Welcome

We are pleased to provide you with the Proceedings of the 109th Abbott Nutrition Research Conference entitled “Mucosal Immune Development and Dietary Influences.” The goal of the Abbott Nutrition Research Conferences is to connect the latest in nutrition science and research with the practice of clinical nutrition. Our belief is that translation of leading-edge science into clinical nutrition practice plays an important role in the future of healthcare.

The purpose of this conference is to explore the components of the mucosal immune system and examine how diet influences its development and function. Because an estimated 70% of the immune system resides in the digestive tract, the mucosal immune system represents a key interface between the environment and the body, and it imparts health benefits beyond the gut.

Specific objectives of the conference include an in-depth review of research related to cellular components of the immune system in the small intestine vs the large intestine; development and regulation of the mucosal immune system; the role of the gut microbiome in immune signaling in health and disease; and the clinical impact of gut-modulating dietary factors such as prebiotics, probiotics, synbiotics, and fatty acids on health outcomes.

The conference tone was set in an introductory overview of the complexity and elegance of mucosal immunology, the gateway to the immune system. The overview was followed by presentations on several related topics: the role of dendritic cells and T cells in mucosal immunity, the immunomodulatory effects of microflora, and the clinical relevance and impact of such dietary factors as prebiotics, probiotics, fatty acids, and early nutrition on mucosal immunity. Robust discussions of these topics were led by several conference participants.

Together, these findings suggest that interactions between the mucosal immune system and the gut microbiome may play a role in certain conditions or disease states, including respiratory health, allergic disease, obesity, and inflammatory bowel disease. Furthermore, the findings indicate that nutritional modulation of the mucosal immune system may have an impact on health, both within the gut and beyond. We hope you find these proceedings informative and that they increase your understanding of the role of nutrition on the development and function of the mucosal immune system.

Divisional Vice President..................................................Robert Miller, PhD
Abbott Nutrition Global R&D and Scientific Affairs

Senior Manager, Science Programs.................................Rosemary Riley, PhD, LD
Abbott Nutrition Health Institute

Senior Medical Director, Pediatric Nutrition.......................Larry Williams, MD
Abbott Nutrition Global R&D and Scientific Affairs
Abbott Nutrition Research Conferences

This Conference is one of a series designed to promote the correlation of findings from recent research on nutrition and health and to stimulate further research by the exchange of information. Abbott Nutrition publishes this report to make the information and concepts developed during the Conference more widely available.

Divisional Vice President
Abbott Nutrition Global R&D and Scientific Affairs
Robert Miller, PhD

Senior Manager of Science Programs
Abbott Nutrition Health Institute
Rosemary Riley, PhD, LD

General Editor
Judith Gussler, PhD

Senior Medical Director, Pediatric Nutrition
Abbott Nutrition Global R&D and Scientific Affairs
Larry Williams, MD

© 2009 Abbott Nutrition
Printed in USA


Abbott Nutrition is privileged to be associated with the production and provision of this information to members of the medical profession. Compilation and publication of this information constitute neither approval nor endorsement by Abbott Nutrition or Abbott Laboratories of the opinions, inferences, findings, or conclusions stated or implied by the authors in the presentations.

Abbott Nutrition
Columbus, Ohio 43215
Division of Abbott Laboratories, USA
Mucosal Immune Development and Dietary Influences

The 109th Abbott Nutrition Research Conference was held in Columbus, Ohio, on November 13 and 14, 2008.

Acronyms and Abbreviations ................................................................. iv

Mucosal Immunology: The Gateway to the Immune System .................. 1
Per Brandtzaeg, PhD, University of Oslo, Norway

The Role of Dendritic Cells in Immunity and Tolerance in the Intestine ...... 14
Stephen McSorley, PhD, University of Minnesota, USA

Flora-Induced Immunoregulation of Allergic and Inflammatory Disease ....... 23
Cathryn Nagler, PhD, Harvard Medical School, USA

Discussion ........................................................................................................... 28
Leader: Cathryn Nagler, PhD, Harvard Medical School, USA

Role of Th1/Th2/Th3/Regulatory T Cells in Mucosal Immune Development ...... 37
Fernando Chirdo, PhD, Universidad Nacional de La Plata, Argentina

Role of Commensal Flora in Mucosal Immune Development ..................... 48
Kathy McCoy, PhD, McMaster University, Canada

Microbial Components as Modulators of Mucosal Immunity...................... 57
Jim Versalovic, MD, PhD, Baylor College of Medicine, USA

Discussion ........................................................................................................... 66
Leader: John Bienenstock, MD, McMaster University, Canada
Early Nutrition, Including the Role of Breast Milk, and Modulation of Tolerogenic and Immunogenic Responses.........................................................71
Ricardo Rueda, MD, PhD, Abbott Nutrition, Spain

Clinical Evidence for the Role of Prebiotics in Mucosal Immune Development and Impact of Respiratory Health and Allergy.................................82
Guido Moro, MD, University of Milan, Italy

Clinical Evidence for the Role of Probiotics in Mucosal Immune Development and Impact on Respiratory Health and Allergy: Probiotics in the Treatment and Prevention of Infantile Allergy .........................................................93
Mikael Kuitunen, MD, PhD, University of Helsinki, Finland

Clinical Evidence for the Role of Dietary Fatty Acids in Mucosal Immune Development: Mechanism of Action and Impact on Respiratory Health and Allergy ..............................................................................104
Paul Noakes, PhD, University of Southampton, United Kingdom

Discussion...............................................................................................................109
Leader: Lee Yuan Kun, PhD, National University of Singapore
### Acronyms and Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AD</td>
<td>atopic dermatitis</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>ATRA</td>
<td>all trans retinoic acid</td>
</tr>
<tr>
<td>CB</td>
<td>human umbilical cord blood</td>
</tr>
<tr>
<td>CCR6</td>
<td>chemokine receptor 6</td>
</tr>
<tr>
<td>CMI</td>
<td>cell-mediated immunity</td>
</tr>
<tr>
<td>CRF</td>
<td>corticotrophin-releasing factor</td>
</tr>
<tr>
<td>CRP</td>
<td>c-reactive protein</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
</tr>
<tr>
<td>DT</td>
<td>diptheria toxin</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EHF</td>
<td>extensively hydrolyzed formula</td>
</tr>
<tr>
<td>EPA</td>
<td>eicosapentaenoic acid</td>
</tr>
<tr>
<td>FcRn</td>
<td>neonatal Fc receptor</td>
</tr>
<tr>
<td>GALT</td>
<td>gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GINI</td>
<td>German Infant Nutritional Intervention</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal (axis)</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin (eg, IgA, IgE, IgM, IgG)</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>lcFOS</td>
<td>long-chain fructooligosaccharides</td>
</tr>
<tr>
<td>LCPUFA</td>
<td>long-chain polyunsaturated fatty acid</td>
</tr>
<tr>
<td>LGG</td>
<td><em>Lactobacillus rhamnosus</em> GG</td>
</tr>
<tr>
<td>LP</td>
<td>lamina propria</td>
</tr>
<tr>
<td>LPDC</td>
<td>lamina propria dendritic cell</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MALA</td>
<td>mucosa-associated lymphoid tissue</td>
</tr>
<tr>
<td>MAMPS</td>
<td>microbe-associated molecular patterns</td>
</tr>
<tr>
<td>MHC</td>
<td>major histo-compatibility</td>
</tr>
<tr>
<td>MIPS</td>
<td>Multicentre Immuno Programming Study</td>
</tr>
<tr>
<td>MLN</td>
<td>mesenteric lymph node</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NALT</td>
<td>nasopharynx-associated lymphoid tissue</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>PAMPS</td>
<td>pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PC</td>
<td>plasma cell</td>
</tr>
<tr>
<td>pDC</td>
<td>plasmacytoid DC</td>
</tr>
<tr>
<td>pIgR</td>
<td>polymeric Ig receptor</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PPs</td>
<td>Peyer's patches</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>SC</td>
<td>(membrane) secretory component</td>
</tr>
<tr>
<td>scGOS</td>
<td>short-chain galactooligosaccharides</td>
</tr>
<tr>
<td>SlgA</td>
<td>secretory IgA (also SlgM)</td>
</tr>
<tr>
<td>SPF</td>
<td>specific pathogen-free</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>Th1</td>
<td>type 1 helper T cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cells</td>
</tr>
<tr>
<td>TSLP</td>
<td>thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitors of metalloproteinase</td>
</tr>
</tbody>
</table>
Mucosal Immunology: The Gateway to the Immune System

Overview of the Innate and Adaptive Mucosal Immune System: Linking Immune Development in the Gut to Other Mucosal Sites and the Periphery

Per Brandtzaeg, PhD, University of Oslo, Norway

The power of our immune system is a result of co-evolution in which commensal bacteria and parasites have shaped the mechanisms of both innate and adaptive immunity. Notably, the adaptive immune system developed rather late in the phylogeny, and most species survive without it. Mammals, however, possess extremely sophisticated adaptive defense mechanisms of both systemic and mucosal type. A great redundancy exists in both immune systems, providing robustness to preserve homeostasis.

Innate and Adaptive Immunity

The attempt of a microbe to invade the body is immediately counteracted by the innate immune defense, which comprises surface barriers (epithelial linings, mucus, ciliary function, and peristalsis), soluble factors (pH of body fluids, antimicrobial peptides, and proteins), phagocytes (granulocytes and macrophages), and dendritic cells (DCs), which are specialized for presenting antigens to T cells. These mechanisms restrict invasion of the body by foreign components and inhibit their persistence within the tissue. Such challenge of the innate system often leads to inflammation but at the same time to activation of the adaptive system, which by its effector T cells, regulatory T (Treg) cells, and B cells aids the recovery from a noxious impact.

In contrast to the antigen-specific surface receptors of T and B cells, which show a random and highly diverse repertoire, the recognition molecules for innate immunity are encoded in the germline. This system is therefore quite similar among healthy individuals but receptor mutations may give rise to disease-promoting polymorphism. Innate responses show no classical memory—that is, re-exposure to a pathogen normally elicits the same type of potentially proinflammatory reaction, although downregulation in the face of subsequent infection has been observed, probably to preserve tissue integrity.
Mucosal Immunology: The Gateway to the Immune System

The innate receptors sense conserved structures that are essential for microbial survival and present in a broad range of microbes, including endotoxin (or lipopolysaccharide [LPS]), teichoic acids, and unmethylated CpG motifs of DNA. Such structures generally are referred to as pathogen-associated molecular patterns (PAMPs), but they also occur in commensal bacteria and should preferably be referred to as microbe-associated molecular patterns (MAMPs). The intestinal microbiota induces distinct programming of the innate immune system, which could partly explain tolerance by the host. Such a tolerogenic response is especially apparent for mucosal epithelia.

The cellular receptors that recognize PAMPs/MAMPs are called pattern recognition receptors (PRRs), many of them belonging to the so-called Toll-like receptors (TLRs). PRRs are expressed by macrophages and DCs, as well as by a variety of other cell types such as T cells, B cells, and epithelial cells. The engagement of PRRs may cause cellular activation (mainly via the transcription factor nuclear factor-kappa B [NF-κB]), which in the case of antigen-presenting cells (APCs) such as DCs leads to maturation accompanied by production of cytokines and upregulation or downregulation of cell-surface molecules according to strictly defined kinetics.

Engagement of other types of receptors on phagocytes, including immunoglobulin (Ig) Fc receptors and complement receptors, triggers phagocytosis and elimination of invading microorganisms. Although pathogens have evolved mechanisms to evade the innate defense (eg, bacterial capsules), they will usually be eliminated when an adaptive response reinforces innate immunity by providing specific antibodies directed against the invading pathogen or its toxins. Thus, innate immunity influences the character of the adaptive response, and the effector arms of adaptive immunity support several innate defense mechanisms. The nonspecific biological amplification collectively triggered by them is referred to as immune reaction, or hypersensitivity/allergy if clinical harm is observed as inflammatory disease.

Strategies of the Mucosal Immune System

The mucosal immune system provides a first defense line that reduces the need for elimination of invading exogenous antigens by proinflammatory systemic immunity. To maintain homeostasis, the mucosal immune system has, through evolution, developed two layers of adaptive noninflammatory defense: (a) immune exclusion provided primarily by secretory antibodies to limit epithelial contact and penetration with host invasion of microorganisms and other potentially dangerous antigens, and (b) immunosuppressive mechanisms to inhibit overreaction against innocuous luminal antigens (Fig 1).

2
The latter strategy, which is referred to as “oral tolerance” when induced via the gut, depends largely on the development of Treg cells in mesenteric lymph nodes to which mucosal DCs carry dietary and microbial antigens and become conditioned for induction of Treg cells. Mucosally induced tolerance probably involves additional suppressive mechanisms, which together with Treg cells, contribute to the fact that overt and persistent hypersensitivity to food is relatively rare. A similar downregulatory tone of the immune system normally exists against commensal bacteria.

Mucosal tolerance appears rather robust in view of the fact that more than a ton of food may pass through the gut of an adult every year. After a meal, intact dietary antigens are taken up in the nanogram range, usually without causing harm.
Mucosal Immunology: The Gateway to the Immune System

However, the neonatal period is critical, both with regard to infections and to priming for allergic disease, because the epithelial barrier and the immunoregulatory network are poorly developed.\textsuperscript{10,11}

Experiments have demonstrated a crucial role of microbial colonization in establishing\textsuperscript{12} and regulating\textsuperscript{13} the epithelial barrier. At least in mice, the beneficial effects of commensal bacteria on the barrier function are largely mediated via PRRs expressed by the gut epithelium, particularly TLRs.\textsuperscript{14,15} Polarized epithelial cells have the ability to dampen the proinflammatory effect of PRR-mediated signals coming from the luminal side.\textsuperscript{5} However, after bacterial invasion, PRR signaling from the basolateral side results in a high level of NF-\kappa B activation, with enhanced release of defensins to combat the infection.\textsuperscript{5}

Secretory Immunity Reinforces the Epithelial Barrier

The mucosal and systemic immune systems differ in many structural, cellular, molecular, and functional ways.\textsuperscript{16} Mucosal immunity is most abundantly expressed in the gut, and the intestinal mucosa of an adult contains at least 80% of the body’s activated B cells—terminally differentiated to plasmablasts and plasma cells (PCs).\textsuperscript{17} Thus, the gut is by far the largest antibody-producing organ in the body.

Most mucosal PCs produce dimeric IgA, which along with pentameric IgM that likewise contains a polypeptide called “joining” (J) chain, can be actively exported by secretory epithelia.\textsuperscript{17} This external transport is mediated by the polymeric Ig receptor (pIgR), also known as membrane secretory component, or SC.\textsuperscript{18} Immune exclusion is performed mainly by secretory (S)IgA, and to a lesser extent SlgM, in cooperation with innate nonspecific defenses (Table and Fig 2). In newborns and people with selective IgA deficiency, SlgM antibodies are of greater importance than in healthy adults.\textsuperscript{19}
Table. Antimicrobial Effects of SlgA Antibodies
Used with kind permission of Springer Science+Business Media.

- SlgA is dimeric/polymeric, therefore, exerting efficient microbial agglutination and virus neutralization
- SlgA performs noninflammatory extracellular and intracellular immune exclusion by inhibiting epithelial adherence and invasion
- SlgA exhibits cross-reactive (“innate-like”) activity and provides cross-protection in the herd
- SlgA (particularly SlgA2) is quite stable (bound SC stabilizes both isotypes of IgA)
- SlgA is endowed with mucophilic and lectin-binding properties (via bound SC in both isotypes and mannose in IgA2)

- SlgA exerts both cross-reactive and infection- or vaccine-induced specific protection against epithelial invasion
- SlgA also can exert noninflammatory effects inside of secretory epithelia (neutralization of virus and endotoxin)
- IgA dimers (pIgA) and IgM pentamers perform antigen (Ag) excretion (clearance of lamina propria)
- SlgA antibodies play no protective role following invasion of infectious agents (systemic immunity must take over)

Fig 2. Different principles of SlgA-mediated contribution to mucosal homeostasis. In addition to immune exclusion at the epithelial surface, the plgR-mediated external transport of dimeric IgA and pentameric IgM (pIgA/IgM) might be exploited for intraepithelial virus or toxin neutralization and excretion of exogenous antigens back to the lumen. However, when infection with persistent pathogen invasion occurs, systemic immunity must take over to eliminate noxious antigens and thereby save life. This involves potent proinflammatory mechanisms such as complement activation by IgG antibodies, cell-mediated immunity (CMI), and cytotoxicity, which all may cause tissue damage.” Used with kind permission of Springer Science+Business Media.
Immune-inductive mucosa-associated lymphoid tissue (MALT) resembles lymph nodes with B-cell follicles, intervening T-cell zones, and a variety of APCs such as macrophages and DCs (Fig 3), but there are no afferent lymphatics.¹⁶
Exogenous stimuli therefore come directly from the mucosal surfaces via a follicle-associated epithelium containing specialized M cells, probably aided by DCs, which may penetrate the epithelium with their processes. In the intestine, induction and regulation of mucosal immunity hence takes place primarily in Peyer’s patches, together with other parts of gut-associated lymphoid tissue (GALT) and the gut-draining mesenteric lymph nodes, but also to some extent at the effector sites to which activated T and B cells home (Fig 3).

Retinoic acid, derived from vitamin A in the diet, exerts a positive impact on both differentiation and gut homing of the precursors for IgA-producing PCs. Moreover, the propensity of the mucosal immune system to generate cross-reactive antibodies is probably explained by the extensive innate drive imposed on it by the abundant commensal microbiota via PPRs. Thus, experiments have documented a role of Toll-like receptors for B-cell differentiation in MALT structures.

Although immunological memory is generated after mucosal priming, this may be masked by a self-limiting SIgA response shielding the inductive lymphoid structures, particularly the Peyer’s patches of GALT. An additional complication is the regionalization of the mucosal immune system with regard to migration of mucosal memory/effector B cells to various effector sites. Nasal vaccines that target nasopharynx-associated lymphoid tissue (NALT) of Waldeyer’s ring and cervical lymph nodes elicit both regional mucosal and systemic immunity but do not regularly furnish the small intestine with activated B cells. Such disparity of mucosal B-cell homing is masked in the intestinal lumen of rodents where much of the SIgA in the upper part of the gut is derived from bile.

Ethical constraints restrict direct tracking of immune-cell migration throughout the human body in vivo. We therefore used deletion of the IgM heavy-chain constant-gene (Cμ) segment as a marker to provide a dispersal signature of an effector B-cell subset (IgD+IgM-CD38+) induced selectively in human tonsils. By DNA analysis, the Cμ deletion identified dissemination of such blasts and their PC progeny to peripheral blood, lymph nodes, and bone marrow, as well as to mucosae and glands of the upper airways. Also the endocervix was often positive while the small intestine was mainly negative, as could be expected from the identified homing-molecule profile of the marker cells—with relatively low levels of integrin α4β7 and the CC chemokine receptor 9 (α4β7int./lowCCR7highCCCR9lowCCR10+ CD62Lhigh). Of further importance, the circulating cells abundantly expressed CD62L (L-selectin) and CCR7, which provided a mechanism for integration of respiratory and systemic immunity. Importantly, lactating mammary glands received precursors for IgA+ PCs, both from GALT and NALT.
The Vulnerable Neonatal Period

IgA-producing PCs generally are undetectable in the mucosae before 10 days of age, but thereafter they increase rapidly. However, IgM-producing PCs often remain predominant up to 1 month of age. Usually, intestinal IgA increases little after 1 year of age. A much faster establishment of secretory immunity often is seen in developing countries with a heavy microbial load. The mucosal PC development reflects the progressive microbial stimulation of MALT. Accordingly, only occasional traces of SlgA and SlgM occur in intestinal juice during the first postnatal period, whereas some immunoglobulin G (IgG) often is present, reflecting paracellular “leakage” from the lamina propria, which after 34 weeks of gestation contains readily detectable maternal IgG. In addition, some IgG may be actively exported by epithelial neonatal FC receptor (FcRn). More importantly, both retinoic acid from vitamin A and butyrate derived from microbial fermentation of oligosaccharides in food (and breast milk) can upregulate the epithelial plgR/SC expression and thereby enhance the SlgA export.

Uptake of SlgA antibodies from breast milk via the neonatal gut mucosa is negligible, however, and of no immunological importance in humans, except perhaps in preterm infants. So-called gut closure normally occurs before birth, but the mucosal barrier may be inadequate up to 2 years of age. Although the mechanisms involved remain poorly defined, SlgA from breast milk and development of the infant’s immune system are two related variables in this process.

Animal experiments have suggested that SlgA-containing immune complexes may be taken up via M cells of GALT and guide the induction of the breastfed infant’s immune system to a homeostatic response. Altogether, therefore, it is not surprising that recent meta-analyses show that breastfeeding protects against allergic disease and several other immune-mediated disorders, driven by exogenous factors in developed societies. The same is true for celiac disease. Notably in this context, plgR knockout mice that lack secretory antibodies show reduced epithelial barrier function and increased uptake of antigens from food and commensal bacteria. They therefore have a hyperreactive immune system and show predisposition for systemic anaphylaxis after sensitization; however, this development is counteracted by enhanced oral tolerance induction as a homeostatic backup mechanism.
Conclusion

Many variables influence mucosally induced tolerance and productive IgA-dependent secretory immunity. Some of these variables are reciprocally modulated to achieve homeostasis. Increased epithelial permeability is an important primary or secondary event in the pathogenesis of many diseases, including allergy, celiac disease, and inflammatory bowel disease. The barrier function is determined by the individual’s age (eg, preterm vs term infant); genetics; mucus; interactions between mast cells, nerves, and neuropeptides; concurrent infection; and the mucosa-shielding effect of SIgA provided by breast milk or produced by the infant’s gut. The remarkable output of SIgA during feeding serves as an optimally targeted passive immunization of the breastfed infant’s gut, as well as a positive homeostatic feedback loop.

Many studies indicate that allergy is associated with delayed or impaired development of the IgA system. This is not surprising because secretory immunity is of great importance for the intestinal barrier function. SIgA not only maintains mutualism with the indigenous microbiota but also forms the first line of defense against commensals and pathogens, as well as other harmful agents. In addition, epithelial integrity depends on interaction with microbial factors (MAMPs) from the environment and particularly from the indigenous microbiota, both by direct engagement of epithelial PRRs and induction of mucosal tolerance via different immunosuppressive mechanisms, including tolerogenic APCs and Treg cells.

References


Q: Dr Brandtzaeg, I think you said that IgA plasma cells probably mature faster in infants in the developing world than in infants in the developed world. Are there studies that show that and show how much faster?

Dr Brandtzaeg: Lars Hanson’s group in Sweden has done studies in Pakistan showing more rapidly increasing levels of IgA in secretions than in developed countries. These studies did not involve biopsies and examination of tissue sections, but the amount of IgA in the secretions reflects a more speedy development of the secretory IgA system [Mellander L et al: J Pediatr 1985;107: 430-433]. Also, Anne Ferguson found high levels of Escherichia coli LPS antibodies in gut fluid from adults in Dhaka (Bangladesh), while in adults in Edinburgh, gut fluids contained higher levels of antibodies to food antigen [Hoque SS et al: Eur J Gastroenterol Hepatol 2000;12:1185-1193]. So the antigen always will be imprinted on the repertoire of antibodies in the secretor IgA system; it is highly adaptive.

Q: You mentioned that in the small bowel there are between 100 and 250 Peyer’s patches. And in the large bowel?

Dr Brandtzaeg: By definition, Peyer’s patches contain five to several hundreds of B-cell follicles and are found only in the small intestine, especially in the distal ileum. The number increases until puberty, and then levels off. The large bowel contains numerous solitary or isolated lymphoid follicles, increasing in numbers distally.

Q: Does that mean there are more plasma cells and more immune cells in the large bowel than the small bowel?

Dr Brandtzaeg: No, they are fairly equally distributed, about the same per length unit of the gut. That means that food must have an impact on the development of mucosal immunity in the upper part of the gut where there are very few or no bacteria. Bacteria are further down in the gut where most of the Peyer’s patches and isolated follicles are found. However, antibacterial antibodies could very well be produced in the upper small intestine, as well, because the activated B cells become disseminated, or home, from the GALT system.

