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Workshop Report

Early nutrition and immunity – progress and perspectives

Philip C. Calder[1]*†, Susanne Krauss-Etschmann[2,3]†, Esther C. de Jong[4], Christophe Dupont[5], Julia-Stefanie Frick[6], Hanne Frokiaer[7], Joachim Heinrich[8], Holger Garn[9], Sibylle Koletzko[10], Gideon Lack[11], Gianluca Mattelio[12], Harald Renz[9], Per T. Sangild[13], Jürgen Schrezenmeir[14], Thomas M. Stulnig[15], Thomas Thymann[13], Agnes E. Wold[16] and Berthold Koletzko[17]

[1]*Institute of Human Nutrition, School of Medicine, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK*
[2]*Clinical Cooperation Group 'Pediatric Immune Regulation', Dr von Hauner Children's Hospital, University of Munich, Linderwurmstrasse, D-80337 Munich, Germany*
[3]*GSF-National Research Center for Environment and Health, Ingolstadter Lanstrasse, D-85758 Neuherberg, Germany*
[4]*Departments of Cell Biology & Histology and Academic Medical Centre, University of Amsterdam, Meiberjdreef, 1105AZ Amsterdam, The Netherlands*
[5]*Department of Pediatric Gastroenterology and Nutrition Unit, Cochin-Saint Vincent de Paul Hospital, University Paris V – René Descartes, Av. Denfirt-Rochereau, 75014 Paris, France*
[6]*Department of Medical Microbiology and Hygiene, University of Tübingen, Elfriede-Aulhornstrasse, D-72076 Tübingen, Germany*
[7]*BioCentrum-DTU, Biochemistry and Nutrition, Technical University of Denmark, Kgs. Lyngby, DK-2800 Denmark*
[8]*Institute of Epidemiology, GSF-National Research Centre for Environment and Health, Ingolstadter Lanstrasse, D-85758 Neuherberg, Germany*
[9]*Department of Clinical Chemistry and Molecular Diagnostics – Central Laboratory, Philipps-University of Marburg, Nans-Meerwinstrase, D-35043 Marburg, Germany*
[10]*Division of Gastroenterology, Dr von Hauner Children's Hospital, University of Munich, Linderwurmstrasse, D-80337 Munich, Germany*
[11]*Pediatric Allergy and Clinical Immunology, Imperial College School of Medicine at St Mary's Hospital, Praed Street London W2 1NY, UK*
[12]*Department of Experimental Oncology, European Institute of Oncology, Via Ripamonti, 20141 Milan, Italy*
[13]*Department of Human Nutrition, The Royal Veterinary and Agricultural University, Rolighedsvej, DK-1958 Frederiksberg, Denmark*
[14]*Institute for Physiology and Biochemistry of Nutrition, Federal Research Centre for Nutrition and Food, Hermann-Weigmannstrasse, D-24103 Kiel, Germany*
[15]*Clinical Division of Endocrinology and Metabolism, Department of Internal Medicine III, Medical University of Vienna, Wahringer Gurtel, 1090 Vienna, Austria*
[16]*Department of Clinical Bacteriology, The Sahlgrenska Academy at Göteborg University, Guldhegsgaten, S-41346 Göteborg, Sweden*
[17]*Division of Metabolic Diseases and Nutritional Medicine, Dr von Hauner Children's Hospital, University of Munich, Linderwurmstrasse, D-80337 Munich, Germany*

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The immune system exists to protect the host against pathogenic organisms and highly complex pathways of recognition, response, elimination and memory have evolved in order to fulfil this role. The immune system also acts to ensure tolerance to 'self', to food and other environmental components, and to commensal bacteria. A breakdown in the tolerogenic pathways can also lead to inflammatory diseases. The prevalence of inflammatory diseases, including atopic disorders, has increased over the last 60 years. The development of tolerance is the result of active immune

Abbreviations: DC, dendritic cells; DC-SIGN, dendritic cell-specific intercellular adhesion moleule-3-grabbing non-integrin; DHA, docosahexaenoic acid; GIT, gastrointestinal tract; IFN, interferon; LAT, linker of activated T cells; *n*-3 LCPUFA, very-long-chain *n*-3 PUFA; NUHEAL, Neutraceuticals for a Healthy Life; OVA, ovalbumin; TGF, transforming growth factor; Th, T helper; Treg, regulatory T.

* **Corresponding author:** Dr Philip C. Calder, fax +44 (0)2380 594379, email pcc@soton.ac.uk

† Authors made an equal contribution to the writing of this report.

mechanisms and both development and maintenance of tolerance are lifelong processes which start very early in life, even prenatally. Profound immunologic changes occur during pregnancy, involving a polarization of T helper (Th) cells towards a dominance of Th2 and regulatory T cell effector responses in both mother and fetus. This situation is important to maintain pregnancy through avoidance of the rejection of the immunologically incompatible fetus. During the third trimester of human pregnancy, fetal T cells are able to mount antigen-specific responses to environmental and food-derived antigens and antigen-specific T cells are detectable in cord blood in virtually all newborns indicating *in utero* sensitization. If the neonatal immune system is not able to down-regulate the pre-existing Th2 dominance effectively then an allergic phenotype may develop. Changes occur at, and soon after, birth in order that the immune system of the neonate becomes competent and functional and that the gut becomes colonized with bacteria. Exposure to bacteria during birth and from the mother's skin and the provision of immunologic factors in breast milk are amongst the key events that promote maturation of the infant's gut and gut-associated and systemic immune systems. The introduction of formula and of solid foods exposes the infant to novel food antigens and also affects the gut flora. Nutrition may be the source of antigens to which the immune system must become tolerant, provide factors, including nutrients, that themselves might modulate immune maturation and responses, and provide factors that influence intestinal flora, which in turn will affect antigen exposure, immune maturation and immune responses. Through these mechanisms it is possible that nutrition early in life might affect later immune competence, the ability to mount an appropriate immune response upon infection, the ability to develop a tolerogenic response to 'self' and to benign environmental antigens, and the development of immunologic disorders. A Workshop held in February 2006 considered recent findings in the areas of oral tolerance, routes of sensitization to allergens and factors affecting the development of atopic disease; factors influencing the maturation of dendritic cells and the development of regulatory T cells; the influence of gut microflora on immunity, allergic sensitization and infectious disease; the role of nutrition in preventing necrotizing enterocolitis in an animal model of preterm birth; and the role of PUFA of different classes in influencing immune responses and in shaping the development of atopic disease. This report summarizes the content of the lectures and the subsequent discussions.

Immunity: Infection: Allergy: Asthma: Atopic disease: Lymphocyte: Dendritic cell: Cytokine: Inflammation: Probiotic: Polyunsaturated fatty acid

The immune system exists to protect the host against pathogenic organisms and highly complex pathways of recognition, response, elimination and memory have evolved in order to fulfil this role. A breakdown in these pathways or their inefficient operation can lead to increased susceptibility to, and increased morbidity and mortality as a result of, infectious disease. The immune system also acts to ensure tolerance to 'self', to food and other environmental components, and to commensal bacteria. A breakdown in the tolerogenic pathways can also lead to so-called inflammatory diseases (Fig. 1): autoimmune disease may result from loss of tolerance to self antigens; allergic disease may result from loss of tolerance to food and environmental antigens; inflammatory bowel diseases may result from loss of tolerance to commensal bacteria within the intestinal tract. The development of tolerance is the result of active immune mechanisms requiring antigen-contact and acting in a T cell-dependent fashion (Faria & Weiner, 2005; Sigal, 2005; Powell, 2006; Taylor *et al.* 2006). However, both development and maintenance of tolerance are lifelong processes which start very early in life, even prenatally (Fig. 2). In this regard early 'education' of the immune system seems to begin *in utero* (Herz *et al.* 2001; Jones *et al.* 2002; Michaelsson *et al.* 2006) and continues after birth, particularly during the first 2 years of life. It is now recognized that profound immunologic changes occur in the mother during pregnancy. These changes involve a polarization of T helper (Th) cells towards a dominance of Th2 and regulatory T (Treg) cell effector responses (Reinhard *et al.* 1998; Raghupathy, 2001). The fetal immune response shows the same polarization (Prescott *et al.* 1999) and this phenomenon has been considered as a Th2/Treg default

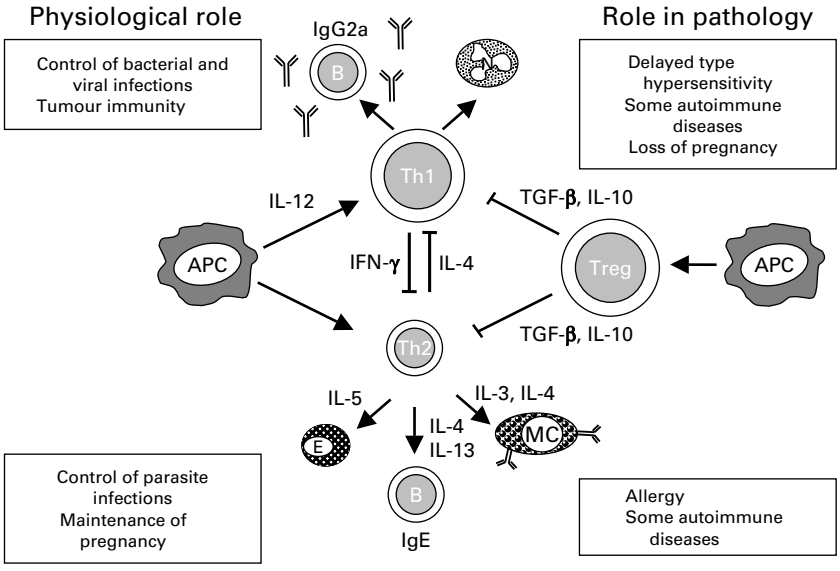

Fig. 1. Schematic representation of the roles of helper T cells in regulating immune responses and in physiological and pathological responses. APC, antigen presenting cell; B, B cell; E, eosinophil; IFN, interferon; MC, mast cell; N, neutrophil; TGF, transforming growth factor; Th1, type 1 helper T cell; Th2, type 2 helper T cell; Treg, regulatory T cell.

