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Probiotics and Immune Response

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Introduction

For many years, probiotic bacteria have been known to confer health benefits to the consumer. In 1989, probiotics were defined as "a live microbial feed supplement which beneficially effects the host animal by improving its intestinal microbial balance" *(1)*. To date, this definition can be extended, as recent developments claim a role for probiotics and their active cellular substances in the intestinal and extraintestinal physiology of the host.

Lactic-acid bacteria (LAB), including *lactobacilli* and bifidobacteria, were shown to prevent adherence, establishment, and replication of several enteropathogens through antimicrobial mechanisms *(2–4)* and modulation of the host mucosal immune response. LAB were also reported to inhibit growth of *H. pylori* in vitro *(5)* and *L.johnsonii* La1 demonstrated a long-term suppressive effect on *H. pylori* in humans *(6)*. Finally, the preventive and therapeutic effect of several LAB on gastrointestinal disorders *(7,8)* and diarrhea caused by rotavirus has been established in several human intervention studies *(9,10)*.

Among the various *lactobacilli*, some strains also show immunostimulatory properties in healthy subjects by enhancing innate immune mechanisms. Schiffrin et al. showed an increase in phagocytosis of peripheral-blood mononuclear cells (PBMCs) after consumption of *L. johnsonii* La1 *(11)*. Moreover, fermented milk products, containing *L. johnsonii* La1 and *B. bifidum* had immune adjuvant effects (significant increase in total and specific serum IgA) when administered in conjunction with an attenuated oral *Salmonella typhi* vaccine *(12)*. Immuno-

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modulation by LAB is dependent on the number of live bacteria which transiently colonize the small bowel. It is well-established that survival in the gastrointestinal tract, especially resistance to gastric pH and bile salts, is a prerequisite for immunomodulatory effects. Adherence to intestinal epithelial cells is also a valuable property *(13,14)* resulting in a competitive advantage for the probiotic to prolong its effect. Although the reports on probiotics as immune-response modifiers are increasing, little is known about the mechanisms on how luminal signals in the intestine, delivered by probiotic bacteria, can modify the activation of the mucosal and thus the systemic immune system *(15–17)*.

This article discusses recent developments in the establishment of human in vitro models to characterize mechanisms of immunomodulation by selected LAB.

The Intestinal Mucosa

Mucosal surfaces represent large areas of interface between the host and the external environment. Physiologically, they can be sterile or colonized, as in the distal gastrointestinal tract. Mucosal mechanisms of defense have evolved common strategies for all mucosal surfaces, but in the case of a colonized mucosa, there are additional characteristics. Although a strong response against invasive pathogens must be mounted, nonresponsiveness or hyporesponsiveness to food antigens or indigenous bacteria must be guaranteed. This lack of immunological response is an active process, based on various mechanisms, which are globally known as oral tolerance.

Both endogenous mediators and luminal factors, including those from bacteria, are implicated in intestinal homeostasis. The integrity of the mucosal barrier is a basic requirement for host survival, both from a nutritional and a defensive point of view. Distinct compartments harboring different cell types and a strongly regulated cellular cross-communication are necessary to allow the establishment and maintainence of tissular homeostasis.

Components of the Mucosal Immune System

Secretory Immune Response

The initiation of mucosal immune response takes place in the small bowel, in lymphoid aggregates, known as Peyer's patches (PP). They are covered by a specialized epithelium containing the M cells dedicated for antigenic sampling *(18)*. The underlying lymphocytes are arranged in prominent follicles containing T- and B-cell compartments. The germinal centers (GCs) of the gut-associated lymphoid tissue (GALT) constitute the main lymphopoietic organ for mucosal B cells.

GC formation depends on luminal antigens, particularly those of microbial origin. Mucosal B cells, which are predominantly committed to IgA production, undergo gene rearrangements, which leads to the diversification of the antibody repertoire. These events take place preferentially in the GALT. Follicular dendritic cells in the GC retain immune complexes on their surface to stimulate B cells with high-affinity receptors and rescue them from deletion by apoptosis. In addition, cognate T cells, predominantly $CD4^+$ $CD40L^+$, provide help to induce class switch *(19)*.

The Lamina Propria

Antigen-specific B and T cells leave the inductive sites and migrate through lymph and blood to finally home back into the mucosa. Re-targeting to the gut is mediated by the selective downregulation of L-selectin and the expression of $\alpha 4\beta 7$ integrin *(20)*. B-cell maturation is completed in the lamina propria (LP) with a commitment to IgA secretion. Dimerig IgA is transported through the epithelium into the intestinal lumen by the polymeric immunoglobulin (Ig) receptor or secretory component.

The LP T-cell population is of thymic origin, representing a high proportion of memory cells and abundant expression of activation antigens. LP $CD4^+$ T cells are generally of the Th2 type with a predominant production of IL-4 and IL-5, thereby promoting IgA secretion.

Intraepithelial Lymphocytes

The epithelial compartment is comprised of two types of cells displaying immune functions: intra-epithelial lymphocytes (IEL) and intestinal epithelial cells (IEC). Upon stimulation, IEL modify the phenotype and function of IEC *(21)*. This modulatory effect implies the interaction of $\alpha E\beta 7$ and E-cadherin, or cytokine secretion, mainly IFNγ. Human and murine IELs are enriched in T cells that express $\gamma\delta$TCR. Both $\gamma\delta$ and $\alpha\beta$TCR express a limited array of diversities, suggesting that IEL may recognize a restricted range of antigens. Furthermore, most of the IEL are $CD3^+CD8^+$, expressing the $CD8\alpha\alpha$ homodimer, which indicates their extrathymic origin *(22)*. The migration of lymphocytes into the epithelial compartment appears to be dependent on the expression of $\alpha E\beta 7$ integrin recognizing E-cadherin at the basolateral membrane of the enterocytes *(23)*. $\alpha E\beta 7$ integrin is induced by TGFβ, which is found in the epithelial microenvironment and is produced by several cell types, including the IEC.

Intestinal Epithelial Cells

It is well-established that IEC constitute a component of the mucosal immune system. They are the first host cells to make contact with luminal antigens and microorganisms, and can exert antigen-presenting functions. Upon stimulation, IEC produce a wide range of cytokines and participate in immediate defenses against intestinal pathogens *(24)*. Although their regulatory activities in encounters with enteropathogens are well-documented, little is known about their role in maintaining homeostasis in the presence of the indigenous microflora.

