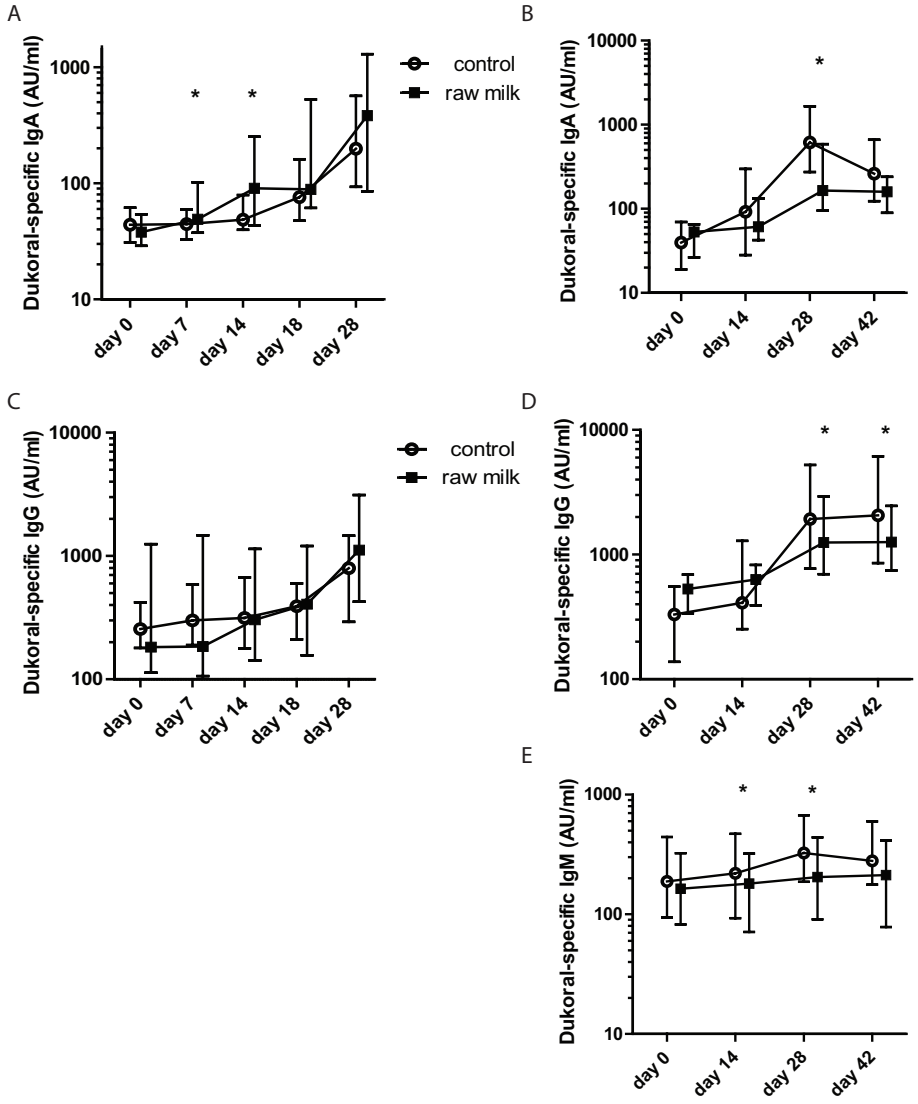

Figure 1: Serum of healthy volunteers was measured on day 0, 7, 14, 18 and 28 in MOSAIC study I (A,C) or on day 0, 14, 28 and 42 in MOSAIC study II. Dukoral-specific IgA (AU/ml) (A,B), IgG (C, D) and IgM (E) were measured in serum. Data are shown as median + interquartile range. Repeated measures ANOVA was performed after 10 log transformation (A,C) or rank transformation of the 10log transformed data (B,D,E). * indicates $p<0.05$ when comparing raw milk versus control vaccination compared to day 0.

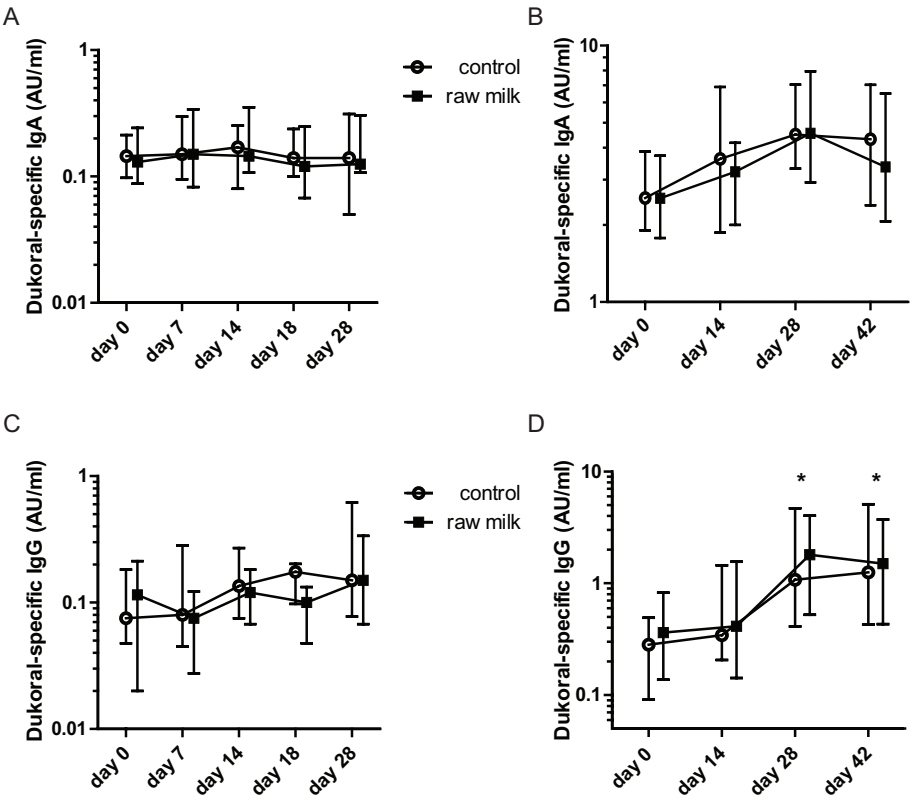

Figure 2: Nasal wash of healthy volunteers was measured on day 0, 7, 14, 18 and 28 in MOSAIC study I (A,C) or on day 0, 14, 28 and 42 in MOSAIC study II (B,D). Dukoral-specific IgA (AU/ml) (A,B) and IgG (C, D) were measured in nasal wash. Data are shown as median + interquartile range. Repeated measures ANOVA was performed after 10 log transformation (A,C,B) or rank transformation of the 10log transformed data (D). * indicates $p<0.05$ when comparing raw milk versus control vaccination group compared to day 0.

Dukoral®-specific antibodies in saliva and fecal water (MOSAIC II)

In the MOSAIC II study, also saliva and fecal water samples were tested for vaccine-specific antibodies (Figure 3). In saliva, levels of Dukoral®-specific IgA and IgG (Figure 3A, B) were determined, while in fecal water, only Dukoral®-specific IgA (Figure 3C) was determined. The control group seemed to have overall slightly higher vaccine-specific IgG levels in saliva, but this was not significant. There was no detectable induction of vaccine-specific IgA levels in both groups. The control group had higher vaccine-specific IgA levels in fecal water compared to the raw milk group at day 14, but this was not significant after Bonferroni correction. There are no differences between the raw milk and the control group in the levels of vaccine-specific antibodies in saliva and fecal water.

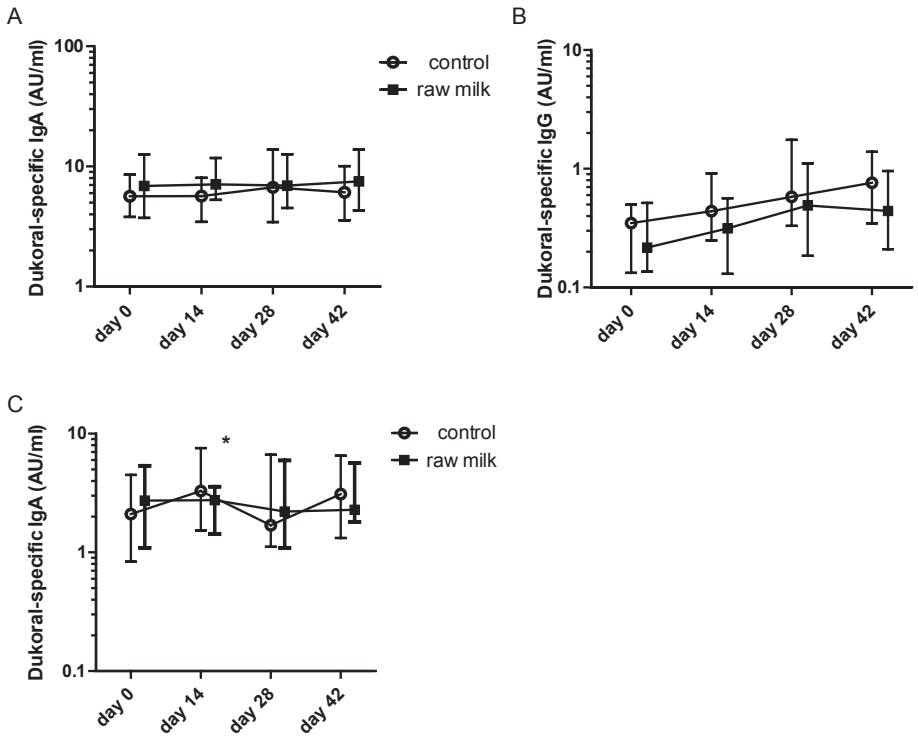

Figure 3: Saliva (A, B) and fecal water (C) of healthy volunteers was measured on day 0, 14, 28 and 42 in MOSAIC study II. Dukoral-specific IgA (AU/ml) and IgG were measured in saliva (A,B) and Dukoral-specific IgA (AU/ml) in fecal water (C). Data are shown as median + interquartile range. Repeated measures ANOVA was performed after 10 log transformation (B) or rank transformation of the 10log transformed data (A,C). * indicates $p<0.05$ when comparing raw milk versus control vaccination group compared to day 0

Dukoral®-specific antibody responses upon oral vaccination combined with pasteurized or ultra-heat treated milk consumption

In order to evaluate whether heating of milk influences the effects seen in MOSAIC I for raw milk, two additional milk groups were investigated as well in MOSAIC II. The subjects in these groups received either the vaccine together with pasteurized milk or with ultra-heat treated milk (UHT). In Supplementary Figure 3, the results of all vaccination groups are shown. For both milk groups similar results were obtained as for raw milk in the case of vaccine-specific IgA, IgG and IgM in serum and IgA in fecal water. UHT was the only group with a tendency for lower levels of vaccine-specific IgG in saliva compared to the control group.

Immunomodulation of airway homing potential

To check if raw milk might promote specific homing of B cells to the airways, we investigated the airway homing potential of IgA^+ and IgA^- B cells of subjects in MOSAIC I. No significant differences are observed between the control and raw milk group. However, Figure 4 shows that for IgA^+ B cells the control group seems to induce more B cells with airway homing potential, while for IgA- B cells the number of B cells with airway homing potential seemed slightly higher in the raw milk group. Altogether, there is no clear induction that raw milk enhances the airway homing potential of B cells.

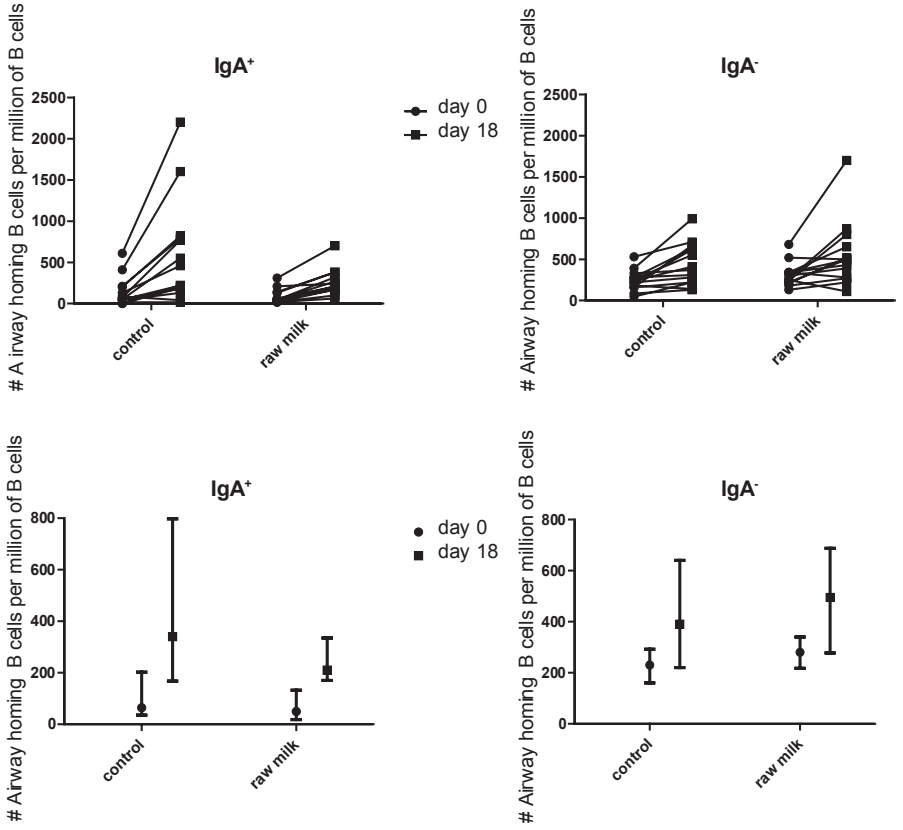

Figure 4: Kinetics of IgA+ and IgA– memory B cells with an airways homing potential. PBMCs of healthy volunteers (n = 14 per group) were analysed on days 0 and 18 in the MOSAIC I study, using flow cytometry. Data are shown as individual responses per group (upper graphs) and as median values with interquartile range (lower graphs). No significant differences are observed in homing potential between the groups.

Discussion

Here, the results of two related subsequent oral cholera vaccination studies are described, in which the effect of nutritional intervention with raw (and processed) bovine milk was studied on Dukoral®-specific antibody production in serum, nasal wash, saliva and fecal water. In the first study, MOSAIC I, raw milk induced a faster vaccine-specific IgA responses in serum, but this could not be confirmed in the follow up study, MOSAIC II. In the second study, the control group tended to have higher vaccine-specific antibodies compared to the raw milk group in serum (IgA, IgG, IgM), nasal wash (IgG) and fecal water (IgA). However, only nasal wash IgG was statically significant after a Bonferroni correction for the number of statistical comparisons. Similarly, in this study also pasteurized milk and UHT milk tended to have non-significant lower IgA and IgM vaccine-specific antibodies in serum compared to the control group. Surprisingly, the control group responded significantly different between the two studies. Therefore, no hard conclusions can be drawn about the effect of raw and processed milk on oral cholera vaccination.

The rationale of the MOSAIC studies was the concept that immunoglobulins, present in (raw) milk, might induce a stronger vaccination response against orally delivered vaccines. Besides, they possibly even increase the response in the (upper) airways as measured in saliva and nasal wash. This effect was hypothesized to be mediated by bovine immunoglobulins that can form immune complexes with the bacteria present in the vaccine.[10,11] In general, immune complexes are better taken up by Waldeyer's ring or in the gut by antigen-presenting cells, which may support the subsequent immune response.[13–15] In the two oral vaccination studies we observed different vaccination responses in the control group. Therefore, the comparison between the control group and the raw milk group is difficult to make, as a difference in results can be due to the raw milk as wells as the vaccine itself. In vaccination studies in children, contradictory results about the presence of maternal antibodies, either via placental transfer or breast feeding are obtained as well. Vaccination studies in children have shown that the presence of maternal antibodies (mostly placental transfer) can inhibit parenteral vaccination responses in these children, as a result of immune exclusion of the pathogen.[22] Immune exclusion of the vaccine in our study is unlikely, because

of the increased kinetics observed in the raw milk group in the MOSAIC I study. In contrast, many studies in which children were withheld from breastfeeding 30-60 minutes prior to and after vaccination, no negative effect of breastmilk on oral rotavirus vaccination responses were observed.[23,24] Besides, oral or parenteral vaccine responses were even enhanced in the breastfed group.[25–27] The raw milks used in the MOSAIC I and the MOSAIC II studies contained cow-derived IgA, IgG and IgM antibodies that were able to bind Dukoral® (Supplementary Figure 2). For all three isotypes, the obtained OD values for raw milk are comparable between MOSAIC I and II. Furthermore, pasteurized milk had comparable levels of Dukoral®-specific IgG, but lower Dukoral®-specific IgA and IgM, while no Dukoral®-specific antibodies of any isotype were observed in the UHT. So, the level of vaccine-specific antibodies in raw milk do not explain the different result obtained in MOSAIC I and II. Supplementary Figure 1 shows that raw milk did not induce different Dukoral®-specific IgG levels in serum between MOSAIC I and II. In contrast, differences were rather seen in the control group, with higher IgA and IgG levels at day 28 in MOSAIC II as compared to MOSAIC I. Furthermore, in the MOSAIC II study, also two groups receiving UHT and pasteurized milk during vaccination were included. In general, these groups showed the same vaccine-specific antibody production and kinetics as the raw milk group (Supplementary Figure 3). This was unexpected, as UHT milk contains no functioning vaccine-specific antibodies and pasteurized milk contains lower levels of vaccine-specific antibodies compared to raw milk, see Supplementary Figure 2. Therefore, the increased kinetic response of vaccine-specific IgA in serum in MOSAIC I, together with the comparable vaccine-responses in the groups receiving raw, pasteurized or UHT milk in MOSAIC II, make it unlikely that antibodies in raw milk caused the difference in the vaccine response in MOSAIC II. Therefore, our hypothesis that the vaccine response is primarily modulated by bovine antibodies forming complexes with the vaccine is not confirmed.

Supplementary Figure 1 shows that, when comparing the increase of vaccine-specific antibodies at day 14 and day 28 compared to day 0 (delta-values), the control group in MOSAIC II had a stronger vaccine-specific IgA and IgG response compared to the MOSAIC I. In contrast, raw milk induced less vaccine-specific IgA in MOSAIC II compared to MOSAIC I. To allow comparison of the antibody responses between MOSAIC I and II, the same pooled serum

standard was used in the ELISA for both studies. When the absolute values (AU/ml) were compared between MOSAIC I and MOSAIC II, the starting concentration at day 0 was comparable between the two studies, whereas differences are observed in the levels after vaccination. This suggests that the difference in outcomes between the two studies is at least in part explained by a truly higher vaccine response in the control group in MOSAIC II. A potential explanation for this observed difference could be that the Dukoral® used for the MOSAIC I study was owned and produced by Crucell (The Netherlands), whereas in 2015 the vaccine was sold to Valneva SE (France). However, the product specifications of the vaccine remained the same. Furthermore, even if the vaccine was changed, similar effects (either higher or lower response by raw milk as compared to control) would be expected in both studies. Both MOSAIC studies were performed in autumn (October-November and October-mid December). Therefore, seasonal variations such as vitamin D status affecting immune responses and Toll like receptor expression, as described by Alvarez-Rodriques et al.[28], are not likely to play a role.

Ageing is known to cause decreasing immune responses to vaccination.[29–31] In both MOSAIC studies, subjects were younger than 50 years old, and the median age and range of the MOSAIC I and II study were similar (Table 1). So, differences in age between the groups also do not explain the observed differences in vaccine-specific antibody production. Another explanation could be gender balance in the groups. Woman are known to have better humoral responses compared to men, as reviewed by Giefing-Kröl.[29] In a study with vaginal cholera vaccination during different menstrual cycle phases, women in the luteal phase produced higher CTB-specific IgA levels in serum.[32] In an oral vaccination study in Haïti, the vaccine efficacy was higher in women than in men, as more men were found to have cholera diarrhoea after vaccination.[33] In the MOSAIC I study, in both the control group and raw milk group 40% of subjects were men and 60% women. In the MOSAIC II study, in the control group and the raw milk group more subjects were female (78% and 70%, respectively). Having relatively more women in especially the control group of MOSAIC II as compared to MOSAIC I, who can be in their luteal menstrual cycle phase, could be a possible explanation for the increased vaccination response in the control group of MOSAIC I versus MOSAIC II.

In this study, oral cholera vaccination was used as a model to study the immunomodulatory effect of raw bovine milk. As oral vaccines, in contrast to parenteral vaccines, follow the same route of uptake as food ingredients, it is conceptually a good model to study immunomodulatory properties of food. In our recent publication, we showed that oral cholera vaccination induces well detectable vaccine-specific antibody responses in serum, and leads to the induction of IgA^+ memory B cells with homing potential towards airways and large intestine.[20] The hypothesis was that raw milk might induce more homing towards the airways, as raw milk consumption reduces asthma prevalence and respiratory tract infections.[1–6] In MOSAIC I, the homing potential of memory B cells in the raw milk group was investigated, however no significant differences of homing potential were observed between the control group and the raw milk group (Figure 4). For IgA^+ memory B cells, raw milk seems to decrease the frequency of cells with airway homing potential at day 18 of the study, while airway homing potential for IgA^- memory B cells seemed increased at day 18 in the raw milk group compared to the control group. Although these differences are not significant, it's nevertheless interesting to speculate that raw milk could induce airway homing using oral cholera vaccination as model.

A limitation of the MOSAIC studies was that raw milk was only consumed at the moment of vaccination, and the evening (0,5 L) and next day after vaccination (0,5 L). This set-up allows only studying the effect of direct interactions with raw milk and the vaccine, and not of long term effects of raw milk consumption. Long term consumption of raw milk before, during and after oral vaccination would allow to study the long term effect of immunomodulatory milk components. Examples of such immunomodulatory factors are bovine TGF-β1 and β2, which are identical to human TGF-β1 and β2 and can lead to the induction of regulatory T cells (Tregs), just like bovine IL-10.[34] Furthermore, TGF-β1 induces IgA switching in mice[35,36] and in humans as reviewed by Cerutti.[21] Therefore, prolonged consumption of raw milk may lead to a more immunoregulatory environment throughout the gastrointestinal tract and Waldeyer's ring. What the final outcome of such an enhanced immunoregulatory environment on oral vaccination will be, is uncertain. On one hand, this could lead to the induction of lower responses to the vaccine, as more Tregs will be induced. On the other hand it could lead to increased vaccine-specific IgA induction, because of increased IgA class switching.

In conclusion, as opposite results were obtained for both MOSAIC studies, it cannot be concluded whether raw bovine milk is able to increase or decrease the production of vaccine-specific antibodies after oral cholera vaccination, when only supplied around the vaccination days. It is recommended for potential follow up studies to focus on an extended nutritional intervention period with (raw) milk before, during and after the vaccination period.

Acknowledgements

We acknowledge all people that were part of the MOSAIC I and II study teams, in particular Daphne Montizaan, Thijs van Osch, Erik van den Brink and Olaf Perdijk for helping with the execution of the study and Alwine Kardinaal for helping with the statistical analysis. We also thank all participants of the MOSAIC I and II studies. This study was financed by FrieslandCampina

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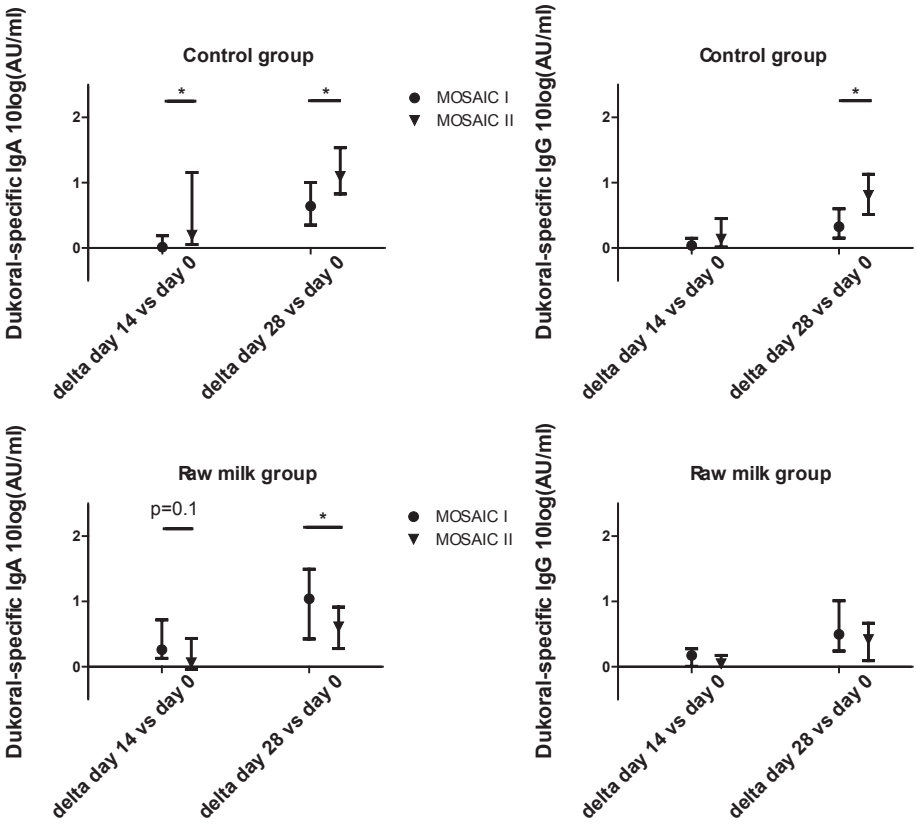
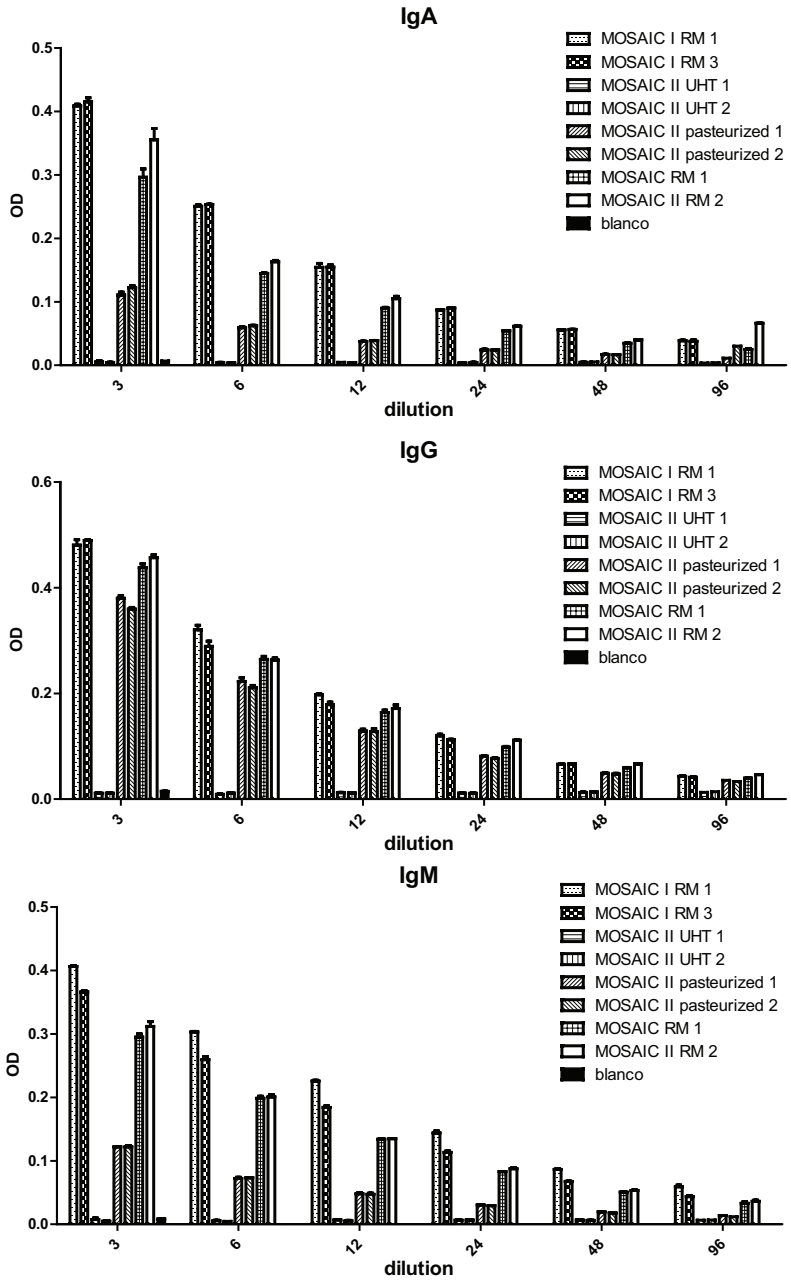
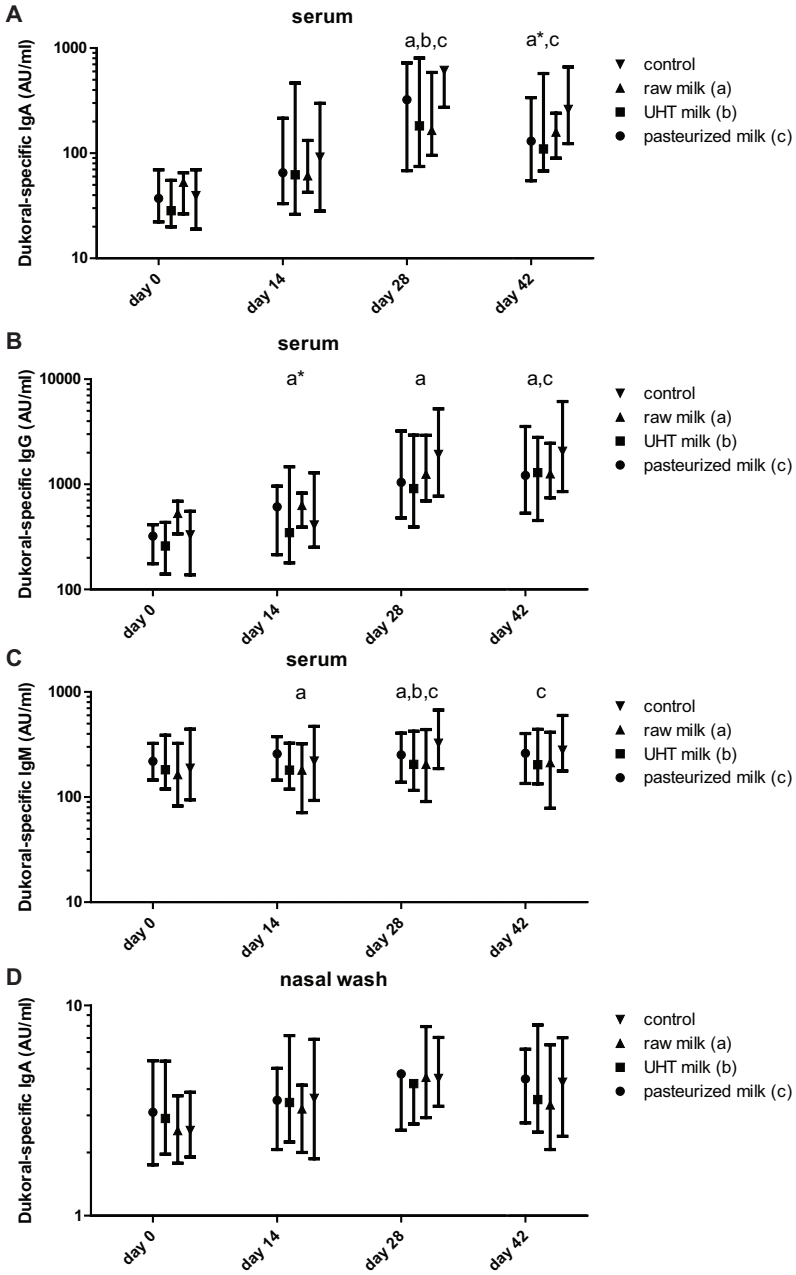