Q: You indicated that immune cells in the large bowel do not home to, say, the respiratory tract. Correct?
Dr Brandtzaeg: I think they may spread out from the GALT system to the upper respiratory tract, but we do not see the homing mechanisms that would be required to take memory/effector B cells from the NALT system—for instance, the tonsils—into the small intestinal lamina propria. They may, to some extent, go into the large intestine, but CCR9 must be expressed, and we do not see that to any substantial extent on cells from this area. Thus, the mucosal immune system is integrated but compartmentalized to a remarkable degree, which is very important for mucosal vaccines.
The Role of Dendritic Cells in Immunity and Tolerance in the Intestine

Stephen McSorley, PhD, University of Minnesota, USA

Anatomical specialization of the mucosa and associated lymphoid tissue suggests that induction of a mucosal immune response will have certain unique features that affect the success of oral vaccines and therapeutics. In particular, the initiation of an effective immune response to oral pathogens vs the induction of tolerance to food antigens is likely to be controlled by signals delivered in mucosal tissues. The failure to rapidly develop an immune response to an intestinal pathogen would have devastating consequences for an infected host. Conversely, the inappropriate development of an inflammatory response to food antigens or normal commensal bacteria could severely hinder nutrient uptake by the intestine and also have life-threatening consequences for the host. Thus, rapid discrimination between harmful pathogens and harmless bacteria or food in the mucosa is essential to host survival.

Antibodies directly recognize intact nonself proteins or organisms and can target their removal from host circulation. In marked contrast, T cells recognize small peptide fragments in the context of major histo-compatibility (MHC) molecules on the surface of antigen-presenting cells. Dendritic cells are highly specialized antigen-presenting cells that can capture foreign or self proteins from peripheral tissues and quickly deliver this material to naïve T cells in draining lymph nodes. Intestinal dendritic cells can initiate an inflammatory response by directly recognizing microbial products, and also can degrade and present antigen to naïve pathogen-specific T cells in mucosal lymphoid tissues. Dendritic cells therefore represent a critical interface between innate and adaptive immunity in the intestine.

Indeed, our laboratory recently demonstrated that intestinal dendritic cells are absolutely required to develop a cellular immune response to oral infection. Bone-marrow chimeras were developed from CD11c-DTR transgenic mice that express the diphtheria toxin (DT) receptor under the control of the CD11c promoter. Thus, CD11c+ dendritic cells express the DT receptor, while other cell types do not. When mice are injected with DT, dendritic cells are rapidly depleted from all secondary lymphoid tissues, including intestinal Peyer’s patches and mesenteric lymph nodes (Fig 1). In the absence of this critical cell population, pathogen-specific T cells fail to respond to oral infection, demonstrating a requirement for dendritic cells in intestinal T-cell activation.
Although dendritic cells can be subcategorized in a variety of ways, recent data suggest that chemokine receptor expression might be a useful marker to define subpopulations in the intestine.\textsuperscript{6,12} For example, chemokine receptor 6 (CCR6) expression defines a subset of dendritic cells that are located in the subepithelial dome region of Peyer’s patches.\textsuperscript{13,14} Our laboratory recently demonstrated that this population migrates toward the infected follicle-associated epithelium immediately following salmonella infection.\textsuperscript{9} Both CCR6\textsuperscript{+} dendritic cell migration and salmonella-specific T-cell activation were dependent on CCR6 expression,\textsuperscript{9} suggesting that migration of this population is critical for initiation of an adaptive immune response in the Peyer’s patches, the major site of bacterial entry.\textsuperscript{15} Thus, the CCR6\textsuperscript{+} subset represents a fairly minor fraction of Peyer’s patch dendritic cells but appears to be essential for immunity to pathogens (Fig 2).

**Fig 1. Depletion of CD11c\textsuperscript{+} intestinal DC.** CD11c-DTR BM chimeras were injected with DT (4 ng/g body weight) and PPs harvested 24 hours later. Plots show (A) PP MHCII\textsuperscript{+} cells from CD11c-DTR DT-treated and untreated chimeras and (B) the mean number ± SD of DCs in the PPs. (DT=diphtheria toxin, DCs=dendritic cells, PPs=Peyer’s patches).\textsuperscript{9} Reprinted with permission of Elsevier.

**Fig 2. Rapid PP DC migration in response to pathogens.** CCR6\textsuperscript{+} DC (green) are found between the T cell area (red) and the outer epithelial layer of the PP (top of dome). Other DCs that do not express CCR6 are found in closer association with the epithelial layer (yellow) or within the T cell area (dark green). B cell follicles are shown in blue and macrophages in brown. Upon detection of salmonella (purple) by epithelial cells, CCR6\textsuperscript{+} DCs rapidly migrate toward the epithelial layer. (PP=Peyer’s patch, DC=dendritic cell)
A second population of Peyer’s patch dendritic cells express the chemokine receptor CX3CR1 and are found tightly associated with the follicle-associated epithelia. Other laboratories have shown that close proximity to the epithelial layer predisposes dendritic cells to generate T-cell immune tolerance rather than immunity. Therefore, antigen processing and presentation by different CCR6⁺ or CX3CR1⁺ dendritic cell populations may be the critical for determining whether an immune response is initiated or turned off in the Peyer’s patches. Thus, differential dendritic cell populations defined by chemokine receptor expression may regulate immunity versus tolerance.

Dendritic cells also are found in the intestinal lamina propria (LP), where they do not express CCR6 but do express CX3CR1. Lamina propria dendritic cells can extend processes between epithelial cell tight junctions and acquire bacteria directly from the intestinal lumen. The process of dendrite formation requires expression of CX3CR1 in vivo. The function of these transepithelial dendrites is not completely clear but can represent a non-Peyer’s patch portal of entry for some intestinal pathogens such as salmonella (Fig 3).

However, the homeostatic function of these intestinal dendritic cells may have more to do with inducing or maintaining tolerance to normal bacterial flora than detecting invasive pathogens. A role for CX3CR1⁺ dendritic cells in oral tolerance seems especially likely given the close association of these cells with epithelial cells which, as previously noted, predisposes dendritic cells to induce T-cell tolerance.

A key feature of dendritic cells in both Peyer’s patches and lamina propria is their ability to migrate within, or to, secondary lymphoid tissues so that they can capture antigens and present them to T cells.
antigens and also encounter naïve antigen-specific T cells. However, the bacterial signals that drive intestinal dendritic cell migration remain poorly defined. It is known that some intestinal dendritic cells directly detect pathogen-associated molecular patterns (PAMPs) via innate receptors, and our laboratory is examining one of these receptors, TLR5. TLR5 is a receptor that recognizes bacterial flagellins and induces an inflammatory response. Flagellin is the main component of bacterial flagella and is produced in large quantities by flagellated bacteria. Bacterial flagellins also are dominant target antigens in mouse and human inflammatory bowel disease, where reactivity to flagellins correlates with increasingly severe disease. The conserved structure of bacterial flagellins across several species ensures that TLR5 detects a diverse array of flagellated bacteria including listeria, salmonella, legionella, and pseudomonas. As with other TLR family members, TLR5 signaling induces NF-kappaB activation, inflammatory cytokine production, and increased expression of MHC and co-stimulatory molecules. Thus, bacterial flagellins are a conserved microbial structure directly recognized by the innate immune system. Our laboratory is examining whether flagellins and TLR5 play a role in directing intestinal dendritic cell migration during immunity and tolerance.

In conclusion, dendritic cells are critical cells that determine whether intestinal immune responses are initiated or whether tolerance to harmless antigens is induced. The role of subsets expressing different chemokine receptors is being examined and may help the development of oral vaccines or future therapies for inflammatory bowel disease.

References
The Role of Dendritic Cells in Immunity and Tolerance in the Intestine


24. Chieppa M, Rescigno M, Huang AY, Germain RN: Dynamic imaging of dendritic...


**Q&A**

**Q:** Dr McSorley, you mentioned oral tolerance and the role of various dendritic cell subsets, but you have not mentioned CD103 and retinoic acid. Can you comment on that?

**Dr McSorley:** We have not worked on CD103 and retinoic acid. Many other people have.

**Q:** Can you put your data in the context of that work, then, which is all in vitro?

**Dr McSorley:** My reluctance to talk about that is partly because we have not defined what these chemokine receptor positive populations CX3CR1 and CCR6...
are, and we do not have a good way to fit them into an established model such as myeloid lymphoid plasmacytoid dendritic cells. It would be fairly easy to go back and characterize the dendritic cell populations like Brian Kelsall did [J Exp Med 1996;183:237-247; Mucosal Immunol 2008;1:460-469], and then fit them out with our chemokine receptors. We think that the CCR6 population in the Peyer’s patches are myeloid cells, but I imagine the CX3CR1 population is CD103 positive, migrates to the mesenteric lymph node, and is part of that population. CD103 positive and negative cells are in the mesenteric lymph nodes, and the CD103 positives are supposed to be the cells migrating from the lymph nodes; so, I guess our population is CX3CR1. However, we have not researched that or whether CX3CR1 would still be expressed in that population in the mesenteric lymph nodes.

I would like to study whether retinoic acid affects the ability of a cell population to activate our salmonella-specific cells. We have looked for Th17 cells, which are regulated by retinoic acid in the Peyer’s patches, and we have not seen any production of IL-17 in our infection model. We also have looked for Treg cells, which are regulated by retinoic acid, and we have not seen much FoxP3 expression either. Maybe it is just our system. Other researchers who have studied those populations have different systems, and perhaps in their studies of oral tolerance are looking at endogenous responses to gut flora. One would see more of that regulation by retinoic acid of Th17 and Treg cells.

**Q:** Would you expect the CX3CR1 dendritic cells to produce different levels of retinoic acid compared to the CCR6, given tolerance vs immunity?

**Dr McSorley:** Yes, that could certainly be a hypothesis. We have not studied that. Some of the participants of this conference probably would be interested in the CX3CR1 population in terms of tolerance, food, and commensals. However, that population is irrelevant to those of us working on infectious disease. We are interested in another population to stimulate an immune response. We primarily think about vaccination and elimination of a pathogen.

**Q:** Do you need to pretreat your mice with antibiotics when studying salmonella infection? What sort of hygiene do you use in housing your mice?

**Dr McSorley:** Some researchers who study salmonella infection pretreat with antibiotics, and I believe when they do that they are looking at something completely different from what we see. We just treat our mice orally with salmonella. They have a normal gut flow. They are kept in a fairly clean room, although it is not germ-free, and we see how they respond.
Q: You said you were surprised to see 80% activation of the cells in the Peyer’s patches within 6 hours of infection. Why were you surprised? Is it because of the complexity? Yet we know how quickly digestion occurs. Is there something more fundamental we miss in thinking about how these things are processed?

**Dr McSorley:** Some people have spent their careers studying such things as Peyer’s patch infection of M cells by salmonella, or the processing of class II peptides in response to given dendritic cells or antigen-presenting cells, and bacteria or protein antigen. I am surprised that, in all that literature, these complex processes happen in such a compressed time scale. Not only do the presentation and actual penetration of the bacteria occur quickly, but also the movement of the cell populations to the area where the bacteria are, and subsequently the movement out of that area to find a T cell and actually find it. When we transferred our T cells, we would see about three antigen-specific cells within the Peyer’s patches, so the dendritic cells would have to find them in the interfollicular region. It still surprises me that all these events—migration, antigen presentation, and the pathogenesis of the bacteria—occur so quickly.

Q: What strain of salmonella do you use? If you use an attenuated strain, do you see any differences in which dendritic cells will pick it up?

**Dr McSorley:** It is a wild-type strain. It will kill the mice within about 5 to 7 days, depending on what dose you give them. We also have used attenuated strains, and the same process occurs. With an attenuated strain, we just see less activation. We think that fewer bacteria get into the Peyer’s patches quickly with an attenuated strain, even if we adjust the system to give higher doses of bacteria. A small number of bacteria do get in and start to replicate; so, over time we get more bacteria. It is roughly the same kinetics with T-cell response, but with a lower percentage. Instead of 80% of the cells being activated, for instance, we may see only 25% or 30%.

Q: But is it the same dendritic cell population, the CCR6?

**Dr McSorley:** We have not studied that, mostly because of technical reasons. If you have a system in which you can see 85% or 90% of the cells activated compared to 30% activated, you do not spend time working with the one that results in 30% activation.

Q: Did you imply that the CCR6− cells may be primarily responsible for the tolerogenic responses in the control? I also am thinking more broadly about these cells. Possibly they are present earlier in life. Let us say that if there is a predominance of the CCR6− in a young infant, maybe he or she is effectively
The Role of Dendritic Cells in Immunity and Tolerance in the Intestine

becoming tolerized by the presence of the commensal pioneers early in life. And later on, the CCR6+ cells come in for defense.

**Dr McSorley:** That would be an interesting hypothesis. I do not know whether there are any data on this. We do not know about the development of these cell populations, not even where the CCR6 population comes from. Some may come from blood, dendritic cell precursors, but they could come from some kind of inflammatory monocyte. We do not know anything about development in terms of when these cells appear, in mice or in humans.

**Q:** Are you studying the relevance to humans and whether those CCR6 or CX3CR1 dendritic cells are present in the human gut?

**Dr McSorley:** I would like to, but as an academic scientist, I work on what they let me work on. I am in the gastrointestinal division; I have good access to patient tissues. If we can funnel money from other projects into that project, we may look into it. Some studies show upregulation of CCR6 and particularly CCL20 expression in patient tissues [Schutyser E et al: *Cytokine Growth Factor Rev* 2003;14:409-426]. Some mRNA studies suggest that the ligand-receptor pair CCL20-CCR6 plays a role in homeostasis, inflammation, and pathology at gut mucosal surfaces.
Increasing evidence suggests that immunomodulatory molecules on the commensal microbiota play an important role in regulating immune responsiveness. Recent work has begun to reveal the broad role of microbiota-derived signals in the regulation of a variety of disorders, including diabetes and obesity. We have shown that mice lacking Toll-like receptor 4 (TLR4), the receptor for bacterial lipopolysaccharides, display aberrant Th2-biased hyperreactivity to food antigens. Intragastric administration of a peanut allergen with the mucosal adjuvant cholera toxin induces allergen-specific IgE, elevated plasma histamine levels, and anaphylactic symptoms in TLR4 mutant mice, but not in MHC matched controls. When the composition of the microbiota is reduced and altered by antibiotic administration (beginning at 2 weeks of age), TLR4 wild-type mice become as susceptible to the induction of allergy as their TLR4 mutant counterparts. Both allergen-specific IgE and Th2 cytokine responses are reduced in antibiotic-treated mice in which the microbiota is allowed to repopulate.1

More recent unpublished work has shown that in TLR4 wild-type mice, a putative regulatory population of plasmacytoid DC (pDC), with constitutive expression of IL-10, is detectable in the mesenteric lymph nodes (MLNs), which drain the gut-associated lymphoid tissue, but not in the spleen. The constitutive expression of IL-10 mRNA by this pDC population is impaired in TLR4 mutant mice. Antibiotic administration eliminates the constitutive expression of IL-10 by MLN pDC, further supporting a role for the microbiota in stimulating the expression of these cytokines. CD4+CD25+FoxP3+ regulatory T cells (Tregs) isolated from the spleen and MLN of TLR4 mutant mice have normal in vitro regulatory function but are impaired in their ability to secrete IL-10 in response to T-cell receptor triggering in vitro. Our findings suggest that TLR4 mutant mice are highly susceptible to allergic responses to food antigens because they lack populations of microbiota-induced regulatory DCs and T cells, which are present in wild-type mice.

The incidence of allergic sensitization to food is increasing dramatically.2 A number of studies already have associated polymorphisms in TLR4, and/or its co-receptor CD14, with an atopic phenotype3-7; TLR4 agonists are under development as immunotherapeutics for the treatment of allergic disease.8 Our animal model data suggest that microbiota-derived TLR4 signals are important developmentally during
Flora-Induced Immunoregulation of Allergic and Inflammatory Disease

the transition to weaning. A relatively short course of broad spectrum antibiotics led to alterations in immune system function by altering the composition of the commensal microbiota; after antibiotic treatment TLR4 wild-type mice developed allergic responses to food antigens similar to those seen in TLR4 mutant mice. Analogously, repeated courses of oral antibiotics during infancy (as are commonly given for the treatment of ear infections) might contribute to the increasing incidence of allergic responses to food. A better understanding of how microbiota-induced TLR4 signals modulate the function of antigen-presenting cells and T cells will inform strategies for the development of TLR4 agonists that mimic these immunoregulatory signals. For example, prophylactic co-administration of this type of TLR4 agonist during antibiotic treatment regimens in infancy would maintain stable TLR4-induced immunoregulatory signals under conditions that alter the composition of the microbiota, and might provide an effective and novel strategy for preventing sensitization to food antigens.

TLR4 signals from the commensal microbiota also influence the Th1/Th17-mediated inflammation associated with the development of colitis in IL-10-/- mice. Spontaneous Helicobacter-dependent colitis is exacerbated in IL-10-/- mice that bear a mutation in TLR4. We found that TLR4-mediated signals play two interrelated roles in the exacerbation of disease in TLR4-/- x IL-10-/- mice. FoxP3+ Tregs accumulate in the colonic lamina propria of TLR4-/- x IL-10-/- mice, acquire the ability to produce interferon g (IFN-g), and fail to protect against disease. In addition, dysregulated control of epithelial cell turnover in TLR4-/- mice results in the persistence of antigen-presenting cells (APCs) bearing apoptotic epithelial fragments in the colonic lamina propria of helicobacter-infected mice. Our data suggest that, in TLR4-/- mice, IL-10 secretion by these APCs holds an inflammatory response in check. In mice that lack both IL-10 and TLR4 mediated signals, aberrant regulatory cell function and dysregulated control of epithelial homeostasis combine to exacerbate inflammation. Taken together with new genome-wide association studies identifying a genetic locus that modifies TLR4 signaling as a novel risk factor for inflammatory bowel disease, our results suggest the possibility of exploring TLR4-based therapies for the treatment of intestinal inflammation.

References


**Q & A**

**Q:** In the food-allergy model, have you tried to deplete plasmacytoid dendritic cells (pDCs)? Also, where are the pDCs?

**Dr Nagler:** That is what we are going to do next. There is no evidence that pDCs are trafficking from the lamina propria or from the Peyer’s patches to the mesenteric lymph nodes. We think there is some other dendritic cell population that initially samples the antigen and carries it to the pDC population, or carries some signal derived from that population to the mesenteric lymph node, which then regulates the response. It is possible that the initial Toll-like receptor 4 (TLR4)-dependent signal is generated by the epithelium, and that it is an epithelial-derived signal that educates the dendritic cells below the epithelium and regulates this pathway. Those questions are all approachable with the models we have now. We can make bone marrow chimeras, do epithelial-specific deletion of TLR4, and do pDC depletion to find out where this response is being generated.
Flora-Induced Immunoregulation of Allergic and Inflammatory Disease

Q: You mentioned that the food-allergy model is TLR4 signal independent. Does that mean Gram-negative bacteria are key in driving the food-allergy response in that model, as opposed to Gram-positive?

Dr Nagler: I do not want to imply a specific role for TLR4 in regulating human disease. TLR4 has been implicated in many different murine allergy models, although the genetics of food allergy are not known yet. It is not inconceivable, but I am trying to present this as a model of the ability of the microbiome to influence immune-mediated responses and the possibility of regulating those responses by manipulating the microbiome.

Q: But do you not think that, in the microbiome, species binding mainly to the TLR4 are Gram-negative?

Dr Nagler: I think that TLR4-mediated signals have both inflammatory and anti-inflammatory roles. So blocking one pathway or another suggests a potential for immunotherapeutics.

Q: Is atopic dermatitis or eczema induced in this food-allergy model?

Dr Nagler: Not in the model. However, that is associated with food allergy.

Q: So you see systemic changes and IgE?

Dr Nagler: Yes. One practical implication of this model that relates directly to your question is found in some soft data that suggest that the increase in food allergy is related to antibiotic use, especially in infancy. So if you were able to modulate at the same time you give antibiotics, or you reduced the use of antibiotics in viral infections, for which they are not needed, and you also provided a TLR4 agonist that could continue the TLR4 immunomodulatory signal at the same time you removed the bacterial pathogen population that is generating the inflammatory response, you perhaps could eliminate the influence of antibiotics on susceptibility to allergy, if that truly exists. This is a potential area of study.

Q: I am familiar with the cholera toxin model, but what happens if you do not use cholera toxin? What happens in C3H/HeJ mice by themselves in your model, and what happens if you treat the C3H/HeJ with antibiotics?

Dr Nagler: I did not show you results without cholera toxin because we did not study that. The allergic response in C3H/HeJ mice treated with antibiotics in unchanged.
Q: You described an MyD88−/− x IL-10−/− model in which TLR4 signal lines protect against inflammatory bowel disease [Rakoff-Nahoum S et al: Cell 2004;118: 229-241]. We can interpret that as meaning that some constitutive group of epithelial cells required signaling between the flora and epithelial cells. Does that fit with your model?

Dr Nagler: No, completely the opposite.

Q: What do you think of that model now?

Dr Nagler: I suggest a unique role for TLR4. Eyal Raz at the University of California at San Diego has unpublished data on TLR9−/− x IL 10−/− mice, and they look like MyD88−/− x IL 10−/− mice. He does not have the big colony data we have, however, and his group did not look at the effect of helicobacter—and the result is clearly changed by the presence or absence of helicobacter infection.

Q: I think your data show nicely that there are a lot of models in which effects are modulated by the flora. In studies by Medzhitov and colleagues [Rakoff-Nahoum S et al: Cell 2004;118:229-241], the hygiene status in which these mice were held probably had a huge effect, and neurovirus infection of many mouse colonies has quite an effect on the epithelial layer.
Discussion

Discussion Leader: Cathryn Nagler, PhD, Harvard Medical School, USA

Dr Nagler: I want to start the discussion with the concept of dysbiosis. This table is taken from a recent commentary by Balfour Sartor [Proc Natl Acad Sci U S A 2008;105:16413-16414].

Table. A Disturbed Balance of Beneficial and Detrimental Bacteria (Dysbiosis) Could Promote Intestinal Inflammation

<table>
<thead>
<tr>
<th>Potentially injurious species in susceptible hosts</th>
<th>Protective species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroides vulgatus, B thetaiotaomicron</td>
<td>Lactobacillus sp</td>
</tr>
<tr>
<td>Escherichia coli (adherent/invasive)</td>
<td>Bifidobacterium sp</td>
</tr>
<tr>
<td>Enterococcus faecalis (nonpathogenic)</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>Bacteroides thetaiotaomicron</td>
</tr>
<tr>
<td>Fusobacterium varium</td>
<td>Faecalibacterium prausnitzii</td>
</tr>
<tr>
<td>Helicobacter hepaticus and other intestinal species</td>
<td></td>
</tr>
<tr>
<td>Bifidobacterium animalis</td>
<td></td>
</tr>
</tbody>
</table>

Sartor suggests that the balance of beneficial vs detrimental commensal bacteria species regulates intestinal homeostasis vs inflammation. He targeted inflammatory bowel disease (IBD), but I suggest that this concept could be applied more broadly and could include diabetes and food allergy. I highlighted Helicobacter hepaticus, which is my favorite inflammation-inciting agent, and Lactobacillus sp and Bifidobacterium sp. So we do not have to restrict the dysbiosis concept to a particular bacterial or viral infection that can influence the composition of the microbiota. Perhaps helicobacter infection does not necessarily act directly as a pathogen—most of us are populated by Helicobacter sp—but perhaps it alters the composition of the microbiome in a way that allows for either a decrease in protective bacterial species or an increase in pathogenic species.

