

Early life: gut microbiota and immune development in infancy

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Received: 15 July 2010 / Accepted: 26 September 2010

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Abstract

The immune system of infants is actively downregulated during pregnancy and therefore the first months of life represent a period of heightened susceptibility to infection. After birth, there is an age-dependent maturation of the immune system. Exposure to environmental microbial components is suggested to play an important role in the maturation process. The gastrointestinal tract is the major site of interaction between the host immune system and microorganisms, both commensal as well as potentially pathogenic. It is well established that the mammalian immune system is designed to help protect the host from invading microorganisms and other danger signals. However, recent research is emerging in the field of host-microbe interactions showing that commensal microorganisms (microbiota) are most likely one of the drivers of immune development and, in turn the immune system shapes the composition of the microbiota. Specific early microbial exposure of the gut is thought to dramatically reduce the incidence of inflammatory, autoimmune and atopic diseases further fuelling the scientific view that microbial colonisation plays an important role in regulating and fine-tuning the immune system throughout life. Therefore, the use of pre-, pro- and synbiotics may result in a beneficial microbiota composition that might have a pivotal role on the prevention of several important diseases that develop in early life such as necrotizing enterocolitis and atopic eczema.

Keywords: immune system, host-microbe balance/interaction, prebiotics, probiotics, synbiotics

1. Introduction

Although it is a common misconception that the immune system of newborns is immunologically naive, the response is biased against production of inflammatory cytokines to prevent destructive immunological reactions between mother and foetus. The bias against pro-inflammatory cytokines leaves the newborn susceptible to microbial infection and contributes to the impairment of the neonatal immune response. Exposure to microorganisms is suggested to play a pivotal role in the maturation of the immune system, and thereby improving the protection against infections and reducing the likelihood of allergy and/or atopy, in accordance with the hygiene hypothesis.

This review highlights the importance of the very early life factors for the development of the mucosal immune system of the gut. With the highest number of immune cells and

the highest concentrations of bacteria in the body, the gut represents the major site of immune education; therefore several strategies are suggested to target the gut microbiota in order to establish preventive effects on immune disorders such as allergy.

2. The immune system (gut)

The major function of the immune system is to defend the host from invading pathogens and other harmful insults. In addition, the immune system plays an important role in both the identification and destruction of tumour cells. It is therefore of key importance to discriminate 'non-self' from 'self' to avoid a destructive immune response against the host's own tissue. The mucosal immune system is the largest immune component in the body and is of key importance in providing protection from the external environment. The gut-associated lymphoid tissue (GALT)

constitutes a major part of the mucosal immune system (reviewed by Mason *et al.*, 2008). The GALT is organised in a complex manner, with a separate inductive site of immune responses, comprising of lymphoid follicles (Peyer's patches), and effector sites, constituted by the lamina propria and epithelial compartments. This lymphoid-associated epithelium includes M cells that samples small particles and effectively transports them from the gut into the organised lymphoid tissue (Neutra *et al.*, 2001). Although the transport of foreign material by M cells is crucial for the induction of protective mucosal immune responses, it also provides an entry route into the mucosa and therefore represents extremely vulnerable parts of the epithelial barrier (Neutra *et al.*, 1996). The vast majority of foreign antigens in the intestine are derived from food and the commensal microbiota with generally no immune response being generated, which is also referred to as 'oral tolerance' (Strober *et al.*, 1998). The exact mechanisms involved in oral tolerance are controversial, but are likely to involve epithelial-lymphoid interactions throughout the intestine (Mowat, 2003). Thus, the GALT can provide efficient protection against pathogens translocating from the gut, while undesirable immune responses to most incoming innocuous antigens are dampened (Neutra *et al.*, 2001).

Innate immune system

The mammalian host is armed with the innate and adaptive immune system. The innate immune system is evolutionary conserved among multi-cellular organisms and provides rapid defence against invading microbes within hours-long before the adaptive immune system mounts an antigen specific response a few days later (Calder, 2007; Janeway and Medzhitov, 2002).

Different layers, i.e. functional levels, of immune defence with increasing specificity can be discriminated. The first layer consists of mechanical, chemical and microbiological barriers. Mechanical barriers include mucus secreted by mucosal tissue in the respiratory and gastrointestinal tract to trap and entangle pathogens. The secretion of antimicrobial peptides by the skin, the gut epithelium and respiratory tract and the low pH and the presence of proteases in the stomach are examples of chemical barrier function.

The commensal bacteria in the gut represent a highly selective ecosystem that has coevolved with its host (Backhed *et al.*, 2005). Commensal bacteria provide an important barrier effect by, for example, the secretion of antimicrobial substances that inhibit the growth of undesirable bacteria or competition with invading organisms for binding sites and nutrients.

Microorganisms that successfully enter the body will be immediately recognised by the cells and mechanisms of the innate immune system, also referred to as the second

line of immune defence (Roitt *et al.*, 1996). This includes soluble factors that are constitutively present (including complement proteins, defensins) or that are released from cells when they are activated (including cytokines and chemokines). Natural killer (NK) cells are also part of the innate immune response, although recent data suggest that NK cells might represent an evolutionary bridge between innate and adaptive immunity (Sun and Lanier, 2009). NK cells control several types of tumours and microbial infections by limiting their development, which may lead to extensive tissue damage. NK cells can rapidly secrete effector cytokines such as IFN- γ and mediates cytotoxicity. The immune function of neutrophils are inferred from the immunologically active molecules they produce and from their accumulation in specific pathological conditions, including at sites of bacterial infection and tissue injury. Neutrophils play an important role in sequestering and clearance of microbial pathogens and repair of tissue injury (Kennedy and DeLeo, 2009). The innate immune system also includes other kinds of cells, including dendritic cells (DCs) as well as their membrane-bound receptors. These surface receptors, so called pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs), specifically recognise conserved molecular structures, called pathogen-associated molecular patterns (PAMPs) that are present in many types of microorganisms (Janeway and Medzhitov, 2002; Medzhitov, 2001). Since these molecular structures can also be found on non-pathogenic, commensal microorganisms, they are referred to more commonly as microbe-associated molecular patterns (MAMPs). The broad expression of recognition molecules by the innate immune system enables the system to act rapidly after an invading pathogen or toxin is encountered. The engagement of PRRs on macrophages, the major phagocytic cell type, causes cellular activation, the release of cytokines and can trigger phagocytosis with subsequent destruction of the pathogens. The signalling molecules released upon PRRs engagement will influence further induction of both innate and adaptive immunity to tailor an effector response to the specific invading pathogen or toxic agent (Table 1). Subtle differences exist between commensal bacteria, probiotic and pathogenic microorganisms with respect to the surface molecules that are present and the interactions that they mediate, determining the final host response (Lebeer *et al.*, 2010).