Host Defense Mechanism

Innate Protection of the Intestinal Mucosa

The single layer of epithelial cells lining the intestinal tract must protect the underlying compartments from both the normal microflora that reside within the intestinal lumen and invading pathogens. Moreover, the intestinal mucosa must cope with environmental antigens, food antigens, or infectious agents, without triggering constant and severe inflammation. The potential for cumulative damage may explain on the one hand the rapid turnover of IEC. Epithelial renewal requires continious stem-cell proliferation and differentiation for normal function of the intestine. On the other hand, the development of a variety of defense mechanisms have evolved, both constitutive and inducible. These evolutionary ancient mechanisms provide innate protection against mucosal injury and rapid mucosal repair in the case of tissue damage. Trefoil peptides secreted to the apical surface of the epithelium interact synergistically with intestinal mucin glycoproteins in the constitution of a physico-chemical barrier *(25)*. These peptides are also implicated in reconstitution of the epithelium after injury. The role of intestinal mucins in innate defense has been highlighted by a recent publication demonstrating the protective effect of *Lactobacillus* species by stimulation of intestinal mucin synthesis *(26)*.

Antimicrobial peptides such as α-defensins, which are secreted from Paneth cells, or β-defensins, which are secreted by the epithelial cell itself, are abundantly found in host-defense reactions in the gastrointestinal tract *(27)*. In humans, only two β-defensins have been identified: hBD-1 and hBD-2. hBD-1 expression in the epithelium appears to be constitutive and is not upregulated by pro-inflammatory mediators, such as lipopolysaccharide (LPS) or TNFα *(28)*. In contrast, hBD-2 was found to be overexpressed in inflammatory conditions *(29)*. Antimicrobial activity of defensins is based on pore formation, membrane depolarization, and interference with bacterial metabolism. In addition, some defensins induce a secretory chloride response in IEC *(30)*; others

display chemotactic activity for T cells, serving as a link between innate and adaptive immunity *(31,32)*. Finally, among the peptides that promote restitution of the epithelium are the growth factors TGFβ, KGF, and HGF produced by epithelial cells and subjacent mesenchymal cells or myofibroblasts *(33–35)*.

The first recognition of a microbial pattern is realized by host cellular-defense molecules, the so-called pattern-recognition receptors (PRR). PRRs are germline-encoded and recognize molecular structures shared by a variety of pathogens *(36)*. In the gut mucosa, PPRs are found on macrophages that are widely distributed beneath the epithelial surface, where they play a key role as they guard the sites of antigen entry. In addition, some newly described PPRs are expressed by the intestinal epithelial cell itself.

A classical PRR is the mannose receptor (MR), expressed on tissue macrophages and immature dendritic cells (DC) *(37)*. MRs recognize the pattern of carbohydrates that decorate the surface of gram-negative and gram-positive bacteria, yeasts, parasites, and mycobacteria *(36)*. Ligation of the MR results either in endocytosis or phagocytosis of the ligand-receptor complex and subsequent clearance of the infectious agent. It appears that PRRs also play a key role in the recognition of microbe-derived glycolipids which are processed by the CD1b pathway *(38)*. Thus, the MR on the one hand mediates the role of antigen clearance in the tissues, and on the other hand plays a role in stimulating clonal immune responses. This is also suggested by the fact that MRs are expressed on immature DCs, which initiate adaptive immune responses. From the current knowledge, it is hypothesized that innate immunity is required to mount long-lived clonal immune responses.

Another class of PRRs, the human Toll-like receptors (TLRs), is related to the Drosophila Toll protein, which is required for ontogenesis and antimicrobial resistance *(39,40)*. Generally, TLRs are type I transmembrane receptors with cytoplasmic domains that resemble the mammalian IL-1 receptor (IL-1R) *(41)*. In vertebrates, TLR2 and TLR4 are implicated in innate immune recognition. Recent evidence suggests that TLR4 is essential for gram-negative recognition (predominantly LPS), whereas TLR2 is involved in cell responsiveness to gram-positive bacteria, including peptidoglycans *(42)*, lipoteichoic acid, and bacterial lipoproteins *(43)*. It is suggested that TLR4 acts as a coreceptor for CD14 in the cellular response to LPS *(44,45)*.

It appears that an additional component, such as the accessory protein MD-2 *(46)* or a proteolytically processed precursor protein, is required for the high-affinity binding of LPS to TLRs. This suggests that different microbial agents may activate different Toll family members, leading to the activation of different target genes.

More recently, the differential expression of TLR2, TLR3, and TLR4 on intestinal epithelial-cell lines and activation of specific signal-transduction pathways after stimulation of IEC with LPS has been reported *(47)*. This provides further evidence that IEC play a front-line role in the recognition and transduction of signals derived from luminal bacteria.

It is well-documented that phylogenetically conserved signaling pathways in innate immunity provide an immediate cellular reaction utilize the nuclear factor NFκB.NFκB is rapidly activated by a large spectrum of agents and cellular stress conditions, including LPS, microbial and viral pathogens, cytokines, and growth factors. Ligation of TLRs was shown to lead to the transactivation of NFκB through an adaptor protein (MyD-88) and activation of the IL-1R associated kinase (IRAK) *(48)*. In addition to its predominant role in innate immunity, NFκB excerts important functions in the adaptive immune system, such as control of lymphocyte proliferation, cytokine expression, and protection from apoptosis. Miettinen et al. studied NFκB transactivation in human primary macrophages by a specific *Lactobacillus* strain (49).

It has been well-established that the way antigen-presenting cells (APC) are primed will determine the faith of the adaptive immune response.

Mucosal B Cells and the Secretory (s) IgA Response

The best-defined effector component of the intestinal mucosa is the production of secretory immunoglobulin A ([s]IgA) against intestinal-damaging agents such as toxins, pathogenic bacteria, and viruses. Through cooperation with innate defense factors, antibodies that reach the mucosal lumen perform "immune exclusion," a noninflammatory process which protects mucosal surfaces. In addition, IgA and IgM antibodies can neutralize invaded pathogens, viruses, and their products by the receptor-mediated transport through the epithelial cell into the intestinal lumen.

Lymphoepithelial GALT interactions, also involving B cells, appear to be crucial for functional properties of the follicular-associated epithelium (FAE), including induction of the M-cell phenotype *(50)*. The antigen uptake by B cells in the M-cell pocket is highly efficient. The prominent germinal centers (GC) of the GALT are the main lymphopoietic sites for mucosal B cells, with a preferential commitment to immunoglobulin A (IgA) production. GC development depends on antigenic challenge, mainly of microbial origin. B cells migrate into the GC according to their affinity for specific antigens, where they undergo somatic mutation of the immunoglobulin genes leading to increased affinity for the specific antigens. In the GALT, immunoglobulin isotype switching occurs predominantly toward the IgA isotype. $CD4^+$ T cells

expressing CD40 ligand and producing IL-4, IL-5, and IL-10 co-localize with B cells in the GC and participate actively in the process of isotype switching.