Does that seem reasonable? The diabetes data I showed by Wen and colleagues were interesting [Nature 2008;455:1109-1113], but the investigators do not know which bacterial species regulates the susceptibility to diabetes or protection against diabetes. Apparently they are different populations, but both are MyD88 independent.
Dr McSorley: What do you think of the inflammatory bowel disease (IBD) literature in regard to that? Most people are using these IBD models. If this dysbiosis concept is true, it would be critical, and we could test it in TLR5 knockout mice, which were reported to develop rectal prolapse [Vijay-Kumar M et al: *J Clin Invest* 2007;117:3909-3921]. Mice in our colony do not develop rectal prolapse, but no one defines these strains when they publish data on them.

Dr Nagler: Another problem with helicobacter infection is that in academic mouse colonies, it is endemic, but it varies from cage to cage. Mice in one cage will be infected, and those in another cage will not. That variation complicates the results of experiments unless researchers do not use large numbers of mice and track the infection mouse by mouse. This is done fairly easily by using fecal DNA.

Dr McSorley: Why helicobacter? Do you look back at what is driving this response?

Dr Nagler: No. However, it has been implicated in colitis in many mouse strains. It is found in human patients; it has been implicated in colon cancer and is known to cause gastric ulcer. The enterohepatic *Helicobacter sp* in humans is not well characterized, but it is a focus in many groups in the ongoing human microbiome project. The association of helicobacter with food allergy is completely unexplored. We are interested in this possible association because it could explain the big increase in food allergies over the last 10 years. As Dr Brandtzaeg mentioned, the microbial deprivation hypothesis, or the hygiene hypothesis as originally presented, was too simplistic. The increase in food allergies must have something to do with various populations of microbes and their influence on each other, which we are only beginning to explore.

Dr Bienenstock: What do you know about helicobacter-negative humans that you would like to leave with us that we can extrapolate to food allergies?

Dr Nagler: This has not been studied, but it could be fairly easily. We could compare people with food allergies with nonallergic family members and see whether helicobacter has anything to do with the allergies.

Dr Bienenstock: Researchers are studying the question of balance or imbalance in the microbiome. Agnes Wold and colleagues, for instance, have data on *Staphylococcus aureus*, which has become a major player in the first couple of years of life [Lundell AC et al: *Clin Exp Allergy* 2007;37:62-71]. They have developed a whole theory about the role of *Staphylococcus aureus* in development of food allergy. Presumably, we can extrapolate that theory to other microbes that also could displace or change the balance of the microbiome.
Dr Nagler: That is why I showed Sartor’s table. It shows that many microbes probably can do this. Understanding what they are and the nature of their interaction is another level of complexity in the influence of the microbiome on the mucous layer. Evidence is emerging that shows how the composition of the mucosa-associated bacteria influences the mucous layer, and how that affects the ability of other bacteria to approach the epithelium and influence immune responsiveness [Young VB, Schmidt TM: Adv Exp Med Biol 2008;635:29-40]. When we understand the microbiome better, we can manipulate it.

Dr Versalovic: It is important to consider the relative balance of these classes of organisms. We are limited now by our lack of knowledge about microbial deficiencies, as well as excesses (see Table, p 28, left column). We have published data about Faecalibacterium prausnitzii (see Table, p 28, right column). It is intriguing, and we have learned from it. As we expand our concept of probiotics and beneficial microbes, we will discover new organisms that have potent immunomodulatory features. Certainly emerging data support this notion of dysbiosis.

Dr Lee: There is balance between the beneficial and detrimental groups of bacteria. One group induces inflammation; the other suppresses inflammation. But in your antibiotic study, Dr Nagler, it is the absence of microbes that leads to inflammatory responses. Can you comment on that?

Dr Nagler: Although I and other people call this “antibiotic decontamination,” all it does is knock down the number of bacteria several logs. We still have not identified most of the bacteria; so I could not say what we have eliminated, but plenty of bacteria are still in there. The antibiotic treatment does remove an important population of bacteria—maybe the mucosal-associated population—or it somehow disturbs the balance in a way that removes the protective population or its access to the immune system. My first step in microbiome analysis in this model would be to find out what we removed in the antibiotic-treated mice to determine the effects on TLR4 signaling, and the exact interaction between the bacteria and TLR4 in this model.

Dr Lee: You use a combination of cholera toxin and antibiotics. Have you changed the antibiotic profile? Did you observe any differences?

Dr Nagler: No. We just used this mixed cocktail and looked for maximum effect. This is similar to the cocktail that Medzhitov’s group used in their studies [Rakoff-Nahoum S et al: Cell 2004;118:229-241].

Discussion
Dr Michael Montalto (Abbott Faculty): Are we to assume that the protective species are all similar within the lactobacillus strain or that some are more potent than others?

Dr Nagler: I think some other people here would be better qualified to address that than I am, but I think probably not. I do not think that all species are welcome. E coli is Gram-negative, for sure, and Dr Bienenstock mentioned Staphylococcus aureus. They are not all what we have considered to be in the probiotic family. I certainly would put B fragilis on the protective side, based on Kasper’s work that I alluded to earlier—that polysaccharide can modulate inflammation [Mazmanian SK et al: Nature 2008;453:620-625].

Dr Versalovic: Saying “lactobacillus” is a bit like saying “T cells.” We know that, within a specific species—Lactobacillus reuteri, for example—that individual strains can potently stimulate the immune system. Others can downregulate and have very potent anti-inflammatory features. The issue is one of resolution. Clearly, there are good examples within the lactobacillus genome of potent anti-inflammatory organisms, but we could flip it the other way when we talk about different strains. The phrase “antibiotic decontamination” itself has a bias that is being reconsidered.

Dr Brandtzaeg: Bringing this back to real life, you said that in less than 10 years, peanut allergies in the United States have at least doubled. If this is true, do you really think that the bacterial composition has changed that much in 10 years, even in the face of increased use of antibiotics? What about looking for some toxic factor? Cholera toxin breaks all mucosal tolerance; you have to use that toxin to induce efficient sensitization through the gut. There is discussion in Europe about a possible toxic sequence of the Cry proteins in the construct of genetically modified food, transferred from the Bacillus thuringiensis (Bt) bacteria that were used to make the construct. Perhaps there is some cholera toxin-like expression in a genetically modified food such as maize, consumption of which has increased enormously during these 10 years. That could be a parallel development—increased intake of genetically modified maize and then increasing peanut allergy—reflecting break of mucosal tolerance by Cry toxin from Bt.

Dr Nagler: I do not think that these numbers about the increase are necessarily reliable. I think the increase is real, but it has not happened over the last 10 years—certainly, within a generation. No one would argue with at least a generational change in peanut allergy in this country. That cannot be explained by genetics, so it has to be environmental, whether it is a microbial component, a mix of microbial components, or toxins.
Discussion

Dr Brandtzaeg: Is there no discussion in this country about genetically modified food? As I said, that could introduce a toxic component.

Dr Nagler: I have not heard any such discussion.

Dr Kuitunen: Why have food allergies increased? In Europe, a lot of elimination diets are prescribed—too many, in my view—because of various symptoms in infants. In a recent study on mice, researchers assessed the effect of ovalbumin transfer via breast milk. The development of tolerance in offspring was induced by the transfer of ovalbumin in the milk [Verhasselt V et al: Nat Med 2008;14:170-175]. These avoidance diets might be one reason for the increase in food allergies. Dr Brandtzaeg, what is your view of the effect of these breast-milk antigens on the development of the IgA system?

Dr Brandtzaeg: In that study, lactating mice were fed albumin. The breastfed mice babies were protected from asthma if the mother was fed oral albumin after delivery. The study also showed that a combination of antigen and TGF-β was needed in the breast milk to induce this tolerance.

That paper has created a lot of discussion around the world. Perhaps we should not be as strict about food avoidance during pregnancy and breastfeeding as some people have advocated. Now people at meetings I have attended are saying, "do not worry about allergen avoidance during breastfeeding and pregnancy; women cannot avoid them entirely anyway." With regard to gluten intolerance, there is a large European Union study on introduction of gluten very early in genetically prone infants to see whether that can delay or inhibit the presentation of celiac disease. Perhaps an early allergen exposure is a natural thing, and especially mixed feeding with allergen and secretory IgA from breast milk.

Dr Rueda: What do you think about the role of hydrolyzed protein in stimulating the immune system, or not stimulating the immune system enough, and consequently increasing the development of allergy, or not, during the first months of life?

Dr Brandtzaeg: Partially hydrolyzed formula, in the opinion of some researchers, could induce tolerance, since it has some intact antigen. Tolerance is an active process; you need antigens to activate the immune system. Clearly, we have to distinguish between prevention and treatment. If we want to treat a patient with severe food allergy, we need to have completely degraded antigens or amino acid-based diets. But in prevention, this discussion is not included. Studies are being conducted to see whether partially hydrolyzed formulas can prevent allergies, but researchers find it difficult to select a study population because most patients who
become allergic lack a family history. They are scattered in the general population, so the researchers would have to treat everyone.

**Dr Montalto:** What happens to the microbiome when a premature infant is born and they hit him or her with a cocktail of vancomycin, gentamycin, and neomycin? Is it wiped out, or do all the bad bugs go away and the good ones stay?

**Dr Nagler:** I do not think there is information on that yet. However, it could have a detrimental effect by removing populations of bacteria that might be beneficial. This needs to be studied.

**Dr Montalto:** Can you envision a designer protective species bug that is resistant to the antibiotics that normally would wipe it out?

**Dr Nagler:** Yes, but microbial constituents might be even more attractive. In our model, for instance, we are looking for a TLR4 agonist that is antibiotic resistant.

**Dr Rueda:** Dr Brandtzaeg, one of your slides showed how the levels of IgA in saliva increased during the first months of life and went on increasing during the first years of life. How do these levels relate to the IgA in the intestine and the rest of the mucosa? Might they be a good indicator of the IgA levels in the rest of the mucosa?

**Dr Brandtzaeg:** Salivary IgA, particularly parotid secretory IgA, probably reflects the immune response of the upper respiratory airways better than the gut. However, we probably cannot use it as a reliable indicator of the development of the mucosal immune system in general. There was a question about selective IgA deficiency, which generally shows a mild phenotype in humans. This deficiency may increase some diseases such as inflammatory bowel disease and celiac disease, but the gut in most people with selective IgA deficiency is okay. They do, however, have increased allergies and upper airway problems. One explanation for why most people with the deficiency do well is that there is compensation with secretory IgM antibodies in the gut, which is not so predictable in the airways. The polymeric IgA receptor, or SC, will pump out IgM as compensation for lack of IgA.

Confusion has been introduced into this field by studies of the IgA knockout mouse, which also has secretory IgM compensation, especially in the gut, as well as a reduced antigen presentation capacity. So this mouse has a defect in proinflammatory potential. The confusion is due to the need to distinguish between that model—the IgA knockout—and our model, which is a knockout of the polymeric Ig receptor, or SC. In our model, all the secretory immune system is blocked and there is no antibody compensation at all.
Discussion

**Dr Robert Miller (Abbott Faculty):** Does research suggest that the microbiome develops differently in infants delivered vaginally than it does in those delivered by caesarian section? If so, are those differences associated with any type of disease?

**Dr Nagler:** In our mouse model, we remove the helicobacter infection by caesarian rederivation of the mice. The disease that is induced in the caesarian rederived mice is not the same as the disease in the naturally infected mice, which suggests not only an influence beyond just removing helicobacter of the caesarian rederivation, but also a difference between perinatal infection and adult infection. Extrapolation from studies of animals after caesarian or vaginal birth suggests there is a big difference in the microbiome. This has not been studied to any great extent.

**Dr McSorley:** A comment about these protective species. I do not closely follow the literature on lactobacillus. Are its anti-inflammatory effects an active process, or do the lactobacilli fill a niche so that the species that would normally take hold are not there?

**Dr Nagler:** As Dr Versalovic pointed out, there are many different subspecies of lactobacillus, all with different characteristics. What model do we use to study them? If you study them in vivo, you know whether it is the interaction of the bacteria with the epithelium that influences the education of dendritic cells, but that is difficult to model in vitro.

**Dr McSorley:** That is an important question with regard to probiotics. The stage of life at which you give probiotics—whether the patient has a fully intact flora at that point—determines whether the probiotic fails or succeeds. It should not matter whether it is actively anti-inflammatory.

**Dr Nagler:** We started the antibiotic decontamination protocol at 2 weeks because it is difficult to modulate the flora in adult mice. We have done some limited experiments with lactobacilli. We have found that we need to start introducing the bacteria before weaning because the composition of both the flora and the gut-associated tissue changes dramatically at weaning. That is why administration of probiotics for treatment of allergy in adults is not efficacious.

**Dr Kuitunen:** Some reports indicated that children born by C-section have more allergic diseases. We are interested in examining this in the cohort we are following for 5 years. We want to supplement the children born by C-section with probiotics because this target group could benefit most. We are analyzing data now that suggest the frequency of caesarian deliveries in northern Europe is about 17%, and
according to some reports, the frequency in the United States is as much as 30%. So this target group is quite large.

**Dr Brandtzaeg:** We have a large cohort of children in Oslo that is followed carefully. In that cohort, egg allergy is eight-times higher among those born by C-section than among those born vaginally, but only when there is a positive family history for allergy [Eggesbø M et al: *J Allergy Clin Immunol* 2003;112:420-426]. Also, a recently published meta-analysis shows that asthma is 20% higher among children born by C-section, so that must have something to do with colonization [Thavagnanam S et al: *Clin Exp Allergy* 2008;38:629-633]. Studies of certain strains of probiotic lactobacilli show stimulation of increased production of IgA; that is one aspect of anti-inflammatory action [Rautava S et al: *Pediatr Res* 2006;60:221-224].

**Dr Rachael Buck (Abbott Faculty):** Following up on the comments of Dr Kuitunen and Dr Brandtzaeg, we know there was delayed bacterial colonization in preterm infants, as well as in full-term infants born by C-section. What are the consequences of delayed colonization besides allergies?

**Dr Versalovic:** We need to rethink how babies are being cared for. I hesitate to say "treated," although I suppose we could call it treatment because we may be dealing with a microbial deficiency syndrome. Certainly there is a population of children that is relatively deficient in specific classes of microbes, and those are the children who may need to be targeted with specific prebiotics, probiotics, or symbiotic regimens. The data emerging from some of the meta-genomic studies support the fact that colonization is delayed in children born by C-section.

**Dr Buck:** Dr Brandtzaeg, would you speculate on the mechanisms involved in the increase of ileal secretory IgA by fructooligosaccharides (FOS)?

**Dr Brandtzaeg:** You are referring to the Japanese study with the mice model [Nakamura et al. *Clin Exp Immunol* 2004;137:52-58]. I asked the senior author at a meeting about the mechanism, and he said he did not know. One possibility is the fermentation to butyrate. Butyrate has quite a positive effect on the IgA pump, but it could be other mechanisms or a combination of mechanisms changing the composition of the flora and so on.

**Dr Buck:** You talked about the increase in the polymeric immunoglobulin receptor.

**Dr Brandtzaeg:** Yes, I alluded to that. Butyrate is an active component in that respect. The senior author was happy when I suggested that fermentation of FOS to butyrate might be the mechanism that increases the ileal secretory IgA level.
**Discussion**

**Dr Buck:** Any thought of just increasing microbial loads, thus acting directly rather than indirectly?

**Dr Brandtzaeg:** Yes, that is one possibility, but we really do not know.
Role of Th1/Th2/Th3 Regulatory T Cells in Mucosal Immune Development

Fernando Chirdo, PhD, Universidad Nacional de La Plata, Argentina

The gastrointestinal tract is in constant contact with food proteins, commensals, and potentially pathogenic microorganisms. To maintain immune homeostasis in this environment, the intestinal immune system has developed redundant regulatory strategies. Soluble factors such as transforming growth factor beta (TGF-β), interleukin-10 (IL-10), thymic stromal lymphopoietin (TSLP), and cells such as tolerogenic dendritic cells (DCs), and a subset of T-helper cells (regulatory T cells or Tregs) play a role in the control of the immune response to avoid overreaction and excessive tissue damage.1

Oral administration of protein antigens induces systemic immunological tolerance, a phenomenon largely known as oral tolerance. Failure in the regulatory mechanism may lead to dietary-antigen-derived pathology such as food allergy or celiac disease. Similarly, aberrant immune response against components of commensal flora may trigger inflammatory bowel diseases such as Crohn's disease or ulcerative colitis.

The microenvironment determines the functions of cells recruited to the tissue, with DCs serving as the master coordinator of the mucosal immune response. These cells have a critical influence in changing the balance between active regulatory mechanisms or driving an inflammatory response. Under steady-state conditions, DCs are central to the induction of tolerance to both self and foreign (dietary and commensal) antigens by inducing Tregs. In the presence of inflammation or pathogenic organisms, DCs are activated to express a full range of co-stimulatory molecules and cytokines, ensuring the efficient stimulation and differentiation of effector T cells.

After oral administration of a model antigen (ovalbumin), lamina propria DCs (LPDCs) exhibit regulatory properties, because in vivo antigen-loaded LPDCs transferred into normal recipients induced a state of immune hyporesponsiveness.2 These LPDCs produce more mRNA for IL-10 and type 1 interferon than spleen DCs and less interleukin (IL)-12, suggesting that there may be a population of DCs in the lamina propria that can preferentially induce T-cell tolerance in the absence of inflammation.
Intestinal DC Subsets

As in peripheral lymph nodes or other tissues, distinct DC (CD11c+) subsets are described in intestinal mucosa in mice. With the use of more and more markers, the initial description CD11b+, CD8α+, CD11b-CD8α- has become much more complex. Now, additional markers are shown to discriminate different functional populations, and the discrimination between DC subsets CD103 positive or negative and CX3CR1 positive or negative are shown to be important in the understanding of different capabilities of intestinal DC subsets. To increase the complexity of this scenario, distinction between DCs and macrophages in the intestine is not completely elucidated. Finally, because there are no clear markers for human DC subsets, description of human mucosal DCs is more difficult than that of murine counterparts.

In addition to the antigen uptake mechanism by M cells (mainly in Peyer’s patches) or antigen passage throughout the tight junctions, intestinal DCs may extend through the enterocyte layer, as nicely described by real-time imaging recording. These extensions are capable not only of sensing the microorganisms in the lumen but also of taking them up and transporting them to the LP. Although the biological relevance of each mechanism is still uncertain, it is clear that DCs can directly take up antigens present in the lumen, process and present orally delivered antigen, and finally stimulate naïve T cells in mesenteric lymph nodes (MLNs). The outcome of this interaction depends on the characteristics of the DC and signals from the environment. Consequently, Tregs or effector T cells (Th1, Th2, or Th17) can be induced. Moreover, as part of their differentiation program, these cells upregulate the homing receptors that direct them to the intestinal mucosa.

Induction of Tregs in the Intestinal Mucosa

It is now clear that extrathymic generation of Tregs occurs in the small intestine. Intestinal DC can induce antigen-specific Tregs, which are mainly involved in the control of immune response against dietary antigen and commensal flora. As we will discuss, retinoic acid (RA) is one main player in the induction of Tregs, but also in determining the homing tropism of the activated cells in the mucosa.

By adoptive transference experiments, Sun et al. demonstrated that FoxP3+ cells can be induced in lamina propria of the small intestine and MLNs, and this population may be traced as long as 8 weeks after transference, implying a continual conversion or accumulation. These cells are antigen-specific and selectively express CD103.
LPDCs are especially suited to convert CD4⁺ T cells to Tregs, and CD103⁺ LPDCs, in particular, are much better than CD103⁻ LPDCs. However, TGF-β supplementation renders CD103⁻ LPDCs equally able to convert FoxP3⁺ T cells. TGF-β also may induce CD103 expression. LPDCs also are able to expand FoxP3⁺ T cells.

RA does not induce FoxP3⁺ expression alone, but upregulates its expression when combined with TGF-β. Furthermore, RA is a potent inducer of CCR9 and α4β7 expression, which mediates the gut tropism of Tregs.

It is clear that Tregs conversion occurs in the intestinal mucosa, and FoxP3⁺ T cells can be observed in LP, Peyer’s patches, and MLN. However, LPDCs, particularly CD103⁺ LPDCs, which enter the MLN from LP, are the more potent inducers of Treg conversion.⁴

**Regulation of Immune Response**

The immune system contains potent mechanisms that eliminate pathogenic microorganisms and infected cells, and parallel regulatory mechanisms that keep effector cells under control during physiological conditions and after the activation elicited by an inflammatory process. In particular, in this inflammatory situation, immune regulation is required to reduce the tissue damage caused by excessive immune activation.

In recent years, the study of suppressor T cells, most commonly Tregs, has been intense. The transcription factor FoxP3⁺, originally thought to be uniquely expressed by Tregs cells, helped to identify the initial descriptions of Treg functions. Humans and mice that do not have functional FoxP3⁺ T cells as a consequence of mutations in the FoxP3 gene suffer from inflammatory and autoimmune disease. This highlights the essential role of Treg cells in control of the immune response.

Thymic-borne Tregs migrate as naïve FoxP3⁺ cells to secondary lymph nodes for activation and differentiation into memory type FoxP3⁺ T cells. Then Tregs can migrate to B-cell areas, nonlymphoid tissues, or sites of Th1 or Th2 inflammation. In addition, naïve FoxP3⁻ cells can be converted to FoxP3⁺ cells by different factors; RA is one of the modulators.

**Murine and Human Tregs**

Human and mice Tregs are different in many aspects regarding the impact of or regulation of FoxP3 expression. In humans, FoxP3⁺ expression is very low and transient in activated T cells. These FoxP3⁺ cells do not suppress T-cell proliferation. Overexpression of FoxP3 induces hyporesponsiveness and suppression of IL-2 production but does not convert T cells in Tregs, suggesting that additional signals are required in humans. TGF-β can induce FoxP3 expression but alone is not sufficient to induce a full Tregs programming in humans. RA is sufficient to convert
Role of Th1,Th2,Th3 Regulatory T Cells in Mucosal Immune Development

FoxP3+ T cells in humans, but in mice TGF-β is required in addition to RA to generate functional FoxP3+ Tregs cells from FoxP3- T cells.

**Mechanism of Suppression by Tregs**

Evidence exists of the suppressive effects displayed by Tregs, but information about the actual pathways determining regulation or suppression of effector T cells is scarce. One of these mechanisms consists of redirecting the DC function to a regulatory/tolerogenic phenotype as a consequence of IL-10 secretion by Tregs. Regulatory DCs later can induce expansion/differentiation of new Tregs. This is an indirect manner to induce regulation, and mechanisms having a direct effect on effector T cells are less evident.

When molecules like cytokines are the functional mechanism of action, one must keep in mind that most of them have a short half-life. Thus, Tregs must be located in close proximity to the target cells, although this does not necessarily imply cell contact.

At present, data suggest that overlapping and, to some extent, redundant mechanisms may be required to obtain the maximal suppressive effect. Mechanisms for the suppressive effects of Tregs can be grouped into four types, as described next.5

**Suppression by inhibitory cytokines.** IL-10, TGF-β, and, more recently, IL-35 have been described as mediators of the suppressive effects by Tregs. All three have suppressive activity but the mechanisms and relevance of each still are unclear. However, different studies have reported that the three are involved in distinct pathogenic or homeostatic situations and have non-overlapping functions.5

Production of IL-10 is clearly demonstrated in the mouse model of colitis, in which secretion by Tregs is essential to control inflammation. The situation for TGF-β is more complex because the biological activity is mostly due to the TGF-β tethered to the plasma membrane of Tregs.

One member of the IL-12 family, IL-35, is preferentially expressed by mouse FoxP3+ Treg cells but not by activated effector T cells. IL-35 also is significantly upregulated in Tregs that are actively suppressing and is sufficient to confer regulatory function to naïve T cells or to suppress T-cell proliferation in vitro, although the pathways for these effects still are undefined.
Suppression by cytotoxicity. Tregs have suppressive effects on target cells by the induction of granzyme A and granzyme B, in human and mouse, respectively, resulting in cytotoxicity.

Suppression by metabolic disruption. One of the mechanisms, largely debated, is deprivation of IL-2 by Tregs through the high expression of CD25 (the IL-2Rα high affinity chain) that starves dividing effector T cells and induces apoptosis. In addition, it recently was observed that release of adenosines can inhibit effector T-cell function by inhibiting the secretion of IL-6 and inducing the production of TGF-β, which in turn favors the generation of Tregs compared with Th17 cells. It also has been proposed that release of cAMP by Tregs through gap junctions can mediate strong suppressive effects on target cells.