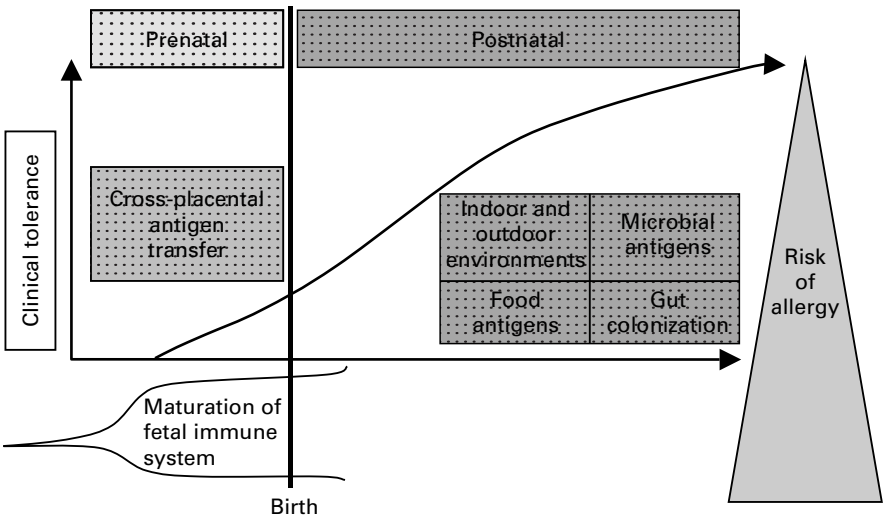

Fig. 2. Schematic representation of the development of clinical tolerance through the pre- and postnatal periods and of how this relates to the risk of allergy.

pathway early in life (Prescott *et al.* 1998). The default pathway is important to maintain pregnancy, through suppression and avoidance of the rejection of the immunologically incompatible fetus (Reinhard *et al.* 1998; Raghupathy, 2001). Th2-driven IgE and IgA responses allow the recognition of ultra-low antigen doses (Jones *et al.* 1999). Since there is significant uptake and transfer of food-derived and environmental antigens via the placental barrier into amniotic fluid (Jones *et al.* 1998, 2002; Szepfalusi *et al.* 2000), the ability to recognize low antigen doses may be important in order to develop early specific T cell responses against such antigens. Thus, the formation of antigen-specific IgE (and IgA) starts prenatally (Bjerke *et al.* 1994; Shah & Bapat, 2006), and this pattern of Ig production is heavily driven through the Th2-biased environment.

Mature $CD4^+$ and $CD8^+$T cells can be detected in the fetus as early as around week 20 in human pregnancy (Thilaganathan *et al.* 1992; Michaelsson *et al.* 2006). Therefore, particularly during the third trimester of human pregnancy, the fetal T cell compartment is ready and able to mount antigen-specific T cell responses to environmental and food-derived antigens. Indeed, such T cells are detectable in cord blood in virtually all newborns indicating *in utero* sensitization (Kondo *et al.* 1992; Warner *et al.* 1996; Jones *et al.* 1996). This seems to be a normal phenomenon although its functional consequences are not clear. One idea is that this is designed as a route to the early development of antigen-specific tolerance, particularly to food-derived antigens. However, if the neonatal immune system is not able to effectively down-regulate the pre-existing Th2 dominance and overcome the presence of low levels of allergen-specific IgE antibodies then an allergic phenotype may develop.

Although the fetus at term may be sensitized to certain antigens, it does lack a fully functional immune system and has a sterile gastrointestinal tract (GIT). Changes occur at, and soon after, birth in order that the immune system of the neonate becomes competent and functional and that the gut becomes colonized with bacteria. Exposure to bacteria during birth and from the mother's skin and the provision of immunologic factors in breast milk are amongst the key events that promote maturation of the infant's gut and gut-associated and systemic immune systems. The introduction of formula and of solid foods exposes the infant to novel food antigens and also affects the gut flora. The nature of the infant's environment (e.g. the presence of older siblings or of pets, the use of nurseries) will determine exposure to novel bacterial and environmental antigens to which it must respond in an appropriate manner. Thus, pregnancy, the suckling period, and the periods during which formula and solid foods are introduced offer windows during which nutrition might affect the immunologic development of the fetus and young infant. Nutrition may:

- be the source of antigens to which the immune system must become tolerant;
- provide factors, including nutrients, that themselves might modulate immune maturation and responses;
- provide factors that influence intestinal flora, which in turn will affect antigen exposure, immune maturation and immune responses.

Through these mechanisms it is possible that nutrition early in life might affect later immune competence, the ability to mount an appropriate immune response upon infection, the ability to develop a tolerogenic response to 'self' and to benign environmental antigens, and the development of immunologic disorders.

In order to discuss these emerging and rapidly developing scientific issues, a 3 d Workshop on 'Early Nutrition and Immunity – Progress and Perspectives' was held in Obergurgl, Austria in February 2006. The Workshop was organized by Professor Berthold Koletzko, Munich, Germany. It brought together experts in the fields of allergy, immunology and nutrition in order to share recent findings and to discuss the evidence relating early nutritional exposures to later immune competence and to the development of immunologic disorders, with a particular focus upon atopic diseases such as allergies and asthma. The overall aim was to share expertise, to evaluate the evidence, and to identify research gaps and opportunities in this area. The Workshop took the form of seventeen expert lectures with extensive discussion of each. The lectures covered oral tolerance, routes of sensitization to allergens and factors affecting the development of atopic disease; factors influencing the maturation of dendritic cells (DC) and the development of Treg cells; the influence of gut microflora on immunity, allergic sensitization and infectious disease; the role of nutrition in preventing

necrotizing enterocolitis in an animal model of preterm birth; and the role of PUFA of different classes in influencing immune responses and in shaping the development of atopic disease. This report summarizes the content of these lectures and the subsequent discussions.

Factors affecting the development of atopic disease

A global increase in the incidence and prevalence of many chronic inflammatory conditions, including autoimmune diseases, allergies, asthma and atopy, and inflammatory bowel diseases, has been observed over the last sixty years or so, particularly in westernized and industrialized countries (Bach, 2002). Although there are genetic predispositions to these diseases (e.g. Barnes, 2006), the presence of certain polymorphisms alone is unlikely to explain the shift in disease prevalence. Thus, in addition to genetics, environmental factors must be taken into consideration; these are listed for atopic disease in Table 1. Epidemiological investigations have identified that early exposure to microbes may be an important protective factor for allergy and asthma, perhaps acting as an 'immune educator'. This is the so-called 'hygiene hypothesis' (Strachan, 1989; Borchers *et al.* 2005; Renz *et al.* 2006), which suggests that the absence of early exposure to certain types of microbes as a result of modern hygienic and medical practices removes the drive to mature Th1 cell effector responses and so allows the dominant early Th2 cell responses to continue unchecked. The role of microbial exposure in educating the immune system away from Th2 predominance may explain why certain probiotic strains of bacteria appear to have a protective role in atopic diseases (Isolauri, 2004; Ogden & Bielory, 2005). Epidemiology has also identified that early exposure to certain nutrients, including very-long-chain *n*-3 PUFA (*n*-3 LCPUFA) may be protective (see Calder & Miles, 2000; Calder, 2003*a*,*b*; see later).

The German Infant Nutritional Intervention was a large, government-sponsored study initiated to compare the effect of three different hydrolysed formulas and a standard cow's milk formula as the only substitute for breast-feeding in children in the first 4 months of life and at high risk of developing cow's milk allergy (identified as parental or sibling allergy). Infants (*n* 2252) in one urban and one rural area of Germany were randomly allocated at birth to one of four masked study formulae: partially hydrolysed whey formula, extensively hydrolysed whey formula, extensively hydrolysed casein formula and standard cow's milk formula. The formulae were anticipated to be the only substitute for insufficient breast-feeding during the first 16 weeks of life (intervention period). A further 3739 infants, either with no heredity for atopy or parental refusal to take part in the intervention, formed an observational cohort. After adjustment for confounding factors, exclusive human milk feeding during the first 4 months of life was associated with a significant reduction in atopic dermatitis at 1 year of age compared to infants who had received cow's milk formula in the intervention group, but not in the observational group (Laubereau *et al.* 2004). Of the different atopic manifestations (atopic dermatitis, asthma, gastrointestinal manifestations and urticaria), only the risk for atopic dermatitis at 1 year was reduced by feeding a hydrolysed formula (von Berg *et al.* 2003). Compared to cow's milk formula, the extensively hydrolysed casein formula significantly reduced atopic dermatitis in both per protocol and intention to treat analyses; the partially hydrolysed whey formula showed a significant reduction in the per protocol analysis, while the extensively hydrolysed whey formula showed no allergy preventive effect (von Berg *et al.* 2003). The effect developed in the first year of life and persisted until the third year (von Berg *et al.* 2003). Stratification for the type of allergic manifestation revealed that the genetic background seems to modify the effect of infant nutrition.