Adaptive immune system

The adaptive or acquired immune response involves lymphocytes. The response is highly specific to the particular pathogen that induced them. A key characteristic of the adaptive immune response is that it provides immunological memory by long-lived cells that persist for some time after the removal of the initiating antigen. The adaptive immune response is constituted by the functional properties of B and T lymphocytes and is directed by antigen-specific surface receptors. The effector cells of

Table 1. Development of the immune system. Newborns have a limited capacity to initiate immune responses and both innate and adaptive immune responses being compromised. The kinetics of the maturation of the immune system varies for the different components (dark grey = immature; light grey = developing; white = adult levels).

	Birth	6 months	1 year	2 years	3 years	4 years	5 years	5-15 years	Adult
Acidic mucins									
Microbiota composition									Changes again while aging
NK cells		> adult levels	> adult levels	> adult levels	> adult levels	> adult levels			
IgG									
IgM									
IgA									
Th1 mediated immunity									Decreases again while aging
T cell independent antibody response									

the B-cell system are terminally differentiated plasma cells, characterised by their ability to secrete immunoglobulins (antibodies). Antibodies exploit different mechanisms to combat invading pathogens. Microorganisms can be 'neutralised' by antibody-binding and thereby preventing their attachment to host cells and complement proteins can be activated, thereby promoting destruction of bacteria by phagocytes. Circulating antibodies comprise of five classes; IgG, IgA, IgM, IgD and IgE. IgM and IgG dominate systemic immunity, while IgA is the dominating antibody class of mucosal immunity. IgE is an important immunoglobulin in the defence against helminths, but can play a major role in allergic reactions also by binding to their specific Fc receptor (Fc ϵ RI) on mast cells and basophils, causing degranulation and release of inflammatory mediators. Intestinal bacteria can trigger T-cell independent IgA class switching in B cells by inducing epithelial cell-secretion of cytokines, like APRIL (a proliferation inducing ligand) (He *et al.*, 2007a; Xu *et al.*, 2007). In addition, intestinal epithelial-cell derived TSLP and IL-10, produced in response to bacterial signals, may orchestrate local B cell responses (He *et al.*, 2007b; Xu *et al.*, 2007).

In contrast to B lymphocytes, T lymphocytes are only able to recognise antigens that have been taken up by antigen-presenting cells (APCs) and displayed on their cell surface in conjunction with major histocompatibility complex (MHC) proteins. T cells can differentiate into discrete subpopulations, each with defined repertoires of effector functions. Helper T cells (Th) are CD4+ and activate both humoral and cellular immune responses, whereas CD8+ T

cells, also called cytotoxic T cells (Tc) show major cytotoxic activity against cells infected with intracellular microbes as well as against certain tumour cells. Within T cells, specific subpopulations exist that downregulate immune responses and are called regulatory T cells (Treg). Naive T cells (Th0) can differentiate into functionally distinct subsets after exposure to an antigen and these cells can differentiate towards either Th1, Th2, or Th17 depending on the nature of the cytokines present at the site of activation; exogenous stimuli and the maturational stage of APCs (Sallusto and Lanzavecchia, 2009; Soroosh and Doherty, 2009). Generally, Th1 cells support cell-mediated immune responses and Th2 cells support humoral and allergic responses. Th1 cells produce IFN- γ that promotes clearance of viruses and intracellular bacteria by activating macrophages and the induction of killer mechanisms, including Tc. A Th2 profile includes mainly IL-4, IL-5 and IL-13 that promotes clearance of extracellular parasites (Romagnani, 2007). Recent studies have indicated that the differentiation of Th cells along two phenotypic pathways, described as the Th1/Th2 paradigm, represents an over-simplification and illustrate the existence of multiple pathways of effector T cell differentiation (Sallusto and Lanzavecchia, 2009). Recently, Th17 cells are described as a distinct lineage that is important in the response to extracellular bacteria but can also direct destructive inflammatory responses that are part of many autoimmune diseases (Sallusto and Lanzavecchia, 2009). There is increasing evidence that there might be lineages, including Th22 and Th9, although it remains to be established whether these represent a true distinct lineage (Sallusto and Lanzavecchia, 2009). The same

authors concluded that the differentiation programs have some part of flexibility and T cells may express opposing cytokines at different times, in different tissues, or enforced by polarising signals that provide tailored mechanisms of protection and immune surveillance against different pathogens (Sallusto and Lanzavecchia, 2009)

3. Development of the immune system

Although newborns have a competent immune system which is able to handle infections and responses to immunisation, it invariably differs to the immune mechanisms present in adults. These differences are due to two main interrelated factors. The first is the development of the immune system; certain aspects of the adaptive and innate immune system are not fully functional at birth and develop thereafter. The second is a consequence of the low exposure to antigen up to birth. Additionally, the immune system of newborns is biased against the production of pro-inflammatory cytokines to prevent adverse immunological reactions between mother and foetus during pregnancy (Levy, 2007).