Suppression through dendritic cells. As mentioned previously, Tregs may influence DCs by altering their maturation and function. CTLA-4, which is constitutively expressed by Tregs, modulates the priming and co-stimulatory properties of DCs, thereby attenuating the DC activation of effector T cells. The co-stimulatory function of DCs can be impaired by decreasing the expression of CD80 and CD86 mediated by CTLA-4 or via IL-10 and TGF-β. More recently, it was proposed that LAG3 (CD223) may block DC maturation because it is able to bind with high affinity to MHC class II molecules and deliver suppressive signals. This correlates with higher suppressive effects of human MHC class II+ Treg cells compared to MHC class II- cells.

Retinoids and Immune Regulation

Regulatory T cells, in particular FoxP3+ cells, suppress the immune system to prevent overactive response and inflammation induced by effector T cells, and other non-T cells. FoxP3+ cells arise in the thymus (nTregs), and in parallel induced Tregs, may arise from naïve CD4+ T cells in the periphery. The factors that determine the development of Tregs—DCs, cytokines, tissue-derived factors, including retinoids—recently have received much attention. RA can be produced from vitamin A by DCs in the intestine by a metabolic pathway involving enzymes such as RALDH1/2 (retinal dehydrogenase). RA, particularly all trans retinoic acid (ATRA), binds to a retinoid nuclear receptor that acts as a transcription factor on many genes. Retinoids induce expression of gut homing receptor in DCs; B and T cells induce IgA switch, reduce TNFα and IL-1 concentration in serum, and increase IL-10. Consequently, retinoids are important regulators of mucosal immunity.

Both human and mouse FoxP3+ Treg cells induced by RA and TGF-β express CCR9 and α4β7, which engage CCL25 and MadCAM1, respectively, and determine gut
Role of Th1/Th2/Th3 Regulatory T Cells in Mucosal Immune Development

homing tropism. These cells also lose the expression of CD62L, which guides them to lymphoid nodes. In addition, RA and TGF-β induce CD103 (αE), which allows interaction with E-cadherin. These changes can occur during the induction of antigen-specific Tregs, as was demonstrated in animal models using ovalbumin (OVA)-specific transgenic mice.

Th17: A Central Player in Intestinal Antimicrobial/Inflammatory Response?

In the normal intestine, Th17 cells contribute to maintaining the epithelial barrier integrity and inhibiting bacterial colonization by producing defensins, whereas in chronic intestinal inflammation high levels of IL-23 produced by DCs may activate their aggressive pathogenic program. In this context, production of other inflammatory cytokines (IL-1, IL-6, TNFα) exacerbates the production of IL-17 by Th17 cells. IL-17A, the most studied member of the IL-17 family, is important in host defense against extracellular bacteria and fungi by stimulating the production of chemokines that recruit and activate granulocytes and macrophages. However, IL-17A also promotes an inflammatory cascade, which can drive autoimmune diseases.

In mice, Th17 cell differentiation is induced by TGF-β plus IL-6. Th17 cells produce IL-21, which acts as an autocrine positive regulator, and among other effects, induces IL-23R. IL-23 supports further differentiation. Since mouse Th17 cells express IL-23R only after induction by IL-6, IL-23 is not involved in the initiation of the Th17 differentiation. On the other hand, human Th17 cell differentiation is driven by IL-6, IL-1, and possibly by IL-23.

Th17 cell differentiation depends heavily on the transcription factors RORγT and RORα in mouse and human, respectively. IL-6, IL-21, and IL-23 activate the transcription factor STAT3, which directly binds to IL-17 and IL-21 genes. STAT3 is also a positive signal for the receptor RORγT and RORα.

Because of the potent inflammatory function of IL-17, Th17 cell differentiation is regulated by negative factors such as IFNγ, IL-4, IL-2, and RA. Recently, IL-27 was identified as an important negative regulator of Th17 differentiation.

In contrast, in humans it is possible to find IL-17 producing cells as well as IL-17 plus IFNγ “double producer” cells—two populations that seem to be characterized by the expression of CCR6 and CCR4 for Th17 and CCR6 and CCR3 for Th17-1.

There is not a unique link between Th17 and IL-17. Not all the functions of Th17 are
in parallel with IL-17 activity. Other cytokines such as IL-21 and IL-22 are involved in the pathogenic cascade that can drive a chronic disorder. IL-21R is expressed on T and B cells, DCs, and macrophages, as well as on epithelial cells and natural killer (NK) cells; its activation drives many different effects. In addition to Th17 cells, other non-T cells such as monocytes and macrophages can produce IL-17.

**Th17 Cells: Gut Homeostasis and Inflammation**

Chronic inflammatory disorders of the intestinal tract (e.g., Crohn’s disease and ulcerative colitis) have unknown etiology, and no cure exists at this time. Disease mechanisms involve a complex multifactorial scenario. It is evident that chronic inflammation is mediated by an aberrant immune response directed against components of the intestinal microflora, but many steps involved in the molecular pathogenesis remain undiscovered. It is recognized that IL-12p40 is involved in Crohn’s disease, leading to the concept that the disease is mainly a Th1 IL-2-driven pathology. Furthermore, anti-IL-2p40 antibody therapy has been used successfully in many patients. However, more recent findings demonstrate that IL-23 is the cytokine implied in the pathology. IL-23 belongs to the IL-12 heterodimeric family, and it is composed of IL-23p19 and IL-12p40 subunits. Consequently, treatment with anti-IL-12p40 antibodies targets IL-23. In addition, IL-23 selectively potentiates the IL-17 expression by Th17 cells, and its deficiency protects against experimental autoimmune encephalomyelitis (EAE) in a mouse model. Many of the Th1-driven autoimmune diseases may in fact be due to IL-23.

Epithelial cells constitute not just a physical barrier, they also take part in the amplification and maintenance of chronic intestinal inflammation in IBD. Intestinal epithelial cells can synthesize cytokines that control survival and activity of lymphocytes and contribute to generating a chemoattractant milieu that sustains the recruitment of inflammatory cells. They express IL-21R and respond to stimulation with IL-21 by secreting the chemokine MIP-3α, which can mediate the recruitment of macrophages, but also of α4β7 T lymphocytes.

By a different pathway, epithelium is involved in inflammatory processes. Epithelial cells can produce IL-32 after induction by a Th1 environment such as IL-12 and IFN-γ and stimulate TNFα and IL-8. In addition, IL-32 synergizes with signaling derived of Nod protein activation to produce IL-1β and IL-6. All these mediators largely contribute to amplification of the inflammatory process.

Moreover, lamina propria fibroblasts play an important role in tissue remodeling and fibrosis. They secrete collagen, but in inflammatory conditions also matrix metalloproteinases (MMPs), which are neutral endopeptidases secreted in the
inactive form and activated in the extracellular environment. Their activity is controlled by specific tissue inhibitor of MMPs (TIMPs). Imbalance between the activity of MMPs and TIMPs contributes to tissue damage and fibrosis. Fibroblasts have IL-21R and respond to IL-21 by secreting large amounts of MMPs, but not TIMPs, from internal deposits. TNFα and IL-1β are inducers of IL-21R expression on fibroblasts, driving the initial steps in the inflammatory process.

References

Q & A

Q: Dr Chirdo, in most of your studies, dendritic cells were isolated from the lamina propria. You also mentioned that there were differences between T cells and cells in the lymph nodes. What about those in Peyer’s patches? Are there many differences between these dendritic cells? Apparently, they function the same way.

Dr Chirdo: It is difficult to answer the question precisely, but there are some differences in the function of dendritic cells in the lamina propria and Peyer’s patches. Subset analysis shows that the composition of the whole cell population is different. The percentage of conventional dendritic cells in the lamina propria differs from those in Peyer’s patches. To me, however, the most important difference is that in the Peyer’s patches, some cells are located in specific places—some are in the dome region, and some are in the interfollicular region. In comparing the whole subset, not too many differences are seen, but specific cells may function only at that specific location. In the lamina propria, however, cells are not restricted to a specific location. Also, lamina propria dendritic cells do nothing in priming; they must migrate to the mesenteric region. In the Peyer’s patches, however, the dendritic cells are able to use priming, and this is a different situation.

Q: Did you say that the cells underneath the epithelium and in the villi are plasmacytoid DCs (pDCs)?

Dr Chirdo: That has been published [Wendland M et al: PNAS 2007;104:6347-6352].

Q: Are the majority pDCs, or all of them?

Dr Chirdo: The authors claim that a large part of the population is close to the epithelium, although not in the epithelial compartment. They estimate that most of the cells are close to the basal membrane.

Dr Brandtzaeg: We have to be aware of the important differences between mice and humans in regard to the distribution of various dendritic cell types. I do not think we always are talking about the same cells in mice and humans, particularly when we are dealing with CD103+ DCs and plasmacytoid DCs. To my knowledge, we have not seen IL-3 receptors, CD45A, and CD103 in the lamina propria of humans, except in the periphery of lymphoid aggregates, which are the markers we ordinarily use. You mentioned mesenteric lymph nodes.

Dr Chirdo: A recent publication shows them in the mesenteric lymph nodes but not
Role of Th1/Th2/Th3 Regulatory T Cells in Mucosal Immune Development


Dr Brandtzaeg: I understand from other discussions that those researchers have seen them in the human lamina propria, but these data are not published.

Q: I have seen rodent studies in which vitamin A deficiency decreased asthma severity, while high levels of vitamin A increased asthma severity. Is there evidence that vitamin A levels differ in diets in Western countries compared to those in non-Western diets, and could vitamin A be implicated in the increases in immune disorders over the last few decades?

Dr Chirdo: I not know about Western vs non-Western dietary differences in vitamin A, but research findings about the effects of deficient and excess concentrations of vitamin A in tissues are contradictory. Both can cause problems. There are research difficulties in studying this. In vitro, we can calculate exactly what concentration of vitamin A we are using, but we do not know what really happens in tissue.

Dr McSorley: The literature suggests that many nutritional deficiencies in developing countries are associated with increased susceptibility to infection. This is the opposite of the model, whereby retinoic acid suppresses Th17 cells and encourages regulation.

Q: Dr Chirdo, you mentioned CX3CR1 cells but not CCR6 dendritic cells expressing the chemokine receptor, which Dr McSorley talked about with regard to the mouse model. Can you comment?

Dr Chirdo: In a big study in Europe, researchers are trying to define the relationships between CCR6 positive or negative and CX3CR1 positive or negative. They seem to belong to different subsets. CD103 positive or negative also are different lineages. One does not develop from the other. The problem for developing different lineages in dendritic cells is that no one knows exactly how these cells differentiate each other.

Q: We have talked a lot about DCs in the gut. In terms of expression of chemokine receptors, are these cells very different in the respiratory tract and other mucosal sites?

Dr Chirdo: The intestinal cells are best known.

Dr Brandtzaeg: We studied the human nasal mucosa and found that the antigen-presenting cells are quite different from those in the gut. Some cells with a clear macrophage phenotype look like normal dendritic cells. In the human gut, there is a band of antigen-presenting cells below the epithelium where there is a transition between monocyte-derived macrophages and dendritic cells. So it is often hard to say whether cells are true dendritic cells, but small subsets of cells in various layers of the gut are clearly dendritic cells.
Dr Chirdo: A recently published paper claimed that all regulatory functions in the intestine are due to macrophages, not to DCs—that the production of IL-10 and induction of Tregs are due to macrophages, not to dendritic cells [Denning TL et al: Nat Immunol 2007;8:1086-1094]. This is the opposite of most other research findings. As Dr Brandtzaeg said, we cannot clearly define what a dendritic cell is and what a macrophage is. At some point, we define this population of DCs according to markers, and the markers we use are CD11C and CD11B. They may be expressed at different levels at the surface, and consequently it is difficult to set the definition of both lineages only on these markers.

Q: Regarding CCR6 and CX3CR1, the data you presented from Chieppa’s paper [Chieppa M et al: J Exp Med 2006;203:2841-2852] were in direct conflict with Dr McSorley’s. Can you reconcile that?

Dr McSorley: I presented my interpretation of the data. Christian Reinecker’s group would interpret them slightly differently and say that the CX3CR1 cells are actually involved in the priming of CD4 cells [Neiss JH et al: Science 2005;307:254-258]. For various reasons, I do not think that is correct, but he could be right.

Dr Chirdo: One of the problems in comparing Reinecker’s paper to Chieppa’s is that although their ends are the same, their methodologies differ. Reinecker and colleagues used a green fluorescent protein (GFP) under the promoter of CX3CR1, Chieppa’s group used the GFP under the promoter of Class II, so that group produced different findings.
Role of Commensal Flora in Mucosal Immune Development

Kathy McCoy, PhD, McMaster University, Canada

Microbial species are able to colonize and populate an incredibly diverse range of different microenvironments on our planet. However, while the density of the microbiota found in soils or other geological or marine habitats typically reach up to $10^8$ microbes/gram, the mammalian lower intestine is home to at least 400 different microbial species, and densities reach up to $10^{12}$ microbes/gram of luminal contents. Our commensal microflora therefore outnumbers the eukaryotic cells in our body by an order of magnitude. This demonstrates that our intestines constitute very good culture media. Mammals therefore coexist, mostly peacefully, with an extremely dense and diverse load of microbes in the lower intestine.

Within the intestine, only a single layer of intestinal epithelial cells physically separates this massive load of bacteria from the inside of our body, which essentially remains sterile. Importantly, the mucosal immune system, which likely contains the majority of lymphocytes in the body, is strategically positioned directly opposing the luminal bacteria. Despite its close proximity to a multitude of bacterial antigens, in most human individuals and wild-type animals the host mucosal immune system enjoys peaceful mutual coexistence with the intestinal microbiota.

It is clear that the mucosal immune system is not ignorant of the presence of intestinal bacteria because a vigorous intestinal IgA response directed against the commensal microflora can be measured and a significant proportion of this IgA is induced through a T-independent pathway. Commensal organisms that penetrate past the epithelial barrier are efficiently killed by macrophages. In contrast, bacteria that are sampled by local intestinal dendritic cells are brought to the mesenteric lymph nodes (MLNs), where an efficient anticommensal IgA response is induced. The MLNs are not absolutely required for induction of IgA, but they act as a barrier to restrict the commensal-laden dendritic cells from entering the systemic immune system, which in turn limits systemic priming and induction of systemic immunopathology.

It is therefore clear that the mucosal immune system is neither ignorant nor tolerant of the intestinal microbiota. In neonates, however, the intestinal environment is sterile. Colonization of the intestine (and other body surfaces) begins only after the newborn is exposed to the external environment. The composition of the microflora is built up by successive waves of organisms that colonize the intestinal tract after birth; once an individual is fully colonized, the composition of microbes remains reasonably stable. This successive colonization of the intestine occurs during the first few years of life, a window of time that also corresponds to a critical period of immune development and maturation. Unfortunately, investigation of the
mechanisms by which early bacterial exposure influences immune development is very difficult to assess in humans because one cannot easily determine or manipulate microbial exposure. However, germ-free mice provide a powerful tool to investigate how the intestinal microflora can shape the developing immune system. Germ-free mice do not harbor any microorganisms in their intestines or other body surfaces, making it possible to use them to directly assess the impact of colonization in vivo. Deliberate colonization of germ-free mice allows the researcher to precisely manipulate the time, dose, and diversity of the bacterial exposure. Germ-free animals are bred and housed in flexible-film isolators that are maintained under positive pressure. Virtually any mouse strain of interest can be rederived to germ-free status through 2-cell embryo transfer into germ-free pseudopregnant recipient females. 

Analysis of germ-free animals has provided clear evidence that the absence of microbial stimulation has profound effects on development of the mucosal immune system. Immediate downstream effects of the absence of intestinal microbiota include reduced numbers and size of Peyer’s patches (PP), decreased intestinal IgA-secreting plasma cells, decreased lamina propria CD4+ T-cell numbers, and alterations in the T-cell content of the intraepithelial compartment. We have found that colonization of germ-free mice leads to a marked increase in CD3+ and B220+ cells in the intestinal lamina propria (Fig 1). The T-cell populations that enter the lamina propria are composed of both CD4+ and CD8+ T-cell subsets (data not shown).

We have found that the number of intestinal CD4+ T cells increases over 100-fold in serial studies over 28 days after germ-free animals acquire a limited defined commensal flora. A smaller CD4+ increase and an oligoclonal expansion of CD8+ cells on colonization also have been observed in the intraepithelial compartment. The total number of B and T lymphocytes in the MLNs also increases dramatically.

Fig 1. Colonization of germ-free mice leads to increased numbers of lamina propria B and T lymphocytes. The small intestine and colon (as indicated) were taken from germ-free C57BL/6 mice (left column) or from germ-free littermates 21 days postcolonization (right column) and analyzed for the presence of B cells (B220) and CD3+ T cells, as indicated. Photographs show sections at x10 magnification.
Role of Commensal Flora in Mucosal Immune Development

following colonization. Exposure to commensal bacteria also induces activation and differentiation of mucosal lymphocyte populations. In B cells there is strong induction of class-switch recombination to IgA, and IgA-secreting plasma cells increase dramatically following colonization of germ-free animals (Fig 2). Accordingly, the total level of SLgA found in the intestinal lumen also increases significantly.

We have developed a flow-cytometry-based method of measuring bacterial antibody binding that is specific for the immunizing strain, and extremely sensitive for all isotypes, with very small antibody and bacterial samples required. Using this method we have found that a specific IgA response is induced against the immunizing strain of commensal bacteria—colonization of germ-free mice with Enterobacter cloacae generated only IgA specific for *E. cloacae* and not for *Escherichia coli* (Fig 3, upper panels). Conversely, monocolonization with *E. coli* induces specific IgA that does not bind to *E. cloacae* (Fig 3, lower panel). Colonization of the intestine with bacteria therefore induces maturation of the mucosal immune system and induction of specific anticommensal reactivity.

---

**Fig 2. Colonization of germ-free mice leads to increased IgA in the intestinal lamina propria.** The small intestine was taken from germ-free C57BL/6 mice (left panel) or from germ-free littermates 7, 14, and 21 days postcolonization, as indicated, and analyzed for the presence of IgA. Photographs show sections at x10 magnification.
We currently are studying the functionality of the large intestinal CD4+ T-cell response generated by colonization. The function of normal CD4+ T cells in the lamina propria is especially important—while activated Th1 lamina propria lymphocytes have been traditionally seen as proinflammatory, it has been shown that CD4+ T cells are required to limit translocation of commensal bacteria,\(^\text{14}\) probably through the activation of biocidal activity in subepithelial macrophages.\(^\text{15}\) A further indirect line of evidence that the CD4 response is functional in maintaining the barrier against commensals is that the induction of lamina propria CD4 cells (germ free → colonized) is increased in strains with no protective antibody secretion, probably as a compensation to increased penetration of intestinal bacteria through the epithelial cell layer.\(^\text{10}\)

The absence of microbial stimulation also has profound effects on development of the systemic immune system. Germ-free mice display systemic lymphopenia, hypoplastic secondary lymphoid structures with reduced B- and T-cell content, and poorly formed high endothelial venules (as reviewed in reference 9), whereas colonization can increase CD4+ T-cell numbers and normalize the splenic
architecture. Exposure to intestinal microbial stimulation also appears to greatly influence the background levels of different antibody isotypes, and germ-free mice display altered levels of spontaneously produced antibodies. While germ-free mice have normal serum levels of total IgM, they have greatly reduced levels of the class-switched antibodies IgA and IgG. The most striking differences in the number of antibody-secreting cells are in the MLNs, which have drastically reduced numbers of IgM-, IgG-, and IgA-secreting cells. In stark contrast, we have found that germ-free mice have elevated levels of natural IgE. Other groups have shown greatly increased IgE positive cells in the PPs of germ-free rats, and feeding with certain bacteria or bacterial cell-wall components led to a decrease in the number of IgE-bearing PP cells. These results suggest that in addition to shaping the developing mucosal immune system, intestinal microflora also can exert a major effect on the IgE response. It is unclear why, of all the class-switched antibody isotypes, only IgE is elevated in germ-free mice. This observation is potentially important, especially considering the epidemiological evidence in humans that the development of allergy is highly influenced by exposure to microbial stimulation early in life, which forms the basis for the hygiene hypothesis. It has been postulated that colonization of the intestine with specific bacterial species during immune development is an important factor in protection from allergic and autoimmune diseases.

It is clear that exposure to intestinal microflora can shape the developing immune system. Using colonization of germ-free mice to model the exposure to bacteria that occurs in all mammals after birth will provide valuable insight into the mechanisms by which the microflora induces maturation and proper regulation of the mucosal, as well as the systemic immune system.

K.D. McCoy is a Canada Research Chair in Gastrointestinal Immunology at McMaster University, Canada, and her research is funded by Canadian Institutes of Health Research.

References


Role of Commensal Flora in Mucosal Immune Development


**Q & A**

**Q:** I am not familiar with one technique you used in your IgA work. You said the western blot showed a lot of cross-reactivity. I am familiar with that technique, but not the flow-cytometry-based method of measuring antibacterial antibodies. It strikes me as a very powerful observation that has a lot of potential ramifications. Much of this research is done with western blots where we get a lot of denaturation, exposure of those binding sites, and cross-reactivity. In situ, however, that probably is not the case. What do you think this means?

**Dr McCoy:** When we looked through our bacterial flow cytometry method, IgA or even IgG anticommensal responses to the surface antigens were specific to each type of bacteria, and there was no cross-reactivity. In the western blots, where we did see cross-reactivity, we tried to determine what the antibodies were actually recognizing. We have not published this yet, but it looks as though when we systemically prime with commensal bacteria to try in a way to model a system in which we cannot handle our commensal flora and it becomes systemic all the time, we get antibodies to bacterial ribosomes. Here we see a lot of cross-reactivity, but probably because those structures are highly conserved between bacterial species. We think these antiribosomal antibodies are protective. So we did cecal puncture and ligation experiments. We punctured the cecum of the animals to release high levels of bacteria into the peritoneum, then ligated to close the punctured cecum. Anticommensal antibodies seemed to be protective and prolonged the life of the animal in response to the commensal bacteria.

**Q:** The results you described are different from those originally described by Cebra’s group [Talham GL et al: *Infect Immunol* 1999;67:1992-2000; Keilbaugh SA et al: *Gut* 2005;54:623-629]. As you know, they compared segmented filamentous bacterium to Morganella morganii and also to a Schaedler’s Cocktail, which is a defined blend of bacteria used to standardize the microbiota used to colonize germ-free rodents. Is it possible that you have simply chosen an organism in which the response is directed wholly against the IgA class, and if you used another organism, it is not?
Dr McCoy: Yes, but we are now trying to isolate as many different commensal organisms as we can in the population. We have done about 10 different isolates, and all the IgA in the gut seems to be specific.

Q: Can you account for the difference between your results and those of Cebra’s group? Better methodology?

Dr McCoy: Maybe. Certainly a different methodology was used in the research you refer to.

Q: Cebra and the Japanese researchers before him [Umesaki Y et al: *Microbiol Immunol* 1995;39:555-562; Umesaki Y et al: *Infect Immunol* 1999;67:3504-3511] showed that the segmented filamentous bacterium has extraordinary capacity. Just a simple monoassociation with that particular organism that is unculturable at the moment, except in germ-free animals, was capable of extending the whole immune repertoire. To me, this is an extraordinary finding. Do you have a similar understanding about the *E cloacae* you are using?

Dr McCoy: Mazmanian and colleagues used polysaccharide A and got good architectural correction in the spleen [Cell 2005;122:107-118]. However, we have not yet done enough monocolonizations to precisely determine what each bacteria may induce. We have not examined enough different bacteria to say whether they all induce the same response or whether different bacteria possess different abilities to mature the immune system. Probably many different bacteria act in concert. If we select one of bacteria, maybe we would see a big response in germ-free mice, but it is hard to say how much that translates into in a real colonization.