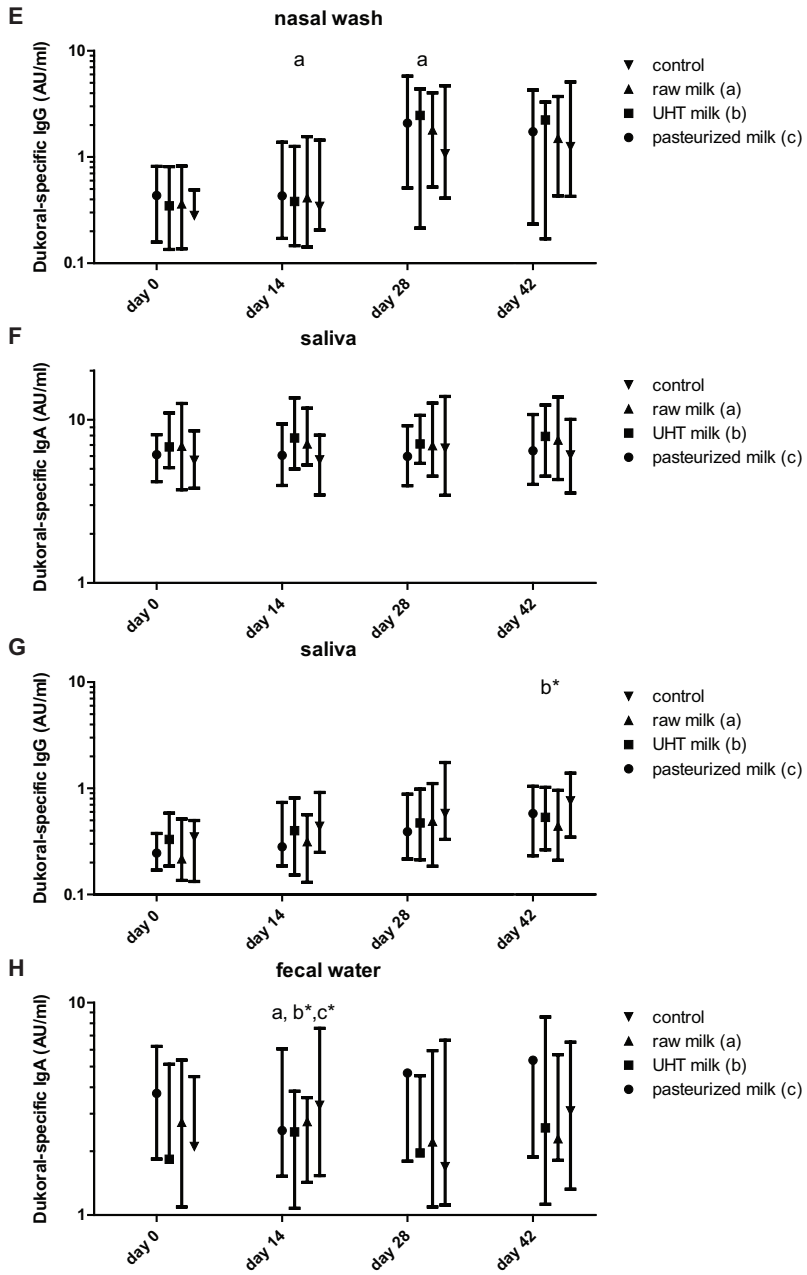
Supplementary Figure 1: Difference in antibody response when comparing MOSAIC I versus MOSAIC II. Delta values were taken from 10log(AU/ml) on day 14 compared to day 0 and on day 28 compared to day 0 for serum IgA and IgG for both the control group and raw milk group. Data are shown as median + interquartile range. Independent t-test was done on normally distributed data. * $p<0.05$



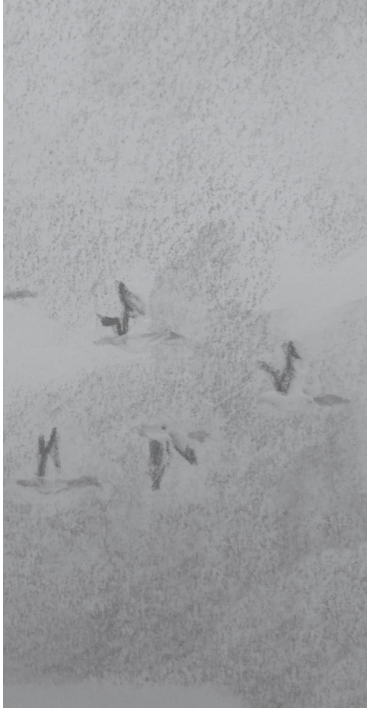
Supplementary Figure 2: Dukoral-specific IgA, IgG and IgM (OD) in cow's milk used for MOSAIC I and MOSAIC II study diluted 3-96 times. RM=Raw milk; UHT=ultra-heat treated milk; 1=study day 0 MOSAIC I & II; 2= study day 14 MOSAIC II; 3=study day 14 MOSAIC I. No statistics was performed.



Supplementary Figure 3: Vaccine-specific antibodies (AU/ml) in serum, nasal wash, saliva and fecal water of healthy volunteers was measured on day 0, 14, 28 and 42 in the MOSAIC study II. In serum Dukoral-specific IgA (A), IgG (B) and IgM (C) were measured, in nasal wash Dukoral-specific IgA (D) and IgG (E), in saliva Dukoral-specific IgA (F) and IgG (G) and in fecal water Dukoral-specific IgA (H) were measured. Data are shown as median + interquartile range. Repeated measures ANOVA was



performed after rank transformation of the 10log transformed data. a,b,c indicate a significant difference ($P<0.05$) of raw milk (a), UHT (b) or pasteurized milk (c) at a specific study day compared to the control vaccination group when comparing that specific study day to day 0. * indicates $0.05<p<0.1$ when comparing milk groups versus control vaccination compared to day 0



Chapter 6

Plasmacytoid dendritic cell and myeloid dendritic cell function in ageing: a comparison between elderly and young adult women

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Abstract

Ageing is associated with a changing immune system, leading to inflammaging (increased levels of inflammation markers in serum) and immunosenescence (reduced immune cells and reduced responses towards pathogens). This results in reduced vaccination responses and increased infections in elderly. Much is known about the adaptive immune system upon ageing, but less is known about the innate immune system. Therefore, the aim of this study was to compare innate immune function of Toll like receptor (TLR)-mediated responses between elderly and young adult women. To this end, elderly and young adult women were compared to study the effect of ageing on the frequency and reactivity to TLR-mediated responses of myeloid- and plasmacytoid dendritic cells (mDC, pDC). Next to this, TLR expression and inflammatory markers in serum were investigated. Elderly women had reduced numbers of circulating pDCs. In addition, pDCs and mDCs of elderly women responded differently towards TLR stimulation, especially TLR7/8 mediated stimulation, compared to young adults. In serum, markers involved in inflammation were generally increased in elderly. This study confirms and extends the concept of both immunosenescence and inflammaging in elderly women.

Introduction

The ageing population is growing rapidly, and more than 30% of all people are expected to be >65 year old in 2050 compared to 10-20% in 2015.[1] This is especially the case in Europe, North America and East Asia.[1] Ageing is associated with changes in the immune system. The lifelong history of infections, changes in microbiota composition, diet, physical activity and stress all contribute to decreased immune function in elderly people.[2] Immune deficiency during ageing occurs at two levels: irreversible primary immune deficiency and reversible secondary immune deficiency of which low nutritional status is an example.[3] Immunosenescence can be seen as an example of primary immune deficiency, in which both adaptive immune responses by B and T cells are reduced, as well as responses of the innate immune system.

Much is known about the effect of ageing on the adaptive immune system, as reviewed by Ventura et al.[4] Numbers of naïve T and B cells are declining during ageing, as well as effector memory T cells. Besides, $CD8^+$ effector T cells are increased, but change phenotypically (e.g. loss of CD8) and regulatory T cells numbers are increased.[4] In contrast, fewer mature B cells are found upon ageing due to declining numbers of progenitors. Serum levels of IgM and IgD are reduced, while IgG and IgA levels are increasing upon ageing.[4] In addition to this, first line immune defences such as fragile skin and antibody production by the mucosal immune system, are decreased in elderly.[5]

Less is known about the effect of ageing on the innate immune system.[6] In ageing reduced responsiveness to pathogens is observed due to reduced expression and activation of pattern recognition receptors (PRRs), such as Toll like receptors.[7] This results in less phagocytosis of pathogens by myeloid cells, resulting in increased levels of C-reactive protein, IL-6 and TNF-α.[8] One of the best documented examples of immunosenescence is the reduced response to influenza vaccination in elderly, which results in only 33% of the cases in protection of elderly, compared to 59% in adults (16-65 years old)[9]. This is partly caused by the fact that the vaccines are optimized for young adults.[10] Elderly (and children) are most vulnerable to influenza infections.[11,12] In addition, influenza infection is associated with an increased rate of pneumonia and other respiratory illnesses, resulting in higher mortality rates in elderly during influenza epidemics.[13,14]

Myeloid dendritic cells (mDCS) and plasmacytoid dendritic cells (pDCs) are two types of human blood DCs that derive from different progenitors and have different functions.[15] MDCs regulate pro-inflammatory responses via inducing T-helper 1 and cytotoxic T lymphocyte responses upon bacterial and viral infections[16]. Plasmacytoid dendritic cells (pDCs) produce type I interferons (IFNs), for example upon Toll like receptor (TLR) 7 mediated activation by influenza virus.[10] Reduced pDCs numbers in elderly may in part explain the increased occurrence of severe influenza infections in this age group.[17]

Inflammaging is another immunological phenomenon associated with ageing. Inflammaging is defined as the increase in inflammatory factors in serum that is seen in ageing.[18] Inflammaging is observed both in diseased elderly as well as healthy centenarians, with high levels of IL-6 being correlated to increased morbidity and mortality.[18] It is hypothesized that after a lifetime of inflammatory immune responses, the immune system in elderly fails to downregulate these responses, resulting in a low-grade chronic inflammation.[19] Whether the increased levels of pro-inflammatory cytokines lead to age-related diseases is a delicate tipping point and probably differs per individual.[18] A possible explanation could be the observation that during ageing the Treg numbers increase, resulting in a suppression of T cell responses (e.g. IFN-γ production) in mice and can subsequently lead to enhanced chronic infections.[20] Inflammaging may relate to the fact that in a relatively short timeframe, during the last century, life expectancy increased far beyond our reproductive age. It has been suggested that the immune system may not have adapted to this elongation of inflammatory exposures.[19]

After menopause, pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) are increased in serum and CD4 T helper cells and B cells are decreased in women, resulting in lower humoral immunity. A consequence of the reduced T helper and B cells numbers is higher susceptibility to infections, which could be linked to reduced estrogen levels in postmenopausal women.[21] Estrogen receptors are found on all kinds of innate and adaptive immune cells, including macrophages and dentritic cells.[22] Furthermore, pDCs of postmenopausal women produce less IFN-α compared to pre-menopausal after TLR7/8 stimulation.[23,24] This effect can be inverted by estrogen supplementation, as

pDCs of postmenopausal women were shown to produce more IFN-α and TNF-α upon TLR7/8 stimulation after 1 month of estrogen supplementation.[24]

The aim of the current study was to compare innate immune function towards TLR-mediated responses between elderly and young adult women. To this end, elderly women and young female adults were compared to study the effect of ageing on the frequency and reactivity of myeloid and plasmacytoid dendritic cells (mDC, pDC) to TLR-mediated responses. Next to this, TLR expression and pro-inflammatory markers in serum were compared.

Material and Methods

In this study, we investigated the effect of ageing on the innate immune system of young and elderly women. Elderly women (65-85 years) n=30 were included for a nutrition intervention study 'NOBLE'. The protocol was approved by the Medical Ethics Committee of Wageningen University, the Netherlands (protocol no. NL57345.081.16), and registered at clinicaltrials.gov (identifier NCT03026244). Furthermore, 15 young women (18-30 years) were included. After providing informed consent, subjects were screened and were included when they were generally healthy, having a BMI 20-30, not smoking and good mental status. Subjects with chronic inflammatory, autoimmune or gastrointestinal diseases or being immune-compromised were excluded from participation. Subjects using hormone replacement therapy, anti-inflammatory drugs (>1x week) or immunosuppressive drugs were excluded. An overview of the demographic information of the two study groups is given in Table 1.

Table 1: Demographics of elderly and young adult study population

Group	Age (years) median + range	Anti-inflammatory medicine (Y/N)	Vitamine D supplementation before study (Y/N)	Blood pressure or cholesterol medication (Y/N)
Elderly women n=30	74.5 (69-85)	1/29	13/17	12/18
Young women N=15	24 (20-29)	1/14	2/13	0/15

Blood sampling

Blood was collected at the study day for serum storage (10 mL tubes; cat. no. 367895, BD) or for PBMC isolation (K2-EDTA; 4 x 10mL; cat.no. 367525, BD). Serum tubes were left at room temperature for at least 30 min before centrifugation at 2000 x g 10 min at room temperature. Serum was aliquoted and stored at -80°C. PBMCs were isolated within 6 hours using 50 ml Leucosep tubes (227290, Greiner Bio-One) filled with Ficoll plaque plus (17-1440-02, GE Healthcare Life Sciences) according to manufacturer's protocol. Remaining PBMCs were cryopreserved and stored in liquid nitrogen.

TLR expression in pDCs and mDCs

Isolated PBMCs were stained with a TLR antibody panel (Table 2) to measure the expression of TLR 2, 4, 7 and 9 *ex vivo*. To measure expression of TLRs, 0.4 x10^6 (young) or 2 x10^6 (elderly) freshly isolated PBMCs/donor were put in a 96 well plate (NUNC PP Sigma-Aldrich 7116) and per well 200 ul FACS buffer (PBS (Lonza BE17-516Q/12) + 2mM EDTA (Merck CBI 108418); 0,5% BSA (Roche 10735086001); 0,01% NaN3 (Merck CBI 822335) was added to wash the cells by centrifuging the plate at 400xg for 3 minutes at 4˚C. First, extracellular surface markers (Table 2) including 5 µl Fc block (564220, BD Pharmingen) were stained for 30 minutes on ice covered in aluminium foil and washed twice with cold PBS. Cells were stained with Fixable Viability Dye FVD520 (65086718, Ebioscience) in PBS and incubated for 20 minutes in the fridge, followed by washing the cells FACS buffer. Afterwards cells were permeabilized by adding IC fixation buffer (00-8222-49, Ebioscience) to each well and incubated for 30 minutes at room temperature, followed by washing twice in Perm buffer (00-8333-56, Ebioscience). The intracellular antibody mix (Table 2) in Perm buffer was incubated for 20 minutes in the fridge, followed by washing the cells twice in Perm buffer. Cells were resuspended in 300 ul FACS buffer and measured for 240s on the FACS CANTO II at medium flow rate, threshold 45.000. As controls the combination of 'fluorescent minus one' (FMO) control was used for the TLR expression and the FMO TLR was replaced with their isotype controls. In order to be able to gate pDCs and mDCs, the 'backbone' antibodies containing live/death, lineage-2, HLA-DR, CD11c and CD123 were used.

Flow cytometry data analysis was performed by using FlowJo software (version 10 TreeStar, Inc.) and gating for mDCs and pDCs was performed as is shown in Supplementary Figure 1, in line with Panda et al.[16] Data were exported as median fluorescent intensity for either all pDCs or mDCs per TLR.

Table 2: Antibodies used for TLR expression and intracellular cytokine measurements.

Antibody	Fluor-chrome	host	isotype	Light chain	company	Catalog number	Panel	Extra/ Intra-cellular
lineage 2	FITC	mouse	IgG1	κ	BD	643397	TLR & cytokine	Extra
HLA-DR	APC-Cy7	mouse	IgG2b	κ	Ebioscience	47-9956-42	TLR & cytokine	Extra
CD123	PE-Cy5	mouse	IgG1	κ	Ebioscience	15-1239-42	TLR & cytokine	Extra
CD11c	PE-Cy7	mouse	IgG1	κ	Ebioscience	25-0116-42	TLR & cytokine	Extra
TLR2	biotin	mouse	IgG2a	κ	Ebioscience	13992282	TLR	Extra
TLR2 ic	biotin	mouse	IgG2a	κ	Ebioscience	13472785	TLR	Extra
streptavidin	BV510				BD	563261	TLR	Extra
TLR 4	BV421	mouse	IgG1	κ	BD	564401	TLR	Extra
TLR4 ic	BV421	mouse	IgG1	κ	BD	562438	TLR	Extra
FVD 520	efluor520				Ebioscience	65-0867-18	TLR & cytokine	Extra
TLR7	PE	mouse	IgG2a		R&D Systems	IC5875P	TLR	Intra
TLR 7 ic	PE	mouse	IgG2a		R&D Systems	IC003P	TLR	Intra
TLR9	APC	rat	IgG2a	κ	Ebioscience	17909982	TLR	Intra
TLR9 ic	APC	rat	IgG2a	κ	Ebioscience	17-4321-81	TLR	Intra
CD16	BV510	mouse	IgG1	κ	BD	740203	Cytokine	Extra
IL-6	PE	rat	IgG1	κ	Ebioscience	12706982	Cytokine	Intra
IL-6 ic	PE	rat	IgG1	κ	Ebioscience	12430183	cytokine	Intra
IFN-alpha	V450	mouse	IgG1	κ	BD	561382	Cytokine	Intra
IFN-alpha ic	V450	mouse	IgG1	κ	BD Horizon	561504	cytokine	Intra
TNF-alpha	APC	mouse	IgG1	κ	Ebioscience	17734982	Cytokine	Intra
TNF-alpha ic	APC	mouse	IgG1	κ	Ebioscience	17-4714-41	Cytokine	Intra

Antibody mixes were made for extra-cellular or intra-cellular staining. ic= isotype control

Intracellular cytokine measurement in pDCs and mDCs

In order to measure intracellular cytokines 0.4 x10^6 (young) or 2 x10^6 (elderly) PBMCs were stimulated in a 12-well plate (CLS3513-50ea, Sigma-Aldrich) (total volume 1 ml) for three hours with RPMI-1640 (Be112-115F, Lonza), TLR1/2 ligand PAM3CSK4 (PAM) 10 µg/ml (L2000, EMC microcollections),

TLR4 ligand Ultra-pure LPS 0,1 µg/ml (3pelps, Invivogen), TLR7/8 ligand R848 3 µg/ml (TLRL-R848-5, Invivogen) or TLR9 ligand CpG 3 µg/ml (TLRL-2216-1 (class 'A'), Invivogen). All TLR stimulations were done in the presence of Brefeldin A (B7651, Sigma-Aldrich) in RPMI-1640 with 5% human AB serum (H4522, Sigma Aldrich). Afterwards, cells were harvested by pipetting and stained in the same way as described for the TLR staining. Cells were resuspended in 250 ul FACS buffer and measured for 200s with FACS CantoII. Flow cytometry data analysis was performed by using FlowJo software (version 10 TreeStar, Inc.) and gating was performed as is shown in Figure 1. As controls, the combination of 'fluorescent minus one' (FMO) control was used for the intracellular cytokine production and intracellular cytokines were replaced with their isotype controls, see Supplementary Figure 2. In order to be able to gate pDCs and mDCs, the 'backbone' antibodies containing live/death, lineage-2, HLA-DR, CD11c and CD123 were used. Gating of DCs and pDC and mDCs was adapted per donor, other gates were set based on FMO + ic controls or negative versus positive population in most donors. Data were exported as percentage cytokine-positive pDCs or mDC as percentage of all pDCs or mDCs.

Cytokine and pro-inflammatory marker measurements in serum

In serum, IL-1β (558279, BD Pharmingen); TNF-α (560112, BD Pharmingen), IL-6 (558276, BD Pharmingen) sCD106 (sVCAM-1; 560427, BD Pharmingen), sCD54 (ICAM-1; 560269, BD Pharmingen) and IL-10 (558274, BD Pharmingen) were measured in triplo by cytometric bead array, according to manufacturer's protocol. Beads were measured for 50 seconds at high speed using a FACS CANTO II. Furthermore, IL-1Ra (CHC1183, Thermo Fisher) was measured by ELISA and C-Reactive Protein (CRP) was measured using C-Reactive Protein kit ELISA (Ebioscience, 88-7502-28) according to manufacturer's protocol.

Statistical analysis

Statistical analysis was performed by using IBM SPSS Statistics version 23. Data were tested for normal distribution using Shapiro-Wilk test. The cytokine production (% of all pDCs or all mDC) of pDC and mDCs per stimulation (RPMI, PAM, LPS, R848 and CpG) were analysed by MANOVA with a pairwise comparison using bonferroni correction. To obtain normally distributed data,

percentages were logit-transformed or rank-transformed, after which outliers (>2SD) were removed. For TLR 2, 4, 7 and 9 expression the median MFI was analyzed per cell type (pDC or mDC). The median MFI was 10log transformed to obtain normal distributed data. A MANOVA-analysis was performed per cell type (pDC or mDC) with a pairwise comparison using bonferroni correction. Cytokine levels (pg/ml) were 10log transformed or rank transformed (in case of IL-1β, TNF-α and sICAM) to obtain normal distributed data. As the value 0 cannot be 10log transformed, this value was artificially put on 0,001 to obtain a value after transformation. MANOVA-analysis was performed with a pairwise comparison using bonferroni correction.

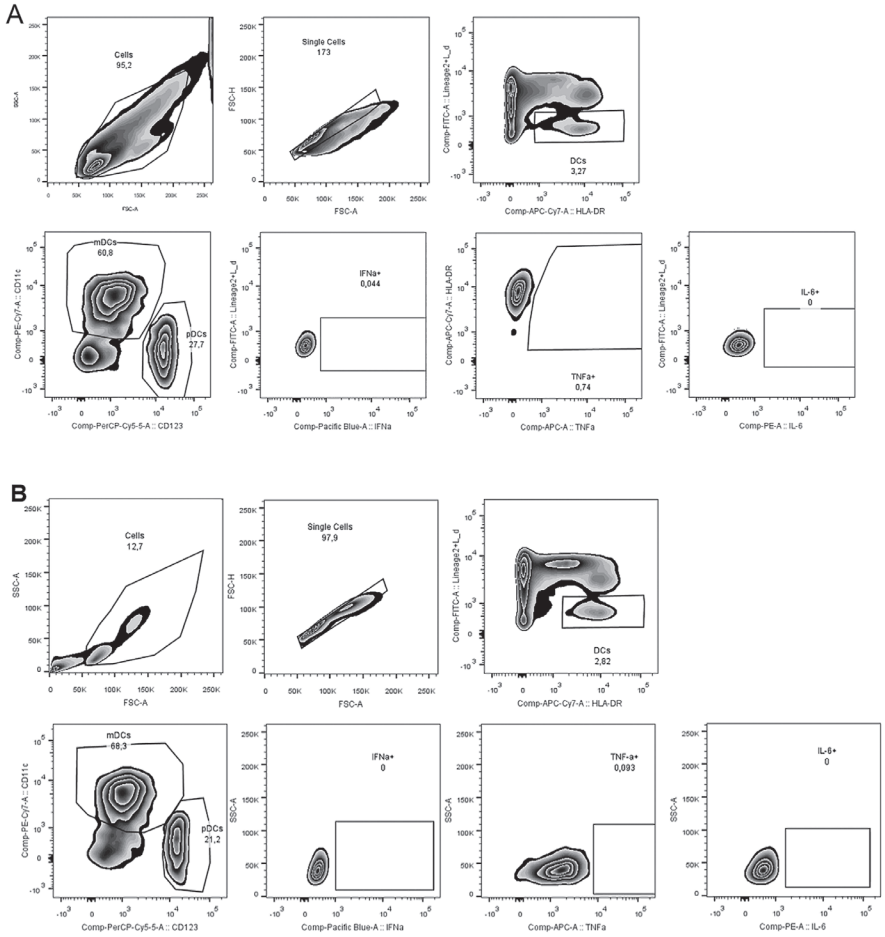

Figure 1: Gating strategy of intracellular cytokine production by a donor from the elderly population (171/002) (A) or a donor from the young adult population (171/young/003) (B) in steady state (RPMI).

Results

pDC numbers, but not mDC numbers, in peripheral blood decrease upon ageing

To study the numbers of mDC and pDC in peripheral blood of elderly and young adult women, PBMC were isolated form freshly drawn blood, and were stained with Lineage markers 2 cocktail, HLA-DR, CD11c, CD123, IFN-α, TNF-α and IL-6. The gating strategy shown in Figure 1 was used to determine the % of pDC and mDC in the total DC population. mDC were defined by expression of Lineage 2^- HLA-DR^+ $CD11c^+$ $CD123^-$, and pDC were identified by Lineage 2^- HLA-DR^+ $CD11c^-$ $CD123^+$. In addition to pDC and mDC, a $CD11c^-CD123^-$ double negative cell population was observed. The relative percentage of pDCs and mDCs as part of the total DC population was determined. As shown in Figure 2, the relative percentage of pDCs in elderly women was significantly lower compared to pDC in young adults. In contrast, the relative percentage of mDCs between elderly and young adults did not differ significantly. As a result, the relative percentage of the double negative DC population was higher in the elderly total DC population. To correct for this, the ratio between %mDC and %pDCs was taken. The ratio %mDC/%pDCs, with a mean value of 6.97±6.06, was higher in elderly than in young adults, which had a mean ratio of 2.91 ±1.26, see Figure 2a. As the relative percentage of mDCs was not different between young adults and elderly, this means that the percentage of pDCs of all DCs was indeed lower in elderly and was not due to changes in the $CD11c^-CD123^-$ double negative cell population. Furthermore, the spontaneous production of intracellular cytokines by mDCs (Figure 2b) and pDCs (Figure 2c) was determined in unstimulated PBMC. A higher number of mDCs of young adults produced TNF-α or IFN-α compared to elderly. In contrast, the percentage of pDCs of elderly producing IFN-α was higher compared to young adults, whereas no differences were observed for IL-6 and TNF-α positive pDCs between young adults and elderly.