Table 1. Potential determinants of atopic disease

Genetics
Family history of atopy
Low birth weight
Exposure to pets
Exposure to allergens
Infections/Microbial stimulation/Gut microflora
Exposure to parasites
Obesity
Dietary fatty acids
Dietary antioxidants
Exposure to tobacco smoke
Exposure to air pollutants – indoor/outdoor

The German Infant Nutritional Intervention study provides a significant amount of data to investigate the effect of other environmental factors on risk of atopic disease. Data from questionnaires at the 12- and 24-month time-points of the intervention and observational cohorts were analysed for an association of pet keeping in the household and doctor's diagnosed atopic dermatitis. Complete data were available from 4578 families (Zirngibl *et al.* 2002). After adjusting for possible confounders, keeping of any pet, and particularly keeping a dog, reduced the risk for atopic dermatitis in the first and second year of life (Zirngibl *et al.* 2002). Keeping cats did not reduce the risk, while small furred animals like hamster, rabbit or guinea pig showed a borderline significant effect in the first year of life. These results confirmed recent studies on the preventive effect of pet keeping on allergic manifestation (Braback *et al.* 2001). To investigate whether delivery by caesarean section is a risk factor for later allergy, infants in the intervention group who received solely human milk during the first 4 months of life and had a 1-year follow-up were investigated (*n* 865). Infants born by caesarean section (17 %) had a greater risk of diarrhoea and sensitization to food allergens, both in adjusted and stratified analyses (by cord blood IgE) (Laubereau *et al.* 2005). Caesarean delivery was not associated with colicky pain and atopic dermatitis (Laubereau *et al.* 2005). It is speculated that the different gut flora present in infants born by caesarean section, or other factors, like antibiotics given to the mother, may put the child at risk for diarrhoea and allergic sensitization.

The main strategy for avoiding adverse allergic reactions to foods among individuals sensitized to those foods has been the avoidance of the incriminated food, although this can be difficult for some foods like cow's milk, which are very common. Avoidance has also been advocated as a way of preventing sensitization from occurring. WHO advocates exclusive breast-feeding of the infant and delayed introduction of solids until 6 months of age, while some countries recommend avoidance of food allergens such as peanut during pregnancy, lactation and the first 3 years of childhood (Department of Health, 1997). However, epidemiological studies consistently fail to show any association between maternal consumption of allergen during pregnancy and lactation, and the development of food

allergies (Tariq *et al.* 1996; Lack *et al.* 2003). Similarly, randomized controlled dietary elimination studies during pregnancy, lactation and infancy, while improving eczema outcome, consistently fail to reduce the prevalence of IgE-mediated food allergies (Kramer & Kakuma, 2003). Findings from murine models of allergic sensitization may shed some light on this human data. Single doses of ovalbumin (OVA), β-lactoglobulin or peanut protein fed to young mice induce long-lasting oral tolerance to the food antigen, whereas low-dose cutaneous exposure through inflamed skin or via the inhalational route leads to allergen-specific IgE elaboration. Thus, it is possible that an imbalance between routes of allergen exposure affects the outcomes of tolerance and allergy. Allergens such as egg, peanut and milk can be measured in the home environment and so exposure through skin or lungs is possible and it has been shown in a prospective birth cohort study that peanut allergy is associated both with severe atopic dermatitis and cutaneous exposure to topical arachis (peanut) oil (Lack *et al.* 2003). It has been observed that in countries in Africa and Asia where environmental exposure to peanut is high, there is nevertheless a low reported prevalence of peanut allergy. One possible explanation is that oral tolerance induction through early infant feeding protects these populations against allergic sensitization, although genetic and other environmental factors may also play a role.

Therapies have been attempted to induce food tolerance in allergic individuals. Isolated and successful experiences using increasing oral doses of cow's milk to induce oral tolerance have been reported (Meglio *et al.* 2004). The sublingual route is currently being investigated for the management of aeroallergen sensitization and has been attempted once with food, namely hazelnut, in adults (Enrique *et al.* 2005). Cow's milk allergy affects 2–3 % of infants less than 1 year of age, and although most children, including those with non-IgE-mediated cow's milk allergy, tolerate cow's milk by 3 years of age, 75 % of children with IgE-mediated cow's milk allergy remain intolerant after 6 years of age, with 15 % of them being still affected after age 8 years. A preliminary study has investigated the response to sublingual immunotherapy of IgE-mediated cow's milk allergy in eight children at risk of permanent disease, i.e. with IgE-mediated cow's milk allergy and above 6 years of age (de Boissieu & Dupont, 2006). The protocol was based on the sublingual administration of an amount of cow's milk below the dose eliciting symptoms during a preliminary food challenge. In this limited study, tolerance to normal amounts of cow's milk was achieved in four out of six children who strictly adhered to the protocol during 6 months. Sublingual immunotherapy was easy to perform and proved to be safe for patients, even at home. This could therefore represent an alternative approach to elimination diets in patients with persisting IgE-mediated cow's milk allergy. Although encouraging, these preliminary results need confirmation with larger studies, aiming also at determining the optimal duration of the procedure. The mechanisms involved in the induction of tolerance remain poorly understood.

The gastrointestinal tract, gut microflora and immunity

Factors influencing the maturation of dendritic cells and the development of regulatory T cells

DC play an important role in the initiation of the immune response. Based on microbial or tissue-specific priming, DC develop different instructive signals and drive the differentiation of naive Th cells into either Th1, Th2 or Treg cells (de Jong *et al.* 2002; Fig. 3). Treg cells act by suppressing the effector function of other immune cells, in particular T cells. They play a key role in immune homeostasis, prevention of autoimmunity and allergy, and in negative regulation of adaptive immune responses against pathogens (Bluestone & Abbas, 2003). Adaptive Treg cells can be induced by specific priming of immature DC or mature regulatory DC.

Lactobacilli are one of the most frequently used strains of probiotic bacteria in the management of gastroenteritis, inflammatory bowel diseases (Bergonzelli *et al.* 2005) and atopic diseases (Gionchetti *et al.* 2000; Kalliomaki *et al.* 2003; Isolauri, 2004; Ogden & Bielory, 2005). It is hypothesized that these probiotic bacteria have immunoregulatory properties and promote mucosal tolerance, which is in part mediated by Treg cells (Kapsenberg, 2003). The ability of different species of lactobacilli to prime human DC has been investigated (Smits *et al.* 2005). *Lactobacillus reuteri* and *L. casei*, but not *L. plantarum*, primed human monocyte-derived DC to drive the development of Treg cells (Smits *et al.* 2005). The development of the Treg cells was dependent on the expression of IL-10, transforming growth factor-β (TGF-β) and programmed death ligand 2 by DC. These Treg cells produced elevated levels of IL-10 and were capable of inhibiting the proliferation of bystander T cells in an IL-10-dependent fashion. Strikingly, both *L. reuteri* and *L. casei*, and not *L. plantarum*, bind the C-type lectin dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN). Blocking antibodies to DC-SIGN inhibited the induction of the Treg cells by these probiotic bacteria, stressing that ligation of DC-SIGN can actively prime DC to induce Treg cells. Analysis of bacterial components that may bind DC-SIGN identified lipoteichoic acid as a likely candidate. Indeed, lipoteichoic acid from *L. reuteri* and *L. casei* but not from *L. plantarum* bind DC-SIGN (M van Oosterwijk *et al.* unpublished results). These data indicate that targeting of DC-SIGN by certain probiotic bacteria may explain their beneficial effect in the treatment of a number of inflammatory diseases, including inflammatory bowel diseases (Bergonzelli *et al.* 2005) and atopic diseases (Gionchetti *et al.* 2000; Kalliomaki *et al.* 2003; Isolauri, 2004; Ogden & Bielory, 2005). In addition, this study shows that different probiotic species may affect the immune system in a different fashion (M van Oosterwojk *et al.* Unpublished results).

IL-2 deficient (IL-$2^{-/-}$) mice are a model for microflora triggered, chronic immune-mediated colitis (Autenrieth *et al.* 1997). IL-$2^{-/-}$, but not IL-$2^{+/+}$, gnotobiotic mice mono-colonized with *Escherichia coli* mpk developed colitis whereas IL-$2^{-/-}$ mice colonized with *Bacteroides vulgatus* mpk did not (Waidmann *et al.* 2003). Furthermore, *B. vulgatus* mpk was able to prevent the *E. coli* mpk-induced colitis (Waidmann *et al.* 2003). Using fluorescent *in situ* hybridization and culture methods it was shown that the anti-colitogenic effect exerted by *B. vulgatus* mpk on *E. coli* mpk could not be explained by a reduction in numbers of *E. coli* in the colon (Waidmann *et al.* 2003) and so may be due to an effect on host immune defence. In specific pathogen-free IL-$2^{-/-}$ mice the *E. coli* mpk-induced colitis was associated with increased interferon (IFN)-γ, TNF-α and CD14 mRNA expression in the colon (Waidman *et al.* 2003). Since DC,