Q: The question about western blot vs flow cytometry was interesting. I think it is important to distinguish these two methods. I assume you use a cocktail and sonicate when you do a western blot. Is that correct?

Dr McCoy: Yes. We sonicate to lyse the bacteria, centrifuge to get rid of the membrane fractions, then ultracentrifuge to collect the ribosomes.

Dr Brandtzaeg: In inflammatory bowel disease, the normal, healthy immune response against the bacterial cytosolic antigens shifts more to bacterial surface antigens. The IgG response—IgG1 in ulcerative colitis, for example—is predominately against the surface antigen. Obviously, the immune system sees various things, in various conditions. If there is invasion of a particular organism, the characteristics of the immune response may vary [Furrie E et al: *Gut* 2004;53:91-98].
Q: You mentioned only live commensal flora were triggering and inducing the IgA response, and the dead probiotics did not do that. How were they killed?

Dr McCoy: They were heat killed.

Q: Have you tried any other method of killing?

Dr McCoy: We are trying to kill the bacteria using peracetic acid. That seems to fix the structure and does not destroy it. We are looking at that now to see whether that method of killing can have inductive effects. It seems to be a bit in between, depending on what immune parameters we look at. It was able to induce some IgA, but it does not induce a big T-cell response. We have a new model in which we colonize germ-free mice, and they become germ-free again because the bacteria can no longer propagate. We are using that model to look at bacteria that are alive, dead, or killed in different ways, and probably how intact the bacterial structure is might make a difference.

Q: Some experiments have been conducted with Toll-like receptor 9 (TLR9). Do you know whether the germ-free mouse expresses TLR9 in a polarized fashion?

Dr McCoy: We have not looked at that.

Q: If the mesenteric lymph node acts as a fire wall in humans—I know this is debated—that could preclude the entry of probiotics into breast milk through the circulation. If the mesenteric lymph node does act as a fire wall, and it is blocking the entry of commensal flora and pathogens into the circulation, how do probiotics enter the breast milk? Is it possible that there is a unique resident microbiota lining the mammary gland?

Dr McCoy: The mesenteric lymph nodes act as a fire wall for live bacteria, so, of course, microbial products become systemic. When we colonize mice, the live bacteria do not affect the systemic immune system; the microbial products probably produce the effect.

Dr Bienenstock: This area is controversial. There is no question that the breast can push out viruses such as cytomegalovirus. Whether or not commensal probiotic organisms appear in human breast milk still remains an important question that has important implications for this discussion.

Dr McCoy: We should look at lactating germ-free or monocolonized mice where it is easier and cleaner to collect the milk.
Microbial Components as Modulators of Mucosal Immunity

James Versalovic, MD, PhD, Baylor College of Medicine, USA

The Human Microbiome

Beneficial microbes and probiotics may modulate mucosal immunity, and our evolving understanding of the human microbiome will provide fundamental insights into key aspects of pediatric nutrition. The human gastrointestinal tract contains complex microbial communities composed of bacteria, yeasts, and viruses. One study highlighted the fact that the human intestine contains between 800 and 1000 bacterial species, with >60% of DNA sequences representing previously unknown microbes, respectively. Even the skin of the human forearm, a region of the body with a less complex microbiota, may contain more than 180 bacterial species. In addition to compositional data, microbial communities may be structured in three-dimensional space. Published studies by Swidsinski et al highlighted the point that specific microbes are not randomly distributed in space, but instead, microbes are differentially distributed in different locations within the intestinal tract. Specifically, staining by fluorescence in situ hybridization of the human colon revealed that Bacteroides fragilis, Eubacterium rectale, and other bacteria inhabit discrete zones in successive layers within the intestinal lumen.

Dynamic fluctuations and microbial population shifts within the human microbiome likely occur in the neonatal period and infancy, and in association with changes in nutritional status or exposure to antibiotics. Palmer et al demonstrated that the composition of intestinal microbial communities varied widely among infants during the first year of life, with complex communities existing by 1 week of age. Despite temporal variation, distinct features of each infant’s microbial community were recognizable for months at a time. At the end of the first year of life, each infant’s gut bacterial population achieves adult-like complexity and a state of relative equilibrium. Thus, the first year of life provides a “window of opportunity” for directed manipulation of the composition and aggregate function of the human microbiome.

Beneficial Microbes and Probiotics

The probiotic concept was described in a treatise by Elie Metchnikoff, noted immunologist, microbiologist, and Nobel Laureate entitled The Prolongation of Life:
Microbial Components as Modulators of Mucosal Immunity

Optimistic Studies and published in 1907. However, this concept essentially lay dormant during the 20th century and throughout the “golden era” of antibiotics and vaccines for the treatment and prevention of infectious diseases. The rise in the number of multidrug-resistant pathogens and the recognition of the role that human microbial communities play in health and disease has generated a recent expansion of interest in probiotics. This phenomenon is apparent in both the numbers of probiotic products now marketed to consumers and the increased amount of scientific research occurring in probiotics. Evidence for the renewed interest in probiotics exists in the revival of the probiotic concept in the last 2 decades by Roy Fuller and a group of scientists working on behalf of the World Health Organization.

Probiotics are living microbes that exert a variety of beneficial effects on the host when consumed in adequate amounts. Beneficial effects are broadly defined by design and may include antipathogenic effects, immunomodulatory features, regulation of cell proliferation, the ability to promote normal physiologic development of the mucosal epithelium, and enhancement of human nutrition. Commensal microbes may actively prevent gastrointestinal infections through production of antimicrobial factors, stimulation of the host immune system, or competition with pathogens for nutrients or host-binding sites (Fig 1).

![Fig 1. Beneficial roles of microbes in the mammalian intestine.](image)

Bacteria Modulate Immune Responses

Several studies have shown that bacteria can regulate innate and adaptive immunity. Studies from our own laboratory showed that secreted factors from lactobacilli could block cytokine production by mouse and human cells. Cytokines are key proteins that lead to the proliferation or migration of specific types of
immune cells, and these proteins also may affect adaptive immunity by influencing antibody production. By developing laboratory assays that evaluate the relative abilities of candidate probiotics to suppress cytokine responses, our laboratory identified vast differences between microbes and their abilities to stimulate or block immune responses. These laboratory tests were used to select specific bacterial strains that were administered to mice in order to explore the abilities of candidate probiotics to regulate immune responses in animals. The lessons that emerged from these studies included the recognition that lactobacilli may suppress intestinal inflammation despite the presence of complex microbial communities in the intestine. Laboratory data indicated that the lactobacilli secreted factors (possibly a combination of factors shed or secreted by bacteria) that regulated immune responses.

In addition to dampening cytokine production and suppressing inflammation, bacteria may stimulate immune responses. Several strains of lactobacilli were found to stimulate signaling and cytokine production by immune cells. Furthermore, new studies in our laboratory have found that probiotics may enhance intestinal antibody responses and boost the immune defenses to infectious challenges. These effects by microbes may be due to secreted factors or substances associated with cell surfaces that ultimately stimulate immune responses.

**Mechanisms of Immunomodulation by Probiotics**

New insights are emerging from various studies that suggest that very small organic molecules are secreted by a variety of beneficial microbes and probiotics. In our own studies, we have uncovered folate derivatives that seem to confer anti-inflammatory features. Other candidate compounds include fatty acids that appear to be secreted by probiotics and are associated with anti-inflammatory effects. Other laboratories have documented that supernatants from bacterial cultures may have potent immunoregulatory effects, and small molecules from different bacterial strains confer immunosuppressive or anti-inflammatory effects. Work from Brent Polk’s laboratory at Vanderbilt has documented the production of specific proteins by lactobacilli that regulate the proliferation and programmed cell death of intestinal epithelial cells. A different group found that cell-wall components (lipoteichoic acids) from *Lactobacillus plantarum* have immunoregulatory effects. Probiotics and commensal bacteria also produce vitamins (eg, vitamin B12 and vitamin K) and essential nutrients (amino acids and folates) that may have important functional consequences for immune function. A variety of microbial compounds have emerged as candidate immunoregulatory molecules that may contribute to the role of the microbiome in the development and maintenance of a healthy immune system.
**Microbial Components as Modulators of Mucosal Immunity**

**Bacteria Regulate Key Immune Signaling Pathways**

Beneficial microbes and probiotics can regulate key signaling pathways in different types of immune cells. In our own studies and in studies from other labs, bacteria have been shown to block the activation of transcription factors that may regulate key immune response genes such as cytokine genes. Probiotics secrete or shed organic factors that somehow transmit signals to immune cells (intestinal epithelial cells, myeloid cells, and lymphocytes), and these signals regulate pathways inside cells that ultimately result in reprogramming gene expression. Recent papers from our laboratory highlight the ability of probiotics to block different immune signaling pathways depending on the nature of incoming immune signals (Fig 2).\(^{15,16}\)

Our latest model suggests that intestinal bacteria provide signals that stimulate immune responses, and probiotics can suppress these signals by effectively diminishing cytokine gene expression. Some cytokines are produced, and these cytokines provide signals to other immune cells. However, probiotics also may block the signals from these human proteins, thereby further regulating immune responses via a two-step mechanism. So beneficial microbes and probiotics have evolved patterns of immunoregulation by production of immunomodulatory factors. These factors may be identified as key microbial components of new functional nutrition strategies in infants and children.

---

*Fig 2. Probiotics and suppression of TNF production by macrophages.*
Conclusion

The human microbiome includes many microbes that together may have a fundamental impact on the development and healthy functioning of mucosal and systemic immunity. As the intestine contains a complex immune system with different types of immune cells, directed manipulation of the human microbiome with probiotics and their secreted components may influence immune function in infants and children. Microbes may stimulate immunity and enhance the body’s defense mechanisms against infectious and dietary challenges. In addition, microbes may suppress inflammation and limit the impact of allergies and chronic inflammatory disorders. Microbe:host interactions in the gastrointestinal tract provide opportunities for directed manipulation of immunity by nutritional strategies that affect the composition and function of the human microbiome.

References

Microbial Components as Modulators of Mucosal Immunity


Q & A

Q: I was intrigued by the result of injecting the probiotic. Did you compare the effect of injection to that of oral administration?

Dr Versalovic: No. I just brought up the point that investigators have explored the impact of different routes of administration on systemic immunity. In our mice studies, we used only orogastric gavage.

Q: But there are data on the effect of injecting probiotics, and I am intrigued by whether injection reproduces the results you get with oral administration. Can you comment on that?

Dr Versalovic: Clearly, the route of administration may have an effect. The oral route of administration has enabled us to see important effects on mucosal immunity, but these effects often are localized to the mucosal immune system. In our rotavirus model using orogastric gavage, for instance, we do not see the induction of rotavirus-specific IgA systemically. Other routes of administration may affect systemic immunity more broadly. A number of studies have been done using subcutaneous routes.

Q: Dr Versalovic, you demonstrated that L reuteri interfere directly with the signal pathway in inflammatory responses, which involve NF-kappaB in terms of kinases and so on. Can we infer that, in the present, these bacteria, which can suppress inflammatory responses, may interfere with immune responses?
Dr Versalovic: If you are asking whether the presence of certain bacterial strains present early in life may suppress the development of immunity, that may be true. Looking ahead, I think we may find important differences in the composition of that microbial community very early in life that may be partly determined by the mother’s genotype, the breast milk her infant gets, or by a combination of the infant formula and the mother’s genetic makeup. We know the host’s genotype has a major impact on bacterial composition in the gut. We knew that when we studied IL-10-deficient animals. We saw a complete deficiency in *L. reuteri* in animals that simply lacked the function of a single gene, albeit an important one, in the immune system. The isogenic animals that were IL-10-intact had plenty of *L. reuteri*. So the question is, is it the deficiency of the microbe that predisposes the animals to disease? That is likely, but we also know from a variety of studies that the host genotype has an impact. Some infants are less capable of fostering the proliferation of certain classes of bacteria; in effect, their immune system does not develop as well as that of other infants. As we begin to do metagenomics and community analyses, we may be able to introduce certain organisms early on in an infant’s life to compensate for deficiencies. However, we have to be careful which probiotic strain we select. Roughly 10% of the strains we examined had potent activity. If we use a very potent anti-inflammatory probiotic, we may cause more harm than good.

Q: You mentioned that some of these strains were isolated from breast milk. I am a bit skeptical. Do you know how this was done? Was the strain actually from breast milk or from skin contaminants?

Dr Versalovic: We know breast milk is not sterile. A number of studies published in the past few years have documented that breast milk does have a bacterial component. *Bifidobacterium* is more common than *lactobacillus*. Currently, we are not performing microbiome studies on breast milk, although that is on the table. There may be some skin and surface contamination in such studies, although these groups of organisms are not commonly found on the skin. A recent interesting paper in *Pediatrics* suggested that commensal bacteria may translocate through the intestine through a leaky barrier in the third trimester of pregnancy. Also, the mouse model suggests that there may be a leaky barrier late in pregnancy, and certain commensal microbes may attach to leukocytes and get into the breast milk. This is controversial, but populations of some organisms have been cultured directly from breast milk.

Q: I am reassured to hear that the strains that you have isolated in your lab are stable and do not change. However, do you know whether several kinds of strains appear in breast milk, or just one or two in each woman?
Microbial Components as Modulators of Mucosal Immunity

Dr Versalovic: We do not know enough about this yet. We are just beginning to understand the nature of the microbiome and glycome in breast milk. New prebiotics will be characterized, and at this point the stability of these bacterial populations is just conjecture.

Q: Were the samples you used isolated by you and your colleagues?

Dr Versalovic: No. The ones I alluded to were isolated by Ivan Casas and his group in Peru. He also did microbiology in this field with a group in Finland.

Q: Were these bacteria cultivated before or after nursing was started? Contamination from the mouth to the infant is an important issue.

Dr Versalovic: That is a good point. There is always some potential surface contamination. I think we are getting a greater appreciation of the fact that microbial communities are in breast milk, and the fact that lactobacillus and bifidobacterium are there suggests that infants are getting these beneficial microbes from mother’s breast milk.

Q: Dr Versalovic, you mentioned Andy Neish’s work looking at NF-kappaB suppression with nonpathogenic strains of salmonella, and you said that your work showed similar data using monocytic macrophage cell lines. Which cell population do you think is being targeted? Is it important that you target epithelial cells and macrophages? Has anyone looked at dendritic cells?

Dr Versalovic: Dendritic cell data are sparse. Some studies have examined effects of probiotics on dendritic cells with respect to different patterns of cytokine production. Probiotics may have targeted effects on macrophages. Epithelial cells have been studied in more depth. Clearly, epithelial cells are a primary target. We have examined cytokine production, and we are beginning to explore signaling pathways in epithelial cells in more detail. These microbes are producing very small molecules, which I think are targeting multiple cell types. I think that the molecules get through the epithelial barrier fairly easily; they are small products. We have other “candidates” I did not share today, such as very small fatty acids. Other small organic compounds that we picked up by mass spectrometry are specifically found in organisms that suppress NF-kappaB signaling.

Many small factors affect epithelial cells, but they also get through the tight epithelial barrier and may affect dendritic cells. I think these factors get through the wall and affect macrophages and lymphocytes in the lamina propria. In that way, commensal bacteria are directly regulating immune responses, beyond just affecting epithelial cells.
Q: Do you anticipate that these immunomodulants or these polyglutamates are actually produced by commensal flora other than the \textit{L. reuteri}?

\textbf{Dr Versalovic:} Yes. We do think that a variety of bacteria produce them. Ultimately, we are interested in metabonomics, so we are interested in the metabolites that these communities produce, which could include a variety of other compounds. So in the end, we may be interested in, not just the metabolites, but the metagenome and the functions that are encoded by that metagenome.

Q: In the experiments in which \textit{L. reuteri} increased IgA antibody, did the course of diarrhea change, such as a decrease in rotavirus?

\textbf{Dr Versalovic:} Yes, we saw a reduction in duration of the diarrhea, whether the mouse pups got either strain—TNF inhibitory or TNF stimulatory. These findings were obtained strictly with the murine rotavirus model. We have not studied other etiologies of acute gastroenteritis in the mouse model.
Discussion

Discussion Leader: John Bienenstock, MD, McMaster University, Canada

Dr Bienenstock: No one has mentioned the nervous system, and it would be unwise, in my opinion, to ignore it. There is no question that nerves can control immune response. A recent paper reports that the vagus nerve can control B-cell production indirectly through parasympathetic or sympathetic systems, and it controls B-cell production of immunoglobulins in the spleen. Other research has shown that if you cut the vagus nerve in animals, or if you stimulate the distal end of the vagus nerve, you can have profound effects on some of the things that, for example, Dr Versalovic talked about regarding gut health. Through an alpha-7 nicotinic receptor, in fact, you can control and regulate TNF production with a lipopolysaccharide injection into animals.

The nervous system can, in fact, interact with bicommmensal organisms. We found that if you feed a probiotic organism such as L reuteri to a conventional rat, a calcium-activated potassium channel is activated in the enteric nervous system. This activation is associated with all of the effects that we see—downregulation of immune response, upregulation of IgA, and downregulation of TNF-alpha production.

Let us discuss timing now. Research in Japan showed that, in the germ-free animal, the hypothalamic-pituitary-adrenal (HPA) axis response to stress was hyperexaggerated. If the investigator then normalized or conventionalized these germ-free animals with either specific pathogen-free (SPF) feces or with a single bacterium—he used Bifidobacterium infantis, but he did it before the animals were 6 weeks of age—he reduced the HPA axis response (adrenocorticotropic hormone [ACTH], corticotrophin-releasing factor [CRF], and corticosterone) to the normal response to stress. This lasted into adulthood. I think this is an important observation. It opens up the whole biology of these systems and shows that there is a window. Dr Nagler, in some of your studies you referred to a window of vulnerability, which suggests that when we feed probiotic organisms or commensal organisms, when we feed, how much we feed, and the timing are all highly significant. Dr Kuitunen, you have had more experience with this than most of us. Do you have any comments about the timing of the feeding of the particular probiotic you used in your experiments in Finland?

Dr Kuitunen: It seems important to give the probiotic to the pregnant woman so that the infant meets these bacteria very early. Susan Prescott’s group published a study 1½ years ago but found no allergy preventive effect. For ethical reasons, however, they could give the bacteria only to the infants, not to the mothers [Taylor
AL et al: *J Allergy Clin Immunol* 2007;119:184-191. We had a clear effect when we started this probiotic feeding a couple of weeks before expected delivery. This outcome points to an early window of opportunity.

**Dr Bienenstock:** There clearly is a window in rodents, but I think the question of whether there is such a window in humans is still open. There is also discussion about whether, if there is a window, it shuts by the age of say 2 years. If we can change the set point of the neuroendocrine system, keep the window open and maintain it into adulthood, and extrapolate that to the immune system or the nervous system, we have an extraordinary opportunity. But if we miss the opportunity, we will not have that effect. Also, this is an epigenetic phenomenon. Studies have looked at this in terms of the HPA axis from an epigenetic point of view, even localizing it down to the promoter for the benefit of the corticoid receptors. This is not trivial.

**Dr Lee:** The literature suggests that for the purpose of affecting allergic responses the window seems to be the first 2 years—in fact, the first year. We have seen 13 or 14 obscure studies that should indicate that effect, but only one or two show a positive effect on people with allergic asthma, or specifically, pollen allergies [Xiao JZ et al: *Clin Exp Allergy* 2006;36:1425-1435]. We do not see others who have achieved any effect on people suffering from allergy.

**Dr Bienenstock:** But that is treatment, not prophylaxis.

**Dr Lee:** That is right, it is not prevention. That is a different method altogether. As you say, the timing is important.

**Dr Noakes:** As already mentioned, timing is important when we talk about treatment versus prevention. A lot of my work has been with Susan Prescott in Australia and Philip Calder in the United Kingdom. We have a number of pregnancy cohort studies going on based on the idea that the window for prevention is within the first 18 months of life. So we are providing different types of omega-3 fatty acids and probiotics during this time. Dr Prescott has published work with Jan Dunstan in which they looked at the effect of interventions with omega-3 fatty acids in a small cohort—40 women in each group [Prescott SL et al: *J Allergy Clin Immunol* 2007;120:200-206; Dunstan JA et al: *J Allergy Clin Immunol* 2003;112:1178-1184]. They saw a general dampening down of Th1-type and Th2-type immune responses overall, rather than skewing in one direction or another. There was a trend toward decreased severity of such conditions as atopic dermatitis in the group that took fish oil, although other larger cohort studies are now going on in which the primary outcomes are clinical outcomes. To reiterate, timing is extremely important in
Discussion

prevention as opposed to treatment. Not to say that there is not a role for probiotics and fish oil in treatment, but from a preventative point of view, we need intervention studies.

Dr Lee: We do not see the effects on the prevention of allergic reactions in adults. Is it a case of colonization of these systems, or is there a change in the maturity of the immune system that results in the differences in the responses in the infant and the adult?

Dr Noakes: Dr Prescott’s group also gave probiotics to infants 6 to 18 months of age. They saw trends for a reduction in the severity of atopic dermatitis in those infants who received the probiotics, as well as improved quality of life [Weston S et al: Arch Dis Child 2005;90:892-897]. I believe, however, that these results were sustained only for the duration of the intervention. So we also have to think about the duration of the intervention and how long the effects last.

Dr Kuitunen: Yes, duration might be important. Just getting one probiotic bacteria for 6 months and then hoping that for the rest of your life you are safe from allergies might be too naïve. We are now analyzing the 5-year results of our cohort study, and it seems that the allergy preventive effects are not as strong as they were at 2 years. We did a 6-month intervention with four probiotics and found that the colonization is also transient. We can see colonization at 3 months and at 6 months, but at 2 years—1½ years after the colonization intervention—we see no differences between those who received the probiotics and those who did not.


Dr Bienenstock: Those were effects on atopic dermatitis, but not on asthma.

Dr Lee: And not on sensitization.

Dr Kuitunen: This is the only study—and it is a small study—showing that the effect is sustained. In this study, in fact, the effect was strong at 2 and 4 years and a little weaker at 7 years. It remains to be seen whether this is the case in larger cohorts.
**Dr Rueda:** A recently published study used the same experimental design as the Isolauri group, and they obtained the opposite results [Kopp MV et al: *Pediatrics* 2008;121:e850-e856]. Another recent study obtained positive results using *Lactobacillus rhamnosus*, giving the probiotic to the mother starting at 35 weeks of gestation and to the infant until 2 years of age. That is a controversial study, however, because if you use the same experimental design as a previous researcher and obtain the opposite results, they might be influencing other factors with that intervention.

**Dr Nagler:** In all these studies in which probiotics were given to pregnant women, has anyone demonstrated that the bacteria that were fed can be recovered from either the breast milk or from the gastrointestinal tract of the infant?

**Dr Bienenstock:** There is no question that they can be recovered from the gastrointestinal tract of the infant. The question is, how long you feed the probiotics? I think the longest after termination of feeding that anyone has found the probiotics—I think it was with LGG—was 18 months. I do not think anyone has suggested that these are permanent colonizers, right?

**Dr Kuitunen:** Right. Studies have been conducted with LGG on adult volunteers, and, in fact, the disappearance is rapid once you stop feeding the probiotics. At 3 weeks it is almost gone [Lassig A et al: SSA Gut Impact: 3rd Platform Meeting on Foods for Intestinal Health. Haikko, Finland: 2007, pp 29-31].

**Dr Nagler:** That same group, I believe, did a study in which they fed pregnant women a specific strain of bacteria and tried to isolate that strain in the breast after birth. I do not know whether they obtained positive results or whether they have published yet.

**Dr Versalovic:** Dr Bienenstock, I want to go back to something you mentioned at the beginning of this discussion—the connection with the enteric nervous system. I do believe in this window of opportunity to produce health benefits in the first year of an infant’s life. I am intrigued by one study that indicates that the window may be as long as 5 or 6 years [Palmer C et al: *PLoS Biol* 2007;5:e1777], but clearly, according to the Stanford metagenomic data set, there is tremendous flux and an opportunity during the first year of life. Flavia Indrio’s group from the University of Bari in Italy has tried to apply probiotics to effects on intestinal motility in infants [Indrio F et al: *J Pediatr* 2008;152:801-806]. They have shown that, with a single strain, they can have a significant effect on gastric emptying and irritability, with a
Discussion

reduction in mean daily crying times. Recently, she shared data from a 4-month follow-up, which showed a reduction in regurgitation in the infants. These data suggest that there may be a real opportunity to affect the function of smooth muscle in the enteric nervous system.