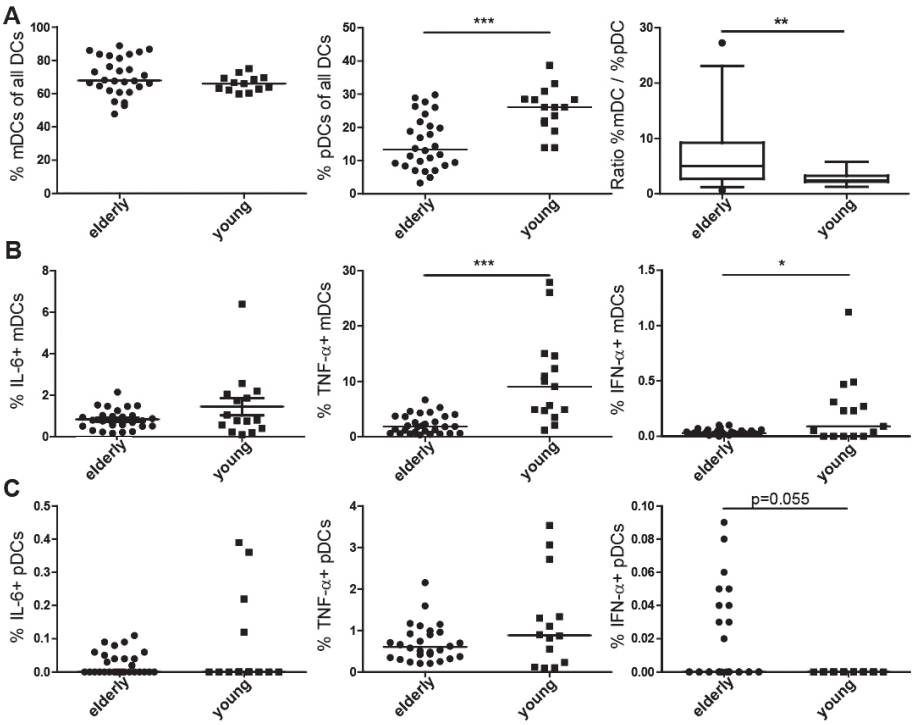

Figure 2: Percentage of pDCs and mDCs and ratio (%mDC / %pDCs) in elderly and young adults (A) and percentage of mDCs (B) and pDCs (C) positive for intracellular cytokine production of IL-6, TNF-α and IFN-α in steady state (RPMI). Data shown as dotplot with median, outliers with >2SD based on transformed data were removed. Elderly n=30; young n=15 donors. Statistics were done with logit transformed data using MANOVA with a pairwise comparison and a bonferroni correction. *p<0.05; **p<0.01; *** p<0.001.

Age-related differences in intracellular cytokine production of pDCs in response to TLR stimulation

It was investigated how pDCs respond to TLR stimulations. To this aim, pDCs were stimulated with TLR1/2 ligand Pam3CSK4 (Pam), TLR 4 ligand LPS, TLR 7/8 ligand R848 and TLR 9 ligand CpG, and intracellular production of TNF-α, IL-6 and IFN-α was determined as described in materials and methods. The gating strategy to detect intracellular cytokine production of pDCs upon TLR stimulation is shown in supplementary figure 1 and of the flow cytometric controls (fluorescence minus one (FMO) and isotype control) in supplementary Figure 2. The relative percentages of pDCs did not change after TLR stimulation, which indicates that the markers used for pDC gating do not change upon stimulation, see Supplementary Figure 3a. Therefore, the relative percentages of pDCs upon TLR stimulation were still lower in elderly compared to young adults.

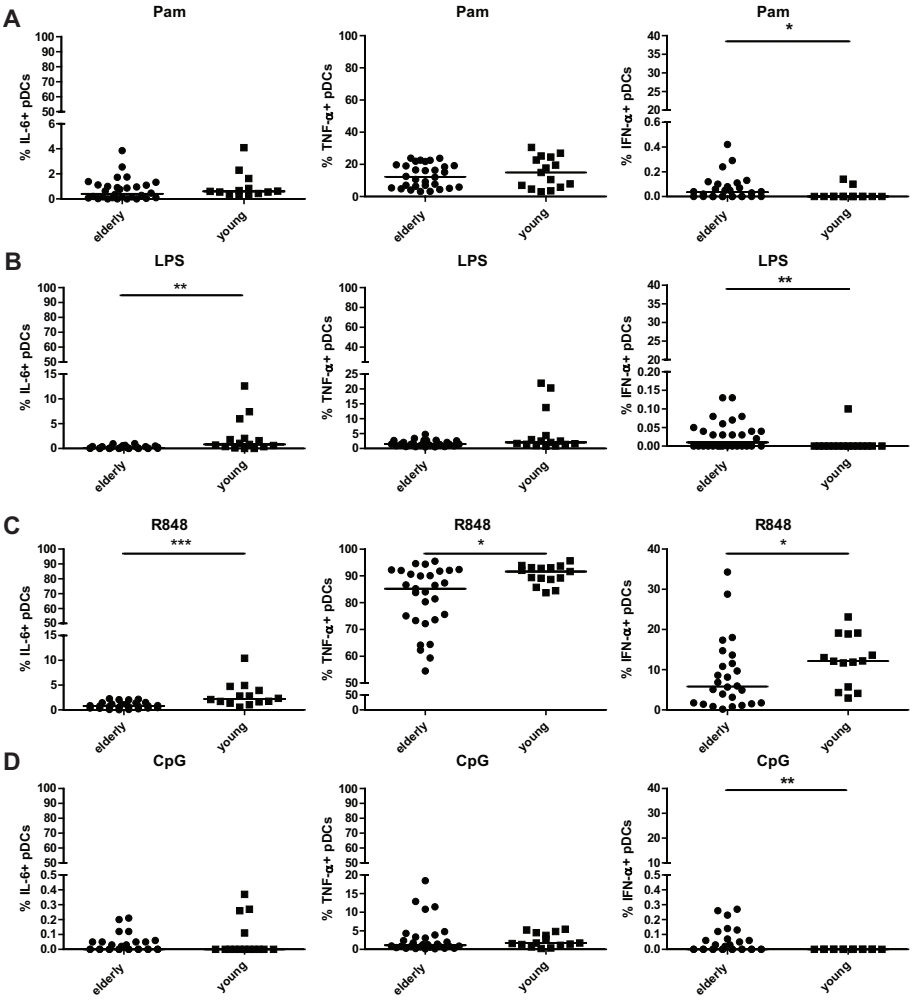

Figure 3: Intracellular cytokine production (IL-6, TNF-α and IFN-α) of pDCs upon TLR1/2 (A), TLR4 (B), TLR7/8 (C) and TLR9 (D) stimulation in young versus older adults. Data shown as dot plots with median, every dot represents a donor, outliers with >2SD based on transformed data were removed. Elderly n=30; young n=15 donors. Statistics were done with logit or rank transformed data using MANOVA per TLR stimulation with a pairwise comparison and a bonferroni correction. *p<0.05; **p<0.01; *** p<0.001.

When pDC were stimulated with TLR ligands, the number of TNF-α$^+$ pDCs was highest for all stimulations. The highest percentage of IFN-α$^+$ pDC was detected after stimulation with R848, while IL-6$^+$ pDC were detected in comparable numbers upon stimulation with Pam, LPS and R848.

R848 was the only stimulus that induced strong IFN-α production by pDC. This response was significantly higher in the young adult women compared to the elderly women tested. For the other stimuli, remarkably, the percentage of IFN-α$^+$ pDCs was significantly higher in elderly pDC than in young adult pDC (Figure 3A-D). For TLR stimulations other than TLR7/8 stimulation, this may partly be explained by a higher spontaneous production of IFN-α in pDC in elderly women (Figure 2C).

Upon TLR4 and TLR7/8 stimulation, young adults had significantly more IL-6$^+$ pDCs compared to elderly (Figure 3B, C). Furthermore, the number of TNF-α$^+$ pDC after TLR7/8 stimulation with R848 was also higher in the young adults. Taken together, in general, pDCs of young adult women responded more to TLR stimulation than elderly women. This was demonstrated most prominently after stimulation with TLR7/8, with statistically significant higher percentages of all cytokine-producing pDC in the young adult women. The fact that elderly women also had lower numbers of circulating pDC indicates that this is an underestimation, as the data are represented as % of pDC.

Age-related differences in intracellular cytokine production of mDCs upon TLR stimulation

The effects of ageing on the percentage of mDCs were less prominent than the effects shown for pDCs. Only after stimulation with TLR4, elderly had a significantly higher percentage of mDCs (Supplementary Figure 3B) compared to young adults. However, this did not seem to affect differences in intracellular cytokine production, see below.

After stimulation with the different TLR stimuli, only the number of TNF-α$^+$ mDCs was significantly higher in young adults after LPS, R848 and CpG stimulation, while after Pam stimulation TNF-α$^+$ mDCs tended to be higher ($p=0.07$) in young adults compared to elderly, see Figure 4. No significant differences in IL-6$^+$ mDCs were observed.

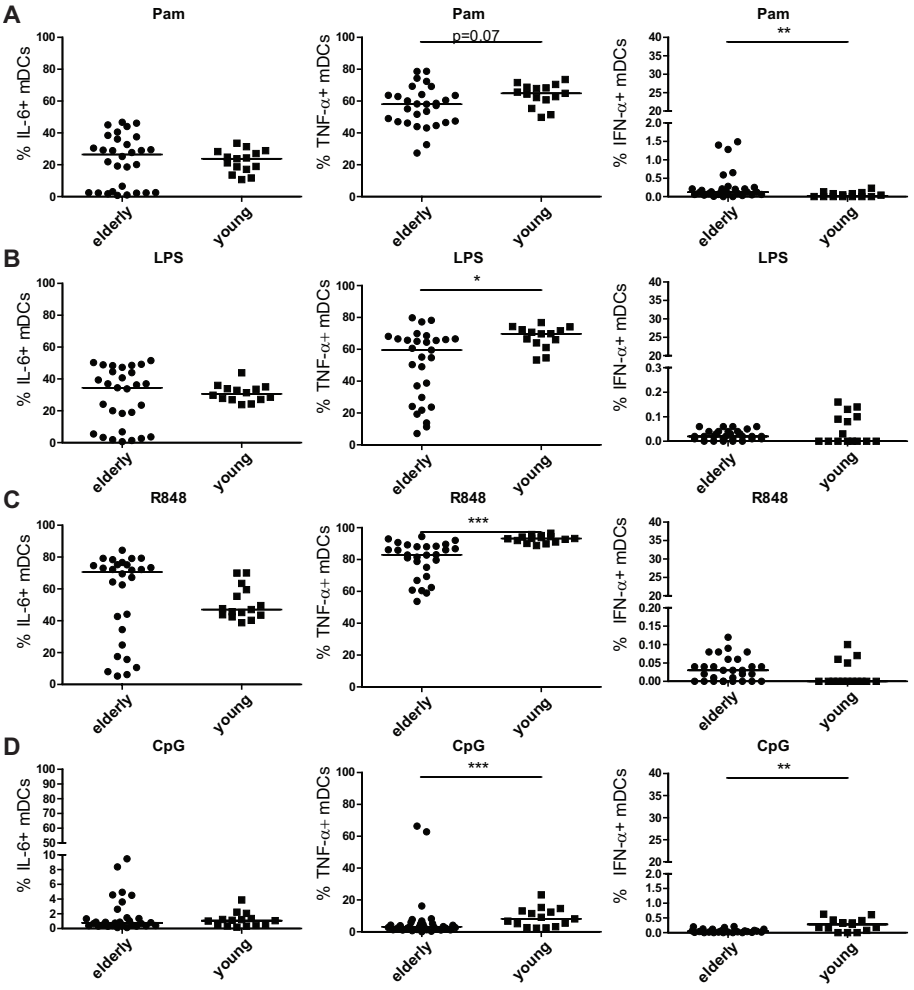

Figure 4: Intracellular cytokine production (IL-6, TNF-α and IFN-α) of mDCs upon TLR1/2 (A), TLR4 (B), TLR7/8 (C) and TLR9 (D) stimulation in young versus older adults. Data shown as dot plots with median, every dot represents a donor, outliers with >2SD based on transformed data were removed. Elderly n=30; young n=15 donors. Statistics were done with logit or rank transformed data using MANOVA per TLR stimulation with a pairwise comparison and a bonferroni correction. *p<0.05; **p<0.01; *** p<0.001.

In contrast, elderly had relatively higher percentages of IFN-α$^+$ mDCs compared to young adults upon TLR1/2 stimulation (Figure 4A). No differences were observed between elderly and young adults in IFN-α$^+$ mDCs after TLR4 and TLR7/8 stimulation. In contrast to pDCs and TLR1/2 stimulation of mDCs, young adults had higher percentages of IFN-α$^+$ mDCs when stimulated with TLR9 ligand CpG (Figure 4D). In all cases, the number of IFN-α$^+$ mDCs was very low.

In conclusion, young adults had more TNF-α$^+$ mDCs compared to elderly upon all TLR stimulations, and more IFN-α$^+$ mDCs when stimulated with TLR9 ligand CpG. However, for the latter this may be partly explained by the increased spontaneous production of TNF-α and IFN-α by mDC as shown in Figure 2B.

Pro-inflammatory markers sICAM, sVCAM and TNF-α elevated in serum of elderly

To determine the inflammatory status of the elderly and young adult women, a panel of pro-inflammatory markers that are known to be associated with ageing[2] was measured in serum samples. The concentrations of pro-inflammatory markers soluble ICAM (sICAM), soluble VCAM (sVCAM) and TNF-α were significantly higher in sera of elderly compared to sera of young adults, see Table 3. Furthermore, the concentration of IL-10 was also increased in elderly women. IL-1Ra showed a trend (p=0.06) to be decreased in elderly women. No differences in IL-6, IL-1β and CRP levels were observed.

Table 3: Concentration of pro-inflammatory markers in serum in median pg/ml with range (minimum to maximum value) in elderly (n=30) and young adults (n=15) women.

	Elderly (pg/ml)	Young adult (pg/ml)	Statistically different
IL-6	3.77 (0.55-10.54)	2.31 (0.00-8.17)	NS
IL-1β	0.22 (0.00-3.59)	0.02 (0.00-1.78)	NS
IL-10	2.93 (0.949-5.71)	1.47 (0.53-5.34)	**
TNF-α	13.57 (0.00-66.93)	0.00 (0.00-48.99)	**
sICAM ng/ml	84.38 (0.00-281.81)	15.10 (0.00-180.48)	***
sVCAM ng/ml	258.48 (104.14-573.42)	153.13 (46.49-328.07)	***
IL-1Ra	58.93 (13.73-3715.71)	87.40 (46.10-179.07)	P=0.06
CRP (ng/ml)	538 (12-12456)	662 (52-29200)	NS

Statistical analysis was performed using 10log or rank transformed data by MANOVA with pairwise comparisons using bonferroni correction. * p<0.05; ** p<0.01; *** p<0.001. NS no significant differences.

Discussion

The results presented here show that elderly women have reduced numbers of circulating pDCs. In addition, pDCs and mDCs of elderly women are less responsive to TLR stimulation, especially TLR7/8 mediated stimulation, compared to pDC and mDCs of young adults. In serum, markers involved in inflammation are increased in elderly compared to young adults.

Immune responses are not equal between men and women.[23,25] TLR7 is X-linked which might explain why women have better TLR7-mediated responses.[22] Therefore, we compared young adult women with postmenopausal elderly women.

Ageing is associated with changes in the immune system, leading to inflammaging and immunosenescence.[18,19] One aspect of immunosenescence is that immune cell numbers decline. Elderly women in this study had less pDCs compared to young adult women, which is in line with many other studies[10,17,23,26–31], although some studies detected no difference upon ageing[32,33]. Reduced numbers of pDCs could lead to increased TLR7/8 mediated infections, such as influenza and RSV infection.[34] The percentage of mDCs in elderly women was similar to young adults in steady state, in agreement with other studies[17,32], although some studies observed a decrease in mDCs percentages upon ageing.[28,33] It is important to look not only to the percentage of pDCs and mDCs but also to the ratio between the two, as there is a third category of double negative $CD11c^{-}$ $CD123^{-}$ cells in the current gating strategy that changes between the two age groups (see Figure 1). Based on FSC/SSC back-gating we hypothesize that these cells could be a mixture of $CD16^{+}CD56^{dim}$NK cells, BDCA-3^{+} mDCs or $CD34^{+}$ DC-like cells.[31,35,36] Future research is needed to further investigate the function of this population in ageing.

Besides looking at percentages of pDCs and mDCs upon ageing, their cytokine production in steady state and upon TLR stimulation was also investigated. In steady state, young adult women had more TNF-α^{+} and IFN-α^{+} mDCs, but comparable IL-6^{+} mDCs, while elderly women seemed to have more IFN-α^{+} pDCs, but comparable levels of TNF-α^{+} and IL-6^{+} pDCs in steady state. These

data partly confirm the study of Panda et al., who also observed higher levels of TNF-α$^+$ mDCs and of TNF-α$^+$ and IFN-α$^+$ pDCs in elderly.[26] In contrast, they also showed higher percentages of IL-6$^+$ mDCs and TNF-α$^+$ pDCs in elderly. A possible explanation could be that Panda et al. used a mixed population of male and female subjects, with 70% females in the young population and 41% females in the old population. It could be speculated that older males have a higher basal cytokine production by mDCs compared to women, as is known for IL-6 levels in blood[37]. This increased basal cytokine production by both mDCs and pDCs of elderly, although only confirmed for IFN-α$^+$ pDCs in this study, could contribute to the increased level of IL-6 and TNF-α in serum observed in general in inflammaging.[8]

Although in general elderly suffer from inflammaging, which is expressed by an increased basal cytokine production, elderly often have reduced cytokine responses after TLR stimulation as reviewed by Kollman et al. and Shaw et al.[7,38] In this paper, we focused on the differences between pDC and mDC responsiveness in elderly and young women.

After TLR7/8 stimulation, elderly women had less IL-6$^+$ and TNF-α$^+$ pDCs and less IFN-α$^+$ pDCs. However, upon other TLR stimulations elderly had more IFN-α$^+$ pDCs, albeit with very low percentages. This difference in reactivity could partly be explained by reduced TLR4 expression. However, this does not account for TLR2 and TLR9, of which the expression was even increased in elderly, see supplementary Figure 4. Our findings confirm other studies in which pDCs were stimulated with influenza or West Nile Virus (TLR7)[17,39], R848 (TLR7/8)[24,26,39] or CpG (TLR9)[17,26]. In these studies, no differences were observed in the amount of IFN-α produced per cell.[17] In contrast to our finding that TLR9 expression in pDCs was increased, Panda et al. observed a similar TLR9 expression on pDCs between old and young adults, and reduced TLR7 mRNA expression in elderly, whereas Garbe et al. found reduced TLR9 expression upon ageing.[26,27]

Upon TLR stimulation, mDCs of elderly women had a similar percentage of IL-6$^+$ mDCs and less TNF-α$^+$ mDCs for all TLR stimulations compared to young women. The percentage of IFN-α$^+$ mDCs was higher in elderly after TLR1/2 stimulation, but lower after TLR9 stimulation. In contrast to Krug et al., IFN-α$^+$

mDCs were observed upon CpG stimulation[40]. For both TLR1/2 and TLR9 stimulation, the percentage of IFN-α$^+$ mDCs was very low and therefore the biological relevance is debatable. In the study of Panda et al. mDCs of young people were more responsive to stimulation with TLR1/2, TLR2/6, TLR5 and TLR7/8 than mDCs from elderly, which is confirmed in our study.[26] This suggest that mDCs of elderly are less able to respond to viral and bacterial infections, leading potentially to more severe infections in elderly.

Of all TLR expression we measured on mDCs, only TLR2 expression was significantly higher in elderly compared to young women (supplementary Figure 5). In contrast, Panda et al. measured a higher TLR 1, 2, 3 and 8 expression on mDC by flow cytometric analysis in young adults, while Jing et al. observed comparable TLR2 and TLR4 expression between young adults and elderly.[17,26]

So although elderly have comparable numbers of mDCs compared to young adults, their mDCs are less responsive to TLR stimulation, which cannot be explained by TLR expression. These phenomena may underlie part of the observed immunosenescence upon ageing.

In serum of elderly women, increased levels of sICAM, sVCAM, TNF-α and IL-10 were detected compared to young women. For TNF-α this is confirmed by many studies[8,33,41,42], although not all[43–45]. An increased level of IL-6 is often observed[8,42,43,45–47], while in our study IL-6 was slightly higher in elderly but not significantly enhanced, like in the study of Beharka et al.[48] Some studies observed no difference between old and young individuals for serum levels of IL-6 and IL-10[33,49] and TNF-α[49]. For IL-10, several studies do not detect differences in serum levels upon ageing[44,45,47,49], while in our study IL-10 was enhanced in elderly women. A trend was observed of increased levels of IL-1Ra in serum, which is in line with other studies.[43,50] An increased level of IL-1Ra could be considered beneficial; IL-1Ra is a natural inhibitor of IL-1β as they bind the same IL-1β-receptors, but IL-1Ra binding does not lead to receptor activation.[51] High concentrations of TNF-α in serum have been associated with Alzheimer disease, atherosclerosis and frailty.[52,42] TNF-α together with IL-1β induces IL-6 production, while the three cytokines together induce the production of CRP, leading to inflammatory responses in multiple sites of the

body.[2,42] A potential explanation for not observing a significant increase in IL-6 and CRP concentration in serum in elderly in this study, might be related to the low levels of IL-1β that were detected as well.[44] An explanation for the increased levels of IL-10 and TNF-α in serum of elderly women could be the decreased levels of estrogen. Estrogen used as hormone replacement therapy in elderly women has been shown to reduce IL-10, IL-6 and TNF-α levels in serum, as reviewed by Giefing-Kröll et al.[53]

Next to cytokines, the adhesion molecules sICAM and sVCAM-1 were elevated in elderly compared to young adults, which is in line with other studies.[47,54,55] Soluble forms of ICAM and VCAM are shed from the cell surface, and their expression is connected to migration of immune cells towards arthero-sclerotic plaques and inflammatory lesions.[55] It has been suggested that sICAM and sVCAM expression is activated by oxidative stress, which is supposed to increase by age.[55] Antioxidant supplementation, which is supposed to mimic the antioxidant effect of estrogen in postmenopausal women, resulted in reduced levels of sICAM and sVCAM.[56] Therefore the increase in both sICAM and sVCAM in our study in the postmenopausal group of women could partly be explained by the loss in estrogen production, which is observed in general. However, increased levels of sICAM and sVCAM are also observed in mixed ageing population.[47,55] Altogether, there is an increase in pro-inflammatory markers in serum of elderly women, confirming the concept of inflammaging. This inflammaging may be related to the reduction in estrogen levels after menopause.

Recent hypotheses have suggested that the decreased response to TLR stimuli in elderly people and the enhanced steady state production of cytokines by blood cells of elderly people may both be linked to miRNAs.[57] Interestingly, miRNAs seem to play an important role in TLR signaling[57] and these miRNAs (e.g. miRNA-21; miRNA-126; miRNA-146) are modulated during ageing.[58] MiRNA-21 increases upon ageing, while miRNA-146 decreases, although concentrations in serum of both miRNAs can be decreased by estrogen hormone replacement therapy (HRT) in postmenopausal women.[59] So, if ageing affects miRNA expression, leading to dysregulation of TLR function (increased pro-inflammatory cytokines), and if miRNA can also inhibit TLR function, the balance of inhibitory and stimulatory miRNA might play a role in

both processes. This would be an interesting topic to study in-depth in future gerontological studies.

In this study differences between elderly and young women are observed. The question remains whether that is a bad thing in itself. One would expect that a lifetime of exposure to environmental factors such as infections and vaccinations would result in an experienced immune system with less naive T cells[21], less CD34 progenitor cells[33,36] and thereby changed numbers in dendritic cells as well. In steady state, it might be a positive situation that pDCs and mDCs of elderly do not produce many cytokines, as it would increase the levels of pro-inflammatory cytokines in serum even further. Reduced responsiveness to pathogens, as mimicked by stimulation by TLR agonists, can be beneficial in case of severe infections of influenza, as immune-pathological effects are reduced[60]. As long as an individual eventually can clear the virus, this does not have to be a problem. It does become a problem when pathogens cannot be cleared. This is often the case in elderly when it comes to mortality caused by influenza infections.[10]

Conclusion

In this study, elderly women were shown to have lower pDCs frequencies, comparable mDC frequencies, lower basal production of cytokines by pDCs and mDCs in steady state, and increased markers involved in inflammation in serum, compared to young adult women. Especially after TLR7/8 stimulation, pDCs and mDCs of elderly are less responsive compared to young adult women. However, no direct link was observed between intracellular immune responses by pDCs and mDCs and pro-inflammatory cytokines in serum. This confirms the concept of both immunosenescence and inflammaging in elderly women.