When failure occurs in this first line of protection, penetrating antigens must be removed from the subepithelial lamina propria (LP) compartment by antibodies that are locally produced from terminally differentiated B cells and T-cell effector mechanisms. This process is enhanced by pro-inflammatory defense mechanisms. Mucosal T-cells must promote this particular B cell differentiation and, under physiological conditions, favor the downregulation of pro-inflammatory immune mechanisms to maintain oral tolerance. Thus, production and secretion of IgA in the LP is regulated by endogenous mediators, such as TGF-β, IL-5, and IL-10—mainly produced by regulatory T cells *(51–53)*—and intestinal bacterial colonization *(54)*. Interaction of B cells with cognate T cells results in IL-2 production and T-cell proliferation, leading to enhanced diversification of mucosal immune responses. This may be an important characteristic to control the continuous antigenic challenge drift of the endogenous microflora. The main IgA subclass of the human jejunum is IgA1, whereas IgA2 is predominant in the colon. This may reflect the distribution of food antigens vs bacterial antigens in the normal gut. In case of bacterial overgrowth, the composition is changed with an increase of IgA2 in the small bowel, suggesting that LPS may play a role in antibody class switch *(54)*. Other trophic factors to promote class switch are TGFβ and vasointestinal peptide (VIP). That TCRγδ$^+$ IEL exert IgA-enhancing effects was demonstrated by the observation that TCRγδ$^{-/-}$ knockout mice have significantly reduced numbers of IgA-producing cells and a poor IgA response, whereas IgG and IgM titers are normal *(55,56)*. In humans, it is hypothesized that TCRγδ$^+$ IEL residing in the epithelium contribute to the large IgA production in the gut.

Host defenses against the autochthonus microflora are still poorly understood. There is recent experimental evidence in mice that a large proportion of the intestinal IgA against commensal bacteria is specifically induced in response to their presence in the microflora, but in a T-cell and germinal-center independent manner *(57)*. This IgA, mainly directed against bacterial proteins, appears to be derived from B1 lymphocytes *(58,59)* which develop in the subepithelial compartment, and is spread all over the LP. The IgA antibodies protect the host from the penetration of commensals, but do not spontaneously appear in the serum. However, in the case of bacterial infection, specific IgG can be produced by T-cell-dependent pathways. The authors hypothesize that specific T-cell-independent IgA forms part of the normal mucosal response against the continuous antigenic load of commensal bacteria

and may represent an ancient evolutionary pathway of the immune system. The question of whether these observations are valuable in humans needs further study.

Stimulation of IgA Production by Probiotics

Fermented milk products containing probiotic bacteria, such as *L. johnsonii* La1 (10^{10} CFU/mL, daily dose), were shown to have immune adjuvant effects, as demonstrated by a significant increase of total serum IgA in adult human volunteers *(16,60)*. Furthermore, consumption of *L. johnsonii* La1 in conjunction with an attenuated oral *Salmonella typhi* vaccine (Vivotif), promoted the specific immune response, as assessed by a significant increase of Ty12a specific serum IgA *(12)*.

It has been reported that the human indigenous microflora is only partially covered by IgA-specific antibodies, and even less so by IgG and IgM *(61,62)*. A significant proportion of the microflora (close to 50%) is not covered by antibodies. These findings seem to indicate that the partial unresponsiveness to the autochthonous microflora may appear after a transient immune response took place, which is also suggested by the gnotobiotic animal model. On the other hand, the effect of ingested bacteria, such as probiotics, for maintaining activation at the GC level is not known. However, they could contribute to it and thereby promote an IgA response, which is not only specific against bacterial antigens but also against bystander antigens sampled through the FAE containing the M cells.

IECs as a Key Target in Mucosal Immune Responses

IEC are considered to be a constitutive component of the mucosal immune system. They participate in the innate defense mechanisms, and moreover, in the initiation and regulation of the mucosal immune response to bacteria by interacting with immune cells of the GALT, lamina propria lymphocytes (LPL), and intraepithelial lymphocytes (IEL) *(63)*. It was shown that IECs may change phenotype as a consequence of stimulation by IEL-derived soluble mediators, such as IFNγ *(64)*. This finding fits in with the concept that activated IECs express higher levels of HLA class II molecules *(65)*, classical class I and nonclassical HLA class Ib molecules, such as CD1d *(66)*, the adhesion molecule ICAM-1, complement factors, and cytokine receptors *(67,68)*. Upon stimulation they are able to produce a wide range of immunomodulatory cytokines *(69–71)*.

The endogenous microflora seems to have a modulatory effect on the mucosal immune homeostasis and therefore on the mucosal mechanisms of defense. The importance of microflora-derived host protection is evident by the higher susceptibility of germ-free animals to intestinal infections. To date, IECs are believed to be implicated in the

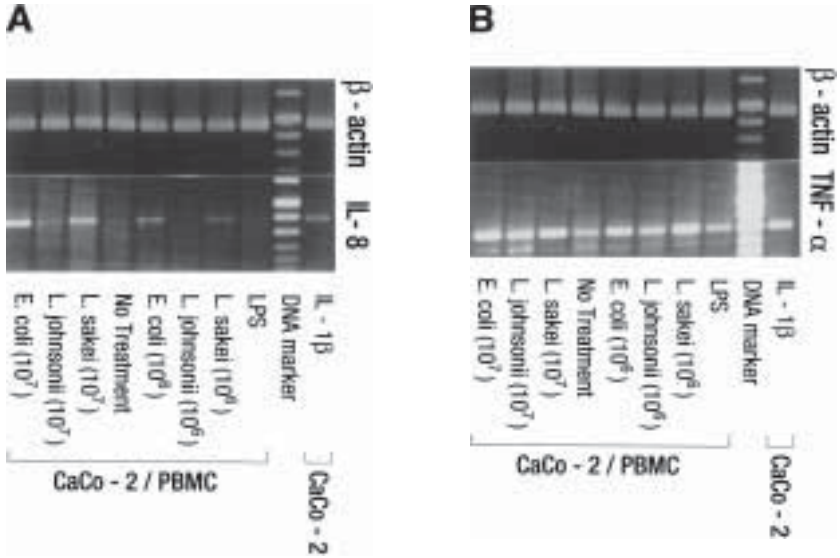


Fig. 1A–B. Differential induction of cytokines and chemokines by nonpathogenic bacteria in leukocyte-sensitized CaCo-2 cells.

Determination of specific gene transcripts for IL-8 **(A)** and MCP-1 **(B)** in CaCo-2 cells after stimulation of CaCo-2/leukocyte co-cultures with nonpathogenic *E. coli*, *L. johnsonii* La1 and *L. sakei* (16 h, 10^6 and 10^7 CFU/mL). *Controls*: LPS (1 mg/mL), IL-1β (10 ng/mL), no treatment (medium). Results represent one of three independent experiments.

recognition of components of the intestinal microflora, including food-derived probiotic bacteria, and the transduction of bacteria-derived signals to resident mucosal immune cells.