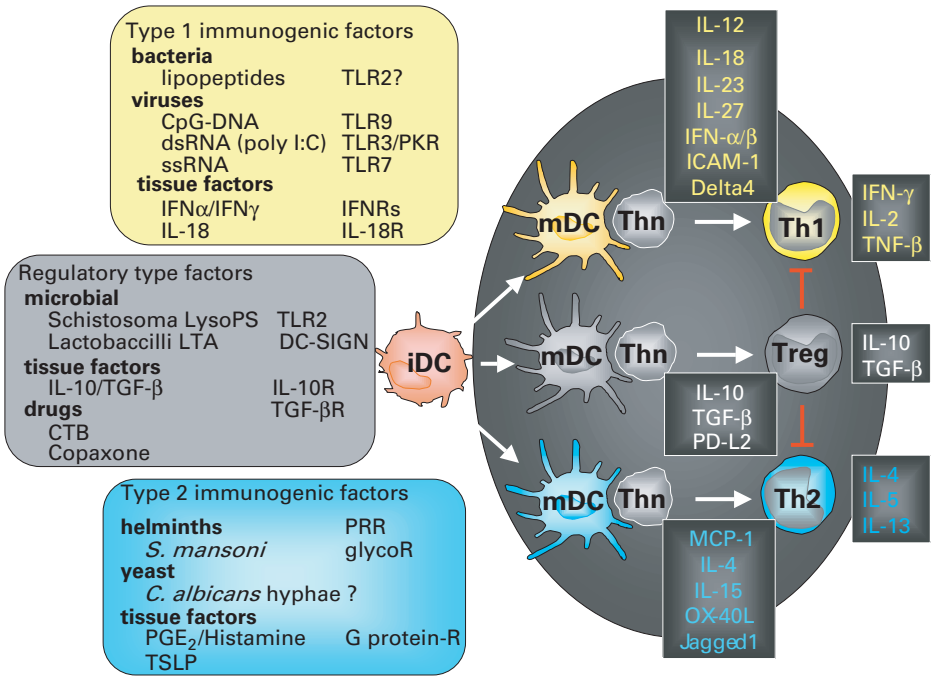

Fig. 3. Schematic representation of factors that influence the T cell polarizing capacity of dendritic cells (DC) and of the expression of molecules (either cytokines or membrane-bound molecules) that drive the development of either type 1 helper T cell (Th1), type 2 helper T cell (Th2) or regulatory T cells (Treg). CTB, cholera tixzin B; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; glycoR, g glyco receptor; ICAM, intercellular adhesion molecule; iDC, immature DC; IFN, interferon; LTA, lipoteichoic acid; LysoPS, lysophosphatidylserine; MCP, monocyte chemotatic peptide; mDC, mature DC; PD-L2, programmed death ligand 2; PGE_2, prostaglandin E_2; PKR, protein kinase R; PRR, pattern recognition receptor; R, receptor; TGF, transforming growth factor; Thn, naive helper T cell; TLR, toll-like receptor; TSLP, thymic stomal lymphopoetin.

transport pathogens to the mesenteric lymph nodes and the spleen for induction of systemic immune responses (Vazquez-Torres *et al.* 1999), it is hypothesized that differential modulation of DC by commensal bacteria might lead to colitogenic, inert or protective effects in $IL2^{-/-}$ mice.

The intestinal mucosa is characterized by the maintenance of a constant equilibrium between the activation and the suppression of mucosal immune responses. The activation of the mucosal immune system is essential to counteract potentially harmful antigens. However, the control of such responses is necessary to avoid undesired immunologic reactions towards 'self' antigens or harmless bacterial and food-derived antigens contained within the intestinal lumen. Although historically epithelial cells have been considered as a passive line of defence protecting deeper tissues from the external environment, recent data have suggested that epithelial cells are key players participating actively in mucosal immune responses. By the virtue of their production of cytokines, growth factors and membrane proteins, epithelial cells play an active role within an integrated immune system. The crosstalk between epithelial cells and DC has been analysed and epithelial cells have been examined for their capacity to release cytokines and chemokines that induce the migration and activation of DC (Rimoldi *et al.* 2005*a*). To mimic the complex intestinal environment an *in vitro* model of a three-player system of DC, epithelial cell monolayers and bacteria has been developed (Rescigno *et al.* 2001*a*). Using this model it was demonstrated that DC play an active role in bacterial uptake across mucosal surfaces (Rescigno *et al.* 2001*a*). Indeed, DC are able to open tight junctions and to sample antigens directly across the epithelial layer, both *in vitro* and *in vivo* (Rescigno *et al.* 2001*a*). However, because DC express tight junction proteins, the integrity of the epithelial barrier is preserved. The capacity of epithelial cells to produce cytokines and activate DC depends on the invasiveness of the bacteria tested. Invasive bacteria (*Salmonella typhimurium*) stimulate epithelial cells to release proinflammatory cytokines and to induce maturation of DC (Rimoldi *et al.* 2005*a*). In contrast, non-invasive bacteria (*L. plantarum*) are unable to stimulate epithelial cells, but can directly activate DC when DC translocate to the apical side of the epithelium. Furthermore, epithelial cells conditioned mucosal DC through the constitutive release of thymic stromal lymphopoietin, resulting in the induction of 'non-inflammatory' DC that released IL-10 and IL-6, but not IL-12, and promoted the polarization of T cells toward a Th2 phenotype (Rescigno *et al.* 2001*b*; Rimoldi *et al.* 2005*a*). This Th2-response was maintained even after exposure to a Th1-inducing pathogen (Rimoldi *et al.* 2005*b*). Thus, these studies indicate that epithelial cells are not simply a barrier to bacteria entering via the oral route, but actively influence the properties of bystander DC and through this may help to maintain gut immune homeostasis.

Gut microflora, regulatory T cells and early childhood allergy

Paucity of microbial exposure during childhood may be the cause of the allergy epidemic in Western countries (Renz *et al.* 2006). Western infants have delayed acquisition

of several gut microbes and a reduced turnover of strains in the microbiota, indicating exposure to a low variety of environmental bacteria (Adlerberth *et al.* 2005; Ahrne *et al.* 2005). The IMMUNOFLORA study has examined how early intestinal colonization affects the development of putative Treg cells and clinical allergy in Swedish infants. The intestinal colonization pattern was assessed by semi-quantitative aerobic culture of rectal swabs obtained at 3 d of age and by quantitative aerobic and anaerobic culture of faecal samples obtained at 1, 2, 4 and 8 weeks, and 6 and 12 months of age. Blood samples were drawn at delivery (cord blood), 3–4 d, and at 4 and 18 months of age, and analysed by flow cytometry for signs of T cell activation and presence of putative Treg cells ($CD4^+CD25^+CTLA\text{-}4^+$). Children were clinically examined for allergy upon first appearance of symptoms and at 18 months of age. On average, the numbers of putative Treg cells did not increase between birth and 4 months of age, but there was a large variation among infants (Fig. 4(a)). The children were divided into two equally sized groups according to the magnitude of their Treg cell population in the 4-month blood sample and the bacterial colonization pattern of the two groups was examined. Colonization by *Staphylococcus aureus* occurred more rapidly in children in whom there was evidence of expansion of the Treg cell population (Fig. 4(b)). No other bacterial group was related to expansion of Treg cells. Approximately half of the *S. aureus* strains produced one or more toxins with superantigen function, i.e. toxins with broad and strong T cell-activating properties. Expansion of Treg cells was seen in children neonatally colonized by superantigen-producing *S. aureus*, but was unrelated to colonization by non-toxin-producing strains (Fig. 4(c, d)). Further, expansion of Treg cells was only seen in children who acquired their *S. aureus* strain in the first days of life, while later colonization had no such effect. Finally, children who developed food allergy during the first 18 months of life were significantly less often neonatally colonized with *S. aureus* compared to other infants. These findings may indicate that a strong T cell stimulation in the neonatal period may trigger maturation of Treg cells and that paucity of such immune activation may predispose to allergy development.

Probiotic bacteria and allergen sensitization in animal models

To test the hypothesis that there is a cause–effect relationship between prenatal exposure to bacterial-derived products and reduced allergic inflammation, an animal model has been developed where pregnant mice are treated with lipopolysaccharide and the development of an experimentally induced respiratory allergic inflammation is investigated later in the offspring (Blumer *et al.* 2005). Perinatal treatment of dams with lipopolysaccharide led to reduced levels of OVA-specific IgG1 and IgE but not of IgG2a in offspring after OVA immunization (Blumer *et al.* 2005). This effect occurred most probably due to a selective reduction of Th2 activities, since after re-stimulation of mononuclear spleen cells from the offspring with OVA significantly reduced levels of IL-5 were observed, whereas IFNγ production was not altered (Blumer *et al.* 2005). After aerosol allergen provocation of the lungs a considerably abolished allergic inflammatory response with respect to inflammatory cell infiltration and histopathology was observed in offspring whose mothers had been treated with lipopolysaccharide (Blumer *et al.* 2005). To develop an allergy-preventing intervention strategy based on these types of investigation, a more appropriate microbial stimulus is