In newborns, different components of the first line of defence are impaired (Figure 1). The epithelial layer shows a higher permeability in both the respiratory and gastrointestinal tracts, indicating that the integrity is not complete. The secretion of components involved in the chemical barrier, including the secretion of proteases and antimicrobial peptides, are not fully developed (Levy, 2007) while the pH in the stomach is relatively high. The composition and glycosylation of the mucus layer, the anatomical site at which the host first encounters gut bacteria, also differs significantly between neonates and adults (Deplancke and Gaskins, 2001).

Granulocytes, including neutrophils and eosinophils, an important subset of immune cells that play a crucial role in the second line innate immune responses, are reduced in number and show a reduced expression of L-selectin, impaired chemotaxis and transmigration (Smith *et al.*, 1992). The activity of antigen-presenting cells in infants is lower than that in adults, displaying decreased production of cytokines (Goriely *et al.*, 2001; Langrish *et al.*, 2002; Stefanovic *et al.*, 1998), reduced chemotaxis, reduced expression of MHC class II and costimulatory molecules as well as reduced capacity in phagocytosis and endocytosis (Wong *et al.*, 2005). Furthermore, NK cell activity is low at birth, although NK cells are predominant during early infancy to early childhood. The high level of NK cell cytotoxicity may make-up for part of the immaturity of the adaptive immune system (Yabuhara *et al.*, 1990).

Neonatal T cells are able to respond to environmental antigens. However, their cytokine production is poor in comparison to the adult, particularly in relation to Th1 cytokines (Bryson *et al.*, 1980; Tang and Kemp, 1995).

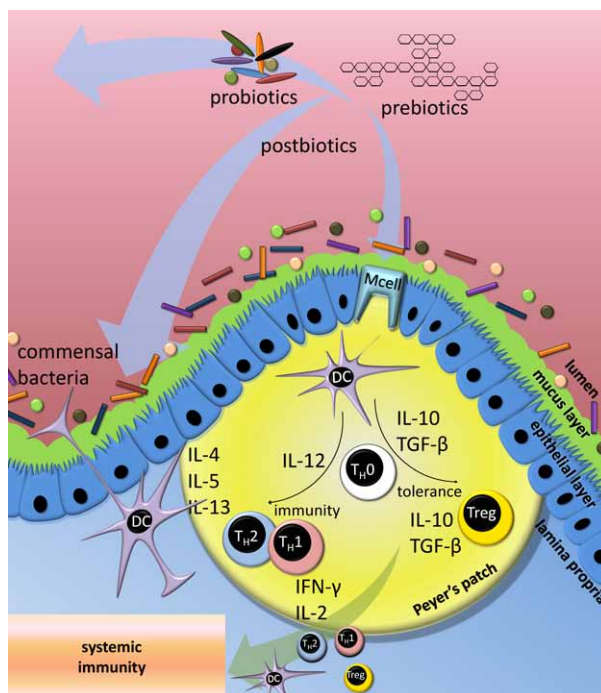


Figure 1. The close interaction between the gut microenvironment and the immune system. Micro-environmental factors derived from gut microbiota or food are playing a key role in the maturation and fine-tuning of the immune system. Dendritic cells play a central role in tailoring the type of immune response depending on the signals provided by the microenvironment. The composition of the microbiota itself can be modulated with probiotics, prebiotics, synbiotics or postbiotics and is a common approach in nutritional intervention studies (Adapted from Schouten, 2009).

Available studies indicated that IFN- γ production is achieving adult levels around the age of five years (Miyawaki *et al.*, 1985; Rowe *et al.*, 2000). In general, antigen-specific immune responses can be generated in infants. The reaction that is generated after vaccination is mostly characterised by a Th2-type response. Although Th1 responses can be generated by some potent vaccines, such as antituberculosis bacillus Calmette-Guérin (Marchant *et al.*, 1999), their response to milder stimuli (such as diphtheria/acellular pertussis/tetanus vaccine) are strongly Th2 polarised (Rowe *et al.*, 2000). Neonatal T cells contain high concentration of T-cell receptor excision circles, indicating high thymic output (Marchant and Goldman, 2005) and high numbers of Treg are present in newborns. In addition, Tc functions are limited, resulting in less proliferation and 'immature' cytokine profiles. The relatively poor capacity of neonatal T cells to produce cytokines is suggested to contribute to impaired responses of other neonatal cell populations that rely on these factors for their function, including NK cell activity as described above (Yabuhara *et al.*, 1990).

The function of B lymphocytes shows great differences between adults and young infants also. Although the number of B cells in the neonate is very high, the maturation of plasma B cells is not yet completed at birth, leading to an impaired antibody isotype switching. As a consequence, the Ig levels in the circulation of the newborn infant are low, apart from IgG from maternal origin. IgA is almost undetectable and IgM levels are also low but increase rapidly with an antigenic challenge. Neonatal B cells are mature in their capacity to produce IgE in presence of exogenous IL-4. Due to the low level of IL-4 produced by neonatal T cells the level of IgE production is limited (Pastorelli *et al.*, 1990). The available studies suggest that adult-equivalent levels of immunoglobulins are achieved by approximately the age of 10 years (Aksu *et al.*, 2006). T-cell independent antibody responses are mediated by B cells in response to cross-linking of surface immunoglobulins; by multivalent antigen along with specific cytokines (BAFF (B cell activation factor) and APRIL) secreted by antigen-presenting cells in response to stimulation with microbial components. T-cell independent antibody responses are generally absent at birth and fully mature T-cell independent B cells responses occur by 3-5 years of age (Wilson, 2008). On the other hand, T cell-dependent B cell responses can be detected shortly after birth to most protein antigens. The cells of the gut immune system develop in proximity to large communities of microorganisms in the intestinal lumen. Available evidence indicated that intestinal bacteria play a crucial role in establishing and regulating the intestinal surface barrier (Hooper *et al.*, 2001; Neish *et al.*, 2000). Intestinal bacteria provide instructive signals for the development of key lymphocyte subsets; they direct class switching in human intestinal B cells (He *et al.*, 2007b); govern the development of intestinal Th17 effector T cells (Ivanov *et al.*, 2008); and suppress the production of Treg cells (Hall *et al.*, 2008). Additionally, intestinal bacteria impact the outcome of systemic immune responses by determining the ratio of Th1 and Th2 effector cells (Mazmanian *et al.*, 2005). In the gut, specific homeostatic mechanisms protect resident immune cells against hyperactivation and accompanying inflammation. Resident APCs express only negligible levels of TLRs (Smith *et al.*, 2001), rendering them quite inert to MAMPs from commensal gut bacteria that normally penetrate into the gut mucosa in small numbers (Macpherson *et al.*, 2005).