In the following section, we will discuss data obtained with different human in vitro models on the molecular mechanisms of bacterial interaction with intestinal epithelial cells.

Modulation of the Mucosal Immune Response by Commensal Bacteria

In Vitro Evidence for Mechanisms of Probiotic Action

Signals for modulation of the mucosal immune homeostasis must be "processed" via the IECs by i) the release of soluble mediators that will translocate through the epithelial layer to neighboring immune cells, ii) the modification of the luminal ecology because of their metabolic activity, and iii) changes in epithelial phenotype and function.

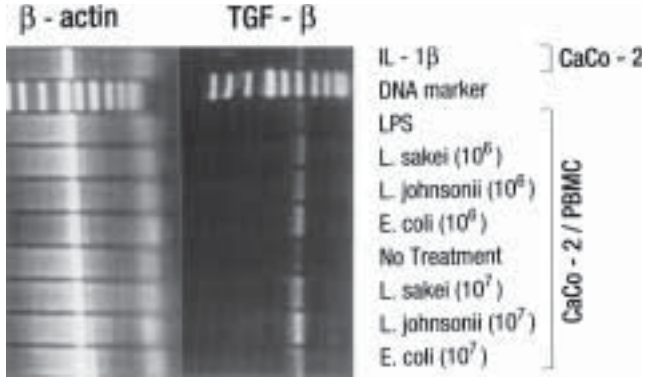


Fig. 2. Significant induction of TGFβ mRNA in leukocyte-sensitized CaCo-2 cells by *L. johnsonii* La1. RT-PCR analysis of TGFβ-specific gene transcripts in CaCo-2 cells after stimulation of CaCo-2/leukocyte co-cultures with nonpathogenic *E. coli*, *L. johnsonii* La1, and *L. sakei* (16 h, 10^6 and 10^7 CFU/mL). *Controls*: LPS (1 mg/mL), IL-1β (10 ng/mL), no treatment (medium). Results represent one of three independent experiments.

Interaction of Nonpathogenic Bacteria with Mixed Mucosal Cell Populations: Human Caco-2/Leukocyte Co-Cultures In Vitro

There is increasing evidence that bacterial signals to the host must be processed by a network of different mucosal cells, resulting in an integrated response that dictates the host reaction against a constantly changing microbial environment in the intestine.

To investigate such interactions, a human in vitro model was established with CaCo-2 or HT-29 cells and peripheral-blood mononuclear cells (PBMC) using the transwell culture technique *(72)*. The immune response to various nonpathogenic bacteria, including the probiotic strains *L. johnsonii* La1 and *L. sakei* LTH 681, was assessed by the determination of cytokine expression in IECs and leukocytes. Furthermore, the role of lymphoid and myeloid subpopulations in the regulation of immune-mediated activation of IEC after bacterial challenge was characterized.

The pro-inflammatory cytokines IL-8 and MCP-1 were induced in CaCo-2 cells upon challenge with nonpathogenic *Escherichia coli* (*E. coli*) and *Lactobacillus sakei* (Fig. 1). In contrast, *L. johnsonii* La1 did not stimulate the production of these cytokines, but upregulated the expression of TGF-β (Fig. 2). Responsiveness of IECs to nonpathogenic bacterial signals was dependent on the presence of PBMCs. In addition, the underlying immune cells also responded in a discriminatory manner to different bacteria, although the bacteria had no direct access to this compartment. As depicted in Fig. 3, *E. coli* and *L. sakei* exclusively induced

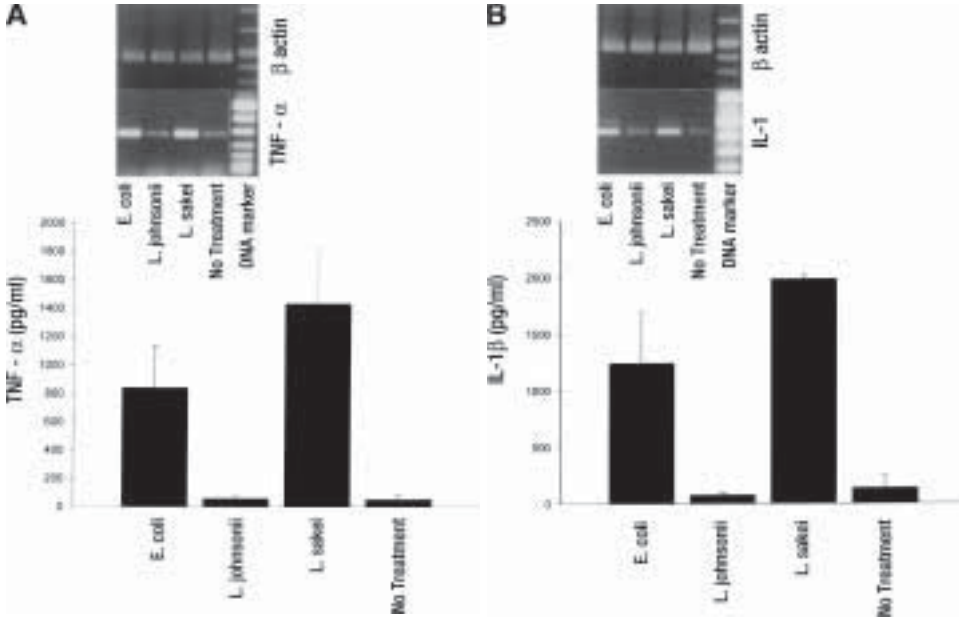


Fig. 3. Absence of secretion of the pro-inflammatory cytokines TNFα and IL1β from *L. johnsonii* La1-challenged CaCo-2/leukocyte co-cultures. Stimulation of CaCo-2/leukocyte co-cultures with nonpathogenic *E. coli*, *L. johnsonii* La1, and *L.sakei* (16 h, 10^6 CFU/mL). Secretion of TNFα **(A)** and IL-1β **(B)** into the basolateral compartment was determined by ELISA technique (bar chart, pg/mL). RT-PCR analysis was used to determine the expression of TNFα **(A)** and IL-1β **(B)** specific gene transcripts in CaCo-2 cells. Values are given as mean +/– SD of triplicates.