Acknowledgements

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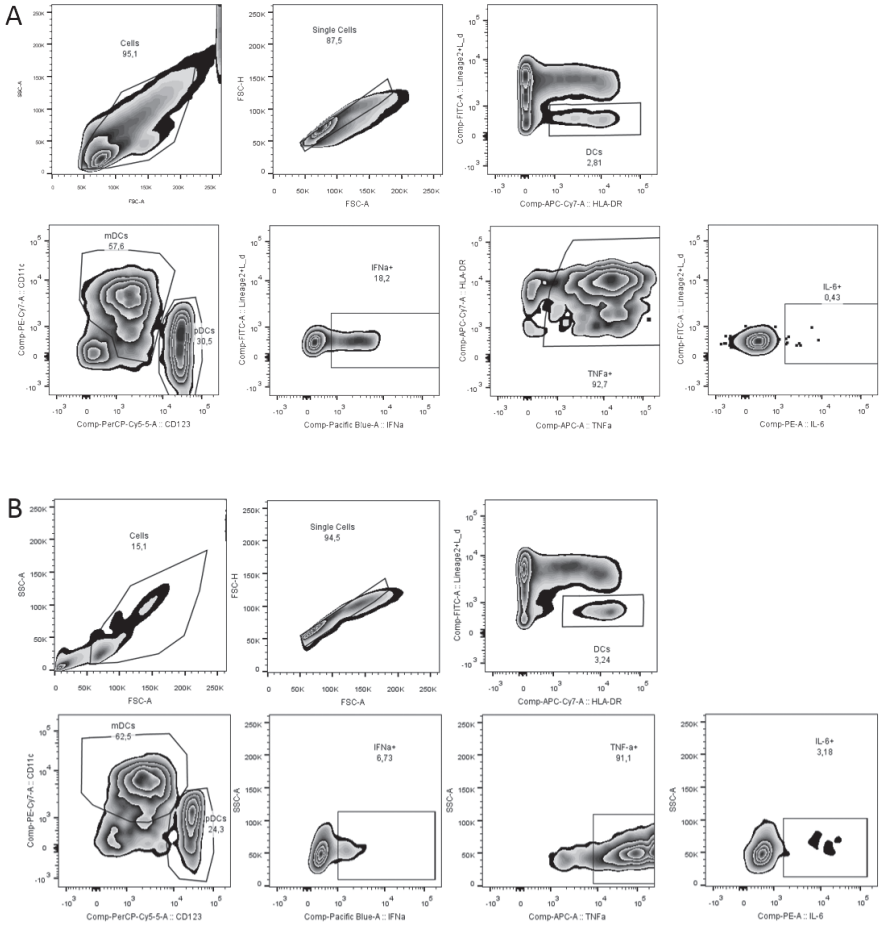
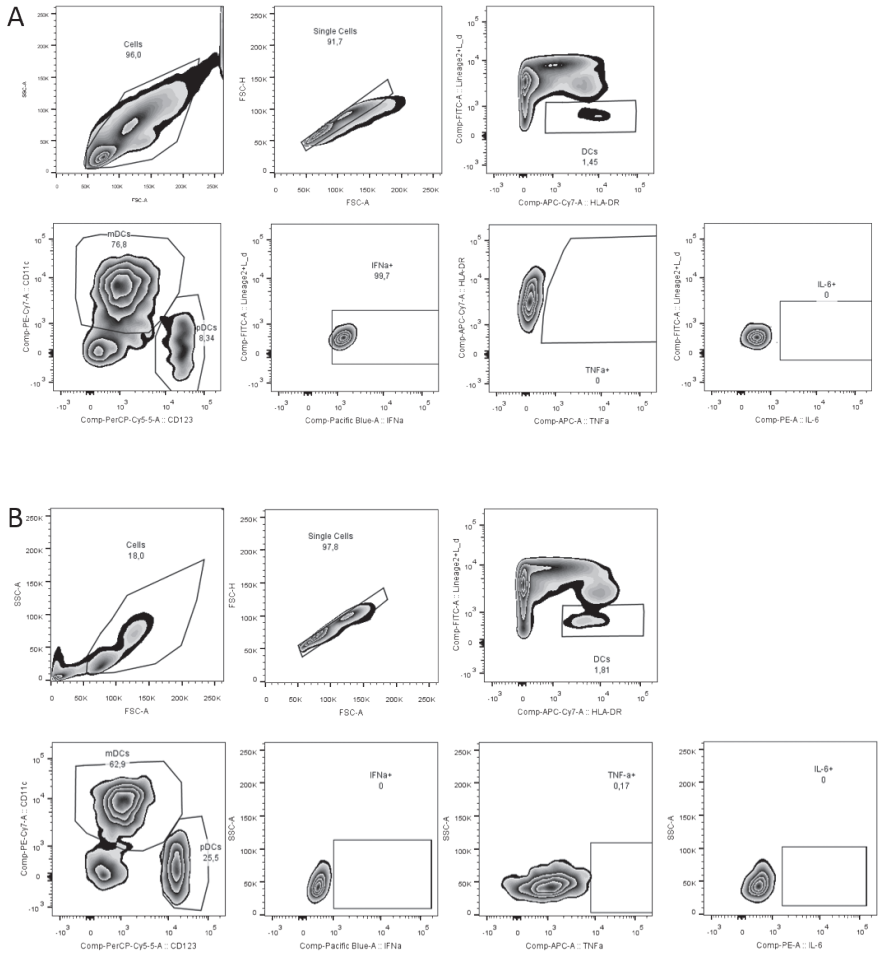
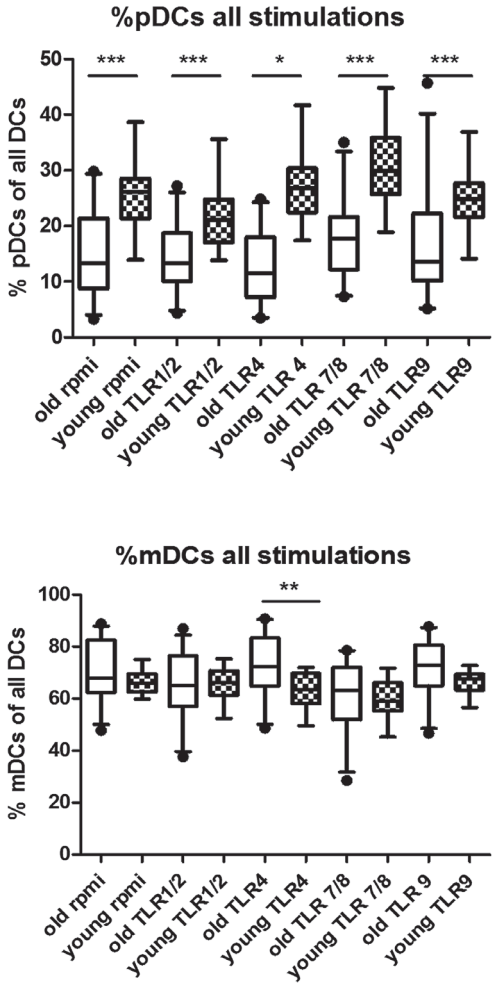


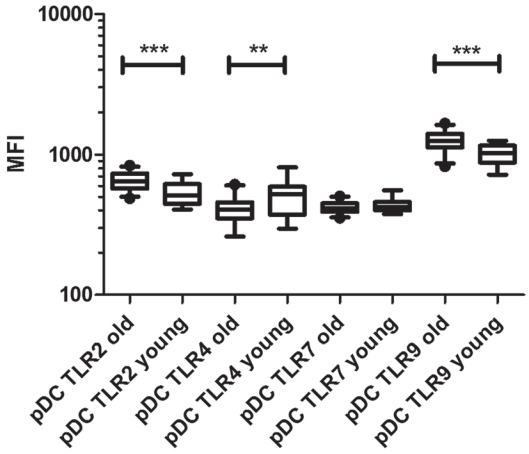
Supplementary Figure 1: Intracellular cytokine production of pDCs by a donor from the elderly population (171/002) (A) or a donor from the young adult population (171/young/3) (B) upon TLR 7/8 stimulation with R848.



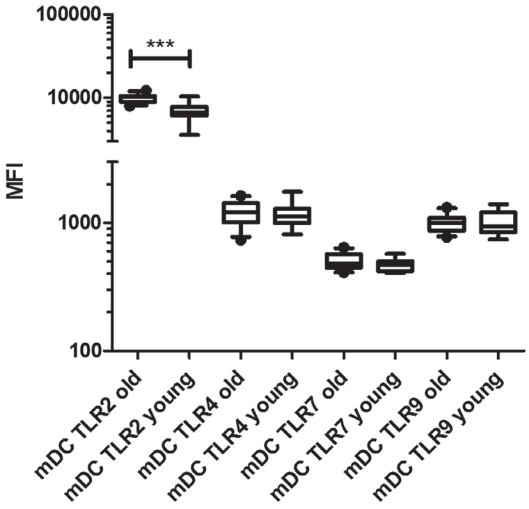
Supplementary Figure 2: Intracellular cytokine production by pDCs of a control sample of a young adult woman (A) and an elderly woman (B); mDC/pDC backbone with isotype controls for IFN-α, IL-6 and TNF-α.



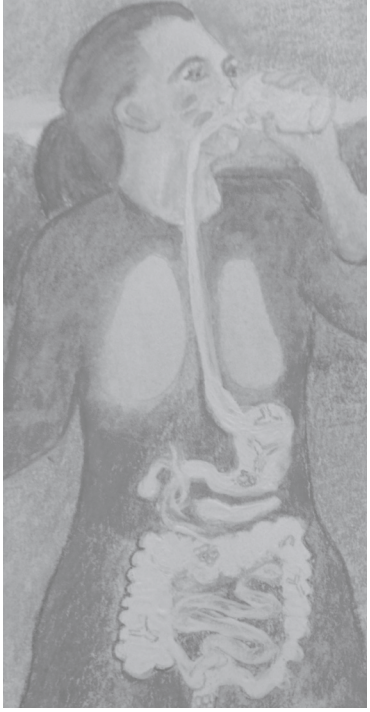
Supplementary Figure 3: Percentage of pDCs (upper graph) and mDCs (lower graph) in steady state (RPMI) and upon TLR stimulation in elderly (white) and young adults (black squares). Data shown as 5-95% whisker plots, outliers with >2SD based on transformed data were removed. Elderly n=30; young n=15 donors. Statistics were done with logit transformed data using MANOVA with a pairwise comparison and a bonferroni correction. *p<0.05; **p<0.01; *** p<0.001.



Supplementary Figure 4: TLR expression on pDCs of elderly and young women measured *ex vivo*. Statistical analysis was done using a MANOVA with a bonferroni correction. * p<0.05; ** p<0.01; ***p<0.001



Supplementary Figure 5: TLR expression on mDCs of elderly and young women measured *ex vivo*. Statistical analysis was done using a MANOVA with a bonferroni correction. * p<0.05; ** p<0.01; ***p<0.001



Chapter 7

Bovine lactoferrin enhances TLR7-mediated responses in plasmacytoid dendritic cells in elderly women

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Abstract

Background

During ageing both the adaptive and innate immune system become dysregulated. Especially plasmacytoid dendritic cells (pDCs) and myeloid DCs (mDCs) have reduced Toll like receptor (TLR)-mediated responses resulting in increased susceptibility to (viral) infections. Consumption of bovine lactoferrin (bLF) has been shown to reduce infections with RNA viruses that can be recognized through TLR7. Galactooligosacharides (GOS) and vitamin D are associated with reduced pro-inflammatory cytokine levels in serum, and increased TLR7/8 responses, respectively.

Objective

A double-blind placebo-controlled nutritional intervention study in elderly women was performed to investigate the potential of bLF, GOS, and vitamin D to restore TLR responsiveness of pDCs and mDCs, and to reduce inflammatory markers in serum.

Design

The nutritional intervention group (n=15) received bLF for 3 weeks, followed by 3 weeks of bLF + GOS, and subsequently 3 weeks of bLF + GOS + vitamin D. The placebo group (n=15) received maltodextrin as placebo for 9 weeks. Blood was collected every 3 weeks, and TLR responses of pDCs and mDCs were measured as well as inflammation-related markers in serum. A RAND-36 questionnaire was filled at the beginning and at the end of the study.

Results

After 3 weeks of bLF supplementation, increased TLR7/8 and TLR1/2 responses were observed in pDCs of the nutritional intervention compared to the placebo group. When the effects of the entire nutritional intervention with bLF, GOS and vitamin D were investigated (day 0 vs day 63), only increased TLR1/2 mediated responses in mDCs were observed, and in serum only sVCAM tended to decrease. Finally, based on the RAND-36 questionnaire physical function tended to improve in the intervention group.

Conclusions
Since especially TLR7-mediated responses in pDCs were enhanced after bLF supplementation compared to placebo, this suggests that bLF may contribute to antiviral responses mediated by pDC in elderly women.

Introduction

During ageing, the immune system becomes dysregulated, as indicated by two phenomena: immunosenescence and inflammaging. In immunosenescence, both the innate and adaptive immune system are dysregulated. Dysregulation of the immune system seems to involve, amongst others, changes in the number and function of lymphocytes and innate immune cells, as well as altered expression of Toll-like receptors (TLRs).[1–3] Because of these compromised innate and adaptive immune responses, elderly people have a decreased ability to respond to infection and vaccination.[2–5] Furthermore, many age-related health disorders, such as osteoarthritis, metabolic diseases, cognitive decline, onset of frailty, and cardiovascular diseases are associated with inflammation, often referred to as inflammaging.[4,6–9] Inflammaging is associated with increased serum concentrations of pro-inflammatory cytokines,[3] acute-phase proteins and soluble adhesion markers[10]. The age-related reduced response to TLR stimulation is best described for myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs).[8,11–14] Interestingly, in relation to anti-viral immune responses, pDCs of elderly people have been shown to produce lower concentrations of antiviral IFN-α and pro-inflammatory cytokines upon TLR7 and TLR9 stimulation, resulting in lowered antiviral immunity.[15,16]

Elderly people are more susceptible to severe influenza and respiratory syncytial virus (RSV) infection leading more often to hospitalization compared to adults[17] and are less responsive to influenza vaccination[8]. Influenza and RSV are both single stranded RNA viruses, and the innate immune response to such viruses is mainly TLR7 mediated.[14,18] Bovine Lactoferrin (bLF) is linked to reduced number of infections by rhinoviruses and hepatitis C which are also recognized by TLR7.[19–21] BLF is an antimicrobial protein that is known to prevent sepsis, fungal infections and enterocolitis in premature infants.[22–24] In addition, bLF has been described to have anti-inflammatory effects.[25,26] Therefore, bLF might be a nutrient from milk that is able to restore TLR7 responses of pDC to viruses. Next to this, elderly have reduced serum concentrations of vitamin D in winter, which has been shown to correlate with reduced expression and responsiveness of TLR7 and TLR8 on monocytes[27]. This suggests that vitamin D may also impact TLR7/8 responsiveness.
In addition to direct effects on immune function, ageing is also associated with

microbiota changes in the gastrointestinal tract. Prebiotic oligosaccharides, such as galactooligosaccharides (GOS), have been shown to increase the concentrations of beneficial *Bifidobacteria* in the gut of elderly in several studies.[28–30] Interestingly, consumption of GOS also reduced the concentrations of circulating pro-inflammatory cytokines.[29]

The study was set up as a double-blind placebo-controlled nutritional intervention study, to investigate the potential of bLF, GOS and vitamin D supplementation to restore TLR responsiveness of pDCs and mDCs and to reduce inflammatory cytokines in serum.

Subjects and methods

Study set-up

The effect of bLF in combination with galacto-oligosacharides (GOS) and vitamin D in elderly women (65-85 years) was studied in a double-blind placebo-controlled pilot study. The protocol was approved by the Medical Ethics Committee of Wageningen University, the Netherlands (protocol no. NL57345.081.16), and registered at clinicaltrials.gov (identifier NCT03026244).

As TLR7 expression is x-linked[31], the study was performed in women. Female subjects (65-85 years) were recruited. After providing informed consent, subjects were screened and were included when they were generally healthy, having a BMI 20-30, good mental status, and non-smoking. Subjects with chronic inflammatory, autoimmune or gastrointestinal diseases or immune-compromised individuals were excluded from participation. Subjects using hormone replacement therapy, anti-inflammatory drugs (>1x week) or immunosuppressive drugs were excluded. Furthermore, subjects were not allowed to use light therapy or go on holiday to a sunny destination. An overview of subject characteristics of the two study groups is given in Table 1. Because of the seasonal effect on vitamin D status, the study was executed in the winter period (January until March 2017).

Table 1: Characteristics of study participants

Treatment	Age (years) median (range)	BMI median (range)	Osteo-arthritis Y/N	Anti-inflammatory medicine Y/N	Vitamine D supplementation before study Y/N	Blood pressure or cholesterol medication Y/N
Intervention (n=15)	74 (70-84)	23.2 (20.3-29.0)	3/12	1/14	6/9	6/9
Placebo (n=15)	76 (69-85)	24.5 (20.8-29.4)	3/12	0/15	7/8	6/9

Study design

Stratification and randomization was performed by a non-blinded person not involved in the study. Women were stratified according to age, BMI, reported arthrosis, use of vitamin D supplements preceding the study, and use of medication for blood pressure or cholesterol. Subjects were randomly assigned to treatment or placebo using a random number generator. Women (n=15) in the nutritional intervention group received 3 weeks supplementation of bLF only, followed by 3 weeks bLF + GOS, followed by 3 weeks bLF + GOS + vitamin D, see Figure 1. In the placebo group, women (n=15) received maltodextrin as placebo for bLF and GOS, and capsules filled with maltodextrin as placebo for vitamin D. Subjects were instructed to maintain their habitual diet, but to stop any vitamin D or prebiotic supplementation during the study, starting from 2 weeks before study start.

Figure 1: Nutritional intervention study design. The intervention group (n=15) received 3 weeks bovine lactoferrin (bLF) (day 0-21), followed by 3 weeks of bLF + Galactooligosaccharides (GOS) (day 21-42) and 3 weeks of bLF + GOS + vitamin D. The placebo group (n=15) received maltodextrin for 9 weeks (day 0-63). At study days 0, 21, 42 and 63, blood was collected.

Study products

In the intervention group, subjects received 3 weeks of bLF (1.026g/day Vivinal Lactoferrin powder, containing an active dose of bLF of 1 g/day; FrieslandCampina Domo, Amersfoort, The Netherlands). This was followed by 3 weeks of supplementation with bLF + GOS (1 g/day BLF as above; 3.67 g/day Vivinal GOS powder, containing an active dose of GOS of 2.64 g/day; FrieslandCampina Domo, Amersfoort, The Netherlands). Both bLF and GOS have an FDA-approved GRAS status. From week 6-9, subjects received bLF + GOS + vitamin D. Vitamin D capsules (Supra D Forte Supradyn, Berlin, Germany) contained 20 µg cholecalciferol (= 800 IE) per capsule. Maltodextrin was used as placebo product, as well as carrier for the nutritional intervention products. As placebo for vitamin D capsules, empty capsules were purchased and filled with ~250 mg maize based maltodextrin (Glucidex, IT19 premium,

Roquette, Nord-Pas-de-Calais, France). bLF and GOS were mixed in bulk amount, with maltodextrin as carrier. The exact content of each component in the final powder to allow proper dosing was checked and confirmed. Powders were analysed for potential microbiological contamination (*Bacillus cereus*, Enterobacteriaceae, yeast and fungi, total plate count, coliforms and *E. coli*). All values were below detection, confirming the microbiological food safety of the powder batches.

Verum ('intervention') and placebo powders were aliquoted in blinded and coded jars in a food-grade environment, in adequate amounts to allow dosing over at least 21 days. After careful instruction, participants dosed the powders at home. One dose consisted of a smoothly streaked plastic scoop that was provided, which was dissolved in water and consumed after the evening meal. Vitamin D or placebo capsules were provided in separate blinded and coded jars, and were swallowed together with the dissolved powder.

Compliance to the intake of study product was monitored by weighing of powders and counting of capsules at start and end of each intervention period. Subjects also recorded the intake of study product daily in a diary.

Rand-36 questionnaire

At the beginning and end of the intervention study participants filled in the RAND 36-item short form survey instrument (RAND-36) questionnaire.[32] The RAND-36 questionnaire is a validated questionnaire for the perception of health and wellbeing. The RAND-36 was analyzed according to the protocol on the subjects physical functioning, limitations in physical health, pain and general health (https://www.rand.org/health/surveys_tools/mos/36-item-short-form/scoring.html). Statistics was done by rank-transforming the data or logit transformation (only general health). A generalized linear model repeated measures ANOVA was performed.

Registration of adverse events

Any adverse events (AE) were registered at each visit, with subjects being asked for any health complaints since the last visit. Furthermore, participants recorded AEs in a diary. The AEs were evaluated by the study physician, and judged as being or not being related to the study product (dietary intervention) or to the study procedures (blood sampling).

Blood sampling

Blood was collected at study days 0, 21, 42 and 63 for serum storage (10 mL tubes; cat.no. 367895, BD) or for PBMC isolation (K2-EDTA; 4 x 10mL; cat. no. 367525, BD). Serum tubes were left at room temperature for at least 30 min before centrifugation at 2000 x g 10 min at room temperature. Serum was aliquoted and stored at -80°C. PBMCs were isolated within 6 hours using 50 ml Leucosep tubes (227290, Greiner Bio-One) filled with Ficoll plaque plus (17-1440-02, GE Healthcare Life Sciences) according to manufacturer's protocol.

TLR expression in pDCs and mDCs

Isolated PBMCs were stained with a TLR antibody panel (Table 2) to measure the expression of TLR 2, 4, 7 and 9 *ex vivo*. To measure expression of TLRs, 2 x10^6 freshly isolated PBMCs/donor were collected in a 96 well plate (NUNC PP Sigma-Aldrich 7116). Per well 200 ul FACS buffer (PBS (Lonza BE17-516Q/12) + 2mM EDTA (Merck CBI 108418); 0,5% BSA (Roche 10735086001); 0,01% NaN3 (Merck CBI 822335) was added to wash the cells. Washing was done by centrifuging the plate at 400xg for 3 minutes at 4˚C. First, extracellular surface markers (Table 2) including 5 µl Fc block (564220, BD Pharmingen) were stained for 30 minutes on ice covered in aluminium foil and washed twice with cold PBS. Cells were stained with Fixable Viability Dye FVD520 (65086718, Ebioscience) in PBS and incubated for 20 minutes in the fridge, followed by washing the cells FACS buffer. Afterwards cells were permeabilized by adding IC fixation buffer (00-8222-49, Ebioscience) to each well and incubated for 30 minutes at room temperature, followed by washing twice in Perm buffer (00-8333-56, Ebioscience). The intracellular antibody mix (Table 2) in Perm buffer was incubated for 20 minutes in the fridge, followed by washing the cells twice in Perm buffer. Cells were resuspended in 300 ul FACS buffer and measured for 240s on the FACS CANTO II at medium flow rate, threshold 45.000. For the panels fluorescent minus one (FMO) controls and isotype controls were included.

Flow cytometry data analysis was performed by using FlowJo software (version 10 TreeStar, Inc.) and gating was performed as is shown in Supplementary Figure S1, in line with Panda et al.[8] PDCs were gated as Lineage2$^-$HLA-DR$^+$CD11c$^-$CD123$^+$ and mDCs were gated as Lineage2$^-$HLA-DR$^+$CD11c$^+$CD123$^-$. Data were exported as median fluorescent intensity for either all pDCs or mDCs per TLR.

Table 2: Antibodies used for TLR expression and intracellular cytokine measurements.

Antibody	Fluorchrome	host	isotype	Light chain	company	Catalog number	Panel	Extra/intracellular
lineage 2	FITC	mouse	IgG1	κ	BD	643397	TLR & cytokine	Extra
HLA-DR	APC-Cy7	mouse	IgG2b	κ	Ebioscience	47-9956-42	TLR & cytokine	Extra
CD123	PE-Cy5	mouse	IgG1	κ	Ebioscience	15-1239-42	TLR & cytokine	Extra
CD11c	PE-Cy7	mouse	IgG1	κ	Ebioscience	25-0116-42	TLR & cytokine	Extra
TLR2	biotin	mouse	IgG2a	κ	Ebioscience	13992282	TLR	Extra
TLR2 ic	biotin	mouse	IgG2a	κ	Ebioscience	13472785	TLR	Extra
streptavidin	BV510				BD	563261	TLR	Extra
TLR 4	BV421	mouse	IgG1	κ	BD	564401	TLR	Extra
TLR4 ic	BV421	mouse	IgG1	κ	BD	562438	TLR	Extra
FVD 520	efluor520				Ebioscience	65-0867-18	TLR & cytokine	Extra
TLR7	PE	mouse	IgG2a		R&D Systems	IC5875P	TLR	Intra
TLR 7 ic	PE	mouse	IgG2a		R&D Systems	IC003P	TLR	Intra
TLR9	APC	rat	IgG2a	κ	Ebioscience	17909982	TLR	Intra
TLR9 ic	APC	rat	IgG2a	κ	Ebioscience	17-4321-81	TLR	Intra
CD16	BV510	mouse	IgG1	κ	BD	740203	Cytokine	Extra
IL-6	PE	rat	IgG1	κ	Ebioscience	12706982	Cytokine	Intra
IL-6 ic	PE	rat	IgG1	κ	Ebioscience	12430183	cytokine	Intra
IFN-alpha	V450	mouse	IgG1	κ	BD	561382	Cytokine	Intra
IFN-alpha ic	V450	mouse	IgG1	κ	BD Horizon	561504	cytokine	Intra
TNF-alpha	APC	mouse	IgG1	κ	Ebioscience	17734982	Cytokine	Intra
TNF-alpha ic	APC	mouse	IgG1	κ	Ebioscience	17-4714-41	Cytokine	Intra

Antibody mixes were made for extra-cellular or intra-cellular staining. ic=isotype control

Intracellular cytokine measurement in pDCs and mDCs

In order to measure intracellular cytokines, 2 x10^6 PBMCs were stimulated in a 12-well plate (CLS3513-50ea, Sigma-Aldrich) (total volume 1 ml) for three hours in the absence or presence of PAM3CSK4 (Pam) 10 µg/ml (L2000, EMC microcollections), Ultra-pure LPS 0,1 µg/ml (3pelps, Invivogen), R848 3 µg/ml (TLRL-R848-5, Invivogen) or CpG 3 µg/ml (TLRL-2216-1 (class 'A'), Invivogen) in the presence of Brefeldin A (B7651, Sigma-Aldrich) in RPMI-1640 with 5% human AB serum (H4522, Sigma Aldrich). Afterwards, cells were harvested by pipetting and stained with Lineage2, HLA-DR, CD11c, CD123, IFN-α, TNF-α and IL-6, as described for the TLR staining. Cells were resuspended in 250 ul FACS buffer and measured for 200s with FACS Canto II. Flow cytometry data analysis was performed by using FlowJo software (version 10 TreeStar, Inc.) and gating was performed as is shown in Supplementary Figure S1. Data were exported as % cytokine-positive pDCs or mDC as % of all pDCs or mDCs.

Cytokine and pro-inflammatory marker measurements in serum.

In serum, IL-1β (558279, BD Pharmingen); TNF-α (560112, BD Pharmingen), IL-6 (558276, BD Pharmingen), sCD106 (sVCAM-1; 560427, BD Pharmingen), sCD54 (sICAM-1; 560269, BD Pharmingen) and IL-10 (558274, BD Pharmingen) were measured by cytometric bead array, according to manufacturer's protocol. Beads were measured for 50 seconds at high speed using a FACS Canto II. Furthermore, IL-1Ra (CHC1183, Thermo Fisher) and cartilage oligomeric matrix protein (COMP) (DY3134, R&D systems) were measured by ELISA according to manufacturer's protocol. CRP was measured with a immunoturbidemetric assay using the c802 module of Cobas 8000 from Roche. 25-OH-vitamine D was measured using chemiluminescent immunoassay using Liaison XL from Diasorin.

Statistical analysis

Statistical analysis was performed by using IBM SPSS Statistics version 23. Data were tested for normal distribution using Shapiro-Wilk test. The intracellular cytokine production (% of all pDCs or all mDC) of pDC and mDCs per stimulation (RPMI, Pam, LPS, R848 and CpG) were analysed by repeated measures MANOVA (RM-MANOVA). To obtain normally distributed data, percentages were logit-transformed or rank-transformed. For TLR expression

the median MFI of TLR 2, 4, 7 and 9 expression was analyzed per cell type (pDC or mDC). The median MFI was 10log transformed to obtain normally distributed data. TLR expression was analysed by RM-ANOVA per TLR per cell type (pDC or mDC). Serum concentrations of pro-inflammatory markers (pg/ml) in serum were 10log transformed to obtain normally distributed data. As the value 0 cannot be 10log transformed, this value was artificially put on 0.001 to obtain a value after transformation. No RM-MANOVA could be performed on pro-inflammatory markers in serum as too many donors would be excluded, therefore an RM-ANOVA per marker was performed. After data transformation, outliers (>2SD) were removed. The transformed data were used for analysis. RM-MANOVA and RM-ANOVA were performed with additional analysis of contrasts (difference and repeated) of time*treatment. All statistical results are therefore a difference over time between the nutritional intervention group and placebo group. As this was a pilot study with a relatively low number of individuals (n=15 per group), we were also interested in statistical trends and not only in statistically significant differences. We considered trends relevant if $0.05 \leq p \leq 0.10$ and indicated this in figures with a # symbol.

Results

Safety and tolerability

The intervention with bLF, GOS and vitamin D was generally well tolerated and safe, as only a few mild study-related adverse events were reported (e.g. flatulence and change in bowel habit), mainly in the intervention group. No moderately severe adverse events related to the study were reported. One non-study related serious adverse event was reported in the placebo group.

Percentages of pDCs and mDCs

To determine if the nutritional intervention affected the numbers of circulating pDCs and mDCs, unstimulated PBMCs were stained with Lineage 2, HLA-DR, CD11c and CD123. Using the gating strategy as depicted in Supplementary Figure S1, the percentage of pDCs and mDCs were determined at each time point. Table 3 shows that, although there were some fluctuations in pDC and mDC percentages over time, no significant changes between the nutritional intervention and placebo group were found.

Table 3: The percentage of pDCs and mDC (%pDC or %mDC of all DCs) in unstimulated PBMCs (median + range) for the nutritional intervention and placebo group at study days 0, 21, 42 and 63.

	Treatment	Day 0	Day 21	Day 42	Day 63
%pDCs	Intervention	11.4% (3.1-45.0%)	12.6% (4.5-40.5%)	14.4% (6.0-34.2%)	13.3% (4.8-30.3%)
	Placebo	14.3% (3.3-27.7%)	13.7% (5.5-31.4%)	13.5% (4.9-31.0%)	15.9% (4.8-22.6%)
%mDCs	Intervention	68.2% (26.9-86.0%)	76.8% (50.3-87.7%)	73.2% (55.4-88.3%)	74.1% (36.1-85.8%)
	Placebo	67.6% (28.0-88.9%)	76.2% (58.9-89.8%)	77.2% (49.5-90.0%)	72.9% (37.7-86.9%)

The effect of nutritional intervention on intracellular cytokine production in pDCs upon TLR stimulation

To determine if the nutritional intervention impacted the antiviral response in pDCs, intracellular IFN-α, IL-6 and TNF-α production was measured after stimulation with TLR7/8 ligand R848. The percentage of IL-6$^+$ pDCs increased significantly (p=0.005) and IFN-α$^+$ pDCs tended to increase (p=0.09) at day 21 compared to day 0 in the nutritional intervention group compared to the placebo group (Figure 2). As the nutritional intervention group only consumed bLF during the first 21 days, this indicates that lactoferrin supplementation increased the response of pDCs to TLR7/8 stimulation. TNF-α$^+$ pDCs tended to increase at day 42 compared to day 0 in the nutritional intervention group after TLR7/8 stimulation, while the placebo group increased from day 21 to day 42.