The important role for microbes in the maturation of the immune system is also being postulated by the so-called 'hygiene hypothesis' (Strachan, 1989). Different epidemiological studies support the hygiene hypothesis and have clearly shown that modifications in the pattern of microbial exposure represent a critical factor underlying the rise in the prevalence of atopic disorders. Moreover, it also suggest that this increase in allergic diseases reflects a decrease in infections during childhood (Singh and Ranjan Das, 2010). In essence, the idea is that modern measures

have deprived infants of adequate immunological stimuli. This reduced immunostimulation resulted in too little Th1 cell activity and therefore, an insufficient level of IFN- γ to cross-regulated IgE-inducing Th2 cell responses, indicating a lack in shifting from Th2 to Th1 activity (missing immune deviation). The composition of the gut microbiota and exposure to food-borne and orofecal pathogens probably have important homeostatic influences, both by enhancing the SIgA-mediated intestinal surface barrier and by promoting oral tolerance through a shift from predominant Th2 cell activity in the newborn period to a more balanced cytokine profile later on (Brandtzaeg, 2010). The role of the commensal bacteria regulating the epithelial barrier integrity and shape the epithelium-associated immune response is discussed later on in this review.

More recently an alternative mechanism has been proposed stating that there is a reduced activity of regulatory T cells (reduced immune deviation) because of the reduced microbial burden (Romagnani, 2007; Yazdanbakhsh *et al.*, 2002). This is called the 'extended hygiene hypothesis' and emphasises the induction of Treg cells as an important part of such microbe-driven immunological homeostasis. Naturally occurring Treg cells with suppressive properties are present in large numbers in foetal MLNs and these are probably part of a systemic tolerance to keep autoreactive effector T cells in check to avoid inflammation and tissue damage.

The intestinal epithelium tightness and the immunoregulatory network remain fragile for a variable period after birth. Animal experiments show that the postnatal development of immunological homeostasis depends on the establishment of a balanced indigenous gut microbiota as well as adequate timing and dosing of the introduction of foreign dietary antigens. The first postnatal year of life seems to be a key period for programming the immune system and the feeding (i.e. breast milk) and other factors to which newborn is subjected (i.e. antibiotics) may have an influence of indigenous gut microbiota and on intestinal epithelial integrity.

Although much fundamental research is being carried out in mice (discussed later in this reviewed), novel ideas are increasingly being supported by experimental human studies and clinical trials. Moreover, different epidemiological studies support the hygiene hypothesis and have clearly shown that modifications in the pattern of microbial exposure represent a critical factor underlying the rise in the immune disorders.

4. The role of gut microbiota in shaping the immune system early in life

Adult gut microbiota

The human gut harbours approximately 10^{14} bacterial cells compared with 10^{13} human cells for the entire body, and constitute an exceptionally diverse and dynamic microbial ecosystem. Although the perception of microbial diversity in the gastrointestinal tract (GIT) has gradually changed over the years, currently the following cultivated groups are considered as predominant in the human GIT: *Bacteroides*, *Clostridium*, *Eubacterium*, *Veillonella*, *Ruminococcus*, *Bifidobacterium*, *Fusobacterium*, *Lactobacillus*, *Peptostreptococcus* and *Peptococcus* (Moore and Holdeman, 1974; Xu and Gordon, 2003). 16S ribosomal RNA gene (rRNA) sequence-based method revealed that two bacterial divisions, the Bacteroidetes and the Firmicutes, are the most common intestinal phyla, accounting for more than 90% of the total community (Eckburg *et al.*, 2005; Ley *et al.*, 2008; Zoetendal *et al.*, 1998). The composition of the gut microbiota is complex, but crucial for human health (Rajilic-Stojanovic *et al.*, 2007). The gut microbiota contribute to human health in many ways such as, host physiology, by enhancing digestive efficiency; aiding the host in food digestion; impacting the bioavailability of nutrients and absorption processes; promoting proper immune development; and limiting pathogen colonisation (Hooper and Macpherson, 2010).

Development of the gut microbiota

It is well known that both the establishment of an optimal microbial community immediately after birth and the maintenance of a balanced intestinal microbiota represent important factors for the development of the immune system (Hooper and Macpherson, 2010; Round and Mazmanian, 2009). The colonisation of the human gut by micro-organisms starts around birth, during birth and immediately afterwards. Bacteria from the mother and the surrounding environment colonise the infants gut. However, it has been suggested recently that the colonisation process or exposure to microbial compounds may start before birth and that infants may also receive microorganisms from the mother during gestation (Hong *et al.*, 2010; Jimenez *et al.*, 2008; Satokari *et al.*, 2009).