TNFα and IL-1β protein secretion from leukocyte-sensitized co-cultures, whereas no induction of these pro-inflammatory cytokines occurred with *L. johnsonii* La1. Secretion of both pro-inflammatory cytokines was polarized and challenged CaCO-2 cells contributed significantly to the production. Those bacteria inducing a pro-inflammatory type of response were also inducers of IL-10, an important immune inhibitory cytokine, shown to downregulate inflammatory cytokines. IL-10 was exclusively secreted by leukocytes (Fig. 4). TNFα, but not IL-1β, was the predominant cytokine implicated in the epithelial-immuno cross-communication as assessed by neutralizing experiments. Differences in the regulation of inflammatory immune response by IEC were observed between an enteropathogenic *E. coli* (EPEC E2348/69) and nonpathogenic bacteria. In contrast to the nonpathogenic *E. coli*, the EPEC strain induced a long-lasting pro-inflammatory signal in CaCO-2 cells. In contrast, TNFα induction by commensal bacteria was of short duration and self-limiting. These results suggest

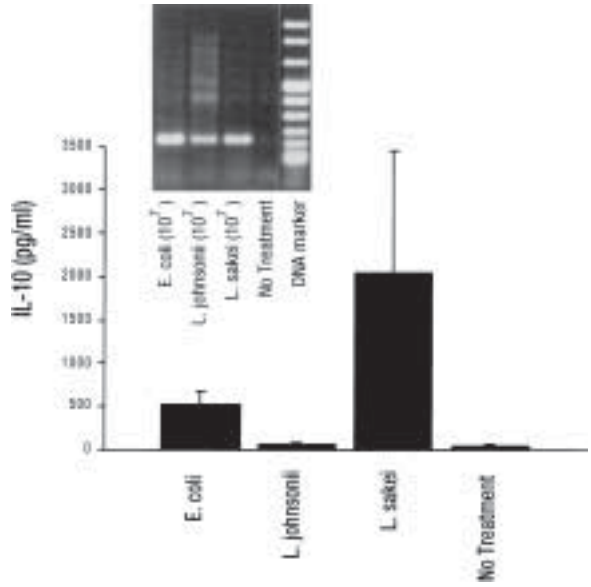


Fig. 4. IL-10 expression by CaCO-2/leukocyte co-cultures. Secretion (pg/mL) of IL-10 was determined in the basolateral compartment upon the stimulation of CaCO-2/leukocyte co-cultures with *E. coli*, *L. johnsonii*, and *L. sakei* (10^7 CFU/mL), respectively (bar chard). Culture medium (no treatment) was used as a control. RT-PCR analysis was used to determine IL-10 mRNA expression in PBMC. IL-10 secretion by underlying PBMC (black bar chard, pg/mL) upon stimulation of CaCO-2/leukocyte co-cultures with *E. coli* (10^7 CFU/mL). IL-10 secretion by leukocyte-sensitized CaCO-2 cells did not occur. Controls (no treatment) did not induce cytokine secretion. Values are given as mean ± standard deviation (SD) of triplicates, and represent one of three independent experiments.

that IEC-transduced signals discriminated between enteropathogens and commensal bacteria.

It was recently shown that enteropathogenic *E. coli* express proteins, such as EspA, EspB, and EspC, which induce signal transduction and cytokine secretion in CaCO-2 cells *(73)*. The nonpathogenic *E. coli* used in this study lacks these and other virulence factors commonly distributed among the species *Escherichia coli (74)*, and therefore did not induce cytokine response in CaCO-2 cells alone, confirming previous observations reported by other groups *(75)*.

The gram-positive food fermenting *L. sakei* exerted a similar proinflammatory activation pattern as the gram-negative nonpathogenic *E. coli* with respect to chemokine (IL-8 and MCP-1) and cytokine expression (TNFα, IL1β and IL-10). *Lactobacillus* species were shown to prevent colitis in IL-$10^{-/-}$ knockout mice. These mice habor decreased levels of colonic *Lactobacillus* species during the neonatal period. Nor-

malization of the colonic *lactobacilli* level was associated with attenuated development of colitis *(76)*. In vitro, *L. johnsonii* La1, an intestinal isolate, revealed no induction of a pro-inflammatory response, but favored TGF-β expression in CaCO-2 cells. TGF-β is a key factor that is implicated in the regulation of intestinal barrier function *(77,78)* and is implicated in the tolerance to the indigenous microflora via bystander suppression *(79)*.

These results strengthen the hypothesis that bacterial signaling at the mucosal surface is dependent on epithelial-immuno cross-communication, which appears to be responsible for the innate reaction that can actually distinguish between different nonpathogenic microorganisms. This discriminative response occurred in both compartments, probably orchestrated by cell-secretory products, which are not yet fully identified. These results also indicate that, depending on the *Lactobacillus* strain, a more pro-inflammatory (*L. sakei*) or more immune-regulatory (*L. johnsonii* La1) type of response might be stimulated at the mucosal site. Interestingly, intestinal *Lactobacilli* such as *L. johnsonii* La1 or a second strain, *L. gasseri*, failed to induce these cytokines in both IEC lines.

The Differential Role of Leukocyte Subpopulation in the Control of Bacteria Mediated Activation of IEC

In subsequent co-culture experiments, using CaCO-2 or HT-29 cells, it could be demonstrated that activation of IEC (in terms of TNFα expression) by *L. sakei* or nonpathogenic *E. coli* required the presence of $CD4^+$ –, $CD8^+$ T cells, or $CD19^+$ B cells, whereas purified monocytes failed to mediate activation. Recognition of commensal bacteria also occurred when the IEC were only pre-exposed to $CD4^+$ T cells or $CD19^+$ B cells, indicating that a "priming" by lymphocyte populations was sufficient. The molecular nature of this "priming" is unknown, and represents a subject of current research.

Monocytes Constitute an Immunoregulatory Subpopulation in Bacteria-Challenged IEC/Leukocyte Co-Cultures

While activation of IEC by *E. coli* or *L. sakei* was driven by lymphoid subpopulations, predominantly $CD4^+$ T cells, co-cultured monocytes demonstrated immunoregulatory functions—e.g., by the downregulation of TNFα. They constituted the main responder cells, secreting initially high amounts of TNFα, GM-CSF, IL-1-receptor antagonist (Ra), and IL-10. At later time-points, the pro-inflammatory cytokines were significantly downregulated, yet secretion of the regulatory cytokines, IL-10 and IL-2Rα remained high.

It was also shown that bacterial-activated IEC modulate cell-surface antigen expression of monocytes/macrophages. $CD14^{high}$ mono-

cytes, derived from peripheral-blood expressed MHC class II molecules and costimulatory molecules, such as B7.1 (CD80) and B7.2 (CD86). Co-culture of these monocytes with HT-29 cells resulted in the downregulation of CD14 and CD54. Thus, in the presence of IEC $CD14^{high}$ monocytes acquired an immunosuppressive phenotype. The modulatory effect on monocytes was even more pronounced after activation of HT-29 cells with *L. sakei*, resulting in a further downregulation of inflammatory and co-stimulatory surface molecules, while CD11b, CD33, CD116 (GM-CSF receptor), and MHC class II were conserved. This particular maturation was not dependent on secreted IL-10, as the addition of rh IL-10 to adherent monocytes cultures in vitro did not reproduce this phenotype (Haller, Detali, in press).