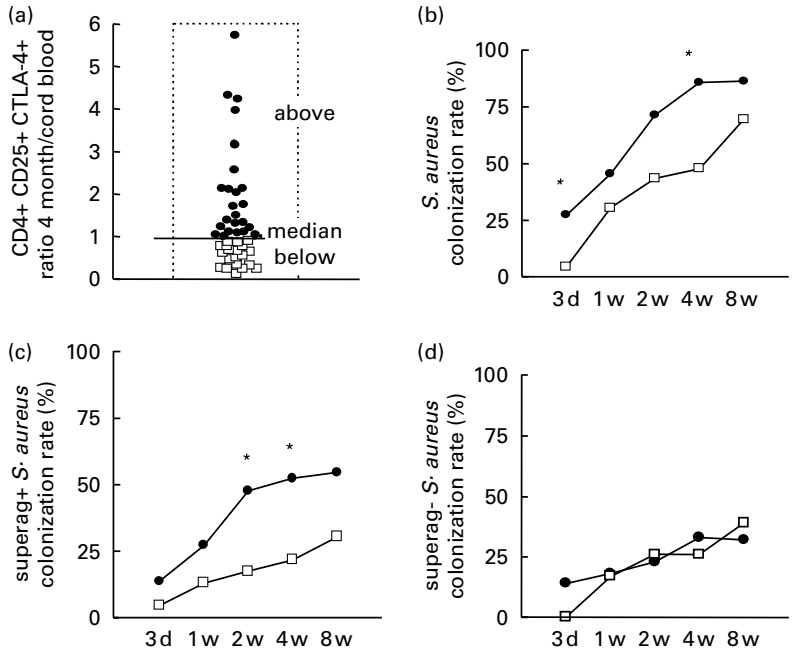

Fig. 4. Putative regulatory T cells in neonatal and infant blood and relationship to gut colonization with *Staphylococcus aureus*. The presence of putative regulatory T cells was determined in cord and 4-month blood samples. On average, no increase occurred between 0 and 4 months of age (a). Children were divided into those who increased their counts of putative regulatory T cells (●; b, c, d), and those who did not (□; b, c, d). The colonization pattern of these two groups is shown with respect to *S. aureus* (b), *S. aureus* strains producing a superantigen (superag +; c) and *S. aureus* strains not producing superantigen (superag −; d). Mean values were significantly different between the two groups: $*P < 0{\cdot}05$. d, days; w, weeks.

needed that should be able to provide sufficient protective signals in the absence of an association with pathological conditions. In addition, a convenient route of application is required. In accordance with these aims, the model was adapted to analyse the effects of oral supplementation of dams with the probiotic bacterium *L. rhamnosus* GG on the allergic airway reactivity in their offspring later in life. Female mice were treated every second day with 10^8 colony-forming units of *L. rhamnosus* GG by intragastric application before mating, during pregnancy and during lactation. Offspring were then intraperitoneally sensitized to OVA and the airway inflammatory response was analysed after aerosolized OVA challenge (Wegmann *et al.* 2005). *L. rhamnosus* GG supplementation of dams had no effects on the sensitization in the offspring irrespective of which Ig isotype was analysed. However, a markedly reduced allergic inflammatory response was observed in the airways as demonstrated by significantly decreased numbers of leucocytes, mainly eosinophils in the bronchoalveolar lavage, reduced numbers of mucus-producing goblet cells and reduced peribronchial and perivascular cell infiltration. In contrast to the results obtained after lipopolysaccharide supplementation, *in vitro* restimulation of spleen cells from offspring of *L. rhamnosus* GG-supplemented mothers revealed a generalized inhibition of T cell responses for all Th cell subclasses indicated by a reduced release of the respective marker cytokines IFN-γ, IL-5 and IL-10. No allergo-protective effects of *L. rhamnosus* GG were observed after long-term treatment of adult mice (H Garn, unpublished results) suggesting that the effects are limited to a 'window of opportunity' defined by an immune system that is still in a very early stage of development. Further studies are currently in progress to elucidate the mechanisms of how effects are transferred from mother to offspring.

Probiotic bacteria and infectious disease in man

There are a number of randomized, double-blind placebo-controlled trials investigating the effect of oral administration of probiotic bacteria on acute infectious diarrhoea in children. In a meta-analysis by Szajewska & Mrukowicz. (2001) the use of probiotics was associated with a significantly reduced risk (RR 0·40) of diarrhoea lasting >3 d and with a significantly reduced duration of diarrhoea (by about 1 d), particularly in rotavirus gastroenteritis. A second meta-analysis confirmed the reduction of duration of diarrhoea by about 1 d (Huang *et al.* 2002). A third meta-analysis (Van Niel *et al.* 2002) ascertained the effect of lactobacilli on diarrhoea. Compared to placebo, diarrhoea duration was reduced by 0·7 d and diarrhoea frequency on day 2 of treatment by 1·6 stools. In a double-blind, randomized, multi-centre study the duration and incidence of infectious diarrhoea in 928 children aged 6–24 months was lower in the group taking yoghurt containing *L. casei* DN-114001 compared to standard yoghurt (Pedone *et al.* 2000). Although the evidence of beneficial effects in adults is less profound (Marteau *et al.* 2001), there are reports of the efficacy of *L. rhamnosus* GG in reducing the incidence of travellers' diarrhoea (Black *et al.* 1989; Oksanen *et al.* 1990; Hilton *et al.* 1997).

In a randomized, double-blind, placebo-controlled study over seven winter months in eighteen day care centres in Helsinki, Finland, 571 healthy children aged 1–6 years received milk with or without *L. rhamnosus* GG (Hatakka *et al.* 2001). Children in the *L. rhamnosus* GG group had fewer days of absence from day care because of illness. The number of children suffering from respiratory infections with complications and lower respiratory tract infections tended to be lower in the probiotic group. Antibiotic treatments for respiratory infection were also reduced. These findings were confirmed by another double-blind, placebo-controlled randomized trial (Weizman *et al.* 2005) in 201 healthy term infants aged 4–10 months using *Bifidobacterium lactis* BB-12 or *L. reuteri* ATCC 55 730 as probiotic for 12 weeks. Both probiotic groups had fewer and shorter febrile and diarrhoea episodes. The duration of respiratory illnesses tended to be lower in the *L. reuteri* group. In an open, randomized controlled pilot study in 360 elderly subjects, a milk fermented with yoghurt cultures and *L. casei* DN-114001 administered during 3 weeks was compared to no intervention (Turchet *et al.* 2003). There was no difference in the incidence of winter infections (gastrointestinal and respiratory). However, duration of all pathologies was about 1 d shorter in the treatment group compared to the control group. In a double-blind, placebo-controlled randomized trial in Sweden, the administration of *L. reuteri* for 80 d resulted in a lower frequency of sick-days (Tubelius *et al.* 2005). The first double-blind randomized placebo-controlled trial providing evidence for effects of probiotics on common cold episodes was published by de Vrese *et al.* (2005). In their study *L. gasseri* PA 16/8, *Bifidobacterium longum* SP 07/3 and *Bifidobacterium bifidum* MF 20/5 were administered over at least 3 months during two winter/spring periods to 479 adults aged 18–67 years. In the probiotic group the common cold episodes were less severe and shorter and less febrile days were observed during the episodes.

An animal model for studies of preterm infant nutrition

Animal models in human infant nutrition have traditionally been dominated by rodent species like the rat and the mouse. These species have advantages in advanced breeding programmes that allow studies of a very mechanistic nature using, for example, knock out strains or other genetic modifications. However, the validity and applicability of an animal model not only depends on advanced breeding programmes, but also developmental, anatomical and physiological similarities to man. Pregnancy length for rodents like rats and mice is short (19–22 d) and the pups at birth are very immature compared with the newborn human infant. The developmental trajectory varies between different organs and tissues and the most nutritionally important organ during early development is probably the GIT. In newborn rat pups the GIT matures gradually during lactation and a particularly rapid maturation takes place during the short period of transition from milk to solid food (weaning) (Fig. 5). This includes anatomical, functional as well as immunological aspects of the GIT. Considering this developmental pattern, rodents represent animal species that are quite different from human infants in whom the GIT is more mature at birth and have a slower gut developmental profile starting long before birth and continuing during the first year of postnatal life (Fig. 5). Finally, there are some marked differences in the anatomy

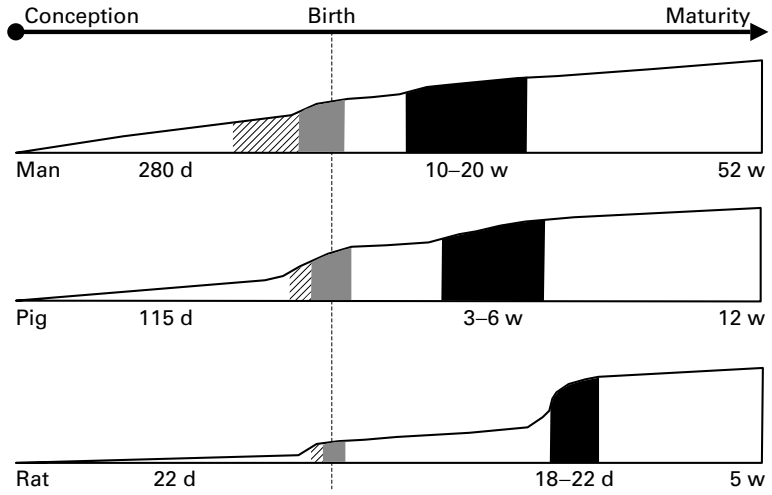

Fig. 5. The timing of gastrointestinal tract maturation in three different mammalian species. In man and other primates, gastrointestinal development is slow and maturation starts early (in fetal life). In most small rodents and carnivorous species, the developmental changes occur relatively fast and late (postnatally around weaning). Gastrointestinal maturation in large domestic animals (pigs, sheep, cattle) is intermediate, i.e. maturation is rapid during the period from shortly before birth to shortly after weaning. Around birth and during weaning, maturation is particularly rapid resulting in a 'birth cluster' (▒) and a 'weaning cluster' (■) of maturational changes. Birth of viable preterm neonates occurs over a wider range of gestational ages in man, compared with most animals (▨). d, days; w, weeks.

of the GIT reflecting that man is an omnivore while rodents are equipped with a rather large sacculated caecum–colon region that better allows intake of a herbivorous diet. Also the baboon, a popular primate model, has dietary habits and a GIT anatomy that reflect a relatively herbivorous diet. To model gut anatomy, physiology and immunology in early life and its response to nutrition, it may be more relevant to use an omnivorous animal species that better reflects GIT and nutritional development in man. The piglet may be a relevant choice. Relative to man, the pig has a shorter gestational length (114–116 d) and a more immature digestive system in the immediate postnatal period but the developmental characteristics of nutrition and GIT development reflect that in human infants at many points (Fig. 5). The pig also has a body size that easily allows extensive surgical manipulation and long-term dietary treatment protocols. Contrary to human infants, however, the pig has no trans-placental transfer of Ig from mother to fetus, and it is fully dependent on intestinal uptake of colostral antibodies from passive immunization (Sangild, 2003). Regardless, cell-mediated GIT and systemic active immunity must develop and mature to mount appropriate responses to dietary and microbial antigens.