Dr Bienenstock: My colleagues and I have evidence in visceral pain from distention of the colon of the same effects with single organisms, showing that they could completely convert the germ-free disorder of motility in adults, following either single organisms or several organisms.

Annick Mercenier and colleagues have published data on the anti-inflammatory effects of probiotics and the anti-inflammatory, downregulatory mutant, which is diamine-deficient and is much more potent than the proinflammatory ones in terms of pain regulation. We believe that this is due to its generation of IL-10, which is antinociceptive. This is one situation in which the whole immune system potentially interacts with the nervous system, although this will prove difficult to unravel.
Early Nutrition, Including the Role of Breast Milk, and Modulation of Tolerogenic and Immunogenic Responses

Ricardo Rueda, MD, PhD, Abbott Nutrition, Spain

The largest component of the immune system is located in the intestine. A key feature of the intestinal immune system is its ability to discriminate between invasive pathogens, for which it generates strong protective immunity against infections, and antigens that are harmless, such as food proteins and commensal bacteria (Fig 1).

The default response to harmless antigens in the gut is the induction of a state of immunological hyporesponsiveness, known as oral tolerance, and this may be the homeostatic mechanism that prevents intestinal disorders such as celiac disease and Crohn’s disease, as well as atopy.1

According to Mowat,1 the way in which antigen is presented to the immune system is the critical factor that controls whether tolerance or immunity results, and most evidence indicates that dendritic cells (DCs) are likely to be the antigen-presenting cells involved (Fig 1). In the presence of inflammation or pathogenic organisms, DCs are activated to express a full range of co-stimulatory molecules and cytokines, ensuring the efficient stimulation and differentiation of effector T cells. However,
DCs are also central to the induction of tolerance to both self and foreign antigens, as in the absence of inflammation, DCs can present antigen to T cells but lack the complete range of co-stimulatory molecules necessary for full T-cell activation. DCs of this kind have been referred to as mature but “quiescent,” and T cells stimulated in this way become unresponsive (anergic) and/or differentiate into regulatory T cells.

Recent work has begun to identify factors responsible for intestinal conditioning of DC function and the subsequent decision between tolerance and immunity in the intestine. These studies have identified how DCs resident in the intestine, depending on how they are conditioned by epithelial cell-derived factors or pathogenic virulence factors, drive the differentiation of different type of T-cell responses, such as forkhead box P3 (FoxP3)+ regulatory T (Treg) cells or T helper 1 (Th1)- or Th17-type responses. Furthermore, recent studies addressing the mechanism of oral tolerance also show that mucosal tissues are replete with a unique subset of DCs that secrete factors such as TGF-β1 and retinoic acid that induce FoxP3+ regulatory T cells. According to these studies, mucosal unresponsiveness might be related to the availability of a factor in the food stream, such as vitamin A.

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. Consequently the interaction between nutrients, microflora, and the intestinal immune system highly influences how these processes take place (Fig 2).

**Fig 2. Interaction between nutrients, microflora, and the intestinal immune system.**

This is in agreement with the hygiene hypothesis of atopic disease that suggests that environmental changes in the industrialized world have led to reduced microbial contact at an early age and have thus resulted in the growing epidemic of atopic
eczema, allergic rhinoconjunctivitis, and asthma. In addition to protection against atopy, protection against infectious, inflammatory, and autoimmune diseases also may depend on healthy host-microbe interactions implicated in the hygiene hypothesis.5

Breast milk, which is generally accepted as the optimal source to feed infants, constitutes a good model to study the interaction between nutrients, microflora, and the intestinal immune system. On the one hand, it is currently accepted that human milk is a source of beneficial bacteria for infant gut development and maturation,6,7 and on the other hand, it also contains a complex mixture of bioactive compounds that have been demonstrated to have a beneficial role on immune function in infancy (Table).8

Table. Immunological Components in Human Milk

<table>
<thead>
<tr>
<th>Constituents that promote tolerance/priming of the infant immune system</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Cytokines: TGF-β, IL-10</td>
</tr>
<tr>
<td>- Antigens</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Antimicrobial components</td>
</tr>
<tr>
<td>- Immunoglobulins</td>
</tr>
<tr>
<td>- Lysozymes/other enzymes</td>
</tr>
<tr>
<td>- Lactoferrin, mucins, other proteins/peptides</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Factors that promote immune development</td>
</tr>
<tr>
<td>- Nucleotides, gangliosides</td>
</tr>
<tr>
<td>- Cytokines: TGF-β, IL-6, IL-10</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Anti-inflammatory components</td>
</tr>
<tr>
<td>- Cytokines: TGF-β, IL-10</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

The role of breast milk in regulating immunological tolerance to allergen exposure even outside the intestine was recently demonstrated through an elegant animal study. This study showed that breast milk-mediated transfer of an airborne antigen to the neonate resulted in oral tolerance induction, leading to antigen-specific protection from allergic airway disease. That tolerance induction did not require the transfer of immunoglobulins, relied on the presence of transforming growth factor beta (TGF-β) during lactation, was mediated by regulatory CD4+ T lymphocytes, and depended on TGF-β signaling in T cells.9

The inclusion of probiotics, as ingredients in nutritional products, has been one of the tools used to try to influence the process of oral tolerance and response upon
infection at the intestinal level. The immunomodulatory effects of probiotics in the intestinal tract were recently reviewed, suggesting that many probiotic organisms are able to influence DCs to induce a nonresponse state, more particularly by encouraging the development of T cells with immunoregulatory properties.\textsuperscript{10}

The influence of probiotics on the process of oral tolerance, and consequently on the prevention of atopy, has been demonstrated by several studies, the most representative one showing that \textit{Lactobacillus} GG was effective in prevention of early atopic disease in children at high risk.\textsuperscript{11,12} However, another group recently showed, through a similar experimental design, that supplementation with \textit{Lactobacillus} GG during pregnancy and early infancy did not reduce the incidence of atopic dermatitis or alter the severity of atopic dermatitis in affected children but was associated with an increased rate of recurrent episodes of wheezing bronchitis.\textsuperscript{13} According to this last study, the effect of \textit{Lactobacillus} GG on the prevention of atopic dermatitis remains controversial. Nonetheless, another recent study has shown that supplementation with another lactobacillus strain (\textit{Lactobacillus rhamnosus}) during pregnancy and early in life substantially reduced the cumulative prevalence of eczema\textsuperscript{14} and had the potential to influence several fetal immune parameters as well as immunomodulatory factors in breast milk.\textsuperscript{15}

On the other hand, protection against viral or bacterial infections is one of the most frequent claims made for probiotic consumption. Different mechanisms have been suggested to explain this antimicrobial activity, including production of antimicrobial compounds, increase in mucine production, reduction of intestinal permeability, and competition with enterotoxigenic bacteria for nutrients and epithelial intestinal cell-receptor binding sites.\textsuperscript{16}

Human milk also contains a wide range of oligosaccharides, which are considered as the first prebiotics in humans, thus constituting also the first soluble fibers to which humans are exposed.\textsuperscript{17} Although several biological functions have been proposed for human-milk oligosaccharides,\textsuperscript{18} one indisputable role is as primordial soluble fibers and selective prebiotics. In addition, human-milk oligosaccharides act as decoys for pathogens and toxins that adhere to oligosaccharides in the surfaces of target cells. Several studies support this particular role for human-milk oligosaccharides,\textsuperscript{19} which is also shown for other glycoconjugates, such as gangliosides. Gangliosides are also present in human milk, and they may function as “unintended” target receptors for bacterial adhesion to the intestine.

Ganglioside-supplemented infant formula has been reported to modify the intestinal ecology of preterm newborns, increasing the bifidobacteria content and lowering that of \textit{Escherichia coli}.\textsuperscript{20} After oral administration, gangliosides can be putative decoys that interfere with pathogenic binding in the intestine, this being the main
mechanism by which these compounds can prevent infection. Recently, the influence of milk gangliosides on DC maturation and effector functionalities also has been reported, suggesting a role, especially for ganglioside GD3, in modulating the process of oral tolerance during first stages of life.\textsuperscript{21-24}

On the other hand, gangliosides also may stimulate the ability to mount an appropriate immune response upon infection. In fact, the influence of dietary gangliosides on several parameters related to the development of the intestinal immune system, such as cytokine and intestinal IgA production, also has been described in animal models.\textsuperscript{20} This is also the case for other ingredients, such as nucleotides, which are shown in animal studies to have a role modulating IgA production and lymphocyte populations at the intestinal level.\textsuperscript{25} Several clinical studies also have shown the role of nucleotides influencing both humoral and cellular responses, and reducing diarrhea incidence and duration when they are incorporated into infant feeding early in life.\textsuperscript{26} The effect of nucleotides reducing diarrhea incidence might be influenced, at least partially, by also influencing the composition of intestinal microflora. Although this topic has remained controversial because of studies reporting contradictory results,\textsuperscript{27,28} a recent study using more robust molecular techniques to assess fecal microbiology has demonstrated that nucleotide supplementation improves the composition of the gut microbiota in formula-fed infants.\textsuperscript{29}

In summary, nutrition early in life might affect later immune competence, the ability to develop a tolerogenic response to “self” and to benign environmental antigens, and the ability to mount an appropriate immune response upon infection; consequently, it also might prevent the development of immunologic disorders (Fig 3).

![Fig 3. Influence of early nutrition on immunity.](image-url)
Breast milk contains a complex mixture of bioactive compounds that greatly contribute to regulation of those abilities. The incorporation of ingredients such as nucleotides, probiotics, and prebiotics into the composition of infant formulas constitutes a good example of the effort to not only provide the nutritional requirements of the neonate, but also to emulate the immunological development observed in breastfed infants both early and later in life.

References


Q & A

Q: In one study, Dr Rueda, you described inhibition of dendritic cells by several species of gangliosides. In another study, you observed an increase of IgA production in your subjects. Will you speculate about the possible underlying mechanisms in these differing observations?

Dr Rueda: We have not studied dendritic cells with gangliosides, so we could not interpret what the mechanism might be. Regarding IgA, although we described it for another milk compound (it was for nucleotides and not for gangliosides), we could see that nucleotides were able to modify the expression of some markers, especially those of B1a cells, which are precursors of plasma cells producing IgA at the intestinal level [Aggett P et al. *Nutrition* 2003;19:375-384]. We also were able to demonstrate that both nucleotides and gangliosides were able to stimulate the production of some cytokines that are involved in the maturation and differentiation from B cells to plasma cells producing IgA. That is the case, for example, with IL-2 and IL-6. We could see that process for both nucleotides and gangliosides, so that might be one mechanism explaining the increase of IgA production promoted by both milk compounds. There might be others, however, that we cannot interpret.

Q: With respect to the profile of oligosaccharides, there is a difference among species. What about in rodent species? What are the oligosaccharide levels and distribution?

Dr Rueda: We have not studied that. As far as I know, there is a varied population of oligosaccharides in sheep and goats, but I am not aware of the specific role or concentration of oligosaccharides in rodents.

Q: Can you comment on the proposed mechanism or hypothesis as to why nucleotides might affect the microbiota? Are they food for the microbiome?

Dr Rueda: I do not have a clear answer. It has been debated whether nucleotides are prebiotics or not. We do not know whether they reach the colon, because they are absorbed earlier in the small intestine. They might be a specific nutrient, but because they do not reach the colon, I do not know how they are able to modify the microbiota.

Q: Is it possible to give a rough gauge of the relative importance of oligosaccharides, nucleotides, and gangliosides on the direct effect on commensal bacteria, which, in turn, affect the host? What about the relative importance of breast-milk components
on direct effects on the host and on commensal bacteria in the host?

Dr Rueda: It is difficult to isolate one from the other, because they probably are related. On the other hand, we cannot forget that it also is difficult to isolate one specific ingredient present in breast milk from the others. There might be complex interactions between them. The only way to do it is in animal studies in which we isolate a specific ingredient and have a control group that does not get this ingredient, and then interpret the potential mechanism. We always have several mechanisms that might affect the intended outcome, however, so such research is difficult.

Q: Yes, it is with conventional animals and humans. Has anyone done this on germ-free animals to illustrate whether there is a direct or indirect effect on commensal bacteria?

Dr Rueda: Dr McCoy previously described experiments using that wonderful technology—germ-free animals. We have not done any experiments in which we fed germ-free mice with a specific compound and studied intestinal microflora because we did not have that focus. We focused mainly on the immune activity regulated by these ingredients and how they modify some immunological parameters at the intestinal level.

Q: Here is a controversial issue: What sort of advice would you give to mothers who are severely affected by allergic asthma, with high levels of IL-4 and perhaps even T cell specificity for allergens in the breast milk? Should they breastfeed their infants?

Dr Rueda: We could talk about that question for an hour. Although some studies indicate that probiotics taken by the mother and/or the infant might have a role in preventing allergies, results do not clearly indicate which probiotic produces this effect. For a mother of a child who is probably at risk to develop atopy, I would advise controlling the feeding of the infant in the first days of life. At this moment, the tools we have are infant formulas with hydrolyzed protein that help prevent atopy. However, it is not clear whether partially hydrolyzed or extensively hydrolyzed formula should be the first option. This is controversial, but at this moment, international pediatric committees recommend feeding infants at risk for atopy a formula with an extensively hydrolyzed protein.

Q: So would you recommend that a highly allergic asthmatic mother avoid breastfeeding?
Dr Rueda: No, no, no!

Q: No? But some people do that when mothers have high levels of IL-4 and T cells.

Dr Rueda: That is not what the international committees are saying. However, I have read that there might be a risk—that if the mother feeds human milk to the infant, it might be more difficult to control that process later after child has developed allergy [Zeiger RS: Pediatrics 2003;111:1662-1671].

Q: In the mouse study you talked about, you referred to the transfer of antigen into the breast milk. How do you think the ovalbumin moved from the respiratory tract into the milk?

Dr Rueda: I do not know. The only explanation I can give is the same one that Dr Versalovic suggested previously for probiotics. Apparently, there is an increase in susceptibility among pregnant women, especially during the last part of gestation, to having some antigens circulating for some time. The antigens can migrate to, for example, the mammary gland. That is the only mechanism suggested, but we do not have evidence for it. In a study in Spain using probiotics, but not ovalbumin, researchers fed mothers a specific strain and then tried to isolate that strain from human milk. As far as I know, their results are not available or published.

Q: We tend to swallow inhalant allergens, as well, so we get a lot of inhalants in the gut. Might that be involved?

Dr Rueda: Yes.

Q: I would like to follow up on a previous question. The American Academy of Pediatrics and, I believe, the European pediatric groups recommend feeding practices for high-risk infants, but they are not clear. These groups seem silent on what to do with a mother who is profoundly allergic and expressing the symptoms of allergy. Should she breastfeed? That is an important question. Dr Bienenstock, what do you think about that?

Dr Bienenstock: Pat Holt in Western Australia is conducting a study of oral immunization of high-risk infants with large doses of antigen. The infants have been followed since birth and so far have not had any problems or adverse effects in this important and as yet unpublished study. The obvious alternative view we may wish to take is that it makes no difference whether a mother breastfeeds or not, and that one should encourage the normal process, which is breastfeeding, in this situation. The evidence is highly controversial, and I do not accept the position that one
should forbid breastfeeding in a high-risk mother. I think we have to entertain the possibility that what is needed is high exposure levels in these infants to promote tolerance to the antigen.

**Dr Brandtzaeg:** I am not referring to the genetic risk of the child. I am referring to the drive for a Th2 response, perhaps depending on high levels of IL-4 in breast milk and, possibly, T cells with specificity for allergens that could be transferred to the infant and start an allergic response. The large immunological studies from Arizona show that highly asthmatic women could actually transfer with breast milk this drive toward the Th2 response when atopic children grow up (around 6 years of age). I know this is a controversial issue, despite being supported by mouse studies [Wright AL et al: *Thorax* 2001;56:192-197; Leme AS et al: *J Immunol* 2006;176:762-769].

**Dr Bienenstock:** This is just a proposal, Dr Brandtzaeg. We are translating the evidence of IL-4 in the breast milk and so on to what we think is going to happen next. I do not believe that the evidence is there.

**Dr Brandtzaeg:** No, it is controversial and not easy, but an audience like this perhaps could give a clear-cut opinion on it. I take the silence as evidence that there is no answer yet.

**Dr Larry Williams (Abbott Faculty):** I will not give you an answer, but I will give you the practical pediatric allergy response. We are talking about risk reduction, not prevention. For an individual mother, it is a question of risk reduction, and none of the techniques that have been suggested for allergen avoidance over the last 20 years is associated with more than a 30% reduction in risk. That means that if the physician takes the authoritative stance “you must do this,” he or she is setting up a 70% chance that those mothers will fail. That is unacceptable. So the science is such that we do not know what to do. My view is that physicians should not be prescribing extensive dietary manipulation of small infants, but that standard practice should reign until we have better science.

**Q:** Is there any evidence that the presence of IL-4 in breast milk is maintained in the infant? It has to go through a lot of systemic paths. Most of it must be destroyed. Would it even be active?

**Dr Rueda:** Not as far as we know.
Clinical Evidence for the Role of Prebiotics in Mucosal Immune Development and Impact on Respiratory Health and Allergy

Guido Moro, MD, University of Milan, Italy

Background and Hypothesis

The broad consensus is that breastfed infants grow and develop differently than artificially fed infants. Breastfed infants have a reduced incidence of allergic or atopic diseases, as well as of infections, in comparison to bottle-fed infants, indicating a major impact of breastfeeding on the development of the immune system.

Increasing evidence shows that the composition of the intestinal microbiota plays a key role in the postnatal development of the immune system. Before birth, the infant’s gut is sterile. During vaginal delivery, the natural colonization of the infant starts with bacteria mainly from the vaginal and intestinal microbiota of the mother. The infant’s diet plays an important role in further development of the intestinal microbiota. During breastfeeding, the composition of the gut microbiota develops within a short period and becomes dominated by bifidobacteria, whereas formula-fed infants develop a flora of a more adult type.

Because of the importance of the intestinal microbiota for the development of the gut physiology and the immune system, many attempts have been made to mimic the intestinal microbiota of breastfed infants in bottle-fed infants. Recently, a mixture of neutral short-chain galactooligosaccharides and long-chain fructooligosaccharides (scGOS/lcFOS) were shown to reduce the incidence of atopic dermatitis (AD) and infectious episodes during the first 6 months of life. This dual protection occurred through the intervention period. The hypothesis of the following study was to evaluate whether these protective effects would last beyond the intervention period.

Study Population and Methods

In a prospective, randomized, double-blind, placebo-controlled study, 259 healthy term infants with a parental history of atopy were fed either prebiotic-supplemented...
(0.8 g/100 mL scGOS/lcFOS) or placebo-supplemented (0.8 g/100 mL maltodextrin) hypoallergenic formula during the first 6 months of life. A total of 102 infants in the prebiotic group and 104 infants in the placebo group completed the study. All infants were examined for clinical evidence of AD and incidence of infections. Following this intervention period, blinded follow-up continued until 2 years of life. Primary end points were cumulative incidence of allergic manifestations (AD, recurrent wheezing, and allergic urticaria). Secondary end points were rate (incidence) of infections (physician-diagnosed infectious episodes, fever episodes, and antibiotic prescriptions) and growth.

Results

Two hundred and fifty-nine term infants were enrolled, and 206 infants completed the first 6-month part of the study. Parents of 152 completers gave consent to participate in the 2-year follow-up trial.

During the intervention period (first 6 months of life), 10 infants in the intervention group (9.8%) and 24 infants in the placebo group (23.1%) developed AD. The severity of AD was not affected by the diet. During the study period, infants in the scGOS/lcFOS group had fewer episodes of infections. They also tended to have fewer episodes of upper respiratory tract infection and fewer infections requiring antibiotic treatment.

Out of 152 participants in the 2-year follow-up, 134 infants (68 in placebo, 66 in intervention group) completed the study period. During this period, infants in the scGOS/lcFOS group had significantly lower incidence of allergic manifestations. Cumulative incidences of AD, recurrent wheezing, and allergic urticaria were respectively 27.9%, 20.6%, and 10.3% in the placebo; 13.6%, 7.6%, and 1.5% in the intervention group ($P<0.05$).

Infants in the scGOS/lcFOS group had fewer episodes of infections overall (respiratory, urinary, gastrointestinal infections, and otitis media), fewer episodes of fever, and fewer antibiotic prescriptions. The reduction in the number of infectious episodes reached a statistical significance for physician-diagnosed overall infections (5.9 vs 4.0 episodes/infant, $P<0.01$); for fever episodes (4.0 vs 2.2 episodes/infant, $P<0.000001$); for upper respiratory tract infections (3.2 vs 2.1 episodes/infant, $P<0.01$); and for antibiotic prescriptions (2.7 vs 1.8, $P<0.05$).

Growth was normal and similar in both groups.
Clinical Evidence for the Role of Prebiotics in Mucosal Immune Development and Impact on Respiratory Health and Allergy

Conclusion

Early dietary intervention with oligosaccharide prebiotics has a protective effect against both allergic manifestations and infections. This effect lasts beyond the intervention period until 2 years of life with the achievement of adequate growth.

Thus, the evidence shows that prebiotics have a significant and biologically relevant effect on the postnatal development of the immune system. The present data indicate that prebiotics can serve as an effective and safe tool to strengthen the immune system during infancy, which may offer a new method to prevent allergy and infections. However, long-term studies are needed to test the hypothesis that the influence of dietary factors on the immune system early in life might have beneficial consequences later in life.

References


**Q & A**

**Q:** How did you decide on this particular blend of prebiotics?

**Dr Moro:** We did not decide on the blend, the company that produced the mixture decided. After several animal studies, they evaluated different ratios between the short-chain galactooligosaccharide (scGOS) component and the long-chain fructooligosaccharide (lcFOS) component, and at the end, they found that the best ratio was nine to one scGOS to lcFOS. Several studies demonstrated that this level—0.8 g/100 mL of infant formula—was best for producing positive effects in infants.

**Q:** You fed both scGOS and lcFOS in your blend, and lcFOS contributed only 10%. How great an impact do you believe that 10% had on the outcome?

**Dr Moro:** That is a good question, because it is a complex topic. The oligosaccharide composition of human milk is completely different from that of the blend. lcFOS, as you know, are not present in human milk. The mixture of scGOS and lcFOS used in these studies resembles the molecular size distribution of human-milk oligosaccharides. The main objective was not to mimic the complex chemical structure of human milk oligosaccharides, but to mimic the bifidogenic effects of human-milk oligosaccharides in formula-fed infants. Some research groups are trying to replicate the oligosaccharides that are present in human milk, but they are very far from the solution.

**Q:** I would like to follow up on a previous question. Dr Moro, you saw significant differences between the formula groups, with or without the oligosaccharides, in high-risk infants. Is there a cohort of infants fed human milk to show how they would have done over the course of time?

**Dr Moro:** No. In our study, we only compared the group receiving the standard formula to the group receiving the scGOS/lcFOS-supplemented formula in high-risk
Clinical Evidence for the Role of Prebiotics in Mucosal Immune Development and Impact on Respiratory Health and Allergy

infants. They were high risk because at least one of the parents was positive for allergy. We did not use a breastfed group for comparison. However, a breastfed group was used in the Multicentre Immuno Programming Study (MIPS). I showed you the results related to allergies and atopic dermatitis. We had more than 300 infants receiving mother's milk, and we used them for comparison. You cannot use breastfed infants as a control group, because then it is not a blinded study—you know those infants are receiving the mother's milk. You can, however, use them as a reference to evaluate whether the results you obtain in the formula-fed groups are similar to the results in breastfed infants. So we used a breastfed group in the MIPS, but not in our allergy study.