Initial colonisation follows successive steps, firstly dominated by facultative anaerobes such as enterobacteria, coliforms and lactobacilli, followed by anaerobes genera such as *Bifidobacterium*, *Bacteroides*, *Clostridium* and *Eubacterium*. This process is influenced by several factors such as genetic background, gestational age, mode of delivery (caesarean section vs. vaginal delivery), type of feeding (breastfeeding vs. formula-feeding) and antibiotic therapy (Adlerberth *et al.*, 2007; Biasucci *et al.*, 2008;

Grönlund *et al.*, 1999; Huurre *et al.*, 2008; Penders *et al.*, 2006b). Additionally, it has been suggested that also stress and diet during late pregnancy play a role in the initial colonisation of the newborn (Heikkila and Saris, 2003; Kirjavainen *et al.*, 2007). The development of the gut microbiota occurs during the first few years of life, a window of time that corresponds to a critical period of immune development and maturation.

The effect of gut microbiota on the maturation of the immune system

Since the gut microbiota is a key source of microbial driven immune regulation, alterations of the normal bacterial colonisation patterns may change the outcome of the immune development and predispose to certain immune-related disorders later in life, such as allergy, obesity or diabetes. Indeed, differences in gut microbiota composition and activity between healthy and atopic children have been shown in several cross-sectional epidemiologic studies (Bjorksten, 2009a; Garrett *et al.*, 2010; Round and Mazmanian, 2009; Vael and Desager, 2009). Allergic infants have been found to be less frequently colonised by bifidobacteria and by lower quantities than healthy non allergic infants. Moreover, they have more adult-type *Bifidobacterium* species, such as *B. adolescentis*, already in early infancy (He *et al.*, 2001; Ouwehand *et al.*, 2001). Whether the observed changes in the microbiota are the consequences of allergy or perhaps initiate the process leading to allergy remains a key question. However, Kalliomaki *et al.* showed for the first time that differences in gut microbiota precede the development of atopy and characterised the difference as a reduced ratio of bifidobacteria to clostridia at neonatal age (Kalliomaki *et al.*, 2001b; Penders *et al.*, 2006a).

Moreover, Ly *et al.* (2006) have shown that c-section (CS) delivered infants besides having a different microbiota composition, are characterised by enhanced production of the cytokine IL-13 after stimulating cord blood cells. This is suggestive for a higher incidence and severity of both asthma and atopy in these infants. Furthermore, it has been shown that maternal intake of probiotics might influence the infant gut microbiota and reduce the risk of eczema (Kalliomaki *et al.*, 2001c; Kukkonen *et al.*, 2007)

Lessons from animal experiments

Despite these observations, which suggest an association between the different factors influencing the infant gut colonisation and the subsequent development of the immune system, it is very difficult to unravel the mechanisms by which early bacterial exposure influences the immune development in humans.

Most of our knowledge regarding the effects of the microbiota on the host immune system derives from studies using germ-free animals. Germ-free mice do not harbour any microorganisms in their intestines or other body surfaces and as such are a powerful tool to (1) investigate how the intestinal microbiota can shape the developing immune system and (2) to directly assess the impact of colonisation *in vivo*. Analysis of germ-free animals has provided clear evidence that the absence of microbial stimulation has profound effects on development of the mucosal and innate immune systems (Cash and Hooper, 2005).

Comparisons of germ-free and conventional colonised mice have revealed that Paneth cells participate in at least two bacterial-induced developmental transitions: the postnatal expansion of the gut epithelial antimicrobial arsenal and the development of a robust villus capillary network (Hooper, 2004). In contrast to conventional mice, the villus capillaries of germ-free mice develop poorly during weaning and remain underdeveloped into adulthood, suggesting that intestinal bacteria are essential for full intestinal blood vessel development (Stappenbeck *et al.*, 2002).

Germ-free animals likewise are providing evidence that commensal bacterial serve as a driving force in the development of the gut adaptive immune system. Comparisons of germ-free and colonised mice have revealed that microbes play an essential role in promoting B cell development in Peyer's Patches and drive production of mucosal IgA (Shroff *et al.*, 1995). Germ-free mice generate reduced amounts of IgA antibodies compared to conventional mice and have decreased numbers of circulating B and T lymphocytes (Cebra, 1999). It has been suggested that a diverse repertoire of bacterial species and antigens is required to fully promote the development of the gut immune system (Cebra, 1999; Rhee *et al.*, 2004). The gut microbiota contribute to the development of intraepithelial lymphocytes (IELs), as evidenced by the fact that numbers of IELs that bear T cell receptors (TCR) consisting of an α and β chain ($\alpha\beta$ IELs) and are 10-fold reduced in germ-free mice compared to conventional mice (Mowat, 2003; Umesaki *et al.*, 1993, 1999), as the absolute and relative numbers of NK cells (Sanos *et al.*, 2009). In addition, whereas IELs that have TCR consisting in a γ and δ chain ($\gamma\delta$ IELs) isolated from conventional mice are constitutively cytolytic, compared to those isolated from the small intestine of a germ-free mouse which lacks cytolytic activity (Lefrancois and Goodman, 1989).

5. Dietary modulation of microbiota – immune system

The diet may affect the composition and activity of the gut microbiota. The microbiota of breastfed infants differ from formula-fed infants with respect to number, species composition and metabolic activity of bifidobacteria that

may be explained by the presence of bifidogenic factors in breast milk (Kunz *et al.*, 2009). Therefore, in nutritional intervention studies, promotion of the development of bifidobacteria is been considered as a relevant approach. The most common approaches to modulate the composition of the infant gut microbiota is via supplementation with prebiotics or probiotics, with breastfed infant microbiota as a gold standard.