In the perinatal period, introduction of enteral food mediates maturational changes in the GIT. The changes are primarily reflected in rapid intestinal growth and changes in functional parameters like digestive enzyme activity, nutrient absorption and GIT immune function. As breast-feeding is initiated and maintained, the intestine continues to develop and adapt to enteral food, bacterial colonization and life outside the uterus. GIT development during this later phase is more gradual than around birth and probably reflects a slow transition from milk-based nutrition towards nutrition based on solid food. Considering the overall developmental programme of the GIT (see Fig. 5), solid food probably induces the most dramatic maturational changes in immature-at birth species like rodents, while the changes are more gradual in pigs and man.

The nutritional and immunological transition from parenteral nutrition in a sterile environment before birth to enteral nutrition in a microbial environment requires a well-coordinated adaptation in many body systems. Following birth at full term, this transition generally occurs without complications, while at preterm birth there are major adaptational challenges related to GIT function, inflammation and immunology (Sangild *et al.* 2006). Preterm birth of viable offspring is possible much earlier in gestation for long-gestation species like man, while in pigs, and particularly rats, preterm neonates are only viable after modest (pig) or very short (rodents) reductions in fetal age (Fig. 5). Recently an animal model based on formula-fed, caesarean-delivered preterm pigs has been shown to mimic the pathological changes in the gut similar to what is known as necrotizing enterocolitis in human infants (Sangild *et al.* 2002, 2006; Bjornvad *et al.* 2005). Further, studies with fetal pigs *in utero* and with germ-free preterm neonatal pigs, have shown that the inflammatory responses depend on bacterial colonization, and appear to reflect an uncoordinated immunological response to bacterial colonization (Bjornvad *et al.* 2005). The physiological similarity of preterm pigs to preterm human infants, and the spontaneous nature of the model, makes this an example of an animal model that can be used to study neonatal nutrition and immunity using novel *in vivo* experimental approaches.

PUFA, immune responses and allergic disease

PUFA and allergic disease

The timescale of the increased prevalence of atopic disease matches the period over which large changes in the type of fat consumed have occurred in western populations. There has been a decrease in the intake of saturated fatty acids and an increase in the intake of the *n*-6 family of PUFA, mainly as the plant-derived linoleic acid. Linoleic acid (18 : 2*n*-6) is by far the main PUFA and the main *n*-6 PUFA in the western

diet. It is the precursor of arachidonic acid (20 : 4*n*-6), the principal substrate for the synthesis of 2-series prostaglandins and thromboxanes (via cyclooxygenase enzymes) and of 4-series leukotrienes (via 5-lipoxygenase). The 4-series leukotrienes are recognized mediators of allergic inflammation: LTB_4 is able to induce vascular permeability, leucocyte chemotaxis, respiratory burst and production of inflammatory cytokines while the cysteinyl leukotrienes are able to promote smooth muscle contraction and mucous secretion (Lewis *et al.* 1990). In addition to pro-inflammatory effects such as induction of fever, pain and vascular permeability, prostaglandin E_2 exerts effects on the Th1/Th2 balance. It decreases the production of the Th1-type cytokines IFN-γ and IL-2, enhances the production of Th2-type cytokines IL-4 and IL-5, and promotes IgE synthesis by B cells (see Calder, 2003*a*,*b*; Prescott & Calder, 2004 for references). On this basis, a hypothesis has been proposed that links increased intake of the *n*-6 PUFA linoleic acid with increased prevalence of allergic disease, via enhanced arachidonic acid and prostaglandin E_2 production (Hodge *et al.* 1994; Black & Sharpe, 1997). There is some epidemiological evidence in support of this hypothesis (Akerblom *et al.* 1985; Poysa *et al.* 1991; Von Mutius *et al.* 1998; Bolte *et al.* 2001, 2005; Trak-Fellermeier *et al.* 2004; Sausenthaler *et al.* 2006).

There is a second major family of PUFA, the *n*-3 PUFA. The major *n*-3 PUFA in the diet is α-linolenic acid (18 : 3*n*-3) which is plant derived. The metabolic derivatives of α-linolenic acid, EPA (20 : 5*n*-3) and docosahexaenoic acid (DHA; 22 : 6*n*-3) are found in significant amounts in fish and other seafood, especially fatty fish like salmon, herring, tuna and sardines. These *n*-3 LCPUFA are also found in 'fish oils' which are commercially available and have been used in many dietary studies in animals and man. Increased consumption of *n*-3 LCPUFA results in their incorporation into immune cells (see Calder, 2001, 2006 for references). This incorporation occurs largely at the expense of arachidonic acid, so decreasing the availability of the substrate for prostaglandin E_2 formation. Indeed, dietary consumption of *n*-3 LCPUFA has been shown to result in decreased production of prostaglandin E_2 and other eicosanoids, including 4-series leukotrienes, by human monocytes and neutrophils (see Calder, 2001, 2006 for references). Thus, it has been proposed that *n*-3 LCPUFA will be protective towards allergic disease (Hodge *et al.* 1994; Black & Sharpe, 1997). There is some epidemiological evidence to support a protective role of dietary *n*-3 LCPUFA (Hodge *et al.* 1996). Furthermore, *n*-3 LCPUFA status was lower in cord blood serum from pregnancies of allergic compared with non-allergic mothers (Yu *et al.* 1996), higher *n*-3 LCPUFA in breast milk were associated with decreased risk of infant atopy (Duchen *et al.* 1998), and *n*-3 LCPUFA status was lower in serum of asthmatic and/or atopic teenagers compared with age-matched controls (Yu & Bjorksten, 1998).

The recognition that *n*-3 LCPUFA act to decrease production of arachidonic acid-derived eicosanoids has resulted in a number of trials of dietary supplementation with fish oil in patients with asthma. The details of these are discussed in great detail elsewhere (Thien *et al.* 2002; Schachter *et al.* 2004). Thien *et al.* (2002) included eight studies published between 1988 and 2000 in a systematic review. They identified that there was 'no consistent effect on forced expiratory volume at one second, peak flow rate, asthma symptoms, asthma medication use or bronchia hyper-reactivity', although they noted that one study in children showed improved peak flow and reduced asthma medication use (Nagakura *et al.* 2000). A more recent report covering twenty-six studies (both randomized, placebo-controlled and others) concluded that 'no definitive conclusion can yet be drawn regarding the efficacy of *n*-3 fatty acid supplementation as a treatment for asthma in children and adults' (Schachter *et al.* 2004), although studies by Broughton *et al.* (1997) in adults and Nagakura *et al.* (2000) in children indicate that there may be subgroups of asthmatic subjects who may benefit greatly from *n*-3 LCPUFA. Clearly more needs to be done in this area. However, since allergies appear to be determined in early life or even *in utero* (Yabuhara *et al.* 1997; Prescott *et al.* 1998; Jones *et al.* 2000; Warner *et al.* 2000; Blumer *et al.* 2005), *n*-3 LCPUFA intervention once allergic disease is established may be too late. It is possible that fatty acids have a stronger impact on fetal and neonatal Th1/Th2 immune responses as compared to immune responses beyond early infancy. Evidence that early exposure to *n*-3 LCPUFA may alter T cell cytokine profiles later on comes from the observation that $2\frac{1}{2}$-year-old children who had been breast fed for the first 4 months of life by mothers with a high intake of *n*-3 LCPUFA had a significantly higher production of IFN-γ upon stimulation of whole blood compared to a control group with low maternal *n*-3 LCPUFA intake during lactation (Lauritzen *et al.* 2005). This observation suggests long-term immunologic effects of early exposure to *n*-3 LCPUFA.

One study that links increased consumption of *n*-3 LCPUFA in early infancy to clinical outcome is the Childhood Asthma Prevention Study. In this study, infants at risk of developing asthma received fish oil providing about 150 mg EPA + DHA/d or placebo from age 6 months. Although there was no effect of fish oil on sensitization to inhaled or ingested allergens, determined by skin prick testing at 18 months of age, there was a significantly decreased prevalence of wheeze ever and wheeze for >1 week and a trend for decreased visits to the doctor for wheeze in the fish oil group (Mihrshahi *et al.* 2003). There was no effect of fish oil on other wheeze indicators, diagnosed asthma, dermatitis or medication use. Further analysis of data at this age revealed that higher plasma levels of total *n*-3 PUFA were associated with reduced wheeze, visits to the doctors because of wheeze, cough during sleep and bronchodilator use (Mihrshahi *et al.* 2004). There was no effect of fish oil on total serum IgE at 18 or 36 months of age (Mihrshahi *et al.* 2004; Peat *et al.* 2004). At 36 months of age there was no effect of fish oil on prevalence of asthma, wheeze, atopic dermatitis or sensitization to ingested or inhaled allergens, although fish oil decreased the prevalence of cough and atopic cough (Peat *et al.* 2004).