Q: In your last study, you saw a reduction in the number of episodes of fever. Do you think it might be a consequence of the reaction of the episodes of infection, or do you think that the prebiotics might have an effect on reduction of fever in the children independently of whether they have infection. To me, fever is a consequence of the reaction of the immune system to the infection; that might not be good.

Dr Moro: We decided to use fever as an outcome measure because it is an objective element to evaluate. Definitions of infection always depend on the person who is evaluating the infants. So we were not involved in the diagnosis of infection; the diagnosis was made by the family pediatrician. This is the reason why we decided to look at fever and number of infections. Fever generally appears in the most severe cases of infection. You can have infection without fever, but when you have fever, you have infection.

Q: Was the scGOS/lcFOS formula extensively hydrolyzed?

Dr Moro: It was somewhere between partially and extensively hydrolyzed, but we can consider it extensively hydrolyzed.

Q: Were you surprised that, in the placebo group, the incidence of atopic dermatitis was relatively high? Twenty-three percent, then 28% at 2 years.

Dr Moro: Yes, but in Italy, when there is a family history of allergy and the infant is not receiving mother's milk, the pediatrician generally starts the infant on hydrolyzed formula. The incidence of atopic dermatitis in these infants (~20%-22%) is similar to the incidence we found in our group receiving the standard formula.

Q: Do you think that the hydrolyzed formula helped lower the incidence?

Dr Moro: No, it did not help lower the incidence, but it probably helped reduce the
severity of the disease. If you look at the results obtained with the SCORing Atopic Dermatitis (SCORAD) score at 3 and 6 months of age, the severity of the score was similar in the two groups—between 9 and 13.

Q: In the MIPS, was the formula hydrolyzed?

Dr Moro: No, a standard starting formula was used. All those infants were normal healthy babies with no family history of allergy.

Q: When you looked at the vaccine responses, you saw no significant differences. However, if you look at the immunoglobulin (Ig) G2 results in the control group vs that in the scGOS/lcFOS-formula group, it looks like there was a difference.

Dr Moro: There was a small difference, but without statistical significance.

Q: Were you concerned about that since IgG2 is the most predominant immunoglobulin?

Dr Moro: Of course, but we were interested in finding significant differences.

Q: Was there was a trend in the $P$ value?

Dr Moro: I cannot tell you whether there was a trend, but for IgG1, IgG2, and IgG3 there were no significant differences. If you look at the results, IgG1 values were higher in the standard-formula group compared to those in the scGOS/lcFOS-formula group, so it made no difference.

Q: In the first part of your presentation, you talked about prebiotics and the intestinal flora. Did you omit any children who had received antibiotics?

Dr Moro: Yes. Infants who were receiving antibiotic for any reason were taken out of the study.

Q: I did not hear you say anything about asthma or the incidence of asthma in this population, and there has been such a difference of opinion about asthma in the prebiotic groups. What was your experience with wheezing or asthma?

Dr Moro: We followed the infants until the age of 2 years, and as you know, asthma usually occurs later. We were only able to evaluate the infants for wheezing, and the rate of wheezing was lower in the first 2 years of life in the group receiving scGOS/lcFOS formula. We are planning to follow the infants until the age of 5 years. Probably next year we will complete the follow-up and see whether there is any
difference in the rate of asthma in the two groups. Everyone is interested in this topic.

Q: At the end of your presentation, you strongly advocated the imprinting theory and a 6-month intervention. Do you see any place for oligosaccharides beyond 6 months, or does the food of Italian babies contain it naturally?

Dr Moro: I think all the conference participants talked about the intervention “window,” but no one knows how long it lasts. We do know that the earlier you intervene, the better the results. In the MIPS, we continued the scGOS/lcFOS supplementation for the first year of life, but we decided that 6 months was a good time to evaluate the effect of the milk because during that time the infants in both groups were receiving only milk. We were able to evaluate exactly what nutrients and how much scGOS and lcFOS they were receiving. But you can continue this supplementation for a year or longer.

Q: It seems like the major outcome of your prebiotic treatment was to elevate the bifidobacteria levels in the feces of the formula-fed infants to the same levels as those in breastfed infants. Would it be reasonable, then, to speculate that any formula that raises the level of bifidobacteria to that of a breastfed infant would be efficacious against allergy?

Dr Moro: I think that you have to evaluate not only the number of positive bacteria you reach, but also the other aspect. That is, if you are giving bifidobacteria, and the number of bifidobacteria is similar to that found in the stools of breastfed infants, that is okay, but you also have to evaluate the number of pathogens—the different environment you are working with. Short-chain fatty acid levels, pH value, IgA levels—all these aspects—are extremely important. To have only the bifidobacteria value, in my opinion, is not enough to evaluate the efficacy of the formula you are using.

Q: Do you think that the pathogen levels, or differences in pathogen levels, affect allergy, as well—not just infectious diseases?

Dr Moro: Yes. There is a clear correlation between some strains such as clostridium and allergy. You also have to evaluate other aspects of the intestinal flora, not only the number of bifidobacteria and lactobacilla.

Q: Your presentation was about prevention, but based on your experience and your results, do you see any place for prebiotics in the treatment of atopic eczema or food allergy? Very few studies have been done on that.
Dr Moro: That is a good question, because prebiotics and probiotics have different roles. Prebiotics can have a preventive effect, and probiotics can have a therapeutic effect. Probiotics work efficiently and positively on diarrhea such as that caused by antibiotics. There is no evidence that prebiotics have such effects during diarrhea, but they may help prevent diarrhea.

Q: My question is about the hydrolyzed formula and the incidence of eczema observed in your control group. Studies such as the German Infant Nutritional Intervention (GINI) Study were not just about feeding a hydrolyzed formula, they were about a regimen—for instance, not feeding solid foods for a period of time and not exposing the infants to any milk-based formula before they entered the study. Have you considered that the feeding of the hydrolysate alone may have produced your results? Were other regimens incorporated into this study so that those things were avoided in your control group, and if so, could those be related to the higher incidence of eczema you observed in that group?

Dr Moro: The infants received only formula for the first 6 months of life. They did not receive any other foods or other substances during the treatment period. After that, they began weaning. There were no problems with the mothers because they were not breastfeeding; they were free to eat whatever they liked.

Q: You talked about the cumulative incidence of eczema in the MIPS 2-year follow-up. In studies such as GINI, the researchers continue to see differences in the cumulative incidence, because a lot of that is driven by what happens in the first 6 months. Is that what you saw in your study, or did you continue to see a point prevalence difference at older ages, as well as early differences?

Dr Moro: We evaluated the difference and saw that the infants receiving the standard formula developed atopic dermatitis earlier than those receiving the scGOS/lcFOS formula. The majority of infants developed atopic dermatitis in the first 6 months of life.

Q: The difference you saw at 2 years was based on what was seen in the first 6 months?

Dr Moro: Yes, in our study on infants at high risk for allergy. In the MIPS, we treated healthy, full-term infants without any family history of allergy infants for the first year of life, and now we are starting the follow-up. We will follow them until the age of 4 or 5 years to see the incidence of atopic dermatitis and other allergies, and after the first year of life, to also see the incidence of infection. We did not find any difference in the rate of infection during the study period, but we will continue to
Clinical Evidence for the Role of Prebiotics in Mucosal Immune Development and Impact on Respiratory Health and Allergy

follow the infants after that.

**Q:** In the multicenter study, did the mothers pick their own choice of infant formula after the first 6 months, or did you specify a hydrolyzed or nonhydrolyzed formula?

**Dr Moro:** The mothers continued with the same formula until the infant was 1 year of age. The only difference was weaning, because the infants were weaned according to different styles of weaning.

**Q:** Studies such as GINI and others of this nature are fascinating because the control populations have such astoundingly different percentages of atopic dermatitis across the various studies. I believe that in the GINI Study of high-risk infants, 15% of those fed conventional cow’s milk formula had atopic dermatitis at 1 year of age. In a similar population in northern Italy, however, 24% of those fed a hydrolyzed formula without scGOS/IcFOS had atopic dermatitis at 6 months of age. Can you speculate whether living north or south of the Alps has an effect, or is something else working there? Is there a selection bias in how patients come into the studies in the two places? Is there a difference in how the entry criteria are written?

**Dr Moro:** When considering atopic dermatitis or other forms of allergy, we have to consider not only what the infant eats, but also where the infant lives. Milan, for instance, is a very polluted city; this is surely one of the main factors in allergy and atopic dermatitis. I am sure that pollution in industrialized cities is creating problems for these types of infants.

**Q:** If I understand correctly, your studies showed a reduction in the incidence of allergy after treatment with prebiotics. When you studied the specific IgE, you did not find any difference, but you saw a difference in the IgG1-specific level for cow’s milk proteins. Might that suggest that the mechanisms of allergy in these infants are mainly mediated by IgG1 and not by IgE?

**Dr Moro:** We found a difference in IgG1 and total IgE. We found a significant difference between the group receiving the standard formula and the group receiving the scGOS/IcFOS formula; total IgE level was significantly higher in the standard-formula group. We did not see a difference in specific IgE levels, only in the level of IgG1.

**Q:** This process is interesting because it suggests that the mechanisms arise mainly in IgG1.

**Dr Moro:** This is intriguing, but we do not know the mechanism behind it.
Q: When we talk about probiotics, we talk in terms of species, but now it is getting to the point where we talk in terms of the importance of a given strain. When we do research on prebiotics, however, we talk more about the total level of bifidobacteria. You saw a substantial increase in bifidobacteria after feeding this particular blend. Did you look at the different species, and was there a difference in species in your standard formula compared to the treatment group?

Dr Moro: Yes, we looked at the different species, but I did not show those results. We were able to demonstrate that, for example, the number of *Bifidobacterium adolescentis* was decreased in the scGOS/lcFOS group compared to the group receiving the standard formula. If you look at the composition of the subspecies of intestinal flora, you would find differences. This is important for atopic disease and other forms of allergies.

Q: Can you speculate on the use of oligosaccharides for premature infants relative to the programming approach that you suggested? Would you recommend them?

Dr Moro: Of course, the best choice is mother's milk and breastfeeding. If the infant is not receiving mother's milk, however, and particularly if he or she is at high risk for allergy, I suggest giving this mixture of prebiotics, not to save the infant, but to decrease the possibility that he or she will develop atopic dermatitis or other forms of infection in the first years of life.

Q: What about premature infants?

Dr Moro: A couple of studies have been done with premature infants. One study in Milan included a limited number of infants [Boehm G et al: *Arch Dis Child* 2002;86:178-181]. Ten premature infants received a standard formula, and another 10 premature infants received the same formula supplemented with the mixture of scGOS and lcFOS. The infants with the supplemented formula received 1 g scGOS/lcFOS/100 mL of milk. The researchers were able to demonstrate a significantly higher number of bifidobacteria in the feces of the supplemented infants without any side effects. Also, Neena Modi will publish a study soon in which she evaluates a larger number of preterm infants, also with positive results.

Q: I noticed that among the baseline characteristics in this study is a difference between vaginal and caesarian births. It seems that you have enough of a population of caesarian births compared to vaginal births. Are you looking at what those differences might hold in any of these studies?

Dr Moro: We did not evaluate the difference between vaginal birth and caesarian
delivery. However, the rate of caesarian delivery was the same in the two groups. But you are right, there is a significant difference at birth. This is why it is important to start oligosaccharide supplementation immediately, because we are able to modify the intestinal flora of an infant born by a caesarian section in a positive way.

**Q:** Do you have information in your studies that you could look at, even if it is retrospective, to see whether you have differences in bifidobacteria but not in prebiotics between vaginal and caesarean births?

**Dr Moro:** We have these data available, because in the study we performed in high-risk populations we evaluated fecal flora at birth, at 3 months, and at 6 months of life. We would have to take out these data and elaborate on them.
Clinical Evidence for the Role of Probiotics in Mucosal Immune Development and Impact on Respiratory Health and Allergy: Probiotics in the Treatment and Prevention of Infantile Allergy

Mikael Kuitunen, MD, PhD, University of Helsinki, Finland

Infections such as hepatitis A, mumps, and measles have decreased during the last 3 to 4 decades, whereas allergies and Th1 type diseases such as diabetes and Crohn’s disease have increased during the same time. The marked increase in allergic diseases is strongly correlated to fewer environmental microbial stimuli, especially during early childhood.

An inverse relationship between exposure to foodborne and orofecal microbes and respiratory allergies has been discovered. Children growing up in a farming environment who are exposed to more microbes via animal-related contacts and consumption of unpasteurized farm milk experience a lower incidence of asthma and allergic rhinitis as well as to atopic sensitization. Several studies show that the microflora of allergic infants is depleted of lactobacilli and bifidobacteria and contain more clostridia than that of nonallergic infants, and this can be seen even before small children develop atopic eczema. The same investigators also have shown that Estonian infants have a gastrointestinal microflora in many ways similar to that prevailing in Sweden in the 1960s. Families such as those in Estonia who adhere to an anthroposophic lifestyle use more biodynamic and organic food and fermented vegetables that contain lactobacilli, and they use fewer antibiotics and have fewer vaccinations than other families. A study from Sweden compared the incidence of atopy as measured objectively with skin-prick testing among pupils from Steiner schools and ordinary elementary schools. The Steiner school children experienced significantly less atopy than those attending ordinary schools, and a significant outcome measure was the use of organic or biodynamic food. This finding led to an interest in supplementation with lactobacilli and other probiotics in the hope of treating and preventing allergic diseases.

A large amount of foreign proteins and microbes flow into the gut, which the gut
Clinical Evidence for the Role of Probiotics in Mucosal Immune Development and Impact on Respiratory Health and Allergy

needs to handle properly. The immune system of the gastrointestinal system—our body’s largest immune system—has developed to perform this task. In addition, the commensal microflora is a large active organ comprising more than 1.5 kg of microbial mass and 10 times more organisms than the number of cells in the entire body. The gut microflora has many important functions. It promotes normal gut needs to handle properly. The immune system of the gastrointestinal system—our body’s largest immune system—has developed to perform this task. In addition, the commensal microflora is a large active organ comprising more than 1.5 kg of microbial mass and 10 times more organisms than the number of cells in the entire body. The gut microflora has many important functions. It promotes normal gut functions, protects from infections, and has effects on systemic metabolism and the immune system. It is important for the development of tolerance. In a mice study, it was shown that germ-free mice do not develop oral tolerance and the addition of bifidobacteria restored their ability to develop oral tolerance.7

Probiotics are defined by the World Health Organization as “live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host.” Probiotics have been shown to digest food proteins to become less antigenic and have caused a decrease in gut permeability.8 They have been shown to clearly affect the immune system in vitro, in animal studies as well as in humans. One widely available probiotic is Lactobacillus rhamnosus GG (LGG), an organism that was isolated by Sherwood Gorbach and Barry Goldin in 1985. LGG is of human origin, adheres to the intestinal epithelial cells, is resistant to acid and bile, colonizes the human gut, and has good growth characteristics. The first small clinical study using an extensively hydrolyzed formula (EHF) and EHF fortified with LGG showed quite dramatic effects from LGG on severity of atop eczema. More recent larger studies show mildly positive results in alleviating atop eczema and food allergy. In the largest study to date, we gave LGG, a mixture of four probiotics (LGG 10x10⁹ cfu/day, L rhamnosus LC705 10x10⁹ cfu/day, Bifidobacterium breve Bbi99 4x10⁸ cfu/day, and Propionibacterium freundreichii ssp shermanii JS 4x10⁹ cfu/day), or placebo for 4 weeks to infants with suspected eczema and cow’s-milk allergy. We assessed the severity of eczema by the SCORAD scoring system. All were put on a milk-free diet and treated with emollients and topical steroids as needed. After the intervention, the eczema was relieved very efficiently, but with no clear differences between groups. However, in infants with IgE-associated eczema, mean reduction in SCORAD of infants in the LGG group was significantly greater when compared to the placebo group.9 The infants were efficiently colonized by the bacteria they were supplemented with, according to assessment of fecal samples. The results from probiotic treatment studies have proven most effective in IgE-mediated eczema and food allergy.10 However, not all studies show an effect from using lactobacilli (Table 1).11
### Table 1. Randomized Controlled Trials on the Effect of Probiotics in Treatment of Childhood Allergy

<table>
<thead>
<tr>
<th>Authors, Country, Published</th>
<th>N of Patients</th>
<th>Age</th>
<th>Eczema in Baseline</th>
<th>Sensitized in Baseline</th>
<th>Intervention and Amount of Probiotics (cfu)</th>
<th>Outcome Measures</th>
<th>Clinical Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosenfeldt et al Denmark J Allergy Clin Immunol 2003;111</td>
<td>A1=20 A2=23</td>
<td>1 to 13 years</td>
<td>SCORAD &gt;15</td>
<td>63%</td>
<td>L rhamnosus 10x1010 and L reuteri 1x 1010 twice daily 6 weeks</td>
<td>SCORAD</td>
<td>Reduced subjective symptoms Reduced SCORAD in sensitized children</td>
</tr>
<tr>
<td>Weston et al Australia Arch Dis Child 2005:30</td>
<td>A=28 P=28</td>
<td>6 to 18 months</td>
<td>Moderate to severe SCORAD ≥25</td>
<td>71%</td>
<td>L fermentum twice daily 8 weeks</td>
<td>SCORAD</td>
<td>Reduced SCORAD in sensitized infants</td>
</tr>
<tr>
<td>Brouwer et al the Netherlands Clin Exp Allergy 2006;36</td>
<td>A1=16 A2=13 P=13</td>
<td>&lt;5 months</td>
<td>Eczema and suspected cow’s milk allergy</td>
<td>38%</td>
<td>LGG or L rhamnosus</td>
<td>SCORAD Sensitization</td>
<td>No effect</td>
</tr>
<tr>
<td>Sistek et al New Zealand Clin Exp Allergy 2006;36</td>
<td>A=29 P=30</td>
<td>1 to 10 years</td>
<td>All sensitized, SCORAD ≥10</td>
<td>100% (food A=66% P=80%)</td>
<td>LGG and B lactis 2x10x1010 daily 12 weeks</td>
<td>SCORAD</td>
<td>Reduced SCORAD in food-sensitized infants</td>
</tr>
<tr>
<td>Fölster-Holst et al Germany Br J Dermatol 2006;155</td>
<td>A=26 P=27</td>
<td>1 to 55 months</td>
<td>Moderate to severe</td>
<td>38% (A=8 P=12)</td>
<td>LGG 10x109 daily 8 weeks</td>
<td>SCORAD</td>
<td>No effect</td>
</tr>
<tr>
<td>Grüber et al Germany Allergy 2007;62</td>
<td>A=54 P=48</td>
<td>3 to 12 months</td>
<td>Mild to moderate SCORAD 15-40</td>
<td>55% (A=62% P=47%)</td>
<td>LGG capsules &gt;5x109 twice daily 12 weeks</td>
<td>Symptom load score SCORAD</td>
<td>No effect</td>
</tr>
</tbody>
</table>

A=active treatment group, A1=active treatment group 1, A2=active treatment group 2, P=placebo group

Four studies have been published in which prevention of allergic diseases was attempted with supplementation of probiotic bacteria (Table 2).^{12-18}
Clinical Evidence for the Role of Probiotics in Mucosal Immune Development and Impact on Respiratory Health and Allergy

Table 2. Randomized Controlled Trials on the Effect of Probiotics in Prevention of Childhood Allergy

<table>
<thead>
<tr>
<th>Authors, Country, Published</th>
<th>N of Patients</th>
<th>Treatment Initiated</th>
<th>Follow-up</th>
<th>Intervention Amount of Probiotics (cfu)</th>
<th>Clinical Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taylor et al Australia J Allergy Clin Immunol 2007;119</td>
<td>High risk A=89 P=89</td>
<td>Newborn babies aged &lt;48 hours</td>
<td>12 months</td>
<td>L. acidophilus (LAVRI-A1) 3x10^9 daily for 6 months</td>
<td>No effect Increased sensitization (Pos. SPT 40% vs 24% P=0.03)</td>
</tr>
<tr>
<td>Abrahamsson et al Sweden J Allergy Clin Immunol 2007;119</td>
<td>High risk A=95 P=93</td>
<td>Pregnant mothers + newborn babies</td>
<td>2 years</td>
<td>L. reuteri (ATCC55730) 1x10^8 from 36 gw to 12 months after birth</td>
<td>No effect on cumulative incidence of eczema (36% vs 34%) Less atopic eczema during 2nd year: 4% vs 14%, P=0.02)</td>
</tr>
<tr>
<td>Kopp et al Germany Pediatrics 2008;121</td>
<td>High risk A=50 P=44</td>
<td>Pregnant mothers + newborn babies</td>
<td>2 years</td>
<td>LGG (ATCCTS3103) 5x10^9 x2/day before delivery, 6 months after birth to lactating mothers, otherwise to bottle-fed infants</td>
<td>No effect–eczema (28% vs 27%) LGG increased current wheeze</td>
</tr>
<tr>
<td>Wickens et al Australia J Allergy Clin Immunol 2008;122</td>
<td>High risk A1=144 A2=152 P=150</td>
<td>Pregnant mothers + newborn babies</td>
<td>2 years</td>
<td>L. rhamnosus HN001, B animalis ssp lactis HN019</td>
<td>L. rhamnosus: &lt; 0.51 eczema at 2 years, Bifido no effect No effect on atopic sensitization</td>
</tr>
</tbody>
</table>

A=active treatment group, A1=active treatment group 1, A2=active treatment group 2, P=placebo, SPT=skin prick test, gw=gestational weeks

The earlier and smaller studies show strong preventive effects from providing probiotics prenatally to the expectant mothers and their infants for 6 months. The incidence of atopic eczema was halved.13 We studied the preventive effects of four probiotics (LGG 10x10^9 cfu/day, L rhamnosus LC705 10x10^9 cfu/day, B breve Bbi99 4x10^9 cfu/day, and P freundreichii ssp shermanii JS 4x10^8 cfu/day) on more than 900 children at high risk for allergy. The study was randomized, prospective, and double blinded. The mothers consumed the combination of probiotics or placebo 4 weeks before delivery, and their infants consumed the same probiotics and a prebiotic (galactooligosaccharide) from birth until 6 months. A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of a limited number of bacteria in the colon, thus improving host health. The main outcome measure was the development of an allergic disease at age 2 years. A 30% decrease in the cumulative incidence of allergic disease at 2 years of age was observed (Fig 1).
The supplemented bacteria were found in the feces of infants, confirming a successful intervention. Another study from New Zealand failed to show any effect when *Lactobacillus acidophilus* was given only to the infants from birth until 6 months, but not to the mothers during pregnancy. The type, dose, and mode of probiotic supplementation, as well as the setting and population studied might explain the differences. The strongest effects have been shown in high-risk infants demonstrating IgE-mediated atopic allergies.

Immune effects in vivo from supplementing with probiotics have clearly been shown. We have demonstrated an increased production of C-reactive protein (CRP) both in treatment of atopic eczema and prevention of allergies. The increased CRP was associated with a decreased risk of eczema and allergic disease at age 2 years (Fig 2).
Clinical Evidence for the Role of Probiotics in Mucosal Immune Development and Impact on Respiratory Health and Allergy

This supports the view that a chronic low-grade inflammation protects from eczema. This increase in CRP was accompanied by an increase in IL-10 and total IgE and IgA, changes in regulatory mechanisms that resemble those seen in helminth infections. Chronic microbial exposure is an important immune modulator that protects from allergies.

Dendritic cells in the gut mucosa bearing receptors for bacterial components extend into the lumen where the stimulation from bacterial components leads to stimulation and changes in cytokine balance. Stronger and repeated or continuous stimulation can lead to permanent changes in immune balance. This has been shown in both treatment and prevention of atopic diseases. Probiotics are safe, and they may have beneficial effects on immune development of infants, as seen in vaccine antibody responses after supplementing with probiotics. They have been shown to result in somewhat fewer infections and less need for antibiotics. They alleviate atopic eczema and reduce the appearance of allergic disease by age 2 years. They do not, however, affect the rate of IgE sensitization. The beneficial effect is strain-dependent. It seems that supplementation for prevention should start with the mother during pregnancy. The concept that supplementing with probiotics leads to immunologic and clinical effects has been proven. The microbial flora is important for the health of humans, and new research within this field, stimulated by probiotic studies, is most welcome.