At day 63, the number of TNF-α$^+$, IL-6$^+$ and IFN-α$^+$ pDCs was the same in the placebo group and the nutritional intervention group after TLR7/8 stimulation. Overall, it can be concluded that bLF specifically increased IL-6 and tended to increase IFN-α production in pDCs (day 0 to day 21) upon stimulation with R848, but that subsequent supplementation of GOS and vitamin D did not further enhance this effect compared to the placebo group. This increased cytokine production was not caused by an increase of TLR7 expression in pDCs of the intervention group compared to the placebo group (Figure 2B).

In order to determine which subset of cytokine producing pDCs was responsible for the increase of IL-6$^+$ and IFN-α$^+$ pDCs, a boolean gating strategy was performed, resulting in all eight combinations of IL-6, IFN-α and

TNF-α production from single to triple positive pDCs. Figure 2C depicts the four combinations of cytokines that were observed to be produced by pDCs. Figure 2C shows that the increase in IFN-α production is mainly due to IFN-α^+TNF-α^+ pDCs, and for IL-6 the majority of positive pDCs are IL-6^+TNF-α^+. This is in contrast to TNF-α, where the majority of TNF-α production is derived from single TNF-α^+ pDCs.

In addition to stimulation through TLR7/8, stimulations of pDCs were performed through TLR1/2 (Pam), TLR4 (LPS) and TLR9 (CpG). In supplementary Table 1, the percentages of pDCs containing intracellular IL-6, TNF-α and IFN-α after stimulation with Pam, LPS and CpG are shown.

In all stimulations, a higher percentage of pDCs is positive for TNF-α compared to IL-6 and IFN-α. Even though the percentage of positive cells was low, a significant increase of IL-6 ($p=0.021$) was observed in the nutritional intervention group compared to the placebo group after Pam stimulation at day 21, after three weeks of bLF supplementation. At day 21, no effects of the nutritional intervention were observed when pDCs were stimulated with LPS and CpG. At other time-points, no effects of intervention were detected in pDC, with the exception of the percentage of TNF-α^+ pDC in response to CpG stimulation, that tended to increase in the intervention group, both from day 0 to day 42 ($p=0.088$) as well as from day 21 to day 42 ($p=0.085$) (data not shown). Overall, these results show that enhanced cytokine production upon TLR7/8 and to a lesser extent TLR1/2 stimulation mainly occurs after 21 days in pDCs and is thus primarily the result of ingestion of bLF.

Intracellular cytokine production in mDCs upon TLR stimulation

Next, the effect of the nutritional intervention on mDC activation was studied. For all TLR stimulations, a higher percentage of mDCs produced TNF-α, compared to IL-6 and IFN-α, see supplementary Table 2. Upon TLR1/2 stimulation, the percentage of TNF-α^+ mDCs from the nutritional intervention-treated subjects increased compared to the placebo treated subjects at day 63 compared to day 0 ($p=0.03$), due to a lower percentage of TNF-α^+ mDCs at t=0.

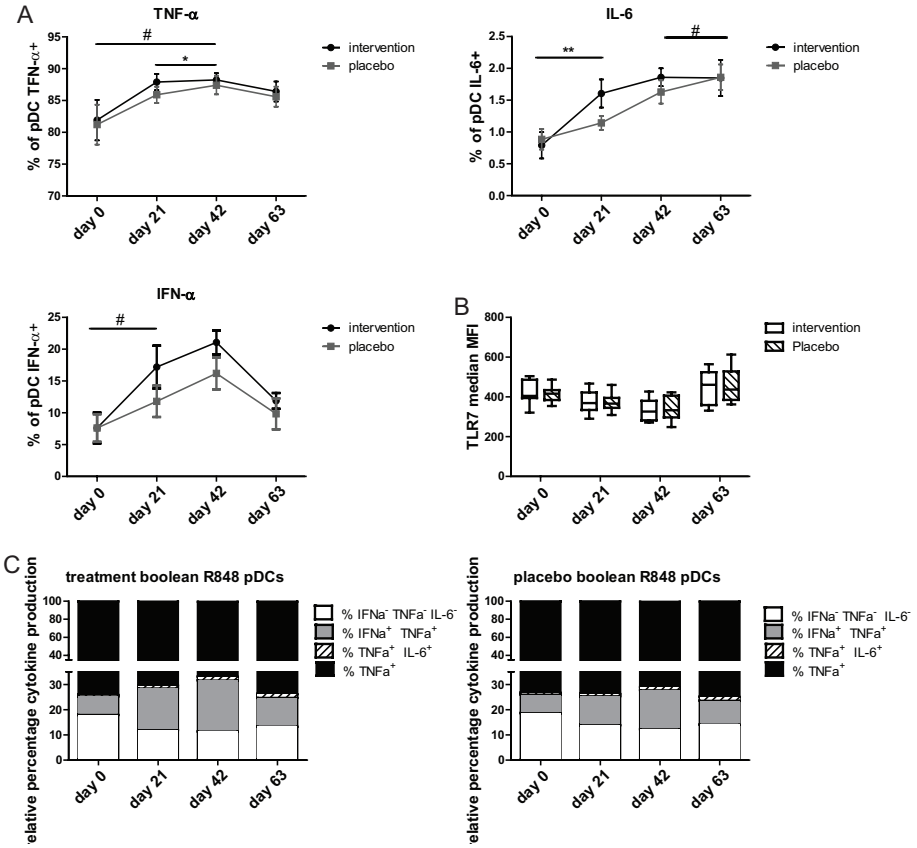

Figure 2: Intracellular cytokine production in pDCs upon R848 stimulation. A) TNF-α, IL-6 and IFN-α positive pDCs (% positive for cytokine of all pDCs) at study day 0, 21, 42 and 63, shown as mean +/- SEM. B) TLR7 expression on pDCs at study day 0, 21, 42, 63. C) Boolean gating of produced cytokine combinations only by pDCs over time. Statistical analysis was done on ranks based on logit transformed data using repeated measures MANOVA. Outliers (>2SD) were removed. * p<0,05; ** p<0,01; # is given for trends, all values indicate a difference between Intervention and placebo group over time. # for IFN-α+ pDCs is obtained by analysing day 0 and day 21 only.

Likewise, TLR9 stimulation with CpG induced more IFN-α^+ mDCs in the nutritional intervention group only when comparing day 63 to day 0 (p=0.029). At the same time, in the nutritional intervention group the percentage IL-6^+ mDCs after TLR9 stimulation at day 63 tended to be increased in the nutritional intervention group compared to the placebo group, as IL-6^+ mDCs in the placobo group decreases more over time. It should be mentioned however, that the IFN-α^+ and IL-6^+ mDC percentages after TLR9 stimulation are very low, and may not be clinically relevant.

In contrast to the results obtained in pDCs, no differences between nutritional intervention and placebo group were observed when comparing cytokine production between day 0 to day 21 (bLF treatment alone), but rather from day 0 to day 63 (after the complete intervention period).

No effect of nutritional intervention on TLR expression in pDCs and mDCs

In order to analyze whether the changes in intracellular cytokine production upon TLR stimulation was due to modulation of expression of any of the TLRs, the TLR expression levels on pDCs and mDCs were measured in unstimulated PBMCs *ex vivo*. No significant differences in expression of TLR 2, 4, 7 and 9 were observed for pDCs and mDCs when comparing the nutritional intervention with the placebo group over time (Supplementary Figure S2), although the expression levels varied over time in both groups. This suggests that the changes in intracellular cytokines upon TLR stimulation or the differences between the intervention and placebo group were not due to differences in TLR expression levels.

Serum markers involved in inflammation

To explore if the nutritional intervention affected serum concentrations of markers involved in inflammation, a wide range of ageing associated inflammatory markers was measured. Figure 3 shows that the only inflammation related marker that tended to decrease after the entire invention period was soluble VCAM (sVCAM), which decreased at day 63 compared to day 0 ($p=0.07$). IL-6, TNF-α, sICAM, IL-1β, CRP, IL-1Ra, and IL-10 did not change during the study. Cartilage oligomeric matrix protein (COMP) as potential prognostic marker for osteoarthritis[33] was also measured in serum, as were vitamin D levels (Supplementary Figure S3). Vitamin D levels did not change during the study. Although the concentration of sICAM and COMP seemed to decrease gradually over time in the nutritional intervention group, this was not significant at any of the time-points

None of the markers was significantly changed at day 21, indicating that 3 weeks of bLF supplementation alone did not have clear effects on serum markers involved in inflammation. The only tendency to an effect on inflammatory markers was observed at day 63 after the addition of GOS and vitamin D to bLF supplementation (sVCAM).

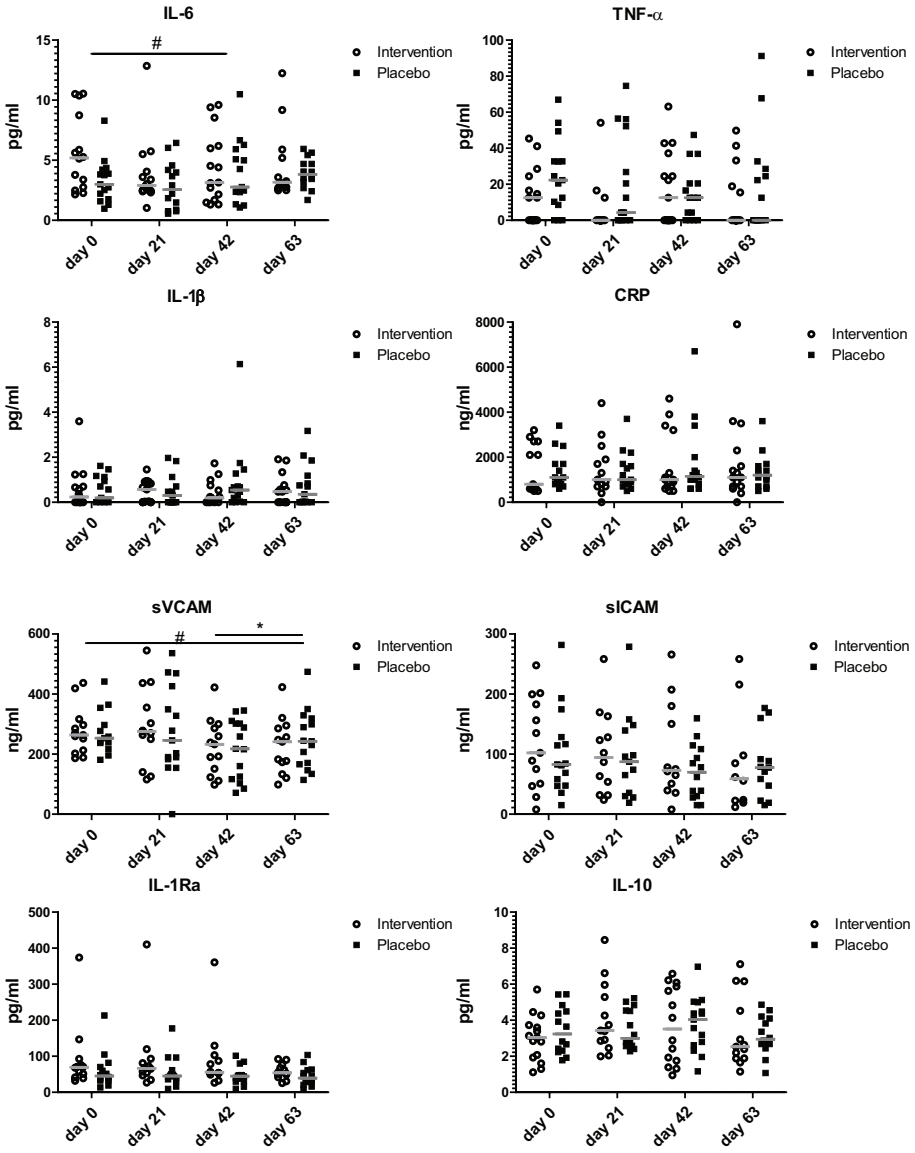

Figure 3: Serum markers involved in inflammation. Concentrations of IL-6, TNF-α, IL-1β, CRP, sVCAM, sICAM, IL-1Ra and IL-10 at study days 0, 21, 42, and 63. Data shown as scatter plots with median value. Analysis was done on the 10log transformed data or ranks (IL-1β and TNF-α) by repeated measures ANOVA,. Outliers (>2SD)were removed. All statistical differences are differences over time between the nutritional intervention group and the placebo group. * p<0,05; or # is given for trends.

RAND-36 questionnaire

Table 4 shows the outcomes of analysis of the RAND-36 questionnaire in the nutritional intervention and placebo group. The questionnaire was only completed before and after the study, and thus compares health status at day 0 versus day 63. A score of 100 is considered very healthy, a score of 0 very unhealthy. Physical function tended to improve in the nutritional intervention group (p=0.09), but no effects were observed on pain, general health and limiting physical health. The results showed no major change when individuals with osteoarthritis (n=6) were excluded from this analysis, indicating that the main effect of supplementation accounted for the whole group and not specifically for individuals with osteoarthritis (not shown).

Table 4: Mean scores and ranks for placebo and nutritional intervention group based on the RAND-36 questionaire.

	timepoint	Placebo		Nutritional intervention		P values (all individuals including OA)	P values (healthy individuals without OA)
		Mean	Rank	Mean	Rank	Time x treatment n=30	Time x treatment n=24
Phys Func	Day 0	95	18,1	85	12,9	0.092	0.087
	Day 63	92	15,5	89	15,5		
Lim. Phys Health	Day 0	95	16	90	15	0.27	0.27
	Day 63	85	14,5	97	16,5		
Pain	Day 0	93	16,7	87	14,3	0.41	0.35
	Day 63	88	15,3	86	15,7		
General Health	Day 0	77	14,9	77	16,1	0.95	0.72
	Day 63	77	15,1	76	15,9		

Phys Func= physical functioning; Lim. Phys health= role limitations due to physical health problems

Discussion

Paradoxically, elderly people have decreased innate antiviral immunity as well as increased levels of inflammatory markers that are produced by the innate immune system. The aim of the intervention study described here was to investigate if these immune defects can be addressed by nutritional intervention with bLF, GOS and vitamin D in elderly women.

This study demonstrates that three weeks of bLF supplementation increased intracellular cytokine production in pDCs in response to TLR7/8 and TLR1/2

activation. Increased effects on TLR activation were only seen in mDC after completion of the intervention study at day 63. The intervention did not induce significant changes in inflammatory markers, as only sVCAM tended to decrease in the intervention group.

The rationale to study postmenopausal women aged >65 was based on several observations. TLR7 is X-linked[31] and women are found to have higher IFN-α production by pDCs compared to men[34]. Furthermore, TLR7 function is reduced in postmenopausal women compared pre-menopausal women and can be improved by hormone replacement therapy, indicating that the reduced TLR7 responsiveness in elderly women can be restored.[12]

pDCs are the primary producers of IFN-α upon influenza infection, which is TLR7mediated.[14] bLF is linked to reduced number of infections by rhinoviruses and hepatitis C that are also recognized by TLR7.[19–21] Therefore, we hypothesized that nutritional intervention with bLF might increase the production of cytokines, including the antiviral cytokine IFN-α by pDCs after TLR7/8 stimulation. Indeed, bLF supplementation alone (day 0 to day 21) enhanced the production of IL-6 and tended to increase the production of IFN-α in response to TLR 7/8 stimulation in pDCs. Subsequent supplementation of GOS and vitamin D did not further enhance cytokine production of pDCs in response to any TLR stimuli used compared to placebo. In contrast, supplementation of bLF during the first three weeks did not result in enhanced responses of mDCs to the same stimuli, nor affected serum markers involved in inflammation.

In many parameters measured in this study, the placebo group shows similar kinetics as the intervention group, which seems a time-related effect and may result in underestimation of the effects of the nutritional intervention. We hypothesized that these time related effects were due to sunlight induced vitamin D. However, vitamin D did not significantly change in the placebo or the nutritional intervention group during the study (Supplementary Figure S3). Hence, it remains elusive which time-related factor contributed to this effect in all subjects during the study period, and it stresses the need of taking along a placebo group.

In elderly, the expression of TLR1 and TLR7 on mDCs and TLR7 on pDCs was reported to be reduced compared to young adults, while the expression of TLR2 on mDCs and TLR9 on pDCs is unchanged.[16,35] Besides, a change in signalling events downstream of TLR activation occurs upon ageing that leads to reduced cytokine secretion upon stimulation.[4,7] The defective TLR function in ageing is illustrated by decreased cytokine production of monocytes and pDCs as well as mDCs of elderly people in response to ligation of TLR1/2[36], TLR4[37], TLR7 and TLR9[8]. In our study, the observed changes in cytokine production by pDCs after bLF supplementation were not due to increased TLR expression levels, suggesting that bLF supplementation may have improved downstream signalling of TLRs. bLF is 69% homologous to human lactoferrin on the protein level[38] and can be taken up by human cells via lactoferrin-receptor (intelectin).[39] We hypothesize that bLF exerts its effect by binding to intelectin or one of the other bLF receptors that are expressed by immune cells. These receptors are low-density lipoprotein receptor-related protein-1 (LRP-1 or CD91)[40,41], CD14 on monocytes in complex with LPS[42], TLR4[43] and CXCR4[41]. It is currently not known whether bLF is taken up and ends up in the blood as the whole protein or that bLF is partially digested and active peptides end up in blood, which subsequently exert an effect on pDCs.

GOS has been shown to exert an effect on inflammatory serum markers via the increase of *Bifidobacteria* levels, which are important for the production of short chain fatty acids (SCFAs), and to reduce pro-inflammatory cytokine concentrations in serum.[29] However, we did not observe a reduction in pro-inflammatory cytokines in this study. This might be because our intervention period was shorter, or because the concentration of GOS was lower compared to other studies.[29,30] Another explanation is that our group size was too small to detect significant changes in these markers.[26] The group size in this study was based on power calculations related to the primary en secondary outcomes of the study (IFN-α, TNF-α and IL-6 production by pDCs upon *ex vivo* stimulation).

Serum concentrations of vitamin D correlate with increased IL-1β, TNF-α and IL-6 production by monocytes upon TLR7 stimulation, while TLR7 expression is inversely correlated with vitamin D levels in serum.[27] In this study we did not observe any increase of TNF-α, IL-6 or IFN-α in stimulated pDC or mDC or

changes in TLR7 expression when comparing day 42 with day 63. A possible explanation is that three weeks of vitamin D supplementation were not sufficient to significantly enhance 25-OH vitamin D concentration in serum (Supplementary Figure S3).

Recent hypotheses have suggested that the decreased response to stimuli through TLR in elderly people and the enhanced steady state production of cytokines by blood cells of elderly people may have the same underlying cause, being miRNAs that regulate activation of myeloid cells downstream of TLRs.[44] If this is the case, an effect on promoting TLR responses in elderly by innate immune training (epigenetic effect) can be expected to also have an effect on the production of inflammatory cytokines. However, even though in this study we did demonstrate increased TLR-mediated responses in pDC and (to a lesser extent) in mDC, no significant decreases of serum concentrations of IL-6, TNF-α and CRP were seen. This is in line with the fact that we did not note any changes in intracellular cytokine concentrations in unstimulated pDCs and mDCs throughout the study, indicating that the activation status of these cells in steady state are not affected by the intervention (data not shown).

It should be noted that the population of elderly women in this study was in general healthy and mobile, with only a few subjects with Osteoarthritis (OA) (6/30). Besides, the average age was relatively low compared to other studies.[45,46] Inclusion of larger study groups including less mobile elderly women or more (non-hospitalized) women with chronic inflammatory diseases might be recommended in future studies to demonstrate effects on inflammatory markers with these ingredients.

In addition to the immune parameters described, the RAND-36 questionnaire was used to assess the health status of the subjects. The RAND-36 questionnaire results showed a trend towards improved physical function in the intervention group, as compared to the placebo. bLF has been shown to prevent arthritis in experimental animal models[47]. COMP is a physiological parameter that is predictive for development of osteoarthritis.[33] The tendency towards improved physical function together with the non-significant decrease in COMP concentrations in the intervention group, might

suggest that nutritional supplementation with bLF, possibly in combination with GOS and vitamin D, could be relevant for prevention of osteoarthritis (OA). However, larger studies including more subjects with OA will be needed to investigate this further.

In conclusion, nutritional supplementation with bLF, GOS and vitamin D is safe and enhances responses to TLR stimuli in elderly women in both pDCs and mDCs, while no clear effects on pro-inflammatory markers in serum were observed, possibly due to the study group size. Especially TLR7-mediated responses in pDCs were enhanced after bLF supplementation compared to placebo, suggesting that bLF may contribute to protection against viral infections in elderly women. The outcomes of this pilot study warrants future studies, that should be powered on the basis of the outcomes described here to confirm and extend our findings on DCs and inflammatory markers in elderly women.

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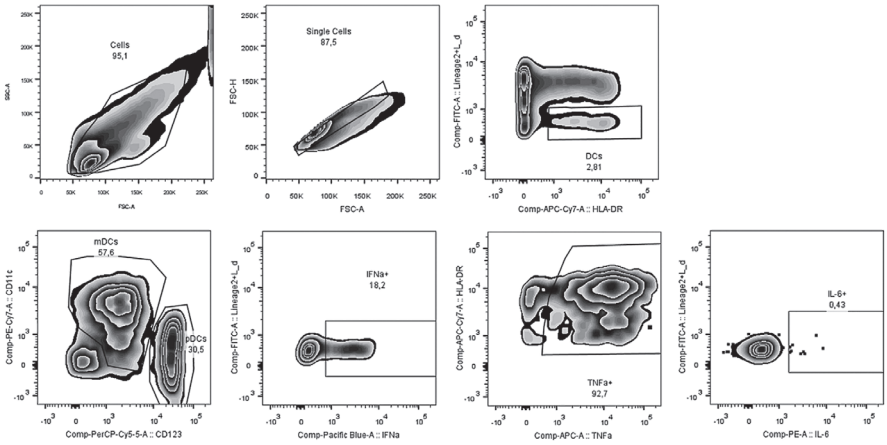
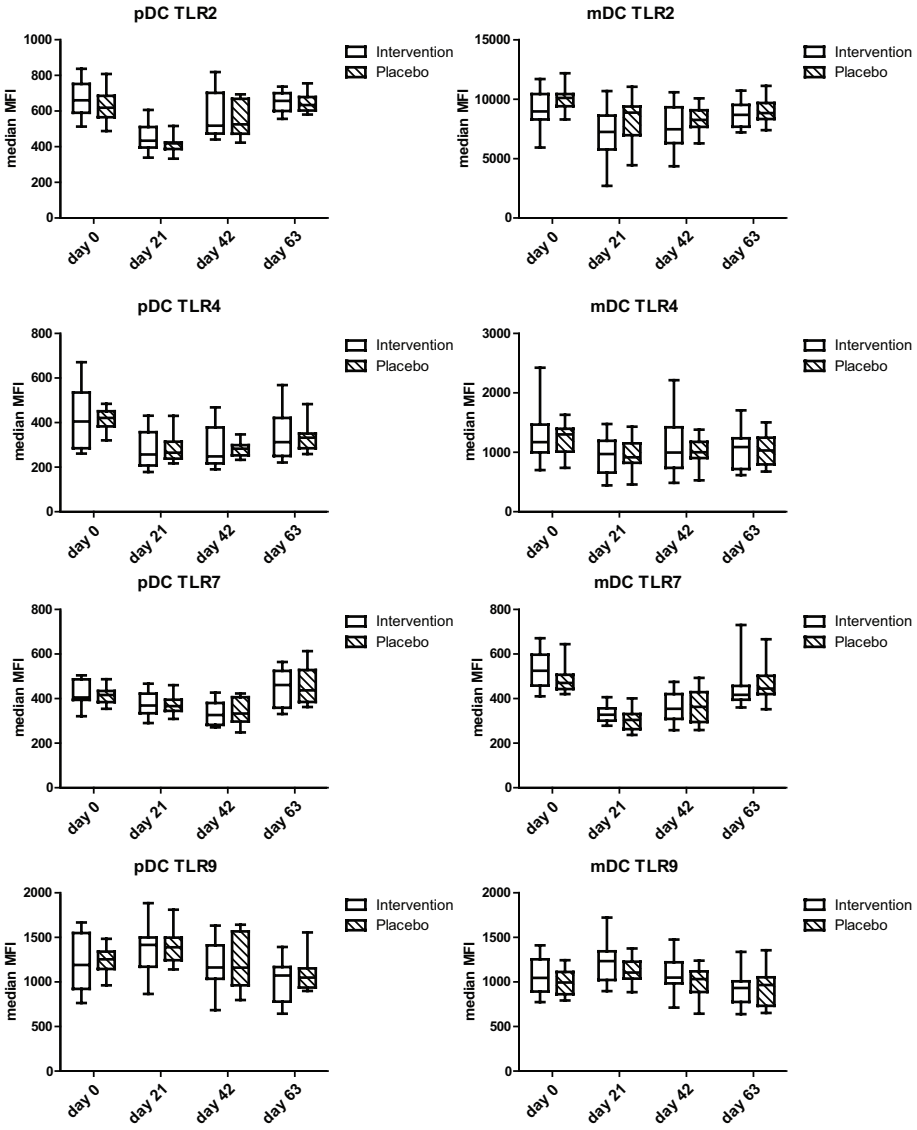
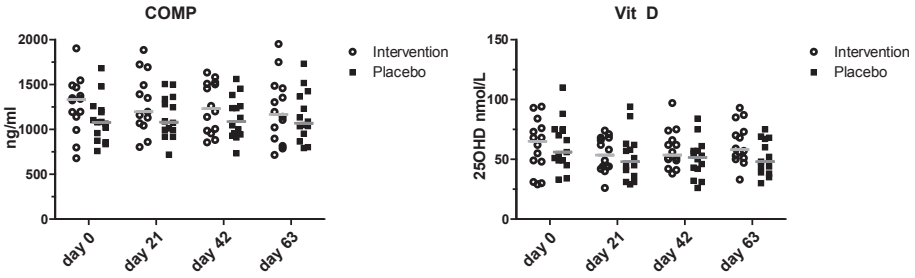


Supplementary Figure S1: Intracellular cytokine production of pDCs by a donor from the elderly population (171/002) upon TLR 7/8 stimulation with R848.



Supplementary Figure S2: TLR 2, 4, 7 and 9 expression *ex vivo* on pDCs and mDC at day 0, 21, 42 and 63 of the study. No significant differences were found.



Supplementary Figure S3: Serum concentrations of Cartilage oligomeric matrix protein (COMP) and Vitamin D (25-OH-vitamin D) at study day 0, 21, 42, 63. Data shown as scatter plots with median value. Analysis was done on 10log transformed data by repeated measures ANOVA. Outliers (>2SD) were removed. No statistical differences found.