Although intervention with *n*-3 LCPUFA in the neonatal period may alter cytokine profiles (Lauritzen *et al.* 2005) and possibly disease outcome in later infancy, the *in utero* period may offer an even greater opportunity to derive benefit from increased *n*-3 LCPUFA exposure. To examine the effect of very early exposure to *n*-3 LCPUFA on T cell cytokine production, mice were fed on diets containing a high amount of either saturated fat (and no *n*-3 LCPUFA) or fish

oil throughout pregnancy and lactation. The offspring were killed at weaning (3 weeks of age). Spleen cells from the offspring of dams fed on fish oil had higher IFN-γ production and lower IL-5 production compared to those from the offspring of dams fed on saturated fat (H Frokiaer, unpublished results). Thus, this animal experiment resulted in the same polarization as observed for children that had received high *n*-3 LCPUFA during lactation (Lauritzen *et al.* 2005).

The effects of increased maternal consumption of fish oil (providing 1·1 g EPA + 2·2 g DHA/d) from week 20 of pregnancy until delivery upon cord blood cytokine levels and production in response to stimulation and upon later disease outcome were reported by Dunstan *et al.* (2003*a*,*b*) in a randomized, placebo-controlled study in women whose babies would be at increased risk of allergic disease. Cord plasma from the fish oil group was less likely to exhibit a detectable level of IL-13 and plasma IL-13 concentrations were significantly lower in the fish oil group (Dunstan *et al.* 2003*a*). Cord plasma IL-13 concentrations were inversely related to cord blood red cell *n*-3 LCPUFA content, especially that of DHA (Dunstan *et al.* 2003*a*). Cord blood mononuclear cell cytokine (IFN-γ, IL-13, IL-10, IL-5) responses to stimulation by various allergens were lower in the fish oil group, although this was significant only for IL-10 production in response to house dust mite or cat hair extract (Dunstan *et al.* 2003*b*). Clinical outcomes in the infants were reported at 1 year of age (Dunstan *et al.* 2003*b*). Infants of mothers in the fish oil group were less likely to be sensitized to a range of allergens, determined by skin prick testing, in particular to egg, and were significantly less likely to have severe atopic dermatitis. The so-called Nutraceuticals for a Healthy Life (NUHEAL) study (Decsi *et al.* 2005) investigated whether supplementation of pregnant women with the *n*-3 LCPUFA (150 mg EPA + 500 mg DHA/d) from week 22 of pregnancy until delivery alters fetal Th1/Th2-related parameters. mRNA transcripts for Th1-associated (IFN-γ, CX chemokine receptor 3), Th2-associated (IL-4, IL-13, chemoattractant receptor homologous-molecule expressed on T-helper-type-2 cells, chemokine receptor 4) and inflammatory (TNF-α, IL-1, TGF-β) proteins were quantified in cord and maternal peripheral blood at delivery in a multicentre (Spain, Hungary, Germany), randomized, double-blind, placebo-controlled trial. Decreased levels of IL-13 and chemokine receptor 4 mRNA and increased levels of TGF-β mRNA were found in the cord blood of the *n*-3 LCPUFA-supplemented group as compared to the group without additional *n*-3 LCPUFA (S Krauss-Etschmann, unpublished results). In maternal peripheral blood obtained at delivery, mRNA levels for IL-1β, TNF-α and IFN-γ were decreased in the group receiving *n*-3 LCPUFA. Similar to cord blood, increased TGF-β mRNA levels were present. Cord blood IL-13 and chemokine receptor 4 mRNA levels correlated inversely with cord plasma DHA levels, while maternal peripheral blood IL-1β mRNA was correlated inversely with maternal plasma DHA at delivery. In a subcohort, it was identified that the frequencies of chemokine receptor 4^{+}T cells and natural killer cells in cord blood were significantly lower in the *n*-3 LCPUFA group. The data obtained by both Dunstan *et al.* (2003*a*,*b*) and from the NUHEAL study indicate that increased maternal intake of *n*-3 LCPUFA during pregnancy does not selectively affect either Th1 or Th2 responses. Rather, it is possible that 'dominant' immune responses (i.e. Th2 responses in neonates and Th1 responses in adults) might be primarily targeted. How this occurs is unclear at present. However, the increased TGF-β mRNA levels of both cord and maternal peripheral blood seen in the NUHEAL study indicate that certain types of adaptive Treg cells might be induced. It remains to be explored whether altered cytokine patterns at birth might translate into clinical consequences.

Very-long-chain n-*3 PUFA, T cell function and T cell signalling*

The influence of various fatty acids including *n*-3 LCPUFA on T cell functional responses and signalling has been studied for many years (see Calder *et al.* 2002; Stulnig, 2003; Stulnig & Zeyda, 2004 for reviews). A number of mechanisms by which fatty acids can affect T cells have been identified (Fig. 6). First, as indicated earlier, arachidonic acid-derived eicosanoids such as prostaglandin E_2 influence the activity of DC, the differentiation of naïve T cells and the activity (proliferation, cytokine production) of Th1 and Th2 cells (Tilley *et al.* 2001). Thus, fatty acids could affect T cell functions through increasing or decreasing production of eicosanoids from arachidonic acid. *n*-3 LCPUFA decrease production of these mediators (see Calder, 2003*b*, 2006 for references). Furthermore, EPA is a substrate for cyclooxygenase and lipoxygenase enzymes giving rise to eicosanoids of altered structure and reduced biological potency (Goldman *et al.* 1983; Lee *et al.* 1984; Bagga *et al.* 2003). Recent studies have demonstrated that EPA and DHA also give rise to a novel family of eicosanoid-like mediators, termed E- and D-resolvins, respectively, that have been shown in cell culture and animal models to be anti-inflammatory and inflammation resolving (see Serhan *et al.* 2004; Serhan, 2005 for reviews). Thus, *n*-3 LCPUFA have the potential to induce complex changes within the mix of eicosanoid-type mediators that are produced. Therefore, the functional outcome of these changes with respect to immunomodulation is not predictable due to parallel pro- and anti-inflammatory effects of the mediators concerned.

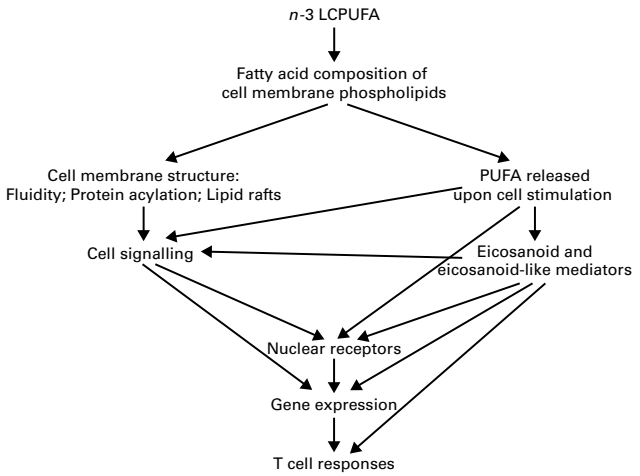

Fig. 6. Mechanisms by which very-long-chain *n*-3 PUFA (*n*-3 LCPUFA) might affect the function of T cells.

A second mechanism for the modulation of immune responses by fatty acids is by direct alteration of gene expression through modification of transcription factor activity (Fig. 6). This can be achieved by either direct interaction with ligand-binding transcription factors, so-called nuclear receptors, or by interference with membrane or cytoplasmic signalling pathways that finally lead to altered transcription factor activation. PPAR γ binds a variety of PUFA, including DHA, and their derivatives and has been shown to be involved in regulation of immune and inflammatory responses (Cunard *et al.* 2002; Valledor & Ricote, 2004). Other nuclear receptors that could be involved in mediating the actions of *n*-3 LCPUFA on the immune system are liver X receptors α and β, that are inhibited by MUFA and PUFA, and retinoid X receptors, the heterodimer partner for several nuclear receptors that is activated by DHA with some selectivity over arachidonic acid (Mata de Urquiza *et al.* 2000). However, nuclear receptors generally lack adequate specificity for *n*-3 LCPUFA to explain the effects on the immune system.