Fig 2. CRP value vs development of allergic disease at age 2 years.\textsuperscript{19,20}
References


Clinical Evidence for the Role of Probiotics in Mucosal Immune Development and Impact on Respiratory Health and Allergy


Q & A

Q: When you used your combination of probiotics as a treatment, you did not get positive results, but when you used the combination as prevention, you had a positive effect. However, you combined the probiotics with oligosaccharides. Do you think that the positive effects were due to the utilization of this mixture of probiotics as a prevention, or to the combination of probiotics with a prebiotic?

Dr Kuitunen: That is a good question. There may have been a symbiotic effect. On the other hand, the dose we gave was small—0.8 g altogether. You gave much more in your studies. We wanted to be safe and not cause any harmful effects such as diarrhea in the children. We cannot conclude from our studies, however, that the positive effect resulted from symbiosis or just from the probiotics.

Q: No one has yet discussed the question of dose. In our rodent studies, we use around 10^8 or 10^9 cfu/day, and these are the same doses used in humans. We do
not understand why we should use the same dose. Very few studies have compared doses in humans in these conditions. I presume you have been using about $10^9$ cfu/day.

**Dr Kuitunen:** Yes.

**Q:** This is sort of the standard dose. My view is that we have to address this question, because a part of the problem we have comparing these organisms may be dose as much as anything else. Do you have any comments about this?

**Dr Kuitunen:** I have not seen any studies in humans comparing different doses, and this should be done. The problem is that we need large study populations. We thought about doing this with two intervention groups, but we would have needed more than 3000 infants. This is too big for anyone to do. This should be studied in smaller animals. In the reported probiotic prevention studies, the dose has been $10^8$ or $10^9$. Researchers use this dose in studies of treatment as well. Dr Moro has told us that the prebiotic dose might affect the results; this suggests that we should probably give higher doses to get a maximum effect because we do not see side effects from higher doses.

**Q:** Since half of these experiment conclusions are translated to humans, we might as well do them in animals. Most of us have never done these experiments, even in animals. Some experiments do show clear dose-response curves in animals on effects on motility. Interestingly, they have a sigmoid-type response, which is amazing. We think there are possibly even bell-shaped curves; it is possible that if you give too great a dose or too little that you will not see an effect. Therefore, I think we should encourage whoever is involved in animal experiments to look at dose responses.

**Dr Kuitunen:** I agree. I am waiting for this to be done. I also encourage animal researchers to do this.

**Q:** When we compare the results of clinical studies done in various diseases, we observe some of the effects you mentioned, or we observe something we did not expect. For example, some allergy studies show a bell-shaped relationship. However, in the case of diarrhea prevention, especially rotavirus-associated diarrhea, we do not see a dose-dependent relationship at all within the range of $10^7$ to $10^{11}$. I suppose you would expect different effects in different diseases, but, as you say, someone should do a study verifying this.

**Dr Kuitunen:** There is a study from the UK that correlated cat allergen levels and
Clinical Evidence for the Role of Probiotics in Mucosal Immune Development and Impact on Respiratory Health and Allergy

sensitization; very small and very high doses of cat allergen decreased sensitization [Custovic A et al: J Allergy Clin Immunol 2001;108:537-539].

**Q:** I have a question about the inflammatory immune profile induced by the probiotics. You talked about increases in IL-6, C-reactive protein (CRP), and IgE. I noticed that those markers increased in both the allergic and the nonallergic infants. Do you think that may explain some of the side effects of the LGG with respect to increased wheezing and bronchitis, and potentially asthma, at the 7-year follow-up?

**Dr Kuitunen:** That might be one explanation, but I find it fascinating that these markers also increased in the nonallergic infants. We do not know how the immune system works when you have a genetically different “drive” to allergy, but we should explore this further.

**Q:** Particularly the increase in IgE in nonallergic infants.

**Dr Kuitunen:** Yes. It is fascinating to compare them to, as I said, the helminth-infested individuals who are not sick. If you treat them, they develop allergy quickly.

**Q:** Some people in the United States are monitoring CRP as a prelude to necrotizing enterocolitis in premature infants, and they want to keep the CRP levels as low as possible. Can you comment on that relative to the infant?

**Dr Kuitunen:** This is mild inflammation, of course. These CRP levels are not close to those we see in clinical symptoms with infections. CRP is a sensitive method that measures very small amounts (<0.4 mg/L). These levels are not related to clinical infections.

**Q:** Most of these prevention trials focus on lactobacilli vs bifidobacteria. Are there data driving the decision by investigators to focus on lactobacilli? A limited number of studies, including yours, have used bifidobacteria either in combination with lactobacilli or something else, or by itself, and they have not shown any efficacy. I wonder whether lactobacilli and bifidobacteria are really different. When you “drill down” even further, only the *L rhamnosus* species shows any benefits. So are there real data, or enough data, to decide about bifidobacteria vs lactobacilli?

**Dr Kuitunen:** Lactobacilli have been proven more effective, but we do not know the right bacteria to study. In one study, investigators gave a nonpathogenic *E coli* and had good effects [Lodinova-Zadnikova R et al: Int Arch Allergy Immunol 2003;131:209-211]. However, it was a retrospective study, designed to work against nosocomial infections; many bacteria-related factors might have influenced the
effect. A new study from New Zealand found no effects from bifidobacteria, but it does not conclude that bifidobacteria do not work [Wickens K et al: *J Allergy Clin Immunol* 2008;122:788-94].

**Q:** Have you heard anything from investigators who choose to study lactobacilli about the reason for their choice?

**Dr Kuitunen:** I think their use of lactobacilli is a matter of supply.

**Q:** So it is not related to potential mechanisms of interaction with the immune system?

**Dr Kuitunen:** I would not say that, but not much.

**Q:** You mentioned that, regarding probiotics, allergy prevention should start late in pregnancy. On the other hand, some data indicate that prebiotics are not needed during pregnancy. Why are probiotics important during pregnancy but prebiotics are not?

**Dr Kuitunen:** The data on prevention using prebiotics are quite limited. We have some preliminary results from our 5-year study that show that infants, especially those delivered by caesarian section, benefit from probiotic therapy. It is important to give these probiotics to mothers before their child is born.

**Q:** In the studies in which giving pregnant women probiotics before birth was efficacious, were most of the infants breastfed? Is there also a potential benefit from the different components of breast milk, as shown by Susan Prescott’s research? Could that account for some of the effects, or do you think the effects are just from the initial colonization from birth?

**Dr Kuitunen:** I think that the breast milk potentially has some effect. As you point out, this has been nicely studied by Susan Prescott’s group. It is an important matter.
Clinical Evidence for the Role of Dietary Fatty Acids in Mucosal Immune Development: Mechanism of Action and Impact on Respiratory Health and Allergy

Paul Noakes, PhD, University of Southampton, UK

Interest is growing in the role of early life events and exposure in the etiology and prevention of disease. Diet and nutrition in pregnancy have a fundamental influence on fetal development, and focus is increasing on the role of key dietary nutrients in subsequent health or disease. This presentation explores the effects of dietary long-chain polyunsaturated fatty acids (LCPUFAs) on early immune development and their potential role in the development or the prevention of immune and inflammatory diseases, in particular allergy and asthma.

The extensive immunomodulatory properties of LCPUFAs are well recognized; the effects are mediated through a number of potential mechanisms. Less is known about these effects in immature and developing systems. In addition to influences on production of eicosanoids, omega-3 (n-3) PUFAs can regulate T-cell function directly through effects on cell membrane fluidity and consequent cell signaling and gene transcription.

Changing patterns of dietary LCPUFA intake are clearly relevant in pregnancy for the developing fetus. The intake of dietary anti-inflammatory n-3 PUFAs has progressively declined in Western diets, with a corresponding increase in proinflammatory omega-6 (n-6) PUFAs. This has resulted in considerably higher dietary intake ratios of n-6:n-3 LCPUFAs (20-30:1).

Epidemiological and experimental data provide a plausible link between these dietary changes and the rise in allergic immune diseases. Although difficult to prove, these associations are supported by the well-described difference in the immune effects of n-3 and n-6 LCPUFA in vivo and in vitro.

Early population studies suggested that the risk of asthma may be higher in children with higher n-6:n-3 diets, either in association with low consumption of fish or high consumption of n-6 rich vegetable oils. Several subsequent studies also have
shown protective effects of fish oil. A well-known Australian study showed that children who regularly consumed oily fish were significantly less likely to develop asthma (odds ratio: 0.26).² Two more recent birth cohort studies also have reported that lower dietary n-6:n-3 intake (ie, higher fish intake) reduced the risk of developing asthma.⁶,⁷ However, this has not been confirmed by all studies, and at least one large study found that higher fish intake was associated with a significantly higher prevalence of asthma (odds ratio: 1.117).¹⁰ In general, because of the limitations of these population-based studies, focus has grown on well-controlled intervention studies, as described below.

A Western Australian (Perth) group undertook the first human intervention study using high-dose fish oil in pregnancy to investigate the effects of n-3 LCPUFAs on early immune development.¹¹ The group gave fish oil (3.7 g n-3 PUFAs/day) or placebo supplements to allergic women (n=98) for the final 20 weeks of pregnancy. Fish-oil supplementation (n=40) achieved significantly higher proportions of n-3 PUFAs in neonatal erythrocyte membranes (mean ± SD, 17.75%±1.85% as a percentage of total fatty acids) compared with the control group (n=43, 13.69% ±1.22%, P<0.001). All neonatal cytokine (IL-5, IL-13, IL-10, and interferon gamma [IFN-γ]) responses (to all allergens) tended to be lower in the fish-oil group (statistically significant only for IL-10 in response to cat).

Although the authors examined the effects of maternal fish-oil supplementation on clinical outcomes in our intervention study,¹¹ the findings cannot be viewed definitively because the study was designed to assess immune function rather than clinical effects, which would have required a larger population size. Dunstan et al¹¹ did note that infants in the fish-oil group were consistently less likely to develop clinical features, including food allergy, recurrent wheeze, persistent cough, diagnosed asthma, angioedema, or anaphylaxis, compared to the control group. Although there was no difference in the frequency of atopic dermatitis at 1 year of age, infants in the fish-oil group had significantly less severe disease (odds ratio: 0.09; 95% CI, 0.01–0.94; P=0.045). Sensitization to egg was also less common in the fish-oil group. Although it is not possible to make conclusions from this, this study has provided justification for larger, long-term studies. Several larger studies in progress in Europe and Australia are specifically designed to assess this, and the results are awaited with great interest.

In addition to the associations mentioned above, the same group also investigated the effects of dietary n-3 PUFA supplementation during pregnancy on a) numbers and function of progenitors,¹² and b) the relationship between neonatal T-cell protein kinase (PKC) expression and subsequent allergic disease.¹³ In short, percentages of
Clinical Evidence for the Role of Dietary Fatty Acids in Mucosal Immune Development

cord blood (CB) CD34+ cell numbers were higher after n-3 PUFA supplementation than after placebo, and significantly more IL-5-responsive CB eosinophil/basophil colony forming units were seen in the fish-oil group, compared with the control group. Furthermore, the authors identified neonatal T-cell PKC isozyme expression (namely PKCζeta) as a potential predictor of allergic disease.

In conclusion, it is probable that if LCPUFAs have clinically relevant effects, these are more likely to appear at a younger age before immune responses and clinical phenotype are established. We and others have shown that immunological abnormalities precede the development of allergic disease and are frequently evident at birth or in the first months of life.14-16 This also may explain why intervention studies in later childhood to reduce symptoms in established asthma have shown only weakly beneficial effects17 or no effect.18 As indicated previously, a number of other ongoing studies will assess the effects of earlier supplementation, from birth or in pregnancy, with higher doses of n-3 PUFAs.

References


Q & A

Q: The research looked at prenatal exposure and intervention. Dr Kuitunen showed what is going on in breast milk with urbanization or change in rural areas. During that time there would be a lot more of not just docosahexaenoic acid (DHA), but also eicosapentaenoic acid (EPA) and some other omega-3 oils. Such exposure to EPA soon after birth through breast milk might have some effect on mucosal development. Has anyone looked at either cultural differences in breast milk levels of fatty acids or supplementing lactating women with EPA and DHA?

Dr Noakes: Koletzko and colleagues reviewed 14 studies from 9 European countries and 10 studies from 7 African countries on fatty acids in mature human milk [Koletzko B et al: J Pediatr 1992;120(4 Pt 2):S62-S70]. They found the average composition data for milk fatty acids surprisingly consistent, despite marked differences in dietary composition in the areas studied. To respond to the last part of your comment, many groups have looked at this, including those of Robert Gibson, Susan Prescott, and Berthold Koletzko.
Clinical Evidence for the Role of Dietary Fatty Acids in Mucosal Immune Development

Q: How critical is EPA as a fatty acid for allergic disease prevention, and will DHA also lower prostaglandin E₂ levels?

Dr Noakes: Traditionally, investigators have looked at EPA, but now with the discovery of resolvins and the fact that DHA can produce D-series resolvins, I think people will now turn their attention to looking at both EPA and DHA.
Discussion

**Discussion Leader:** Lee Yuan Kun, PhD, National University of Singapore

**Dr Lee:** I am sure you agree that we have had four exciting presentations to bring us up to date on studies of probiotics, prebiotics, and fatty acids as dietary components on the subjects we have been talking about. I would like to start the discussion by making a provocative statement for our experts here to comment on and see where we end.

Can one go so far as to say that what we have observed is nothing other than the presence of specific strains of commensal microorganism in our gastrointestinal tract and their relative ratio to each other? The role of prebiotics and oligosaccharide in breast milk and infant formulas and of fatty acids in the diet is to modify and modulate the intestinal microbial profile, which eventually imparts health benefits on us. Can we say, it ultimately points to the microbial profile in the intestinal tract? Without good knowledge of the microecosystems in our intestine, perhaps it is not productive to talk about prebiotics, nutritional components, and other subjects we are discussing here.

Can we have input from some of our expert participants as to your view on our present knowledge of the intestinal microbial profile—the interactions between microbes and us, leading to the facts about probiotics and prebiotics? This subject has been touched on briefly by Dr Kuitunen. Dr Kuitunen, are we at a stage at which we can interpret the effects of probiotics and prebiotics? Perhaps you can explain the contradictory observation in probiotic studies and some prebiotic studies. Is it because we did not take into consideration the microbial profile of these individuals?

**Dr Kuitunen:** In the microflora, there are several hundred types of bacteria. I doubt we can make permanent large changes in the microflora by giving one germ or a couple of germs. Maybe prebiotics affect a larger amount of good bacteria in the gut, but we do not know enough about the balance of the microflora that should be targeted. This issue is not settled, and how do we study all of these 500 foreign types? Which should we give, what is deficient, and what do we have too much of?

**Dr Brandtzæg:** As Dr Kuitunen showed, we do not have a consensus about the mechanisms of prebiotic and probiotic effects. Multiple mechanisms probably produce these effects, and that confuses the issue. We use several readouts, but we do not know which readout will be the proper one for the effect we are looking for.
**Discussion**

**Dr Lee:** Can we go so far as to say that we cannot draw any conclusions at all, because we do not know enough?

**Dr Brandtzaeg:** I say that we can be optimistic, because there are some positive results. However, it is too early to conclude.

**Dr Rueda:** I think we need more studies to determine how the intestinal microbiota are able to metabolize specific substrates, what metabolites are produced by the intestinal microbiota, and at the end, what signal pathways are derived that affect different functional outcomes. This is not easy to demonstrate, but I think that these types of studies might give us some idea of what is happening regarding the effects of prebiotics and probiotics in the gut, at least related to one specific substrate and to some of the microbial species affected by that substrate.

**Dr Brandtzaeg:** You look at signal pathways before you know anything about the receptors employed. That is a little premature. It would be nice to know more about the receptors. Is there an in vitro study that can be used to dissect the Toll-like receptors or whatever would then create the signal pathways?

**Dr Rueda:** There are in vitro studies [van der Werf MJ, Venema K: *J Agric Food Chem* 2001;49:378-383]. They are not in vivo, but when you select the microorganisms that are affected by a specific substrate and you know which pathways are affected, you might have an idea. Of course, you cannot interpret the rest of the interaction with the rest of the microflora, but I think that might help.

**Dr McCoy:** I agree that we need to know a lot more about the real composition of the microflora, and that is difficult. Maybe the increased accessibility to high-throughput sequencing platforms will provide answers. We do not even know the whole microflora of mice. That research has just started. With more information, we might be able to look at the consortia of bacteria in the gut, how they interact with each other, and which are luminal and which interact on the mucosal surfaces.

**Dr Lee:** It does not mean that what we have done so far is a waste. We have taken the first steps to understand the whole picture of the interactions. Where do we go from here?

**Dr Kuitunen:** I referred to the early study by Agnes Wold and coworkers that showed that Pakistani children had a lot of change in *E coli* [Adlerberth I et al: *Acta Paediatr Scand* 1991;80:602-610]. That was a nice theory, but in a study in several European countries in which fecal bacterial profiles were measured and related to allergy, the results were disappointing. There were no clear indications of what was
missing or what should be present. So we have a long way to go.

Dr Lee: Why do you think they could not draw conclusions from this study? What can we learn from that?

Dr Kuitunen: How many bacteria should you study to be able to show differences? That study offered no clear direction to go, and it was done in many countries with different environments.

Dr Bienenstock: Part of the problem is that there is a lot of in-the-box thinking—a lack of attention to all the variables that could be affected. I do not want to keep plugging the same business, but to avoid attention to the effects of systems such as the nervous system and the endocrine system on the immune system is just closing your eyes.

Another problem is that fecal samples only tell you what is in the colon; they do not tell you anything about what is in the small intestine. If the small intestine is where most of the action is in terms of effects on the immune system, then we need a lot more information about what is in the small intestine. I would start to sample in animals because of the difficulties in sampling from humans—it is highly interventional. Nevertheless, the conclusions that have been drawn about the human microbiome need to be applied to the small intestine as well.

Dr Lee: As you say, it is difficult to work on humans. At times, however, what happens in animals does not really reflect what happens in human. Do you think there is a good animal model to allow us to perform this kind of study?

Dr Bienenstock: No, I am just referring to normal animals, not to animal models.

Dr Brandtzaeg: I am on the advisory board for a large international multisite microbiome project, the MetaHIT consortium, and I try to question the members, but they only want to sample feces. That is the only thing this big project is focusing on. It is impossible to change their attitude and take any other approach, despite the fact that fecal bacteria are not representative of the entire intestinal tract. Can you sample the small intestine using a noninvasive method?

Dr Bienenstock: Noninvasively? It is not possible.

Dr Lee: Would the Abbott faculty like to share anything from the point of view of your industry? I think this is important, because you are the ones who will apply this information to a product. I am sure that at times the research is confusing. One day, someone will say that a strain has an effect, and at another time, you see reports
Discussion

that conclude there is no effect. How are you going to tell your customer that, in fact, the product you are promoting has an effect?

Dr Miller: I think this gets to the heart of what I heard at this conference. You have it down to some beautiful pathways, but you know the complexity behind them. We talk about the number of species, but then we talk about the fact that bacteria live in colonies of biofilms that have no relationship to cultured bacteria. The bacteria are in biofilms that are potentially like organs that the inside can pull to the outside and have no relationship in the way they interact. This is one of the reasons why you cannot get a decontaminated gut; you never will do that.

The neuroendocrine system and its influence on motility is fundamentally unknown, but we know that probiotics can influence these systems. Some of the research results on motility are impressive, and they may then link back to the neuroendocrine axis. We also saw some beautiful clinical studies that we can take away from this conference, but it is still hit and miss.

I am going to propose something, and I hope it will be beneficial. The question is whether to do clinical trials in, for instance, Milan instead of other parts of Europe because of the environment, or in Pakistan instead of Western Europe. I am just outlining how to get to this “in-between” of clinical research and the reduction of science such that we have at least more predictability or a hypothesis we can act toward. When a company like Abbott puts out a product, we would like to know that the underlying research has reproducibility so that the information is relevant whether the product is in this city, that country, or this culture. I think we have learned some fundamental elements at this conference.

Dr Lee: Can our experts tell us where we are? How do we reach the point at which we are confident that the data apply broadly to multiple countries and populations?

Dr Moro: I think we are just at the beginning of the story, because we have discovered the importance of intestinal microflora in the last few years. No one was aware of it before. Now we know we can modify the composition of intestinal flora with some interventions such as prebiotics and probiotics. We do not know what type of probiotic is best, and we do not know the dosages, how long to give it, and the side effects. We do not know which type of prebiotic is best, and we do not know the dosage, but we do know that we can influence the composition of the intestinal flora, and we know that that influence can change the clinical history of the infant.

Dr Lee: This is the first step, and it points to a direction. We know we are on to something.
We know that probiotics work because we see their positive effects. The question is, should we look beyond that because we need to know more to have better control and a better grip on an important subject?

**Dr Kuitunen:** We know there is quite good evidence that probiotics work in infectious diarrhea. There are also some promising results in the fields of allergy and inflammatory bowel disease. I think the most important thing as we enter this area of research is that no matter whether the work is in vivo, in humans, in animals, or in vitro, that we have some kind of proof of concept. We are at the beginning, but we have chosen a path that, I hope, will be fruitful.

**Dr Montalto:** There are two sides to every coin, and we have seen pros and cons of research results. We have seen an attempt to understand different methodologies and different mechanisms. They tend to be, in some cases, isolated to certain factors, disregarding other possible components. So it is hard to know the specific mechanism. There are a lot of possibilities. I think we are trying to have some assurance that we are going to improve patient outcomes with what we do, and, first, do no harm. I think there are many chapters yet to be written in this whole story. We have a lot of research to do, but good research will lead to more research that will answer more questions. I think we need more long-term funding and repetitive trials, because one trial is not definitive. As Dr Kuitunen’s presentation showed, sometimes you get the effect, and sometimes you do not. I think we will have to have a preponderance of evidence that what we want to do with foods will be the right thing for small children, older people, and everyone in between.

**Dr Kuitunen:** I also think that we should be very cautious, as scientists, about giving advice too early because this advice may not prove to be right. As Dr Bienenstock pointed out, we should refrain from black-and-white thinking. Probiotics are not all good or all bad; they have both sides. It is the same with prebiotics and the interventions we heard about today.

**Dr Lee:** The problem is that the consumer wants an answer.

**Dr Rueda:** About Dr Miller’s previous comment, we are focused mainly on the interaction between the microflora and the immune system. I think that neuroimmunomodulation is going to change a lot of different concepts, and studying that interaction will get us closer to understanding some of these mechanisms.

**Dr Brandtzaeg:** There has been little discussion about helminths and microbial cell-wall products. Would that be a more potent approach than studying streptococci
and lactobacilli? We see striking results in inflammatory bowel disease with the pig pinworm and actually better results than with traditional medicine. Are there other approaches that might be better than lactobacilli and fecal bacteria?

**Dr Lee:** There are reports on parasites in the prevention of atopic diseases. They show interesting results, but the question is, can we get a safe parasite? We have lactobacillus and bifidobacteria, which are harmless, if not beneficial, so they are safe for us to use. Do we have a safe parasite to use? If we can have that, perhaps it is worthwhile for us go in that direction. Can we consult the clinicians here about whether this is possible?

**Dr Bienenstock:** Why do we need a live parasite? I think that is Dr Brandtzaeg’s point. We heard Dr Versalovic talking about immunomodulins and products that Mazmanian and Kasper have been describing with exopolysaccharides. There is no reason to think that this pinworm may produce the sort of molecules that Dr Versalovic described with *L reuteri*. That is thinking out of the box. That approach is crucial to our study. Ultimately for academic researchers, however, the question is, who is going to fund this? This area of gut microbiota and mucosal immunology is just starting to become mainstream. We also are waiting for the technology that is coming. That technology is amazing in terms of the quantity of information we will have and the ability to analyze, not only the human intestinal microbiome, but also the mouse microbiome. At the present rate of progress, the next couple of years will bring us enormous possibilities to study these things.