Supplementary table 1: Intracellular cytokine production pDCs upon stimulation with Pam, LPS, R848 and CpG at day 0, 21, 42, 63. Data shown as median % + range, no outliers were removed. Intervention statically(*) increased ↑ or decreased ↓ compared to placebo over time or a trend was observed (#).

stimulation	cytokine	Treatment	Day 0 (%)	Day 21 (%)	Day 42 (%)	Day 63 (%)	Day 0 vs day 21 (p-value)	Day 0 vs day 63 (p-value)
Pam	TNF-α	Intervention	12.5 (3.2-19.72)	17.35 (4.04-30.62)	18.86 (3.35-31.31)	15.89 (4.27-27.16)		
		Placebo	8.16 (3.11-23.90)	6.88 (2.34-27.27)	6.37 (3.73-29.75)	7.25 (3.86-27.96)		
	IL-6	Intervention	0.2 (0.0-2.55)	0.64 (0.08-1.19)	0.68 (0.23-2.81)	0.93 (0.24-2.18)	0.021↑ (*)	
		Placebo	0.91 (0.0-1.75)	0.34 (0.1-3.38)	0.65 (0.12-2.64)	0.97 (0.1-4.66)		
	IFN-α	Intervention	0.0 (0.0-0.42)	0.0 (0.0-0.05)	0.05 (0.0-0.13)	0.05 (0.0-0.11)		
		Placebo	0.08 (0.0-0.24)	0.0 (0.0-0.06)	0.01 (0.0-0.3)	0.01 (0.0-0.15)		
LPS	TNF-α	Intervention	1.59 (0.54-4.76)	1.7 (0.07-6.31)	1.39 (0.12-4.82)	1.44 (0.25-4.83)		
		Placebo	1.4 (0.0-8.29)	1.3 (0.21-17.28)	0.75 (0.18-18.06)	1.77 (0.12-6.02)		
	IL-6	Intervention	0.07 (0.00-2.96)	0.35 (0.0-1.18)	0.3 (0.05-1.8)	0.41 (0.06-2.68)		
		Placebo	0.24 (0.00-4.22)	0.1 (0.03-6.41)	0.16 (0.0-4.8)	0.3 (0.04-2.6)		
	IFN-α	Intervention	0.00 (0.0-0.08)	0.0 (0.0-0.05)	0.0 (0.0-0.06)	0.0 (0.0-0.08)		
		Placebo	0.03 (0.0-0.13)	0.0 (0.0-0.07)	0.0 (0.0-0.06)	0.0 (0.0-0.62)		

R848	TNF-α	Intervention	84.62 (54.49-98.49)	88.24 (77.13-94.92)	89.08 (81.01-95.8)	86.47 (73.4-95.48)		
		Placebo	86.44 (59.34-94.58)	87.08 (76.84-94.0)	88.99 (76.02-93.32)	85.65 (73.29-94.53)		
	IL-6	Intervention	0.58 (0.0-2.26)	1.62 (0.39-3.42)	1.68 (1.04-2.92)	1.56 (1.01-5.44)	0.005↑ (*)	
		Placebo	0.89 (0.0-2.08)	1.1 (0.44-1.86)	1.64 (0.47-3.17)	1.7 (0.79-3.25)		
	IFN-α	Intervention	5.0 (0.19-34.29)	12.46 (1.14-49.27)	21.87 (10.3-32.76)	13.36 (0.31-17.02)	0.09↑ (#)	
		Placebo	5.68 (0.04-28.79)	10.78 (0.61-37.75)	14.83(4.73-37.89)	6.68 (0.88-33.29)		
CpG	TNF-α	Intervention	0.87 (0.13-12.87)	1.55 (0.26-13.2)	2.99 (0.27-7.21)	1.85 (0.12-15.90)		
		Placebo	1.29 (0.16-18.46)	1.34 (0.18-8.87)	2.45 (0.4-5.33)	1.06 (0.19-7.1)		
	IL-6	Intervention	0.0 (0.0-0.21)	0.02 (0.00.24)	0.07 (0.0-0.23)	0.07 (0.0-0.19)		
		Placebo	0.03 (0.0-0.37)	0.03 (0.0-0.14)	0.04 (0.0-0.18)	0.06 (0.0-0.76)		
	IFN-α	Intervention	0.0 (0.0-0.26)	0.0 (0.0-0.04)	0.05 (0.0-0.95)	0.0 (0.0-0.69)		
		Placebo	0.0 (0.0-0.46)	0.0 (0.0-0.6)	0.0 (0.0-0.36)	0.03 (0.0-0.15)		

Supplementary table 2: Intracellular cytokine production mDCs upon stimulation with Pam, LPS, R848 and CpG at day 0, 21, 42, 63. Data shown as median % + range, no outliers were removed. Intervention statically (*) increased ↑ or decreased ↓ compared to placebo over time or a trend was observed (#).

Stimulation	cytokine	Treatment	Day 0 (%)	Day 21 (%)	Day 42 (%)	Day 63 (%)	Day 0 vs day 21 (p-value)	Day 0 vs day 63 (p-value)
Pam	TNF	Intervention	18.8 (0.77-41.8)	15.95 (9.96-42.7)	16.5 (9.65-42.7)	24.0 (10.2-43.1)		0.03 ↑(*)
		Placebo	28.2 (0.66-44.5)	16.6 (9.76-35.1)	15.7 (5.31-30.3)	29.0 (10.3-36.2)		
	IL-6	Intervention	1.21 (0.08-5.28)	3.77 (1.43-4.91)	3.65 (1.77-5.59)	2.61 (1.35-3.9)		
		Placebo	1.59 (0.05-5.49)	4.54 (1.32-10.1)	3.77 (2.01-7.24)	3.33 (1.75-4.89)		
	IFNα	Intervention	0.06 (0.0-1.24)	0.02 (0.0-0.21)	0.02 (0.05-0.71)	0.08 (0.02-0.32)		
		Placebo	0.05 (0.0-0.99)	0.05 (0.01-0.22)	0.2 (0.05-0.37)	0.05 (0.02-0.1)		
LPS	TNF	Intervention	54.71 (7.1-79.82)	74.51 (58.92-85.76)	75.31 (55.64-84.86)	76.72 (56.64-86.41)		
		Placebo	65.02 (11.27-78.22)	77.52 (60.41-85.23)	77.46 (60.22-86.28)	76.46 (57.8-82.75)		
	IL-6	Intervention	33.75 (1.88-50.33)	32.83 (15.15-47.41)	36.18 (21.72-50.74)	40.06 (26.03-53.18)		
		Placebo	36.94 (0.6-51.51)	32.21 (24.68-44.81)	39.1 (18.84-48.08)	43.74 (28.85-54.02)		
	IFNα	Intervention	0.03 (0.01-0.06)	0.03 (0.0-0.11)	0.09 (0.04-0.28)	0.08 (0.01-0.16)		
		Placebo	0.02 (0.0-0.13)	0.04 (0.01-0.09)	0.11 (0.02-0.18)	0.05 (0.0-0.12)		

R848	TNF	Intervention	81.29 (53.72-96.19)	89.47 (77.92-95.69)	90.75 (79.07-95.85)	86.67 (71.72-94.31)	
		Placebo	86.75 (60.47-94.44)	91.82 (81.12-93.33)	92.47 (81.65-96.01)	87.47 (76.1-93.8)	
	IL-6	Intervention	63.47 (5.3-79.31)	46.24 (26.46-65.58)	49.99 (33.96-64.48)	55.16 (32.66-69.66)	
		Placebo	72.24 (2.33-84.3)	42.58 (31.93-50.87)	49.1 (25.79-63.71)	54.26 (33.01-66.04)	
	IFNα	Intervention	0.03 (0.0-0.12)	0.02 (0.0-0.12)	0.10 (0.00-0.29)	0.06 (0.01-0.18)	
		Placebo	0.02 (0.0-0.08)	0.03 (0.0-0.14)	0.08 (0.02-0.15)	0.04 (0.0-0.09)	
CpG	TNF	Intervention	2.9 (0.36-8.22)	2.45 (0.9-13.09)	3.28 (0.5-9.05)	5.13 (0.84-16.07)	
		Placebo	2.68 (0.21-18.09)	2.35 (0.52-14.1)	3.25 (0.47-7.92)	3.77 (0.4-17.37)	
	IL-6	Intervention	0.65 (0.33-9.47)	0.60 (0.16-1.99)	0.85 (0.29-4.31)	0.73 (0.25-1.5)	
		Placebo	0.88 (0.13-8.37)	0.77 (0.07-6.12)	0.87 (0.14-4.12)	0.59 (0.16-2.93)	0.098$^{\downarrow}$ (#)
	IFNα	Intervention	0.02 (0.00-0.21)	0.04 (0.0-0.13)	0.10 (0.03-0.22)	0.08 (0.04-0.19)	0.029$^{\uparrow}$ (*)
		Placebo	0.05 (0.00-0.20)	0.05 (0.00-0.13)	0.16 (0.05-0.26)	0.07 (0.02-0.11)	

Chapter 8

General discussion

In this chapter (**chapter 8**) I will discuss the main outcomes of our research and their relevance by placing the results in a broader perspective. I will focus on four themes: *1). Induction of trained immunity by diet 2). Modulation of respiratory immunity by raw bovine milk; 3). Immunomodulation in elderly by bovine milk components; 4). The potential role of trained immunity throughout life.*

Induction of trained immunity by diet

In **chapter 3** we have showed that raw bovine milk and bovine IgG isolated from raw colostrum can induce trained immunity in human monocytes. The trained immunity induced by bovine IgG that enhanced TLR7/8-mediated innate immune responses might contribute to protection against infections. In addition to binding and in some cases neutralisation of pathogens[1] bovine IgG could thus exert a dual effect *in vivo*. This can potentially explain why a reduced prevalence of respiratory tract infections is observed in children consuming raw bovine milk.[2] The first mechanistic effect could be the induction of trained immunity by bovine IgG, leading to enhanced responses against pathogens that induce TLR7-mediated immune responses, such as infections by RSV and Influenza.[3,4] The second mechanistic effect by which bovine IgG can reduce the prevalence of respiratory tract infection is by the complex-formation with respiratory pathogens.[1] This may enhance uptake of the pathogens by FCγRII-positive cells. Antigens are better taken up by antigen-presenting cells when they are forming a complex with previous secreted immunoglobulins[25–27], hence bovine IgG complex-formation with pathogens can lead to increased immune responses. In addition to bovine IgG, raw milk was shown to induce trained immunity by TLR1/2 stimulations. As TLR2 is also involved in innate immune responses to RSV[5], this could be relevant for protection against RSV infections in infants and elderly as well, although the consumption of raw milk cannot be recommended as it has potential health risks because the microbiological quality cannot be safeguarded.

In these *in vitro* experiments, we also observed induction of trained immunity by bovine lactoferrin in a limited number of donors, although in most of the donors lactoferrin did not have this effect. In **chapter 7**, we describe a

nutritional intervention study with bovine lactoferrin, in which we observed an increased responsiveness of pDCs to TLR7/8 stimulation. We did not perform the same experiments as in **chapter 3** to confirm the induction of trained immunity *in vivo* in monocytes or pDCs. In addition, trained immunity is not confirmed for pDCs, although theoretically this could be possible as pDCs are innate immune cell and not as short lived as neutrophils for example.[6]

Raw milk and bovine IgG are one of the few food components that are now known to be able to induce trained immunity. Recently it was shown that a Western diet can induce trained immunity in an *in vivo* mouse model.[7] Four weeks of a Western diet-like chow induced innate immune reprogramming that lasted even after an additional 4 weeks of normal chow. Next to this, low-density lipoprotein (oxLDL) particles induced in blood as a result of Western diets, are known to induce trained immunity in human cells *in vitro*.[7,8] However, it should be noted that whereas bovine IgG does not reach the circulation upon ingestion[9], oxLDL is chronically present in the blood and can also continuously stimulate the immune system.

In contrast to trained immunity that could be induced by consumption of raw bovine milk or bIgG, trained immunity induced by a Western Diet has a negative effect. Here, trained immunity enhances the inflammatory state by a chronic exposure to oxLDL and thereby contributes to progression of atherosclerosis.[10] Atherosclerosis is linked as well to a cumulative infectious burden and not so much to infection by a specific pathogen.[10] This results in the concept that infections during childhood, ultimately contribute to the development of atherosclerosis.[11] Next to a cumulative effect of infections, chronic infections form a risk for atherosclerosis development and other cardiovascular diseases as well.[12] In the latter case, trained immunity might be a beneficial mechanism to protect the body against recurrent infections.[10] So in conclusion, trained immunity by diet can have both positive and negative health effects. In the case of raw bovine milk and bovine IgG the potential health effect may contribute to protection against infections, whereas Western diet induced trained immunity promotes chronic inflammation.

Future perspectives for the induction of trained immunity by diet

The aim of **chapter 3** was to identify whether raw milk or its components were able to induce trained immunity. We did not study yet the underlying mechanisms that are responsible for the induction of trained immunity by raw bovine milk and bovine IgG. Therefore, we have no clear explanation why raw bovine milk or which raw milk component induces trained immunity for TLR1/2 stimulation. In addition, we saw trained immunity induction in some subjects for lactoferrin but this was not consistent. What is the causal factor that results in trained immunity induction by a dietary component in one person, but not in another person? Does the epigenetic status of a person, based on previous infections plays a role or not? Or did the diet of the blood donors already influence the responsiveness of their isolated monocytes? In order to answer these questions whole-genome transcriptome and epigenome studies need to be performed. Furthermore, the effect of bovine IgG i*n vivo* could be studied by a nutritional intervention study in humans. In this study, either an Influenza vaccination or even an Influenza challenge, which induces TLR7-mediated immune responses[4], could be administered to the subjects to study the induction of trained immunity. In addition in this study, *ex vivo* trained immunity could be studied by stimulation of isolated monocytes or macrophages with TLR stimulations. Altogether, more in-depth investigation is need to understand how dietary components induce trained immunity and to understand how this knowledge in can applied in e.g. enhancing vaccination responses.

Modulation of respiratory immunity by raw bovine milk

To be able to study immunomodulation of airway immunity by raw bovine milk, we used oral vaccination as a model. Oral Influenza vaccination might have been the most relevant model, but influenza vaccines are only available as injection or nasal vaccines[13]. Nasal vaccines are not fully compatible with the concept of studying direct interaction of raw milk and the mucosal vaccine, hence we selected an oral vaccine. We selected the oral cholera vaccine Dukoral® as it is not commonly used in the Dutch population and it is known to induce vaccine-specific antibody production in serum and mucosal secretions, such as saliva, nasal wash and feces.[14–17] In **chapter 4**[18],

we characterized the kinetics of IgG and IgA antibody responses in serum, and nasal wash, kinetics of CTB-specific memory IgA and IgG B cells and kinetics of tissue homing potential of memory B cells. **Chapter 4** is based on the control group in the MOSAIC I study that only received the oral cholera vaccine. In the MOSAIC I study a group of subjects receiving the vaccine in raw milk was included, these results are described in **chapter 5**. In addition, a 'raw milk long duration' group was included that ingested the vaccine in raw milk with a spoon and instructions were given to expose the oral cavity with milk before swallowing. Sips were taken every 30-45 seconds, so ingesting the vaccine took approximately 30 minutes in total. We hypothesized that exposing the vaccine in raw milk in the oral cavity would increase the uptake by tonsils of Waldeyer's ring and hence improve homing towards the upper airways.

Based on the outcomes of MOSAIC I a follow up study was devised that only focused on induction of vaccine-specific antibodies in serum and mucosal samples (MOSAIC II). In **chapter 5**, we focussed on the difference between raw bovine milk and control vaccination on vaccine-specific antibody production in serum, nasal wash, saliva and fecal water. Based on the outcomes of the two MOSAIC studies, I will discuss in the next paragraphs the effect of raw bovine milk on airway homing potential and on inducing vaccine-specific antibodies in mucosal samples in an oral cholera vaccination model.

Immunomodulation by raw milk on production of vaccine-specific antibodies in upper respiratory tract

In the MOSAIC I study (**chapter 4**) the peak of Dukoral®- and CTB-specific IgA and IgG antibodies in serum was at day 28, as well as Dukoral®-specific IgG in nasal wash. The levels of vaccine-specific antibodies in saliva and fecal water were very low in the MOSAIC I study and did not significantly change over time. For the MOSAIC II study, described in **chapter 5**, it was therefore decided that an additional time point should be added, which was day 42. In addition, protocols were adapted to obtain saliva and to process nasal wash differently. In the MOSAIC I study saliva was collected by passive drooling. This resulted in highly variable saliva volumes and consistency per donor and led to missing samples once the optimal ELISA protocol was obtained. Therefore, in the MOSAIC II study we switched to actively collect saliva by

chewing on a cotton swab in the Salivette® system. This resulted in higher saliva volumes and less mucus in the samples, which made it better possible to measure vaccine-specific antibodies in saliva samples **(chapter 5)**. In MOSAIC I, it turned out that the method used to collect nasal wash diluted the vaccine-specific antibodies a lot. Hence, it was tried to freeze-dry the nasal wash samples, which unfortunately resulted in a phenomena of back-melting. For the MOSAIC II study freeze-drying did work out (2ml nasal wash in a 15ml tube). Samples were reconstituted in ELISA buffer resulting in 3 fold concentrated nasal wash sample and increased vaccine-specific antibody detection compared to the MOSAIC I study (Figure 2 **chapter 5**).

In serum we measured Dukoral®-specific IgA, IgG and IgM, which is mixture of antibodies specific for *Vibrio cholerae* strains O1 Inaba and O1 Ogawa and CTB. In MOSAIC II we did not measure CTB-specific antibodies, as CTB- and Dukoral®- specific IgA and IgG responses were comparable in serum in the MOSAIC I study (**chapter 4**).[18] For most antibody responses the peak is observed at day 28 and remains this a high at day 42 or declines at day 42, while other studies report a peak at day 17-21[19,20] or study only until day 21[21]. In MOSAIC II study we were interested in the production of vaccine-specific antibodies not only in serum, but in saliva, nasal wash and fecal extracts as well (**chapter 5)**. Czerkinksy et al. followed the kinetics of anti-CTB antibodies in serum and in saliva (unstimulated parotid saliva) after oral cholera vaccination at day 0, 14 and 28.[22] They found increased CTB-specific IgG in serum from day 14 to day 42 and on day 56 no significant elevated antibody-titer was observed. However, CTB-specific IgA (serum and saliva) or IgG (saliva) were significantly increased from day 21 to day 56. In our study, no vaccine responses was induced in vaccine-specific IgA production in saliva and vaccine-specific IgG had a peak at day 28 and not at day 42 **(chapter 5)**. A possible explanation could be that the oral cholera vaccine used in their study is more potent and that for high titers in saliva a third vaccination might be needed. In another Dukoral® study the fold increase of CTB-specific IgA and IgG is much higher in serum than in nasal wash. In serum CTB-specific IgA is at maximum 37 fold increased and CTB-specific IgG 19 fold, while in nasal secretions CTB-specific IgG is more induced (6.2x) compared to CTB-specific IgA (2.5x).[15] This is comparable with our study for serum (at day 28 IgA 20x; IgG 10x; IgM 1.5x) and for nasal wash (IgA control 2.6x and IgG 12x at day 28). In another

study by the same group, CTB-specific antibody responses after oral Dukoral® vaccination (day 28) in serum, bronchoalveolar lavage (BAL) fluid and urine IgA levels were only significantly induced in serum (8.6x). In urine the CTB-specific IgA levels were increased 2.5x and BAL 1.6 x, while IgG levels were induced at a more comparable levels for serum (3.8x), BAL and urine (3.0x).[23] In our study vaccine responses are also much stronger in serum compared to nasal wash or saliva as measurements for upper airways. In the study of Shamsuzzaman et al. IgA specific for CTB, *Vibrio cholerae* O1 LPS (present in Dukoral) and *Vibrio cholerae* O139 LPS were measured in feces and only CTB-specific IgA was significantly increase, while IgA specific to O1 LPS was only increased 2 fold.[24] In another study, higher titers for CTB-specific IgA were found compared to cholera membrane preparation in fecal extract as well.[21] In our study we only measured Dukoral®-specific IgA in fecal water. Dukoral® consists mainly of bacteria and a little CTB, nevertheless we obtained a vaccine response for vaccine-specific IgA in the control vaccine group, albeit at a much lower level than in the previously mentioned studies. However, we observed no vaccination response for IgA in fecal water in the milk group. In addition, we did not observe a vaccine-response in both groups for nasal wash IgA. In contrast, the raw milk group does have a vaccine-specific IgG response in nasal wash. However, no clear conclusions about the effect of milk could be drawn as the control groups were responding different in the two MOSAIC studies (**chapter 5**, Supplementary Figure 1). We did not find a satisfactory explanation for the observed difference in response by both the controls groups, as well as for the raw milk groups. Therefore, it would be good to repeat the experiment with the same vaccine, the control group and raw milk group and to compare the data with the previous studies.

Other studies comparing the effect of human or bovine milk on vaccination responses find varying effects. In children the presence of maternal antibodies by both placental transfer and from breast milk has mixed effects, resulting in lower vaccine responses in some studies as reviewed by Edwards[25], no effect[26,27] or an increased effect on vaccine responses[28–30]. In the study of He et al. subjects received bovine colostrum for 7 days or placebo and they were orally vaccinated with a Salmonella vaccine (Vivotif®) at day 1, 3 and 5 to mimic an infection.[31] No significant differences were observed between the groups. However, (non-significant) higher numbers of anti- *S. typhi* Ty21a

IgA antibody secreting cells were observed in the colostrum group compared to the control group ($580/10^6$ vs $109/10^6$, respectively). So, supplementation of 7 days was not long enough to induce an altered vaccine-response towards oral Salmonella vaccination, unfortunately the effect after a longer period of time was not investigated. When comparing this set-up to our study, the incidental raw milk consumption only on the two vaccination days and the following day was probably too short to induce an altered vaccine response after oral cholera vaccination.

In cholera patients supplementation with bovine colostral antibodies did not neutralize cholera toxin and did not reduce diarrhea compared to a control treatment with water.[32] No results were shown for prophylactic use of bovine antibodies on cholera induced diarrhea. In a mice study bovine lactoferrin (bLF) was shown to block binding of CTB to its GM-1 ganglioside receptor[33], which is expressed on intestinal epithelial cells.[34,35] Furthermore, bLF is known to induce local IgA switching in mice[36], which should result in higher sIgA levels in fecal water and that was not observed. When using bLF fortified infant formula, the incidence of respiratory tract illnesses and diarrhea-related illnesses were lower for children receiving breast milk or bLF fortified formula, compared to control infant formula.[37] Lactoferrin and bovine IgG are present in raw milk and in pasteurized milk, but not in UHT milk[38], see also Supplementary Figure 2 **chapter 5** for IgG. As we observed similar results for all milk groups, it is unlikely that lactoferrin or bovine IgG exerted a modulatory effect on the oral cholera vaccination responses at the level of both antibody production as well as at the level of promoting airway homing potential, as will be described below.

The effect of raw bovine milk on airway homing potential of B cells

In order to study modulation of tissue-specific homing potential of peripheral blood cells, FACS panels to detect specific homing marker combinations had to be established. Our hypothesis was that oral vaccination induces airway homing as well as homing towards the small intestine and possibly to the colon, and that consumption of raw milk might preferentially increase airway homing potential. In **chapter 4** we studied the tissue-specific homing potential of B cells after oral cholera vaccination, as well as the presence of CTB-specific B cells. The peak of CTB-specific IgA^+ and IgG^+ memory B cells in peripheral

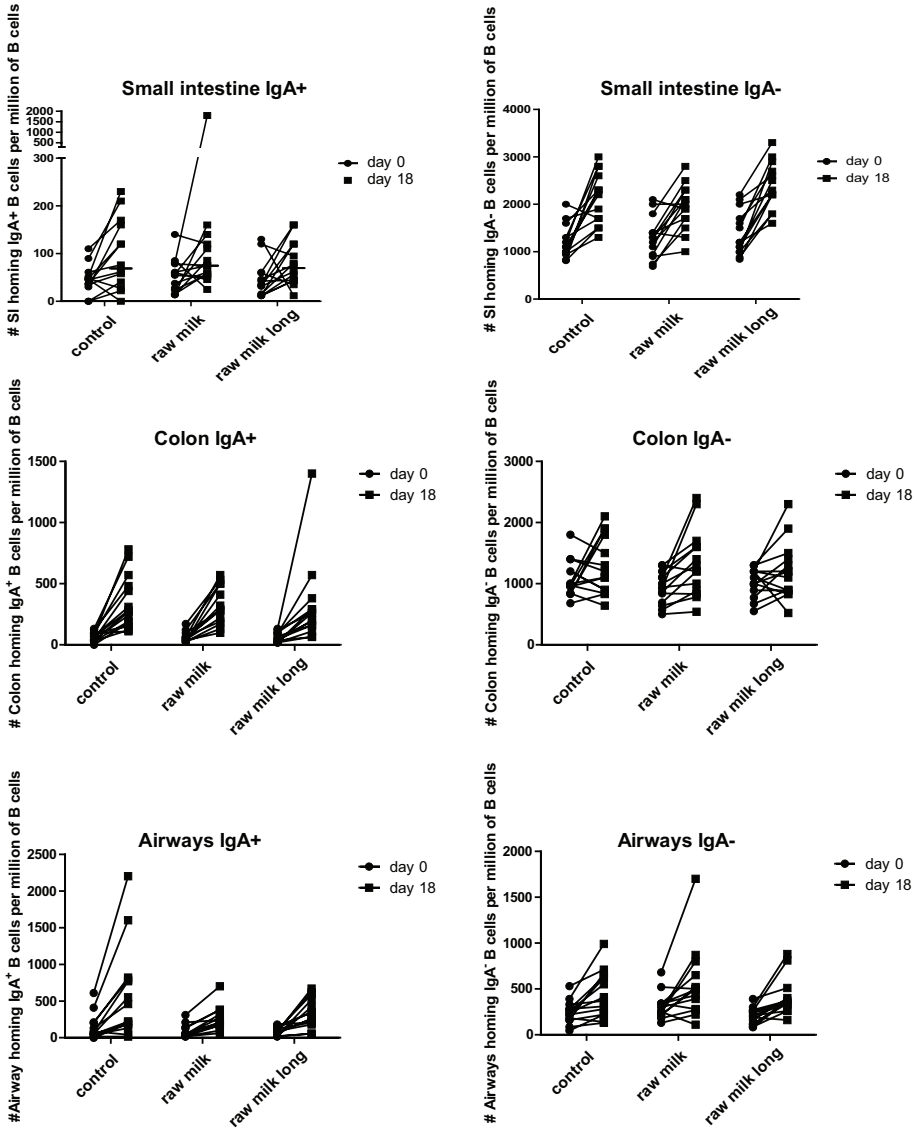


Figure 1: Kinetics of IgA$^+$ and IgA$^-$ memory B cells with an small intestine (CLA–Integrinβ7$^+$ CCR10–CCR9$^+$), colon (CLA–Integrinβ7+CCR10+CCR9–) or airways (CLA–Integrinβ1$^+$ Integrinβ7– CCR10$^+$) homing potential. PBMCs of healthy volunteers (n = 14 per group) were analyzed on days 0, 14, 18, and day 28, using flow cytometry. Data are shown as before-after dot plot. No significant differences are observed in homing potential between the groups.

blood was at day 18. Day 18 was 4 days after the second vaccination and at the same day a peak of memory B cells with distinct tissue homing profiles were observed. Interestingly in the control vaccination group, IgA^+ memory B cells preferentially expressed markers that enable homing to the airways ($CLA^-Integrin\beta1^+Integrin\beta7^-CCR10^+$), and to the colon ($CLA^-Integrin\beta7^+CCR10^+CCR9^-$), while IgA^- (mainly IgG^+) memory B cells primarily expressed markers associated with homing to the small intestine ($CLA^-Integrin\beta7^+CCR10^-CCR9^+$), see **chapter 4**.[18] In addition, we analysed homing potential of IgA^+ and IgA^- memory B cells in the raw milk and the raw milk long duration group, which is shown in Figure 1. Homing potential by both IgA^+ and IgA^- memory B cells towards the small intestine, colon and airways is increased at day 18 compared to day 0 for both milk groups, as well as the control vaccine group, reflecting the response to the oral vaccine. No significant differences were observed in homing potential between the three groups.

Having said that, for IgA^+ memory B cells the raw milk long duration group showed a strong increase from day 0 to day 18 for airways homing potential, although these levels were lower than the control vaccine group. While for IgA^- memory B cells an increased homing potential towards the small intestine was induced, which was higher than the raw milk group and comparable to the control vaccine group, albeit not significantly different. The raw milk group induced slightly more IgA^+ memory B cells with a colon homing potential, compared to the raw milk long duration group, but not compared to the control vaccination group. Raw milk induced a slight non-significant increase in IgA^- memory B cells with airway and colon homing potential, compared to the other two groups. When we compare these results with the results from the control group only (**chapter 4**), the homing profile of IgA^+ memory B cells to have airway and colon homing potential is largely confirmed for the two milk groups. For IgA^- memory B cells a small intestine homing preference was observed in the control, which is confirmed for both milk groups, although raw milk seems to induce some more airway homing potential as well. Although we noted some small differences, the overall conclusion is that raw milk either as bolus or with a long interaction with an oral cholera vaccine does not significantly enhance airway homing potential of memory B cells.