Human milk

Human milk is the first dietary exposure in infancy. It is considered the best nutritional option for growth and healthy development of the newborn, since it contains a wide range of health protective compounds including carbohydrates, nucleotides, fatty acids, immunoglobulins, cytokines, immune cells, lysozyme, lactoferrin and other immune-modulatory factors (Boehm and Moro, 2008; Goldman and Smith, 1973; Penttila, 2010; Puccio *et al.*, 2007; Walker, 2010). Recently the presence of a new immune-modulatory factor in human milk, the microRNAs (miRNAs) identified as a potential biomarker for several diseases has been suggested (Kosaka *et al.*, 2010). Human milk oligosaccharides (HMOs) have been shown to stimulate specific intestinal microbiota, to block pathogen adhesion sites in the gut and/or acts as soluble pathogen receptors analogs (Andersson *et al.*, 1986; Coppa *et al.*, 2004; Cravioto *et al.*, 1991; Newburg *et al.*, 1990).

Moreover, in the last years, breast milk has been shown to be a continuous source of bacteria to the infant gut as well; including staphylococci, streptococci, bifidobacteria and lactic acid bacteria (Collado *et al.*, 2009; Gueimonde *et al.*, 2007; Heikkila and Saris, 2003; Martin *et al.*, 2003, 2009; Perez *et al.*, 2007). It is estimated that an infant consuming approximately 800 ml per day will ingest about 1×10^5 - 1×10^7 commensal bacteria while suckling (Heikkila and Saris, 2003; Martin *et al.*, 2003). However, the origin of the bacteria present in breast milk is highly controversial. It is generally accepted that the infant acquires the mother's faecal microbiota during the delivery and transfer these bacteria to the breast skin and from there to the milk ducts while breastfeeding. Although skin contamination is almost unavoidable, several studies have shown that the bacteria present in the breast skin differs from that of breast milk (Martin *et al.*, 2003, 2009). It has been suggested that at least some of the bacteria present in the maternal gut can reach the mammary gland through an endogenous route (the so-called entero-mammary pathway) involving maternal dendritic cells and macrophages (Martin *et al.*, 2004). This hypothesis has been confirmed by Perez *et al.* (2007). The same authors showed that fresh human milk contains a number of viable bacteria ($<3 \log \text{cfu/ml}$) and a wide range of free bacterial DNA signatures, including bifidobacterial DNA, which may program the neonatal immune system. The translocation of bacteria from the gut to the mammary

gland via mesenteric lymph nodes has been shown in animal models (Fernandez *et al.*, 2004; Perez *et al.*, 2007).

Due to the beneficial effects of human milk in the maturation of the immune system of the newborn, several attempts have been made to develop an infant formula that stimulate a similar gut colonisation to that of breast-fed infants. The main strategies to modulate the gut microbiota of infants have been the administration of prebiotics, probiotics, synbiotics and postbiotics.

Prebiotics

Prebiotics are non-digestible oligosaccharides that reach the colon intact and are known for their ability to selectively stimulate the growth and activity of bacteria that exert positive health effects (Gibson and Roberfroid, 1995). Non-digestible HMOs are one of the major constituents in human milk and regarded as important immunomodulatory components. HMOs are structurally very complex and have a huge diversity (Bode, 2006; Stahl *et al.*, 2001). It has been hypothesised that the large structural variety is to combat the large variety of pathogens that the infant may encounter. HMOs have been shown to act as decoy pathogen receptors, binding to pathogen structures that are essential for adhesion and infection. Furthermore, HMOs are suggested to modulate the intestinal glycosylation pattern, thereby effecting pathogen adhesion (Angeloni *et al.*, 2005).

The beneficial effects on the immune system are commonly ascribed to the stimulation of the growth and metabolism of protective commensal intestinal bacteria (Boehm *et al.*, 2005; Langlands *et al.*, 2004). An increase in the number of beneficial bacteria will provide antimicrobial effects by direct competition with pathogenic bacteria for available binding sites on intestinal epithelium and for nutrients. *Bifidobacterium* species and *Lactobacillus* species are also able to produce antibacterial substances that can inhibit the growth and survival of pathogens (Gibson and Wang, 1994). Different studies have reported that oligosaccharide mixtures were able to modulate the microbiota of bottle-fed infants, making the composition of the microbiota more similar to the bifidobacteria-dominated microbiota in breastfed-infants (Knol *et al.*, 2005; Moro *et al.*, 2002). Although the effects on bifidobacteria and lactobacilli were shown in animal studies as well, the relationship between modulation of the microbiota and immune system was not consistent in all experiments (Vos *et al.*, 2006, 2007). In some experiments, oral supplementation with oligosaccharides induced measurable increases in bifidobacteria and lactobacilli without effecting Th1-related delayed type hypersensitivity (DTH) responses, and vice versa. It has been hypothesised that oligosaccharides may also directly interact with cells of the immune system in a microbiota-independent mechanism (Vos *et al.*,

2010). Available evidence suggests that HMOs may have systemic effects in infants as they have been found in urine (Chaturvedi *et al.*, 2001; Obermeier *et al.*, 1999). In line with these observations, the immunomodulatory effect is not restricted to the gastrointestinal tract. Beneficial effects have been observed on parameters of allergy, infection and inflammation in both animal studies and clinical trials with oligosaccharides (Arslanoglu *et al.*, 2007, 2008; Moro *et al.*, 2006; Van Hoffen, 2009; Vos *et al.*, 2006, 2007). Supplementation with a specific oligosaccharide mixture, scGOS/lcFOS (Immunofortis) in infants at a high risk of allergy reduced the cumulative incidence of atopic dermatitis (Moro *et al.*, 2006). Interestingly, infants that received scGOS/lcFOS displayed reduced incidence of infections as well (Arslanoglu *et al.*, 2007). This protective effect against atopic dermatitis and infections was still evident at the age of 2 years (Arslanoglu *et al.*, 2008). Direct effects of HMOs on immune cells have been described, possibly via the interaction with specific sugar receptors on immune cells (Bode *et al.*, 2004; Eiwegger *et al.*, 2010; Naarding *et al.*, 2005). HMOs have been shown to interfere with leukocyte recruitment to sites of inflammation and to inhibit cell-cell interactions of leukocytes and lymphocytes via selectins (Eiwegger *et al.*, 2010). Specific human milk oligosaccharides inhibit leukocyte rolling and adhesion to endothelial cells (Bode *et al.*, 2004). Oligosaccharides have been shown to interfere with Th1/Th2 skewing in cord blood-derived mononuclear cells and to impact the Th2-type immune response of allergen-specific T cells from peanut allergic individuals (Eiwegger *et al.*, 2004, 2010). Furthermore, *in vitro* evidence was obtained for epithelial transport of human milk-derived oligosaccharides (Eiwegger *et al.*, 2010). Information on whether transfer of oligosaccharides into the bloodstream occurs is limited or absent. Interestingly, Naardig *et al.* have demonstrated that HMOs are able to bind specifically to the lectin receptor DC-SIGN expressed by dendritic cells. DC-SIGN has been described to interact with a variety of pathogens, including HIV-1, and binding of HMOs inhibited the transfer of HIV-1 to CD4+ T lymphocytes (Naarding *et al.*, 2005). These data suggest that HMOs act systemically and modulate immune responses in a microbiota-independent manner.