A third mechanism of action involves the incorporation of *n*-3 LCPUFA into membrane phospholipids and subsequent modulation of membrane structure and function (Fig. 6). The fluid mosaic model of cellular membrane structure suggests that incorporation of highly unsaturated fatty acids, such as *n*-3 LCPUFA, will alter membrane microviscosity. Indeed, incorporation of both EPA and DHA into lymphocyte membranes alters their fluidity and this is strongly associated with the effect of those fatty acids on T cell proliferation (Calder *et al.* 1994). Lipid rafts are specialized microdomains of the plasma membrane that are involved in signal transduction. They are particularly rich in sphingolipids and cholesterol and the phospholipid side chains are usually highly enriched in saturated fatty acids (Pike, 2003). Cytoplasmic proteins that are covalently modified by saturated fatty acids (palmitoyl or myristoyl moieties) and cell surface proteins that are attached via a glycosyl phosphatidylinositol anchor are highly concentrated within lipid rafts. Many proteins involved in signal transduction, e.g. Src family kinases, are acylated and are predominantly found in lipid rafts (Pike, 2003). Therefore, lipid rafts are implicated in signalling processes elicited by a variety of cell surface receptors including the T cell receptor/CD3 complex. Though the exact nature of lipid rafts is still a matter of discussion, many studies suggest lipid rafts are one of several dynamic substructures of the plasma membrane that are crucial for lymphocyte activation (Zeyda & Stulnig, 2006). PUFA treatment alters lipid rafts in a particular manner. Acylated proteins that are anchored to the inner (cytoplasmic) lipid leaflet are displaced from rafts when T lymphocytes are treated with *n*-3 LCPUFA and to a somewhat lower extent also with *n*-6 PUFA whereas glycosyl phosphatidylinositol-anchored proteins remain located in lipid rafts (Stulnig *et al.* 1998). Thus, PUFA selectively alter the protein composition of the inner membrane lipid leaflet. Notably the extent of displacement of acylated proteins from lipid rafts correlates with impairment of calcium signalling indicating a functional impact of these alterations (Stulnig *et al.* 1998). PUFA readily attach to the sn-2 position of membrane phospholipids of both lipid rafts and the plasma membrane proper thereby altering raft protein localization (Stulnig *et al.* 2001). Animal data with fish oil-rich diets revealed that *n*-3 LCPUFA are readily incorporated into raft lipids thereby reducing the raft content of sphingomyelin that is also involved in stabilizing lipid rafts (Fan *et al.* 2003). Hence, incorporation of PUFA into membrane lipids is a likely mechanism for protein displacement from rafts. A number of acylated proteins play important roles in T cell signalling including Src family kinases and the adapter protein linker for activation of T cells (LAT). Phosphorylation of LAT is the most upstream step that is inhibited by *n*-3 LCPUFA treatment of T cells (Zeyda *et al.* 2003) and it appears that LAT displacement from lipid rafts is a molecular mechanism mediating inhibition of T cell responses by *n*-3 LCPUFA, at least *in vitro* (Zeyda *et al.* 2002). Importantly, animal studies have shown that dietary fish oil affects early signalling events in T cells (Sanderson & Calder, 1998) and have linked alterations of T cell lipid rafts by dietary *n*-3 LCPUFA with functional changes (Fan *et al.* 2004).

In vitro, animal feeding and human supplementation studies have frequently investigated the effects of *n*-3 LCPUFA on T cell functional responses (see Calder *et al.* 2002 for a review). Both *in vitro* and animal feeding studies have reported that *n*-3 LCPUFA inhibit T cell proliferation, production of IL-2 and IFN-γ, and surface expression of CD25 (Calder *et al.* 1991; Calder & Newsholme 1992*a*,*b*; Yaqoob *et al.* 1994; Sanderson *et al.* 1995; Wallace *et al.* 2001). These effects are consistent with the inhibition of T cell signalling by *n*-3 LCPUFA referred to earlier. High-dose fish oil supplementation studies in healthy adult human subjects also report decreased *ex vivo* T cell proliferation and IL-2 and IFN-γ production (Meydani *et al.* 1991; Gallai *et al.* 1993), although several studies report no effect on these outcomes (e.g. Yaqoob *et al.* 2000) and one study has reported that moderate dose fish oil increases *ex vivo* T cell proliferation and IFN-γ production (Trebble *et al.* 2003). The studies of Dunstan *et al.* (2003*a*,*b*), Lauritzen *et al.* (2005) and the NUHEAL researchers indicate that early exposure to *n*-3 LCPUFA may either decrease or increase production of cytokines, including IFN-γ, by T cells. Thus, the actions of *n*-3 LCPUFA may differ according to the exact situation. This may be related to the dose used, since *in vitro* studies have clearly demonstrated strong dose-dependent effects (Calder *et al.* 1991; Wallace *et al.* 2001). Indeed, Wallace *et al.* (2001) reported an enhancement of IL-2 production by murine splenocytes *in vitro* with low concentrations of EPA and DHA and an inhibition with high concentrations. The mode of presentation of the fatty acid may also be important, and the mode of stimulation of the T cell (mitogen, antibody, antigen-presenting cell) is also likely to be a contributory factor to different findings. One important factor in determining long-term *in vivo* effects could be that *n*-3 LCPUFA may influence the number and/or activity of certain subpopulations of cells which could affect subsequent maturation and/or polarization of the immune system.

Conclusions, research gaps and research opportunities

The prevalence of allergic disease has markedly increased. Today in some European countries one in ten children suffers from asthma, and one in three shows allergic sensitization (Kabesch *et al.* 1999). The period of time over which this increase has occurred coincides with changes in many lifestyle factors, including nutritional habits and dietary patterns, which may have altered exposure to factors that influence maturation

of the immune system and microbial colonization of the gut. Strategies that alter the pattern of the gut microbiota, such as provision of certain species and strains of probiotic bacteria or modification of the diet to encourage growth of those types of bacteria, may alter immunologic signals that induce tolerance to food and environmental antigens. Several studies have associated dietary intake of various types of fatty acid with risk of the development of allergic diseases, but overall the available data are equivocal. Better assessment of PUFA status by use of biomarkers, such as serum and cell contents, which reduce the intrinsic imprecision of dietary intake assessments, and improved understanding of the role of modification of genes encoding fatty acid-metabolizing enzymes are expected to improve elucidation of the relation between PUFA status and atopic disease manifestations (Schäffer *et al.* 2006).

Since epidemiological studies indicate that the route of birth delivery is important for the priming of allergies, the type of initial bacterial colonization of the newborn may direct immune responses towards a phenotype that is protective against or increases the risk of developing atopic disease. To elucidate the role of the bacterial environment and/or the composition of the neonatal gut flora in the first days of life in the priming of the neonatal immune system, it will be necessary to characterize the precise interaction of microbes with the immune system. Thus, further to the role ascribed to DC-SIGN, additional receptors involved in the recognition of probiotic bacterial strains will have to be identified. In addition, the corresponding bacterial ligand molecules need to be identified as well as the ensuing effector T cell responses. In this respect, not only probiotic bacteria, but also commensal bacteria without the properties of probiotic strains, need further investigation. The extent to which whole bacteria can be replaced by single bacterial components is unclear at present. Nevertheless, it might be easier to define pharmaceutical properties of single molecules as compared to whole living organisms. Although a clear association between early colonization with *S. aureus* and the frequency of Treg cells has been demonstrated, the contribution of other commensal gut bacteria and/or their components to the regulation of immune responses warrants further elucidation. How colonization with *S. aureus* could lead to an increased activity of Treg cells is unclear at present. Since it has been shown that gut epithelial cells participate in the regulation of enteral immune responses, establishing methods to investigate a three-partner communication amongst bacteria, immune cells and other cell types at the enteral interface will provide an opportunity to study the complex interactions between nutrition and immunity.

Mouse models and epidemiological studies indicate that the balance of routes of allergen exposure (e.g. oral *v.* skin *v.* inhaled) determine if food allergens are tolerated or not. Hence, the measurement of environmental food allergens in relation to the risk of developing atopic and allergic manifestations, together with detailed descriptive studies of infant and childhood diets and weaning practices in different parts of the world, and the relationship of these feeding patterns to immunological and clinical outcomes need to be studied. Furthermore, the characterization of genetic haplotypes that modulate the effect of dietary exposure on atopic and allergic manifestations, and their effect sizes, will have to be determined. The evaluation of sublingual immunotherapy for induction of oral tolerance towards cow's milk shows promising results, but needs further investigation in larger studies.

While human studies allow the identification of associations between bacterial exposure and occurrence of allergic disease, animal models provide tools to investigate the underlying mechanisms. Thus, data from epidemiologic studies indicating that a farming environment during early or even prenatal life may protect from allergies (Braun-Fahrlander, 2000; Alfven *et al.* 2006) have been transferred into a mouse model. Perinatal application of both lipopolysaccharide and oral *Lactobacillus* GG to pregnant mice is associated with reduced pulmonary allergic responses in the offspring thus confirming the observations made in man. How these effects can be transmitted from the mother to the fetus needs further investigation.

Administration of *n*-3 LCPUFA to pregnant women has been suggested to modulate neonatal immune responses towards an immune phenotype that may be protective towards allergy. Although preliminary experimental evidence exists for this, it remains to be determined whether these immune changes translate into clinical consequences. Furthermore, there are indications that maternal and neonatal immune responses are affected differentially by *n*-3 LCPUFA depending on the underlying predominant immune response. It is possible that effects of these fatty acids are mediated at least in part indirectly through subsets of regulatory immune cells, such as adaptive or naturally occurring Treg cells or natural killer T cells.

PUFA exert their immunomodulatory activities at several levels. Among these, lipid rafts have recently gained much attention. Lipid rafts are specialized microdomains within the plasma membrane that ensure proper T cell signalling. Recently, it was shown that lipid rafts can be modified by PUFA treatment in such a way that proteins are dislocated from the inner leaflet of the plasma membrane. Since a number of these proteins are involved in T cell signalling, these findings explain to some extent the functional changes of T cells after *n*-3 LCPUFA treatment. However, the relative contribution of PUFA metabolites, altered gene expression and modified lipid raft composition to the resulting T cell response as well as other immune cells is unclear at present.

In summary, the Workshop showed that the field of early nutrition and its impact on early childhood immune responses is a highly active one and that significant progress has been made. Several scientific issues were identified to be of high interest for future research. It is most clear that it is highly important to identify the appropriate time window that allows modulation of immune responses and subsequent disease risk. Further studies as outlined earlier will increase scientific knowledge and potentially allow the development of precise disease prevention strategies.

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