The selection of homing markers

The existence of a "common mucosal immune system" has often been discussed in the past, mostly based on expression of VCAM-1 on endothelium of different mucosal tissues[14,39,40]. One of the observations for this common mucosal immune system is the fact that oral vaccination does not only give a response in the intestine, but also in other mucosal tissues. However, based on recent literature, there seem to be distinct niches of mucosal responses, e.g. intranasal vaccination will lead to vaccine-specific antibodies in the nasal cavity and genital tract, but not in the gut. While vaccination in the genital tract will only induce a local response.[14]

The combination of homing markers to determine homing to specific target tissues is based on expression of selectins, integrins and chemokine receptors expressed by lymphocytes, which can bind to tissue-specific ligands and locally produced chemokines and lead to extravasation of lymphocytes into the tissues. However, ligands of one tissue can be expressed by other tissues as well, therefore a combination of multiple homing markers is needed to get the best selectivity. In the MOSAIC I study described in **chapters 4 and 5** we selected integrin β1, integrin β7, CCR9, CCR10 and CLA as homing markers.[41–51] With these markers the following tissue homing profiles were made: airways ($CLA^-Integrin\beta1^+Integrin\beta7^-CCR10^+$), colon ($CLA^-Integrin\beta7^+CCR10^+CCR9^-$), the small intestine ($CLA^-Integrin\beta7^+ CCR10^-CCR9^+$) and skin ($CLA^+Integrin\beta1^+Integrin\beta7^-CCR10^+$). Both Integrin β1 and Integrin β7 can form a dimer with Integrin α4. Integrin β7 can also form a heterodimer with Integrin αE, but this is mainly expressed on $CD8^+$ T cells and not on peripheral blood B cells[39]. Integrin β1 can bind to multiple integrin α's, such as integrin α5, α4 (CD49d), α3 (CD49c) and αL (CD11a), however in peripheral blood the most common combination is Integrin α4β1[52,53]. In this study it was therefore assumed that Integrin β1 and Integrin β7 represent the expression of the heterodimer Integrin α4β1 and α4β7 leading respectively, to homing potential to non-intestinal tissues or the gut.

Considering airway homing there is no clear homing marker known for B cells to distinguish the lower and upper respiratory tract. In contrast, T cells have CCR3 as a distinct marker for nasal mucosa homing[51]. CCR3 can bind to CCL28, which is also a ligand for CCR10, so apparently B and T cell have

bifurcated homing profiles towards the upper respiratory tract. CCL28 is expressed in the respiratory tract together with VCAM-1[54,50], besides CCL28 is expressed in especially the colon, tonsils and lactating mammary glands[55,42]. CCL27 can bind to CCR10 positive lymphocytes as well[48,43] and is expressed in the skin. The skin homing marker CLA can bind E-selectin, which is expressed in the skin, as wells as on oral mucosa and inflamed tissues[45–47,56]. The homing profiles of B cells that can home to airways and skin are quite comparable, and are based on expression of CCR10 in combination with α4β1 without α4β7. They are differentiated by expression of the skin homing marker CLA. Until recently people thought that in healthy skin B cells were absent, but B cells expressing functional CLA were discovered in the skin of sheep[57] and later in human skin[58,59]. However, CLA^+ B cells are found in human tonsils as well[39]. Furthermore, patients suffering from upper respiratory tract infections show a strong increase in CLA and CD62L expression.[56] As the present study was performed in healthy donors we have made the assumption that CLA expression in this study does not reflect homing to the oral cavity.

In our studies we were limited to a maximum of 8 markers per FACS panel, therefore it was not possible to include CTB in the homing panels. For future studies it would be interesting to include CTB in the homing panels to study vaccine-specific homing potential of B cells.

In the current study the peak of both CTB-specific IgA and IgG memory B cells is at day 18, indicating that the kinetics of the homing potential of memory B cells could be induced by the vaccine, but there is no formal proof of this. In addition, it would be good to include more B cells markers indicative for memory (CD27)[55,60–62] or plasmablast ($Cd38^{high,}$ $CD138^-$) B cells.[56,63] CCR6 could be used as maturation marker of memory B cells as well.[64,65] CD19 is maintained on peripheral B cells, including memory B cells, but also on plasmablasts and subsets of plasma cells. Based on CD19 expression only, memory B-cells will also include a small number of $CD19^+$ plasmablasts.[66] The B cells in the present study are primarily $CD19^+$ memory B cells, as shown in **chapter 4** Supplementary Figure S2. The data are thus complementary to the homing potential of $CD19^-$ plasmablasts. Furthermore, the homing panels could be extended with CD3 and CD4 as T cell and T helper cell markers, just as CCR3[51] and G protein-coupled receptor-15 (GPR15)[56], which are known

as homing receptors for T cells towards the nasal mucosa and colon (and potentially the skin), respectively. GPR15 was expressed by plasmablasts, but was not specifically enhanced in intestinal sites.[56] In this study, we did not select CD62L as it was quite ubiquitously expressed and supposed to be a homing marker for lymph nodes as it can bind to peripheral node addressin (PNad) and sites of chronic inflammation.[46,56,67] Therefore we selected CCR7 as lymph node (and spleen) homing marker.[42] In contrast, plasmablasts of people that suffered from upper respiratory tract infections 7-9 days before, had a high expression of CD62L (L-selectin), CLA and CD49f (Integrin α6) and intermediate expression of CCR10.[56] CD62L was also expressed by *S. pneumonia*-specific plasmablasts from patients with pneumococcal pneumonia and (parenteral) pneumococcal vaccinated volunteers, while CLA was only expressed by the patients.[67] The role of CD49f is not yet clear, but the expression on memory T cells distinguishes circulation through skin and lung versus gastrointestinal tissues.[68] Furthermore, in a recent mouse study it was shown that parenteral tuberculosis vaccination induced memory T cells that are restricted to the blood vessels of the lung. In contrast, after pulmonary tuberculosis infection T cells are faster able to enter the lung parenchymal tissue and airways. Whether systemically induced T cells were able to enter the lung parenchyma and airways was dependent on CXCR3 expression.[69] Whether CXCR3 is important in human airway homing is currently not known. In conclusion, to improve the homing profile for airway homing potential of human peripheral blood lymphocytes, the inclusion of CD62L, next to CLA and CCR10 would be beneficial.

Oral vaccination as model to study immunomodulation by food

Although we were not able to detect consistent results when studying the immunomodulatory effect of raw bovine milk consumption on oral cholera vaccination, oral vaccination can be a relevant model to study immunomodulation by food. One of the reasons to use oral vaccination as model to study immunomodulation by food is because of regulations by the European Food Safety Agency (EFSA). EFSA officially has to approve health claims on food products and they established guidelines for immune related claims.[70] In order to use a health claim on food products and -ingredients there should be wide scientific agreement on the effect, as is the case for article 13 claims. This is the case for intensively studied ingredients like

vitamin A, vitamin D, iron, folic acid and other vitamins[71], which have been investigated for decades. General scientific acceptance is more difficult for new food components, like prebiotics and probiotics. Next to this, in contrast to pharmaceutical products, medical end points are not sufficient for health claims on food components. Food components should have an causal effect on biomarkers of a specific disease or disease process. However, it is often difficult to determine a causal biomarker for a disease and causal biomarkers are therefore subject of scientific debates.[72–75]

Oral vaccination might therefore be a good model as the vaccination induces a clear biomarker, being vaccine-specific antibodies, that may be regarded as a health related effect. If food has an immunomodulatory effect on this vaccination response compared to a proper control group, a causal relationship between the food and the a production of vaccine-specific antibodies can be established.

The interaction of a food component with an oral vaccine can occur on multiple levels. A food component can interact directly with the vaccine, as was hypothesized for bovine IgG in our study. If this is the case, it should be verified that the interaction is not specific for only that particular vaccine, but that the results can be extrapolated to other stimulations or infections. Next to this, it should be kept in mind that a direct interaction with the oral vaccine potentially leads to immune exclusion. In addition to direct interaction with the vaccine, food components can also have an effect on the local microbiota composition and microbial metabolite production (e.g. pro-biotics and pre-biotics) in whole gastrointestinal tract. The role of microbiota on health and specifically on respiratory health, being described as the gut-lung axis, is slowly discovered.[76] As discussed in **chapter 2 and 4** the gut-lung axis could in the case of oral vaccination especially be relevant for the induction of homing towards the airways. The active form of vitamin D, 1,25-dihydroxyvitamin D3, can induce expression of CCR10 in human B cells, which in theory can direct B cells to mucosal sites, including lungs.[77]

Besides a direct effect of microbiota, short chain fatty acid production by microbiota can affect DC and T cells in the respiratory tract[78,79], which can potentially be modulated by prebiotic compounds tested in this model. The

gut-lung axis was also shown to be dependent on the presence of intestinal microbiota to provide TLR-stimulation, as antibiotic treatment before influenza infection led to increased viral load in the lungs of mice and reduced influenza-specific antibody production.[80] This mechanism seems quite similar to what occurs during establishment of trained immunity as an unrelated bacterial TLR stimulation (LPS in this case) leads to improved non-related anti-viral responses in the lung. A more detailed discussion on this topic is given in the section 'the potential effect of trained immunity throughout life'.

Furthermore, depending on the hypothesis of the mechanism by which a dietary components can exert a health effect, the duration of food supplementation should be sufficient. Is a single dose of a component enough to exert an immunomodulatory effect or is supplementation for multiple days or weeks needed? The advantage of oral vaccination is first of all that a direct effect with food is possible as both follow the same route of uptake. Oral vaccination induces responses in serum, which is mildly invasive to collect and therefore the time points of sampling have to be correct to measure a response. This can be challenging as the kinetics of for example expression of homing markers on lymphocytes are different compared to antibody responses (**chapter 4**). In addition, oral vaccination induces responses in saliva, nasal wash, feces and urine, which can be collected non-invasively. The advantage is that these samples can be collected more easily on a day to day basis, even at home, making it possible to detect a big variety of biomarkers which can have different kinetics (e.g. cytokines, antibodies, microbial metabolites).

Future perspectives on modulation of airway immunity by raw bovine milk and its ingredients

For future studies, extending the flow cytometry panels with homing markers, B cell maturation markers and vaccine-specific markers is recommended. Besides, it should be considered to take mucosal biopsies to investigate the local responses in the mucosal immune system, as is done in some studies[81]. These studies revealed differences in magnitude and kinetics of IgA, IgG and IgM production when comparing duodenal biopsies and peripheral blood[24,81] or in salivary glands [22]. Nowadays it is difficult to obtain ethical permission to collect invasive samples like taking biopsies, but it might be worthwhile to investigate. At this point we can only speculate about the homing into target

tissues of peripheral B cells and we can try to measure an increase in local vaccine-specific antibody production by B cells in mucosal tissues. Taking biopsies would therefore give more insight to investigate the presence of vaccine-specific antibody-secreting B cells in mucosal tissues and to investigate whether these are mainly plasmablasts, plasma cells or memory B cells.

In our study, a substantially longer duration of nutritional supplementation of raw milk might have resulted in a stronger immunomodulatory response against the oral vaccine. For example start 4 weeks before oral vaccination with raw bovine milk consumption (1 glass/day) and continue until day 42 of the study, compared to no (raw) bovine milk consumption. This interaction will then not be dependent on the complex formation between bovine IgG and the vaccine, but rather the immunomodulatory capacity of all milk components combined.

To determine which components of raw milk are responsible for the reduced incidence of asthma, allergies and respiratory tract infection in children future research is needed. One of the issues that should be solved is how to purify raw milk components (e.g. bovine IgG and lactoferrin) on an industrial scale in a way that ensures microbial safety to use the raw components in infant formula. Once these processes are established on a small scale, a long-term nutritional intervention study in neonates could be performed using a group on regular infant formula, a raw milk component enriched-infant formula and a breast-fed group. Ideally, this would be longitudinal intervention study of six months, with follow-up measurements to study prolonged effects of intervention on infection and allergy. In the first six months the effect of type of milk consumption can be compared for their effect on the national vaccine programme (Table 1).

In addition, serum measurement can be done to measure the cytokine profiles in blood and antibody-titers against the vaccines. In this way the potential effects of bovine IgG directly on airway infections can be determined or the effect of potential trained immunity induction can be investigated. Next to this, it would be interesting to investigate the effect of adding BCG vaccination as first vaccination in the national vaccination programme to test its effect on vaccination responses in children and the development of asthma and allergies later on in life.

Table 1: The national vaccination programme (2018) in the Netherlands, according to National Institute for Public Health.

Age	Vaccination 1	Vaccination 2
6 - 9 weeks	DKTP-Hib-HepB	Pneumococcus
3 months	DKTP-Hib-HepB	
4 months	DKTP-Hib-HepB	Pneumococcus
11 months	DKTP-Hib-HepB	Pneumococcus
14 months	MMR	Meningococcal ACWY
4 years	DKTP	
9 years	DTP	MMR
Girls of 12 years	HPV	HPV

D(K)TP: diphtheria, (pertussis), tetanus, poliomyelitis; Hib: Haemophilus influenza; HepB: hepatitis B; MMR: measles, mumps and Rubella; HPV: human papilloma virus

Can food support the immune system in elderly?

Ageing is associated with a changing immune system, leading to inflammaging (increased levels of inflammation markers in serum) and immunosenescence (reduced immune cells and reduced responses towards pathogens). This results in reduced vaccination responses and increased infections in elderly. Immune deficiency during ageing occurs at two levels: irreversible primary immune deficiency (e.g. immunosenescence) and reversible secondary immune deficiency of which low nutritional status is an example.[82] Food contains macronutrients like proteins, fat, carbohydrates and micronutrients like minerals and vitamins. Immune cells use glucose, amino acids and fatty acids for energy generation.[83] Upon infection reactive oxygen species (ROS) can be produced and their damaging effect on others cells has to be confined by anti-oxidants. Next to vitamin C and E as antioxidants, there are several antioxidant enzymes which use metal ions at their active site.[84] Other food components that are known to affect the immune system are poly unsaturated fatty acids such as omega-3 fatty acids, flavonoids, carotenoids and pre- and pro-biotics.[85] Hence malnutrition of both macro and micro nutrients results in reduced functioning of the immune system, and is often accompanied by susceptibility to infection.

Low nutritional status as well as obesity are associated with frailty in elderly.[86] Examples of inadequately consumed nutrients in elderly people in relation

to health risk are proteins, omega-3 fatty acids, dietary fibre, calcium, potassium, magnesium, vitamin A precursor (carotenoids), vitamin B-6, B-12, D and E, as reviewed by Shlisly[86]. This might lead to loss of skeletal muscle (sarcopenia) due to inadequate protein intake[86], neurological deterioration and low neurogenesis due to too low vitamine B-12 and E, polyphenols and polyunsaturated fatty acids intake, as reviewed by Poulose et al.[87]

Next to this, micronutrients like zinc are proven to affect immunity in elderly by decreasing incidence of infection[88]. Furthermore, low vitamin D levels can result in decreased calcium storage in bone that can lead potentially to osteoporosis.[86] Therefore, a healthy diet in combination with nutritional supplementation might improve healthy ageing.

In **chapter 7** we performed a double-blind placebo controlled nutritional intervention study with bovine lactoferrin (bLF), GOS and vitamin D in elderly women. Therefore, I will first discuss some background about this components and their effects on the immune system.

bLF is a milk protein that is relatively resistant to gastrointestinal digestion, as 60% of administered bovine LF is unaltered in the stomach of human adults[89]. Bovine Lactoferrin (bLF) has been described to reduce the number of infections by rhinoviruses and hepatitis C, which are also recognized by TLR7.[90–92] The exact mechanism by which bLF reduces rhinovirus infection is unknown[90], in case of Hepatitis C virus direct binding of LF with the virus is known to occur[93–95].

bLF is also shown to prevent sepsis, fungal infections and enterocolitis in premature infants with very low birth weight[96–98], and has also been described to have anti-inflammatory effects.[99,100] In vitro, using the human THP-1 cell line, LF reduced the production of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 upon TLR4 stimulation.[99] Bovine Lactoferrin in combination with ribonuclease supplementation was found to decrease IL-6 and TNF-α serum concentrations in postmenopausal women[100]. Bovine lactoferrin can be taken up by human cells via lactoferrin-receptor (intelectin)[101]. Besides intelectin, other lactoferrin receptors are known in humans, such as low-density lipoprotein receptor-related protein-1 (LRP-1 or CD91) expressed in many

tissues, where bLF is one of the many ligands.[89,102] Furthermore, human LF can bind to CD14 on monocytes in complex with LPS[103], TLR4[104] and recently binding of bovine LF to CXCR4 was confirmed[102]. Many of these receptors are expressed by a variety of tissues and immune cells in humans. However, currently it is unknown to what extent bLF is taken up *in vivo* and by which mechanisms this occurs. Nevertheless, we hypothesized that whether bLF is taken up or exerts an effect indirectly via interaction with the mucosal immune system, it can exert a modulatory effect on the immune system of elderly.

Galactooligosaccharides (GOS) are formed during the hydrolysis of lactose by the β-galactosidase enzyme in a process called transgalactosylation.[105] The effect of GOS on levels of pro-inflammatory cytokines in serum that are produced by PBMCs in elderly are ambiguous. In one study the levels of IL-6 and TNF-α are decreased after GOS supplementation[28], but in a follow-up study by the same group no significant changes in IL-6 and TNF-α concentrations were observed after GOS supplementation[29]. In both these two studies IL-10 production was significantly increased upon GOS supplementation for 10 weeks (5,5 g/day). In another study 15g/day GOS supplementation for 12 weeks in obese or overweight individuals (45-70 years) resulted in an increase in *Bifidobacteria*, but no effect on IL-6 and TNF-α concentrations in serum was found.[106] GOS could exert its effect of decreasing pro-inflammatory cytokines in serum, via the increase of *Bifidobacteria* levels, which are important for the production of short chain fatty acids (SCFAs). These SCFA play a role in inflammation, and could lead to reduced pro-inflammatory cytokine concentrations in serum. However, in the study of Canfora et al. no effect on SCFA concentrations were found, albeit the 15 g GOS/day.

In addition to bLF and GOS, we investigated the effect of further supplementation with vitamin D. Serum concentrations of vitamin D correlate with increased IL-1β, TNF-α and IL-6 production by monocytes upon TLR7 stimulation, while TLR7 expression is inversely correlated with vitamin D.[107]

Immunomodulation in elderly women by bovine milk components

It is assumed that elderly people have reduced number of immune cells, reduced TLR responsiveness, which are examples of immunosenescence and in addition, they suffer from inflammaging.[108,109] To investigate whether this was

the case in the elderly women included in the NOBLE nutritional intervention study described in **chapter 7**, we compared innate immune function towards TLR-mediated responses in elderly and in young adult women (**chapter 6**). In addition, we measured the concentration of serum markers involved in inflammation in both groups (**chapter 6**). For **chapter 6** we compared the 30 elderly women that participated in the NOBLE food intervention study at day 0, with 15 young adult women of which blood was taken in the same week. Elderly women had lower pDC frequencies and comparable mDC frequencies compared to young adult women. Next to this, a lower basal production of cytokines by pDCs and mDCs in steady state, and increased markers involved in inflammation in serum were observed in elderly compared to young adult women. Especially after TLR7/8 stimulation, reduced numbers of pDCs and mDCs positive for pro-inflammatory cytokines were observed in elderly compared to young adult women. This confirms the concept of both immunosenescence and inflammaging in elderly women.

Therefore, we performed a double-blind placebo controlled nutritional intervention study with bovine lactoferrin (bLF), GOS and vitamin D with the aim to restore TLR responsiveness of pDCs and mDCs, and also to reduce inflammatory cytokines in serum in elderly women (**chapter 7**). Nutritional supplementation with bLF, GOS and vitamin D enhanced responses to TLR stimuli in elderly women in both pDCs and mDCs. Especially TLR7-mediated responses in pDCs were enhanced after three weeks of bLF supplementation compared to placebo. As immune responses to Influenza and RSV infection are TLR7 mediated, this could imply that nutritional supplementation with bLF can enhance anti-viral immunity towards Influenza infection by increasing the responsiveness of pDCs.[4] This would be beneficial as elderly suffer more often compared to adults of severe Influenza and RSV infections leading to hospitalization and even increased death rates.[4,110,111]

In **chapter 7** we also measured the concentrations of anti-inflammatory (e.g. IL-1Ra, IL-10) and pro-inflammatory (IL-6, TNF-α, IL-1β, CRP, sVCAM, sICAM) serum markers in the intervention and placebo group during the nutritional intervention study. Only a trend of decreased sVCAM concentration was observed in subjects that completed the whole nutritional intervention (bLF + GOS + vitamin D) compared to the placebo group. This

was unexpected as we expected that one of the effects of the intervention would be to decrease serum concentration of IL-6, TNF-α or CRP, as these are often studied inflammatory markers in serum in elderly and with respect to effects of dairy consumption.[112,113] bLF and GOS were also supposed to reduce the production of pro-inflammatory cytokines and therefore lower levels in serum were expected.[100,114] One explanation could be that 6 weeks instead of 10-12 weeks of GOS supplementation was too short or that 2,6 g/day GOS in our study **(chapter 7)** was insufficient to affect pro-inflammatory cytokine concentrations in serum. Next to this, the duration of the vitamin D supplementation was too short to exert an effect, which is in line with the unchanged concentrations of vitamin D in serum in both placebo and nutritional intervention groups.

In addition, the study group size was based on parameters for pDC activation via TLR7, and one of the possible explanations for not finding clear effects on inflammatory markers might be related to the group size. Therefore we performed a new power calculation based on our data **(chapter 7)** of inflammatory markers and the effect of LF supplementation in the study of Bharadwaj[100]. Based on these calculations we conclude that the group size to establish effects on serum levels inflammatory markers was not sufficient to show significant effects, and should at least have been twice as large.

Another possible explanation could be that the elderly included in this study were generally too healthy. Although, in **chapter 6** elderly women were found to have higher serum concentrations of IL-10, TNF-α, sICAM, sVCAM and tended to have lower IL-1Ra levels, compared to young women. Although the increase of pro-inflammatory cytokines in serum is a hallmark of inflammaging[108,109,115], increased levels are found in healthy centenarians as well.[116–119] Therefore, it is suggested that in general increased levels of pro-inflammatory cytokines are related to ageing, but are not necessarily linked to reduced health as long levels do not exceed a certain individual threshold or tipping point.[115] After this tipping point, various diseases may develop such as osteoarthritis, metabolic diseases, Alzheimer disease, onset of frailty and cardiovascular diseases.[120,121]

When taking elevated inflammatory markers into account that do not cause

detrimental health effects, our study population of elderly women are maybe too healthy to find a result of immunomodulation by food on serum markers. We selected elderly women according to certain exclusion criteria such as having no chronic inflammatory disease, no immune deficiencies, no use of anti-inflammatory drugs, no hormonal replacement therapy and being mobile enough to come to the study location and being cognitively able to participate in the study. Based on this, during the inclusion of the subjects for the NOBLE study, 8 elderly people out of the 47 were excluded and 9 other people were not selected because of too low age. This is different from the strict restraints from the SENIEUR protocol used for selection of healthy elderly in immuno-gerontological studies[122], which resulted in inclusion of only approximately 10% of residents in a home of the aged[123]. This leads to a bias towards inclusion of an above average healthy elderly study population. Although our selection was not as strict as the SENIEUR protocol, there is some bias towards healthy elderly without neurodegenerative disease, mobility issues or morbidities. It might be that nutritional supplementation is more effective, either when consumed for a longer period (months) or in a crosssection of the entire age group rather than a strictly selected group.

Future perspectives on immunomodulation in elderly by bLF

The outcomes of this study warrant future studies with larger group sizes to extend our findings on DCs and inflammatory markers in elderly women. Next to including more subjects per group, the inclusion criteria should be adapted so that less mobile elderly women or more (non-hospitalized) women with chronic inflammatory diseases can be included as well. In these groups there might be a larger window to observe effects of immune modulation by food ingredients.

Elderly people have different immunological needs compared to children, although both need additional protection against viral infections like Influenza. In **chapter 7** we showed that bLF supplementation can increase TLR7/8-mediated responses in pDCs. As pDCs are crucial for anti-viral immunity[4,124] it would be very interesting to extend the set-up of this nutritional intervention study. Elderly women should receive bLF supplementation alone or placebo for 3 weeks and afterwards they are challenged with Influenza virus or another option is to vaccinate people with the seasonal Influenza vaccination. In this way we can confirm whether the increased TLR7/8-mediated responses

after bLF supplementation are functional by means of lower viral load after Influenza infection or higher vaccine-response.

In addition, the responsiveness of different subtypes of DCs can be compared in elderly. In **chapter 6**, we shortly discussed that with the current gating strategy for pDCs ($CD11c^-$ $CD123^+$ $HLA\text{-}DR^+$ $lineage2^-$) and mDCs ($CD11c^+$ $CD123^-$ $HLA\text{-}DR^+$ $lineage2^-$), a third population ($CD11c^-$ $CD123^-$ $HLA\text{-}DR^+$ $lineage2^-$) was observed. It should be kept in mind, that pDCs are one type of dendritic cells, while mDCs consists of multiple types of dendritic cells ($CD16^+$ (65-75%); $CD1c^+$ (10-20%); $CD141^+$ (3-5%))[125]. Furthermore, especially $CD16^+$ mDCs are involved in inflammation as they produce more cytokines compared to CD1c+ mDCs.[125] At the moment we can only speculate what these $CD11c^-CD123^-HLA\text{-}DR^+lineage2^-$ cells are. When using backgating to the FSC/SSC the $CD11c^-CD123^-$ population seems a mixture of $lineage^{low}$ positive lymphocytes and in some donors $CD16^+$ NK cells that could be $CD56^{dim}$, which is included in the lineage 2 cocktail.[126] Furthermore, DC like cells are present, which could be BDCA-3 positive mDCs that are $CD11c^{low}$ and $CD123^{dim}$ [127,128] or $CD34^+$ DC like cells[125] based on FSC/SSC analysis. Whether $CD34^+$ cells are mDCs is debatable, as $CD34^+$ cells are $HLA\text{-}DR^+$ $lineage^-$, but lack CD11c and CD86 expression, therefore they are likely to be progenitor cells.[125] CD11c in mDCs and CD123 expression in pDCs are both upregulated after TLR4 and TLR7 stimulation, while overnight incubation without stimulation reduced CD11c and CD123 expression.[129] We kept our cells for 3 hours in RPMI medium as a control for the TLR stimulations, so maybe after 3 hours CD11c or CD123 could be downregulated as well resulting in a double negative population. As we do not know what type of cells these cells are exactly and what their function is in ageing, it would be very interesting to investigate this subset of cells in future gerontological studies.

The potential effects of trained immunity throughout life

In this section I intend to bring together my own data and data and concepts from literature to set forth a speculative theoretical framework about the potential effects of trained immunity throughout life. In my opinion trained immunity is involved in immune responses from early life to late life. Understanding its function can lead to unravel the complex immune responses

to subsequent pathogen infections and vaccinations. Furthermore, it can lead to understanding when we need artificial induction of trained immunity and when not.

The ability to induce trained immunity *in vitro* by raw bovine milk and bovine IgG (**chapter 3**) may help to prevent infections in early and late life. Raw milk induced trained immunity for TLR1/2 stimulation. TLR1/2 recognizes Triacyl lipopetides[130] and TLR2 is activated by many ligands present on Gram-positive bacteria as well as RSV[5,131]. Bovine IgG induced trained immunity for TLR7/8 stimulation. This could be relevant for enhancing protection of young children to Influenza and RSV infections, but also to protect elderly to Influenza infections.[110,111] Hence, bovine IgG would be a good candidate to use as nutritional supplement for elderly and infants to prevent severe infections by RSV and Influenza. RSV infection in early life is thought to have a causal relationship with development of asthma.[132] In addition, rhinovirus infection (also TLR7 and TLR2 mediated[91]) is associated with the risk of developing asthma.[133] If it would be possible to prevent only a part of the severe rhinovirus and RSV infections by milk components, this might have a beneficial effect on the development of asthma as well. Therefore, I speculate that trained immunity may be one of the mechanisms by which raw milk consumption reduces the odd ratios against asthma, and respiratory tract infections in children.

Trained immunity can have a role throughout adult life as well, by inducing cross-protection after vaccination. Today, vaccines known to induce trained immunity are Bacille Camette-Guérin (BCG), measles, smallpox vaccine *Vaccinia,* and Influenza vaccination.[134–137] In this thesis, we did not look into the potential cross-protective effect of oral cholera vaccination, based on trained immunity. Some studies suggest that oral cholera vaccination provides cross-protection against *Salmonella*[138] and *via* CTB it induces 50-70% cross-protection against heat-labile toxin from ETEC.[139,140] However, these forms of cross-protection are mediated by antibodies produced by B cells that are part of the adaptive immune system. We did observe *in vitro* a trained immunity effect of raw bovine milk, although we cannot draw conclusions about the potential trained immunity effect of raw bovine milk upon oral cholera vaccination as described in **chapter 4 and 5**.