Probiotics

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (WHO/FAO, 2001). The use of probiotics in the paediatric setting has tripled in the past five years, with bifidobacteria and lactobacilli as the most common bacterial groups used (Wallace, 2009). There is a considerable and evolving body of evidence for possible short term benefits of some probiotic strains in the treatment of infants and young children with infectious diarrhoea, as well as in prevention of antibiotic-associated diarrhoea (Salminen and Isolauri, 2008). The other postulated effects of probiotics, such as

prevention of common childhood infections, reduction in food allergy, atopic dermatitis, and primary prevention of atopy, even if promising, remain to be convincingly established (Kullen and Bettler, 2005). Multiple mechanisms of action have been suggested but for most probiotics, little is known about their precise mode of action and will mostly depend on the strain used. A body of evidence is accumulating that commensal microbiota is regulating the epithelial barrier integrity and shape the epithelium-associated immune response (Dotan and Rachmilewitz, 2005). Recent *in vitro* and *in vivo* studies have shown that *Lactobacillus* and *Bifidobacterium* exert direct effects on intestinal epithelial barrier function that are evidenced by decreased intestinal permeability and enhanced intestinal epithelial resistance (Garcia-Lafuente *et al.*, 2001; Madsen *et al.*, 2001; Parassol *et al.*, 2005). In rat models of chronic stress, hemorrhagic shock and sepsis, effects of probiotics on barrier function have been demonstrated although the mechanisms have not been elucidated (Qin *et al.*, 2005; Zareie *et al.*, 2006). In a murine model of dextran sodium sulfate (DSS-) induced colitis, the probiotic *Escherichia coli* Nissle 1917 was shown to protect against colitis (Ukena *et al.*, 2007). Evidence is also derived from *in vitro* studies that have demonstrated a protective effect of probiotic bacteria on epithelial barrier integrity (Ko *et al.*, 2007). Recently, *in vitro* studies with *Lactobacillus plantarum* suggested a role for TLR2 as a regulator of epithelial integrity (Karczewski *et al.*, 2010). In addition, it is suggested that the interaction between probiotic strains and the intestinal epithelium is a key determinant for the cytokine production by enterocytes and probably in the initiation of the immune modulation. Therefore, in view of the importance of the Th1/Th2 paradigm to our understanding of the development of the immune system, the consumption of probiotic strain could produce an immunomodulatory effect through upsetting the Th1/Th2 equilibrium, and prevent or treat allergies or infectious diseases, among others. This assumption has led to high numbers of clinical studies investigating the effect of probiotic supplementation on the risk of allergy and infections (Kalliomaki *et al.*, 2010; Wolvers *et al.*, 2010). So far, probiotics have shown more promise, albeit limited, in the primary prevention of allergic disease rather than in the treatment of established disease (Johannsen and Prescott, 2009). Several studies have shown that various prenatal and postnatal probiotics may reduce the risk of eczema, while others have shown no benefit, probably explained by the significant heterogeneity between studies; including wide variations in the strains used, the methods and timing of administration and the age and assessment of allergic outcome (reviewed by Kalliomaki *et al.*, 2010; Tang *et al.*, 2010). The current state of the art most probably reflects the inherent complexity of the allergic syndrome, the difficulty in taking confounding factors into account (Prescott and Bjorksten, 2007), the varying characteristics and potentials of different probiotics strains, and the still-insufficient understanding of how specific probiotics may

counteract different types of immune dysfunction found in allergic diseases *in vivo*.

Similarly, no general conclusion can be drawn regarding the role of probiotics on preventing or treating infectious diseases. Different studies have been conducted in order to determine its efficacy in managing and preventing infections: (1) infectious diarrhoea in infants and children, (2) traveller's diarrhoea, (3) necrotizing enterocolitis in infants, (4) *Helicobacter pylori* infection, (5) respiratory tract infections in adults and children, (6) ear, nose, and throat infections, and (7) infectious complications in surgical and critically ill patients. However, no general conclusions can be drawn due to the different types of infections that have been subject to clinical studies with different probiotics, and the lack of consistency among studies focusing on one specific infection, in study design, applied probiotic strains, outcome parameters, and study population, along with the still limited number of studies. Sufficient consistent data exist for the management of infectious diarrhoea in infants and traveller's diarrhoea, antibiotic-associated diarrhoea, and necrotizing enterocolitis for which it could be concluded that certain probiotics, under certain conditions, and in certain target populations, are beneficial in reducing the risk of infection (reviewed by Wolvers *et al.*, 2010). Certain probiotics may also reduce the risk of various symptoms of respiratory tract infections in adults and children, including ear, nose, and throat infections, although data are currently far too limited to distil any clinical recommendations in this area (Hojsak *et al.*, 2010; Rautava *et al.*, 2009). For future studies it is recommended that researchers provide adequate power, identify pathogens, and report both clinical outcomes and immune biomarkers relating to putative underlying mechanisms.