One important function of antigen-presenting cells (APC) is the stimulation of T cells. Mixed-lymphocyte reactions (MLR) can be used to assess the potential of APC to stimulate proliferation of allogeneic lymphocytes, which is dependent on the expression of MHC class II and costimulatory molecules. In fact, IEC or *L. sakei* activated IEC co-cultured macrophages were deficient in triggering a substantial lymphocyte response.

Intestinal macrophages, which constitute 10–20% of mononuclear cells in the lamina propria, differ markedly in phenotype and function from peripheral-blood monocytes *(80)*. The classical monocyte-specific surface antigens CD14 (LPS receptor), CD11b (complement receptor 3, CR3), CD11c (complement receptor 4, CR4), and CD16 (FcgIII receptor) are expressed at low levels in the normal intestinal mucosa. This is believed to be one mechanism to prevent activation induced by trace amounts of translocated bacterial products *(81,82)*. Under pathophysiological conditions, such as active inflammatory bowel disease (IBD), an increased fraction of macrophages with a monocytic-like phenotype appears in the LP *(83–85)*. It was reported that $CD14^{high}$ macrophages in IBD mucosa represent a newly recruited subset of intestinal macrophages extravasated from the peripheral blood, with an increased potential for the production of proinflammatory cytokines compared to the resident $CD14^{low}$ population, and therefore may be involved the development of IBD.

We demonstrated that IEC actively contribute to the development of $CD14^{low}$ macrophages with immunosuppressive functions. Under the influence of bacterial-activated IEC, peripheral-blood monocytes changed to a $CD14^{low}$ $CD11b^{high}$ $CD11c^{low}$ phenotype, and the pan-myeloic differentiation marker CD33, reliable for the identification of intestinal tissue macrophages *(81)*, and CD116 (GM-CSF receptor) remained high. Thus, IEC co-cultured macrophages showed similarities to LP macrophages. The absence of increased numbers of apoptotic

or necrotic cells during co-culture indicated that the loss of $CD14^{high}$ cells was a result of monocyte differentiation and not caused by cell death. Notably, commensal bacteria further promoted the induction of immunosuppressive macrophages by IEC, demonstrating that commensal bacteria, especially probiotics, are beneficial for the host.

Activation of Human PBMC by Probiotic Bacteria: Evidence of Natural Killer (NK) Cells as Primary Targets

Although the interaction between commensal, nonpathogenic bacteria and blood leukocytes seems to be an unusual event, it may occur in definite microenvironments of the mucosal immune system. A limited bacterial translocation through the epithelial barrier to the LP has been reported in humans *(18)*.

In vitro, several species of *lactobacilli* have been shown to induce cytokines, such as TNFα and IL-12 in human peripheral blood mononuclear cells (PBMC) *(49,86)*}. IL-12, like IFNγ constitute important cytokines implicated in innate immunity. Early production of IL-12 by macrophages will contribute to effector-cell maturation of both, natural killer (NK) and $CD8^+$ T cells, leading to a Th1-biased immune response *(87–89)*. The majority of circulating NK cells are $CD3^-CD56^+CD16^+$; a minority are $CD3^-CD56^+CD16^-$ *(19,22)*. Morphologically, they are large granular lymphocytes (LGL) and have the ability to migrate into tissues. They are predominantly found in the liver, but can also extravasate to mucosal sites *(90)*. NK cells provide immediate defense against tumors, viral infections, and intestinal pathogens, and thus have important functions in innate immunity *(87,91,92)*.

Although PBMC are only partially representative of immunocompetent cells in intestinal mucosal compartments, phenotypical similarities with respect to germline-encoded receptors involved in the recognition of bacterial antigens on lymphocytes and macrophages, such as pattern-recognition receptors *(36,39)*, could constitute the link between both populations and may provide important indications on functional aspects of the mucosal immune response to luminal bacteria.

We showed that a gram-negative commensal *E. coli* and the gram-positive probiotic strains *L. johnsonii* and *L. sakei* induced a different cytokine pattern in human PBMC. Whereas all bacteria induced TNF-a secretion, differences were observed in respect to the induction of the Th1-like cytokines IL-12, IFN-g and the inhibitory cytokine IL-10. *L. johnsonii* and *L. sakei* strongly induced IFN-g and IL-12, but not IL-10. In contrast, *E. coli* and LPS preferentially stimulated the synthesis of IL-10, but not IFN-g or IL-12 (Figs. 5 and 6). These results are in agreement with the reports by Miettinen et al. *(49)* and Muller-Alouf et al. *(93)* comparing different nonpathogenic and pathogenic gram-positive bac-

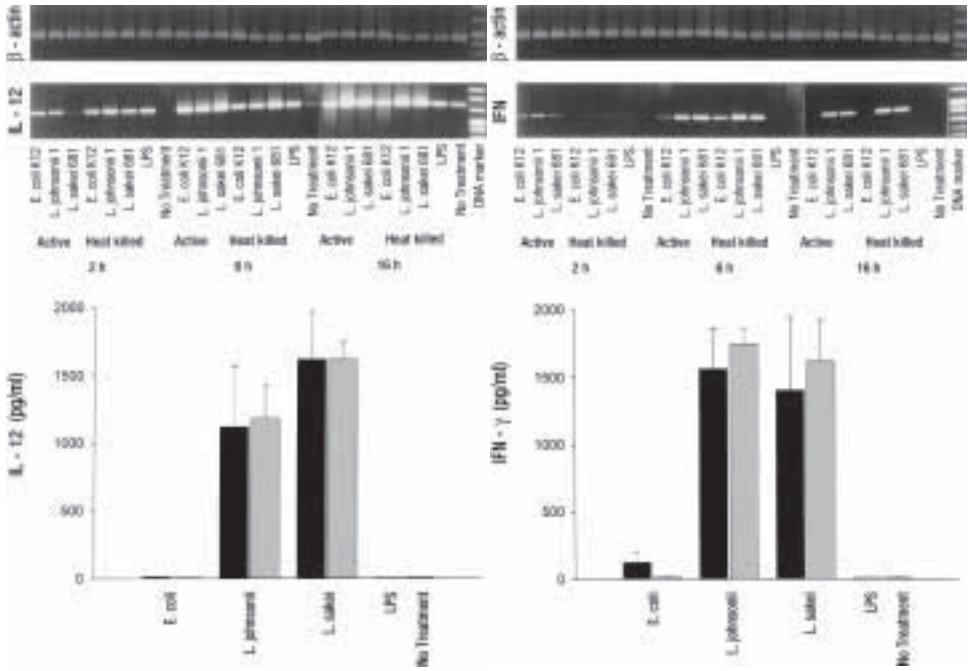
teria with respect to the induction of cytokines in PBMC. These in vitro data may also reflect the common immunosuppression observed in patients undergoing endotoxemia *(94)*.