Another theoretical concept where trained immunity might play a role is the 'gut-lung axis', as discussed in "Oral vaccination as model to study immunomodulation by food' and in **chapter 2**. In **chapter 2**, some mouse studies are discussed, which show that there is a cross-talk between commensal microbiota and airway immunity. Microbiota or TLR agonists (TLR 2, 3, 4 and 9) were needed to clear Influenza infection.[80] In mice, treatment with aerosolized *Haemophilus influenzae* lysate protected against a wide variety of bacterial and fungal infections.[141] Another study showed that injecting intra-peritoneal *E.coli*-derived LPS in germ-free mice led to an abrogated IL-10 production and resulted in resistance against *Klebsiella pneumoniae* infection.[142] These are all examples how TLR-stimulation induced in the gut, or maybe TLR-agonists that are taken up in blood, can induce a protective immune response against an non-related pathogen in another tissue, such as the lung. In the study of Evans et al. they termed the observed effect stimulated innate resistance.[141] Stimulated innate resistance lasted only for 4 to 24 hours after challenging, less at other time points and was not dependent on neutrophils, mast cells or macrophages and only worked when inhaled. Therefore, I think that stimulated innate resistance is more a state of 'short' immune priming and not trained immunity. Especially the other studies in which TLR-signalling in the gut results in protective effects in the lungs, could be mediated via trained immunity on the innate immune cells. Although, eventually the anti-viral or anti-bacterial response is a combination of the innate immune system and T and B cells. In contrast to these observations, we have shown in **chapter 3** that stimulation with TLR-agonists led to tolerance of monocytes and not trained immunity. A potential explanation could be that we used a high concentration of TLR-agonist directly on monocytes, while low concentrations of TLR-agonists do induce trained immunity, as shown by Ifrim et al.[143] Hence, it could be speculated that due to gut microbiota a low concentration of TLR-agonists (e.g. LPS) ends up in the blood. These TLR-agonists provide TLR stimulation of monocytes/macrophages that induces trained immunity rather than tolerance. Altogether, trained immunity could play role in this gut-lung axis.

Upon ageing every individual has it's unique 'immunobiography', being the type, intensity and sequence of infections and antigen exposure, including own gut microbiota, throughout life.[144] As described above, cumulative

infections can lead to an enhanced inflammatory state induced by trained immunity leading eventually to atherosclerosis. Recently, Arts et al. proposed that trained immunity could have both a role in initiation as well as aggravating symptoms of many autoinflammatory and autoimmune diseases.[145] This could be correlated to the development some of the age-related non-communicable diseases as well. Can trained immunity induce inflammaging? Inflammaging is not solely mediated by monocytes and macrophages, but is mediated by non-immune cells as well.[144] Therefore, trained immunity is definitely not the only mechanism involved in inflammaging. Nevertheless, trained immunity can play a role, especially via the increased number of DAMPs present during ageing, like uric acid crystals and oxidized fatty acids that are constantly present in the blood.[144] As such this is different from the situation of milk proteins like IgG that are not taken up into circulation[9] but are expected only to interact with the immune system in the mucosa of the gastrointestinal tract. In contrast, a life-long activation of the immune system could potentially lead to immune tolerance of immune cells resulting in reduced clearance of pathogens and therefore more severe infections? Whether a trained immunity response or tolerance response will be induced is unclear, but probably involves the immunobiography of an individual. Trained immunity does not have to be only detrimental during ageing. Trained immunity could have a beneficial role by improving vaccination responses in elderly as is suggested for poly bacterial vaccines[146] or the mucosal administration of TLR ligands as adjuvants.[147] Next to this, trained immunity for example induced via consumption of bIgG could potentially reduce influenza infections, as described above.

In conclusion, trained immunity could have beneficial effects early in life, while later in life it can theoretically have both detrimental and beneficial effects in elderly. Whether trained immunity has beneficial effects for an individual depends on their immunobiography. Hence, in future research the complex role of immunobiography should be integrated more in the immune responses observed in an individual.

Conclusions

In this thesis we focussed on the immunomodulation by raw bovine milk and its ingredients. We showed that raw bovine milk as well as isolated bovine IgG can induce trained immunity in human monocytes **(chapter 3)**. This can serve as mechanistic concept for protection against infections.

Oral cholera vaccination induces a clear vaccination response in serum, nasal wash, and saliva. Furthermore oral cholera vaccination (**chapter 4**) induces a large intestine and airway homing profile in IgA^+ memory B cells, while in IgA^- memory B cells a homing profile towards the small intestine was induced. As opposite results were obtained for both MOSAIC studies, it cannot be concluded whether raw bovine milk is able to increase or decrease the production of vaccine-specific antibodies after oral cholera vaccination **(chapter 5)**. In the current experimental set-up, raw milk did not induce an increase in airway homing potential.

The aim of **chapter 6** was to compare innate immune function towards TLR-mediated responses between elderly and young adult women. Elderly women were shown to have lower pDCs frequencies, comparable mDC frequencies, lower basal production of cytokines by pDCs and mDCs in steady state, and increased serum markers involved in inflammation, compared to young adult women. Especially after TLR7/8 stimulation, reduced numbers of pDCs and mDCs positive for pro-inflammatory cytokines were observed in elderly compared to young adult women. This confirms the concept of both immunosenescence and inflammaging in elderly women. In **chapter 7** we performed a double-blind placebo-controlled nutritional intervention study, to investigate the potential of bovine lactoferrin, galacto-oligosaccharides (GOS) and vitamin D to restore TLR responsiveness of pDCs and mDCs and to reduce inflammatory cytokines in serum in elderly women. Nutritional supplementation with bLF, GOS and vitamin D is safe and enhances responses to TLR stimuli in elderly women in both pDCs and mDCs. No clear effects on pro-inflammatory markers in serum were observed, probably due to the group size in this study that was set up to study pDC responses as primary outcome. Especially TLR7-mediated responses in pDCs were enhanced after bLF supplementation compared to placebo, suggesting that bLF may contribute to protection against viral infections in elderly women.

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Summary

Both infants and elderly people have compromised immune systems. For infants human milk provides support for the immune system. Infants need immunological support, as their immune system needs time to develop. Furthermore, infants are faced with microbial colonisation and changed nutrition from breast milk towards solid food. In addition to commensal bacteria, infants are increasingly exposed to food- and airborne pathogens as well, which can cause infections in the intestine and airways. As especially, RSV and Influenza infections in the airways can be quite severe, it would be beneficial to improve airway immunity by dietary modulation. Next to this, the epithelial barrier of infants is not fully closed, especially just after birth. As a result intact food an microbial components can cross the epithelium into the mucosa. The infant should not develop allergies or anti-inflammatory immune responses towards these macronutrients and is supposed to induce oral tolerance against both food components as well as to commensal bacteria.

Elderly people often suffer from non-communicable inflammation-related inflammatory diseases as a result of immunosenescence and inflammaging. Next to this, elderly respond less to vaccination and are more vulnerable to infections. The ageing population is growing worldwide and therefore the burden of age-related disease on the global health care system is increasing as well. Hence, it would be beneficial when people become older without developing these diseases, which is termed healthy ageing. The **main objective** of this project was to study the immunomodulatory effect of raw bovine milk and its ingredients in nutritional intervention, oral cholera vaccination and trained immunity and its possible relevance for different stages in life.

Consumption of raw bovine milk is associated in epidemiological studies with a reduced incidence of asthma, allergies, rhinitis and respiratory tract infections, which are all related to immunity in the airways. However, not much is known about the mechanisms by which raw milk can exert this enhanced airway immunity. Therefore, the aim of this thesis was to investigate to which extent raw bovine milk can modulate immune responses to improve immunity in the airways. **Chapter 2, 3, 4 and 5** are relevant for modulating the immune responses in children and adults, by raw bovine milk and it's components.

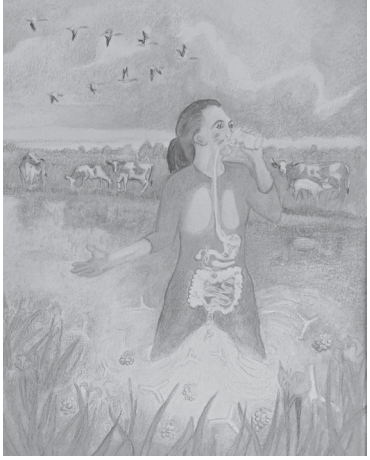
We started this thesis by giving a literature overview of potential mechanisms by which milk components modulate immune function in the upper respiratory tract as well as the gastrointestinal tract (**chapter 2**). Furthermore, in **chapter 3** we investigated the potential of raw bovine milk, milk fractions, lactoferrin and bovine IgG to induce trained immunity. We could demonstrate that raw bovine milk as well as isolated bovine IgG can induce trained immunity in human monocytes. This can serve as mechanistic concept for protection against infections.

Next to this, we characterized the immune responses upon oral cholera vaccination in **chapter 4,** including expression of homing markers that enable homing towards the airways. Oral cholera vaccination induces a clear vaccination response in serum. Furthermore, oral cholera vaccination induces a homing marker expression profile consistent with homing to the large intestine and airways in IgA^+ memory B cells. In IgA^- memory B cells a homing marker expression profile towards the small intestine was induced. In **chapter 5**, we investigated the immunomodulatory effect of raw milk, pasteurized milk and ultra-heat treated milk on oral cholera vaccination responses in blood and mucosal tissues, including saliva and nasal wash in two vaccination studies. Oral cholera vaccination induces a clear vaccination response in serum, nasal wash, and saliva. Opposite results in modulatory capacity of milk on the oral cholera vaccination response were obtained for both MOSAIC studies. Hence, it cannot be concluded whether raw bovine milk is able to increase or decrease the production of vaccine-specific antibodies after oral cholera vaccination. We also compared homing potential towards the airways between the control and raw milk vaccine group. In the current experimental set-up, raw milk did not enhance an increase in airway homing potential.

The aim of **chapter 6** was to compare innate immune function towards TLR-mediated responses between elderly and young adult women. Elderly women were shown to have lower pDCs frequencies, comparable mDC frequencies, lower basal production of cytokines by pDCs and mDCs in steady state, and increased serum markers involved in inflammation, compared to young adult women. Especially after TLR 7/8 stimulation, reduced numbers of pDCs and mDCs positive for pro-inflammatory cytokines were observed in elderly compared to young adult women. This confirms the concept of both

immunosenescence and inflammaging in elderly women. In **chapter 7** we performed a double-blind placebo-controlled nutritional intervention study, to investigate the potential of bovine lactoferrin, galactooligosaccharides (GOS) and vitamin D to restore TLR responsiveness of pDCs and mDCs and to reduce inflammatory cytokines in serum in elderly women. Nutritional supplementation with bLF, GOS and vitamin D is safe and enhances responses to TLR stimuli in elderly women in both pDCs and mDCs. No clear effects on pro-inflammatory markers in serum were observed, probably due to the group size in this study that was set up to study pDC responses as primary outcome. Especially TLR7-mediated responses in pDCs were enhanced after bLF supplementation compared to placebo, suggesting that bLF may contribute to protection against viral infections in elderly women.

In **chapter 8** I discuss the main outcomes of our research and their relevance by placing the results in a broader perspective. I will focus on four themes: *1). Induction of trained immunity by diet; 2). Modulation of respiratory immunity by raw bovine milk; 3). Immunomodulation in elderly by bovine milk components; 4). The potential role of trained immunity throughout life.*



Samenvatting

Zowel kinderen als ouderen hebben een minder goed functionerend immuunsysteem. Bij baby's zorgt borstvoeding voor ondersteuning van het immuunsysteem. Dit is nodig omdat het immuunsysteem nog verder ontwikkeld moet worden. Daarnaast hebben jonge kinderen te maken met kolonisatie door microbiota en is er een verschuiving van borstvoeding naar vast voedsel. Naast de kolonisatie door commensale bacteriën hebben kinderen ook meer te maken met voedsel- en luchtwegpathogenen. Deze kunnen respectievelijk zorgen voor darm- en luchtweginfecties. Vooral infecties met RSV en het influenza virus in de luchtwegen kunnen ernstig zijn, daarom zou het gunstig zijn om luchtwegimmuniteit te verbeteren met behulp van immunomodulatie door voeding. Verder is de darmbarrière nog niet helemaal dicht in jonge kinderen, zeker net na de geboorte. Hierdoor kunnen voedsel en microbiële componenten het darmepitheel passeren. Normaliter ontwikkelt een kind hier geen allergieën of anti-inflammatoire responsen tegen, omdat orale tolerantie geïnduceerd wordt tegen deze voedselcomponenten en commensale bacteriën.

Ouderen hebben vaak last van niet-overdraagbare ontstekingsziektes als gevolg van het verouderen van het immuunsysteem (immunosenescentie) en de toename van ontstekingmerkers door veroudering ('*inflammaging*'). Daarnaast reageren ouderen minder goed op vaccinaties en zijn ze vatbaarder voor infecties. Het aandeel van ouderen in de populatie groeit wereldwijd en daardoor nemen de lasten van ouderdomsziektes op het wereldwijde gezondheidssysteem toe. Vandaar dat het zeer gunstig zou zijn als mensen gezond ouder worden zonder ouderdomsziektes te ontwikkelen, iets wat '*healthy ageing*' genoemd wordt. Het hoofddoel van dit project was om het immunomodulatoire effect van rauwe koemelk en haar ingrediënten te bestuderen tijdens een voedingsinterventiestudie, een orale choleravaccinatie en op *trained immunity* en de potentiële relevantie hiervan gedurende verschillende levensstadia.

Consumptie van rauwe koemelk is in epidemiologische studies gecorreleerd met een verminderde kans op het ontwikkelen van astma, allergieën, rhinitis (bv. hooikoorts) en luchtweginfecties. Al deze aandoeningen zijn gerelateerd aan luchtwegimmuniteit. Daarom was het doel van deze thesis om te onderzoeken in welke mate rauwe koemelk immuunesponsen kan moduleren

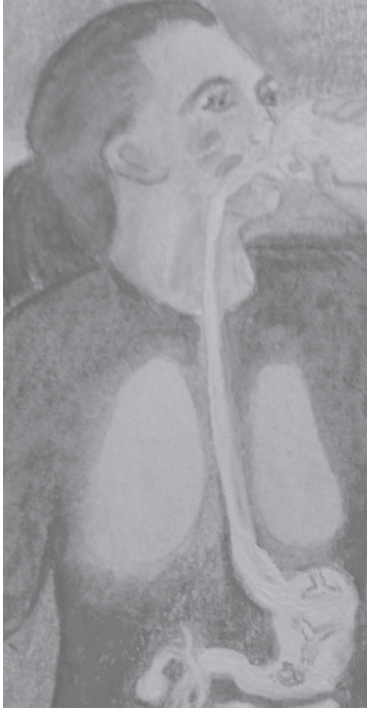
die luchtwegimmuniteit kunnen verbeteren. **Hoofdstuk 2, 3, 4 en 5** gaan over het moduleren van immuunresponsen door rauwe koemelk in kinderen en volwassenen.

In deze thesis beginnen we met het geven van een literatuuroverzicht van de potentiele mechanismen op welke manier melkcomponenten immuniteit moduleren in de bovenste luchtwegen en in de darmen (**hoofdstuk 2**). In **hoofdstuk 3** hebben we onderzocht of rauwe koemelk, melkfracties, lactoferrine of koeien-IgG *trained immunity* kunnen induceren. We hebben kunnen aantonen dat zowel rauwe koemelk als koeien-IgG *trained immunity* kunnen induceren in monocyten van mensen. Dit kan een mechanistisch concept zijn voor bescherming tegen infecties.

Verder hebben we de immuunresponsen gekarakteriseerd die door orale choleravaccinatie worden geïnduceerd (**hoofdstuk 4)**. Onder andere hebben we de expressie van merkers voor *homing* (migratie van bloedcellen) gekarakteriseerd die migratie van immuuncellen naar de luchtwegen mogelijk maken. Orale choleravaccinatie induceert een duidelijke vaccinatierespons in het serum. Daarnaast induceert orale cholera vaccinatie de expressie van *homing* merkers die overeenkomen met een migratieprofiel naar de dikke darm en de luchtwegen voor een specifieke populatie immuuncellen, de IgA^+ geheugen B-cellen. In IgA^- B-cellen daarentegen is expressie van *homing* merkers gevonden die duiden op migratie naar de dunne darm. In **hoofdstuk 5** hebben we het immunomodulerend effect van rauwe koemelk, gepasteuriseerde koemelk en ultra-verhitte koemelk onderzocht op orale choleravaccinatie. Dit hebben we gedaan in bloed en diverse mucosale weefsels, zoals speeksel en een neusspoeling in twee vaccinatie-studies. Orale cholera vaccinatie induceert een duidelijke vaccinatierespons in serum, neusspoeling en speeksel. In de twee vaccinatiestudies vonden we tegengestelde resultaten wat betreft het modulerend effect van melk. Daarom is het niet mogelijk om te concluderen of rauwe koemelk voor een toename of afname in vaccin-specifieke antistoffen zorgt na orale choleravaccinatie. Verder hebben we de *homing*-potentie naar de luchtwegen vergeleken tussen de controle en de rauwe koemelk groep. In de huidige studieopzet resulteert de inname van rauwe koemelk niet in een toegenomen luchtweg-*homing* capaciteit.

Het doel van **hoofdstuk 6** was om de functionaliteit van Toll-like receptor (TLR) responsen door het innate (aangeboren) immuunsysteem te vergelijken tussen jonge en oudere volwassen vrouwen. Oudere vrouwen hebben lagere plasmacytoïde dendritische cel (pDC) frequenties, vergelijkbare myeloïde dendritische cel (mDC) frequenties en een lagere basale productie van cytokinen door pDCs en mDCs. Verder hebben oudere vrouwen een verhoogde concentratie van ontstekingsgerelateerde merkers in serum vergeleken met jongere vrouwen. Vooral na stimulatie van TLR7/8 zijn het aantal pDC en mDCs die pro-inflammatoire cytokinen produceren verlaagd in oudere vrouwen vergeleken met jonge vrouwen. Dit bevestigd het concept van immunosenescentie en *inflammaging* in oudere vrouwen. In hoofdstuk 7 hebben we een dubbelblinde, placebogecontroleerde studie uitgevoerd om het effect van koeien-lactoferrine (bLF), galacto-oligosaccharides (GOS) en vitamine D te bestuderen op het herstellen van TLR responsen in pDCs en mDCS. Daarnaast onderzochten we of supplementatie van deze componenten ontstekingsmerkers in serum van oudere vrouwen verminderden. Voedingssupplementatie met bLF, GOS en vitamine D is veilig en verhoogd de responsiviteit van TLR stimuli in pDCs en mDCs in oudere vrouwen. We vonden geen duidelijke effecten op ontstekingsmerkers in serum, waarschijnlijk omdat de groepsgrootte te klein was om verschillen in serummerkers te meten. De groepsgrootte was berekend op basis van pDC-responsen, omdat dit de primaire uitkomst was. Vooral TLR7-gemedieerde responsen in pDCs waren toegenomen na bLF-supplementatie vergeleken met placebo supplementatie, wat suggereert dat bLF kan bijdragen aan bescherming tegen virale infecties in oudere vrouwen.

In **hoofdstuk 8** bediscussieer ik de belangrijkste uitkomsten van ons onderzoek en hun relevantie door de resultaten in breder perspectief te plaatsten. Ik focus op de volgende thema's: 1) Inductie van *trained immunity* door voeding; 2) Modulatie van luchtwegimmuniteit door rauwe koemelk; 3) Immunomodulatie door koemelkcomponenten in ouderen; 4) De potentiële rol van *trained immunity* gedurende het leven.



About the author

About the author

Marloes van Splunter was born on July 23rd 1989 in Spijkenisse, or "Spike City" as it is colloquially known. Here, she graduated in 2007 at the CSG Blaise Pascal. During her high school years she already demonstrated being an ambitious student, as she participated in the "Next Generation Science Exchange", together with students from five schools from the Rotterdam area and five schools from the city of London. Eager to return to London later that year, she also took part in the "Student Summit Climate Change" in the Natural History Museum in London.

In 2007, Marloes finished her secondary education and decided to come to Wageningen to enrol for a BSc study in Biology, with a major in "Human and Animal Health". Especially the field of Immunology had Marloes' special interest, and not only because of her own severe peanut allergy. Under supervision of then-PhD candidate Gerco den Hartog she performed a BSc thesis entitled "Immunomodulation by *Mytilus edulis*" in the group of prof. Huub Savelkoul.

Apart from her passion for science, Marloes really likes to organise things, and to contribute to student life. Therefore, she became a very active member of the study association B.V.W. 'Biologica', in which she joined various committees and became a board member, during which she served for half a year as president of the board. Furthermore she joined the education committee of the study Biology, where she discussed the Biology curriculum together with university staff and students. As there is more to student life than the study, and Marloes is also an eager sportswoman, she became active in the basketball association Sphynx, where she played in the competition team and again became active as a board member.

Despite Marloes' many extracurricular activities, she finished her BSc in approximately three years. After this, she continued with the MSc Biology, with a major in Cell Biology. As an MSc student Marloes performed two theses, first a minor thesis in the Virology chair group, entitled "*West Nile virus*

evasion of the host antiviral response" under supervision of dr. Gorben Pijlman, and later a major thesis entitled *"Identification of antibodies specific for IgT or IgD in common carp (Cyprinus carpio)"* in the chair group of Cell Biology and Immunology under supervision of dr. Maria Forlenza. Furthermore, Marloes spent seven months abroad for an internship at the Swiss Institute of Allergy and Asthma Research (SIAF) in Davos, Switzerland. Here, she worked under the supervision of Willem van de Veen and Mübeccel Akdis on the characterization of allergen-specific B cells during immune tolerance induction in a bee venom-specific immunotherapy model. In 2013, Marloes graduated as a Master of Science.

However, Marloes' hunger for immunological knowledge was not yet satisfied, as such she decided to return to the chair group of Cell Biology and Immunology, where she worked as a PhD candidate for the last five years to elucidate the role of raw milk and its ingredients on the humane immune system. The results of this endeavour can be read in this thesis. During her PhD she worked together with the dairy company Friesland Campina B.V. and the research institute NIZO food research. Apart from her immunological studies, Marloes decided again to be actively involved on an organizational level, as she co-organised the Wageningen PhD Symposium, became a member of the Education Committee of the graduate school Wageningen Institute of Animal Sciences (WIAS) and became the chairperson of the Wageningen PhD council. In the final year of her PhD trajectory she delivered two babies: The first is Waldemar, the most lovely, smart and handsome baby of the whole universe. The second delivery, which was at times more painful, is the thesis you are currently holding in your hands.

Written by Jeroen van Splunter-Berg

Overview of completed training activities

Conferences and symposia (12 ECTS)

Seminar, How to write a worldclass paper, Wageningen, The Netherlands	2013
Conference, NVVI Mucosal Immunology, Lunteren The Netherlands	2014
Conference, 3rd International conference on Food digestion, Wageningen, The Netherlands	2014
Conference, Annual conference Dutch society for Immunology (NVVI) (P)	2014
Conference, World Immune Regulation Meeting, Davos, Switzerland (P)	2016
Conferences, 10th European Mucosal Immunology Group Meeting, Copenhagen, Denmark (P)	2016
Conference, NVVI Networking in immunity, Lunteren, The Netherlands	2017
Symposium, WIAS Science Day, Wageningen, The Netherlands (O)	2014-2017
Symposium, Wageningen PhD Symposium, Wageningen, The Netherlands	2015, 2016
Symposium, Immunity in early life, FrieslandCampina, Wageningen, The Netherlands	2015
Symposium, Mucosal Immunology: cells living on the edge, Rotterdam, The Netherlands (O)	2015

Presentations at conferences/symposia are indicated with a 'P' for poster presentations and with a 'O' for oral presentations.

Disciplinary courses and interdiciplinary courses (9 ECTS)

Advanced immunology course, Utrecht, The Netherlands	2014
Epigenesis and epigenetics, Wageningen, The Netherlands	2015
Innate Immune memory workshop, Hinxton, United Kingdom	2015
Masterclass Mucosal Immunology: cells living on the edge, Rotterdam, The Netherlands	2015
Design of experiments, Wageningen, The Netherlands	2016
Basic statistics, Wageningen, The Netherlands	2017
Statistics for Life science, Wageningen, The Netherlands	2017

Professional skills support courses (8 ECTS)

Ethics and philosophy of science, Wageningen, The Netherlands	2014
Teaching and supervising thesis students, Wageningen, The Netherlands	2014
WIAS introduction course on essential skills, Wageningen, The Netherlands	2015
Techniques for Scientific writing and presenting, Wageningen, The Netherlands	2015
Project and time management, Wageningen, The Netherlands	2016
Brain training, Wageningen, The Netherlands	2016
Career assessment, Wageningen, The Netherlands	2016
The final touch: writing the general introduction and discussion, Wageningen, The Netherlands	2016

Research skills training (2 ECTS)

Lab training trained immunity at Radboud UMC, Nijmegen, The Netherlands	2014
FACS bootcamp, Utrecht, The Netherlands	2015
FACS advanced course, Utrecht, The Netherlands	2015

Didactic skills training (26 ECTS)

Lecturing	Cell Biology I	2013-2016
Supervising practicals	Cell Biology I	2013-2016
Supervising theses	Supervising 6 MSc thesis	2014-2017

Professional memberships and management skill training (7 ECTS)

Organisation of seminars and courses	Organising Wageningen PhD Symposium	2015
	Convener Wageningen PhD Symposium	2016
Memberships of boards and committees	WIAS Associated PhD Students	2015-2017
	Education Committee WIAS	2015-2017
	Wageningen PhD Council (Chairperson)	2015-2017

Education and training in total 63 ECTS

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). One ECTS equals a study load of 28 hours.

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List of publications

M van Splunter, E van Hoffen, E G Floris-Vollenbroek, H Timmerman, E Lucas-van de Bos, B Meijer, L H Ulfman, B Witteman, J M Wells, S Brugman, H F J Savelkoul, and R J J van Neerven, "Oral cholera vaccination promotes homing preference of IgA^+ memory B cells to the large intestine and the respiratory tract", published in *Mucosal Immunology;* doi: 10.1038/s41385-018-0006-7

O Perdijk, **M van Splunter**, H F J Savelkoul, S Brugman and R J J van Neerven, "Cow's milk and immune function in the respiratory tract: potential mechanisms", published in *Frontiers in Immunology;* doi: 10.3389/fimmu.2018.00143. eCollection 2018.

M van Splunter, T L J van Osch, S Brugman, H F J Savelkoul, L A B Joosten, M G Netea, R J J van Neerven, "Induction of trained immunity in human monocytes by bovine milk and milk-derived IgG", *manuscript submitted*

M van Splunter, O Perdijk, H Fick-Brinkhof, A Feitsma, E G Floris-Vollenbroek, B Meijer, S Brugman, H F J Savelkoul, E van Hoffen, R J J van Neerven, "Bovine lactoferrin enhances TLR7-mediated responses in plasmacytoid dendritic cells in elderly women", *manuscript submitted*

M van Splunter, O Perdijk, H Fick-Brinkhof, E G Floris-Vollenbroek, B Meijer, S Brugman, H F J Savelkoul, E van Hoffen, R J J van Neerven, "Plasmacytoid dendritic cell and myeloid dendritic cell function in ageing: a comparison between elderly and young adult women", *manuscript submitted*

M Akdis, O Palomares, W van de Veen, **M van Splunter**, C A Akdis**, "**T_H17 and T_H22 cells: A confusion of antimicrobial response with tissue inflammation versus protection", *Journal of Allergy and Clinical Immunology, 2012, p*: 1438-49; doi: 10.1016/j.jaci.2012.05.003

Acknowledgements

>200 blood donors
>25 L of blood
> 40x10^9 PBMCs isolated
>62.400 50ml tubes
Many thousands of pipet tips
Very many hours behind the FACS

Dear all,

These numbers give some insight what I have been doing these past five years during my PhD in the Cell Biology and Immunology group. Although it was challenging at some moments, I really enjoyed these years at the E-wing, aka "the best wing' of Zodiac. This PhD thesis would not have been possible without the support of many people in different ways, for which I am very grateful. Even though I might forget someone, I would like to specifically thank some people personally in the following paragraphs.

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