Therefore, better alignment of clinical designs would allow us to reduce controversy in the area of probiotics for infant nutrition and promote rapid progress in this promising field. Moreover, it could lead to the recommendation of specific probiotic strains properly selected for allergic manifestations or infectious diseases in well-designed target populations as an efficient tool in the fight against allergic or infectious diseases

Synbiotics

The mixture of pro- and prebiotics, the so-called synbiotics, has been suggested to have a synergistic effect by ensuring the viability of the delivered probiotic bacteria and stimulating the growth or metabolism of health-promoting bacteria, and thus improving the host welfare (Collins and Gibson, 1999; Gibson and Roberfroid, 1995). So far the studies evaluating the role of synbiotics in modulating the immune system are limited, although several authors have shown the beneficial role of synbiotics on the prevention and/or treatment of allergy and infections. Prenatal and

perinatal supplementation of GOS in combination with probiotics have shown to reduce the incidence of eczema at 2 years, although failed to show long term effects at 5 years of life (Kuitunen *et al.*, 2009; Kukkonen *et al.*, 2007). Moreover, it has been suggested that IgE-associated infants might benefit from a synbiotic mixture containing scGOS/lcFOs and *Bifidobacterium breve* M-16V (Van der Aa *et al.*, 2010b). Furthermore, the supplementation with this specific mixture showed a potential preventive effect on asthma-like symptoms and possibly on subsequent development of asthma (Van der Aa *et al.*, 2010a). Recently several synbiotics mixtures have been shown to have a beneficial role in preventing infectious diseases. Picaud and colleagues have suggested a beneficial role of a specific synbiotic mixture on the incidence of infectious diseases and growth of infants (Picaud *et al.*, 2010). Supplementation with synbiotics also resulted in a significant reduction of dysentery, respiratory morbidity and febrile illness in children 1-3 years of age (Sazawal *et al.*, 2010b). Moreover, a lower number of iron deficient preschoolers and high weight gain after supplementation with the same synbiotic mixture (Sazawal *et al.*, 2010a).

Fermentation products

The use of fermentation products or metabolites produced by bacteria may offer a different approach for modulation of the microbiota and immune system, which may share some features with pro- and prebiotics, yet give rise to a new concept. Several studies have indicated the potential of end products from bacterial fermentation of non-digestible carbohydrates, such as short-chain fatty acids to stimulate the gut barrier (Willemsen *et al.*, 2003). This concept has been used in infant formula by the fermentation of formula based on cow's milk proteins with lactic acid producing bacteria. Fermented milk based infant formula does not contain viable bacteria such as in probiotics based infant formula but contain products resulting from the fermentation process, of which these properties are of specific interest. Fermented milks contain a wide range of active components, which are still not comprehensively characterised. In parallel, milk carbohydrates can produce a wide variety of oligosaccharides. In addition to fermentation products, fermented formula also contains a wide range of components originating from lactic acid producing bacteria, either from cell walls (such as peptidoglycans, exopolysaccharides, lipoteichoic acid) or from bacterial DNA (Meydani and Ha, 2000). These compounds, which are usually present in small amounts, are thought to exhibit specific properties, mostly in the area of immunostimulation (Hatcher and Lambrecht, 1993; Meydani and Ha, 2000).

In preclinical studies, bacterial fermentates were shown to exhibit both tolerogenic and immunogenic properties. Fermentation products induced maturation of DCs associated with increase in IL-10 production and prolonged

survival (Hoarau *et al.*, 2006). Furthermore, DCs treated with fermentation products were able to induce regulatory T cells (Martin *et al.*, unpublished observations). Animal studies indicated the potential of fermented products to stimulate mucosal immune responses and thus enhancing the response against pathogens (Vinderola *et al.*, 2007a,b). Clinical studies with neonates and infants receiving fermented milk based infant formula reported different clinical beneficial effects (reviewed by Agostoni *et al.*, 2007) such as, stimulating the growth of bifidobacteria; increasing antipoliiovirus IgA response (Mullie *et al.*, 2004); and in reducing the severity of acute diarrhoea (Thibault *et al.*, 2004).

6. Conclusion

The immune system of infants is not fully functional at birth, rendering them highly susceptible to infections. After birth, there is an age-dependent maturation of the immune system in which different key functions are being fine tuned when exposed to direct stimulation from environmental signals not previously encountered during foetal life. Commensal microorganisms are most likely one of the drivers of immune development and, in turn the immune system shapes the composition of the microbiota. Due to the complexity of the microbiota and its interaction with the immune system, it is difficult to define the specific mechanism underlying the described functional effects of pre- and probiotics, especially in the case of systemic immune modulation. The host defence may be influenced by the modification of the intestinal microflora, resulting in an increase in the proportions of bifidobacteria and lactobacilli. This interpretation is also supported by the reported relationship between allergic disease and the composition of the intestinal microbiota early in life (Bjorksten, 2009b; Garrett *et al.*, 2010; Kalliomaki *et al.*, 2001a; Round and Mazmanian, 2009; Vael and Desager, 2009). The increase in these bacteria may lead to competition with pathogenic bacteria for binding sites on the intestinal epithelium and for nutrients, thus inhibiting survival of pathogenic bacteria. These beneficial bacteria, or fragments thereof, may also cross the intestinal barrier and activate immune cells (Berg, 1985). Moreover, specific fermentation products, including short chain fatty acids, are produced by *Bifidobacterium* species that have direct anti-pathogenic and immune modulating effects (Ishizaka *et al.*, 1993; Millard *et al.*, 2002). Further understanding of the complex interaction between commensal microorganisms and immune system will provide more insight how to regulate the development of the immune system an early stage to prevent the development of diseases such as allergy and infections.

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