The analysis of activation antigens CD69 and CD25 (IL-2 Ra chain) in bacteria-stimulated PBMC bulk cultures indicated that only NK cells, upregulating both markers, were activated by bacterial treatment, although the PHA control suggested that all lymphocyte subsets ($CD4^+$, $CD8^+$, $CD19^+$) responded normally to a mitogenic stimulus. Low expression of CD69 on $CD8^+$ cells after bacterial treatment could be attributed to a contamination with $CD8^+$ NK cells, rather than a specific activation of $CD3^+CD8^+$ T cells.

Induction of a proliferative response in PBMC by bacteria was observed with all strains. However, when lymphocyte subpopulations were isolated, only $CD3^-CD56^+$ NK cells responded with proliferation. A variety of NK-cell receptors, implicated in activation or inhibition of NK-cell effector functions, such as proliferation or cytolytic activity, have been described recently *(95,96)*. Therefore, a phenotypic characterization of the responsive NK subpopulation would provide further information on the specificity of the interaction with bacteria.

The co-culture of purified NK cells with bacteria-primed macrophages revealed that expression of CD25 is strongly promoted in the presence of an accessory cell, indicating the requirement of cell-contact-based signals for activation. This could be mediated by interaction of costimulatory molecules, such as CD28, CD16, or the CD94 receptor complex, which were shown to be expressed on human NK cells and to have key roles in expansion and effector functions *(97–99)*. The dependence on accessory cell function was also reflected by the selective induction of IFN-γ secretion from NK cells in the presence of *L. johnsonii* La1 primed macrophages or co-culture with macrophages and *L. johnsonii* La1. The synergistic effect on NK-cell activation observed in the combination of macrophages and bacteria is likely to be based on additional secretion of monokines, which engage constitutively expressed monocyte-derived cytokine receptors on NK cells *(62)*.

Although secretion of cytokines required the presence of accessory cells, a direct interaction between bacteria and NK cells, leading to activation, could be demonstrated. This interaction was particularly intense with *L. johnsonii* La1 compared to *E. coli*, and could be linked to different bacterial cell-surface determinants, which may constitute the molecular basis for specific immunomodulatory properties. It is reported that *lactobacilli* interact with asialo-GM1 receptors on epithelial cells *(100,101)*. Expression of this receptor is also reported on murine NK cells *(90)*, and may also constitute a putative receptor on human NK cells to mediate activation by bacteria *(102)*. The increased phagocytic activity observed in PBMC to healthy volunteers after oral admin-



Figs. 5 and 6. Expression of IL-12 and IFNγ by PBMC upon stimulation with nonpathogenic bacteria. RT-PCR and ELISA analyses were used to determine IL-12 and IFNγ production by PBMC (1×10^6/mL) after stimulation with heat-killed (gray bars) and live (black bars) bacteria (*E. coli*, *L. johnsonii* La1, *L. sakei*, 1×10^6 CFU/mL) or LPS (1 μg/mL). Gene transcription (IL-12*p*40, IFNγ) was determined after 2, 6, and 16 h. Protein secretion (IFNγ, IL-12*p*70) was analyzed after 16 h of bacterial stimulation. No antibiotics were added to the cultures. Values are means +/– SD of triplicate measurements, and represent one of three independent experiments.

istration of *L. johnsonii* La1 suggests that this stimulation could take place within the normal homeostasis of the immune system. Thus, activation of monocytes/macrophages seems to be a common denominator for both, the in vivo observation following LAB ingestion and the in vitro data showing that *L. johnsonii* La1-primed monocytes mediate NK-cell activation and subsequent IFN-γ secretion.

NK cells play an important role in innate immune resistance, particularly through synthesis of IFN-γ. It has been clearly established that IL-12 and IFN-γ mediate protective functions against intracellular pathogens by inducing monocyte/macrophage activation *(103)*. Our data suggest that NK cells, as well as the macrophage, are primary targets for bacterial stimulation. This theory is consistent with previous observations that IFN-γ production in response to bacteria requires NK cells but not T cells. IFN-γ production in vitro by activated NK cells is highly dependent on the presence of IL-12, which induces effector

maturation and expansion of NK cells and CD8+ T cells. Those cells which first encounter a foreign antigen play an important role in determining whether a Th1 or Th2 biased immune response is mounted to an antigenic challenge. IL-12 is implicated in the mechanisms of an innate immune response, and, at the same time, shifts the immune response toward initiation of adaptive immunity *(88)*.

It is essential that NK-cell activity remains under stringent and finely tuned control. The system of inhibitory and stimulatory receptors, and the cytokine micro-environment, allows the control of NK-cell responses *(104,105)*. The role of NK cells in the recognition of commensal bacteria signals, in part mediated by monocytes, has not been established thus far. However, the fact that induction of a distinct immune response in human PBMC by nonpathogenic bacteria was demonstrated should encourage further work to understand the physiology of nonpathogenic bacterial interaction with host cells.

Conclusions

To date, human in vitro models, although reductionist, are commonly accepted as valuable tools in the study of molecular mechanisms. Application of well-reflected in vitro models will provide the opportunity to characterize, in molecular terms, the "beneficial effect" of a given probiotic strain to the host. Immune effects by probiotics have been criticized as "inconclusive" *(106)* and probiotic research focused on in vitro tissue culture or animal models "impressive, but with unclear significance for the human physiology" *(107)*. This is actually true for many research fields. The ultimate proof will come from correctly designed human intervention studies. However, to provide a molecular basis for the observed effects *in situ*, fundamental research will refer to adaequate animal or in vitro models.

The results presented here provide molecular evidence on the beneficial effect of specific probiotic strains on intestinal immune homeostasis. IECs permanently interact with the luminal content of the gut, including commensal bacteria, and a cellular network of professional immune cells. Recent experimental data suggest that IECs play an important role in processing nonpathogenic bacteria-derived signals to the mucosal immune system. This is achieved by differential expression of molecules involved in cell-to-cell contact or by the secretion of soluble mediators, such as cytokines/chemokines that will attract specific immune-effector cells. Thus, one appropriate function of IECs is to adapt the physiological reactivity of the host tissues to a highly changing intestinal content. A dysfunction of this interphase could promote discordance between the luminal signal and the initiated response. As a consequence, pathological conditions arising from exaggerated responses to non-dangerous signals, such as food antigens, resulting in

food allergy or chronic inflammation (i.e., IBD) may occur. Fine-tuning of mucosal responses probably depends not only on IEC function, but also on an intricate cell-to-cell cross-communication, where intra-epithelial and lamina propria lymphocytes (LPL) are further participants.

L. johnsonii La1 demonstrated a low potential to induce a pro-inflammatory response in human IEC lines, but favored the induction of TGFβ, a key factor implicated in the regulation of intestinal-barrier function. The in vitro results support the observation of a current human study in healthy volunteers, that immunostimulation by *L. johnsonii* La1 was not of a pro-inflammatory type, as acute phase proteins and soluble IL2- and IL6 receptor (sIL2r/sIL6r) in serum did not increase above control levels during and after consumption of the probiotic *(108)*.

New techniques such as cDNA-chip technology, laser-capture microdissection, and real-time PCR, applied on tissue cultures, animal models, or human biopsies will provide comparative data on target genes modulated by nonpathogenic, probiotic bacteria. This will broaden our knowledge on the molecular basis of "beneficial effects" at the mucosa, especially as the induction, up- or downregulation of target genes can be monitored in parallel and by microdissection techniques associated with specific cells within the intestinal mucosa.

This knowledge provides the food industry with a unique possibility to improve gut hoemeostasis through nutritional interventions. The recent report by Hooper et al. already indicates that colonization of germ-free mice with a commensal resulted in a significant modulation of intestinal functions implicated in nutrient absorption, mucosal defense, and xenobiotic metabolism *(109)*.

References

1. Fuller, R. (1989). *J. Appl. Bacteriol.* **66**,365–378.
2. Coconnier, M.H., Bernet, M.F., Chauviere, G., and Servin, A.L. (1993). *J. Diarrhoeal. Dis. Res.* **11**,235–242.
3. Bernet, M.F. (1994). *Gut* **35**,483–489.
4. Chauviere, G., Coconnier, M.H., Kerneis, S., Darfeuille-Michaud, A., Joly, B., and Servin, A.L. (1992). *FEMS Microbiol. Lett.* **70**,213–217.
5. Midolo, P.D., Lambert, J.R., Hull, R., Luo, F., and Grayson, M.L. (1995). *J. Appl. Bacteriol.* **79**:475–479.
6. Michetti, P., Dorta, G., Wiesel, P.H., Brassart, D., Verdu, E., Herranz, M., et al. (1999). *Digestion* **60**,203-209.
7. Hove, H., Nordgaard-Andersen, I., and Mortensen, P. B. (1994). *Am. J. Clin. Nutr.* **59**,74–79.
8. Jiang, T., Mustapha, A., and Savaiano, D.A. (1996). *J. Dairy Sci.* **79**,750–757.
9. Saavedra, J.M., Bauman, N.A., Oung, I., Perman, J.A., and Yolken, R.H. (1994). *Lancet* **344,**1046–1049.
10. Kaila, M. (1995). *Arch. Dis. Child.* **72**,51–53.
11. Schiffrin, E.J., Rochat, F., Link-Amster, H., Aeschlimann, J.M., and Donnet-Hughes, A. (1995), Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. *J. Dairy Sci.* **78**,491–497.

12. Link-Amster, H., Rochat, F., Saudan, K.Y., Mignot, O., and Aeschlimann, J.M. (1994). *FEMS Immunol. Med. Microbiol.* **10**,55–64.
13. Bernet, M.-F., Brassart, D., Neeser, J.-R., et al. (1994). *Gut* **35**,483–489.
14. Tuomola, E.M. and Salminen, S.J. (1998). *Int. J. Food Microbiol.* **41**,45–51.
15. Perdigon, G., Alvarez, S., Rachid, M., Aguero, G., and Gobbato, N. (1995). *J. Dairy Sci.* **78**,1597–1606.
16. Marteau, P., Vaerman, J. P., Dehenin, J. P., Bord, S., Brassart, D., Pochart, P., et al. (1997). *Gastroenterol. Clin. Biol.* **21**,293–298.
17. Matsuzaki, T. and Chin, J. (2000). *Immunol. Cell Biol.* **78**,67–73.
18. Neutra, M.R. (1999). *Curr. Top. Microbiol. Immunol.* **236**,17–32.
19. Liu, Y.J. and Arpin, C. (1997). *Immunol. Rev.* **156**,111–126.
20. Berlin, C., Berg, E.L., Briskin, M.J., Andrew, D.P., Kilshaw, P.J., Holzmann, B., et al. (1993). *Cell* **74**,185–185.
21. Cerf-Bensussan, N., Quaroni, A., Kurnick, J.T., and Bhan, A.K. (1984). *J. Immunol.* **132**,2244–2252.
22. Regnault, A., Cumano, A., Vassalli, P., Guy-Grand, D., and Kourilsky, P. (1994). *J. Exp. Med.* **180**,1345–1358.
23. Cepek, K.L., Shaw, S.K., Parker, C.M., Russell, G.J., Morrow, J.S., Rimm, D.L., et al. (1994). *Nature* **372**,190–193.
24. Eckmann, L., Jung, H.C., Schürer-Maly, C., Panja, A., Morzycka-Wrobleska, E., and Kagnoff, M. (1993). *Gastroenterology* **105**,1689–1697.
25. Kindon, H., Pothoulakis, C., Thim, L., Lynch-Devaney, K., and Podolsky, D.K. (1995). *Gastroenterology* **109**,516–523.
26. Mack, D.R., Michail, S., Wei, S., McDougall, L., and Hollingsworth, M.A. (1999). *Am. J. Physiol.* **276**,941–950.
27. Hecht, G. (1999). *Am. J. Physiol.* **277**,351–358.
28. O'Neil, D.A., Porter, E.M., Elewaut, D., Anderson, G.M., Eckmann, L., Ganz, T.,et al (1999). *J. Immunol.* **163**,6718–6724.
29. Singh, P.K., Jia, H.P., Wiles, K., Hesselberth, J., Liu, L., Conway, B.A., et al. (1998). *Proc. Natl. Acad. Sci. USA* **95**:14,961–14,966.
30. Lencer, W.I., Cheung, G., Strohmeier, G. R., Currie, M.G., Ouellette, A.J., Selsted, M.E., et al. (1997). *Proc. Natl. Acad. Sci. USA* **94**,8585–8589.
31. Chertov, O., Michiel, D.F., Xu, L., Wang, J.M., Tani, K., Murphy, W.J., et al. (1996). *J. Biol. Chem.* **271**:2935–2940.
32. Lillard, J.W., Boyaka, P.N., Chertov, O., Oppenheim, J.J., and McGhee, J.R. (1999). *Proc. Natl. Acad. Sci. USA* **96**,651–656.
33. Dignass, A.U. and Podolsky, D.K. (1993). *Gastroenterology* **105**,1323–1332.
34. Donnet-Hughes, A., Duc, N., Serrant, P., Vidal, K., and Schiffrin, E.J. (2000). *Immunol. Cell Biol.* **78**,74–79.
35. Goke, M., Kanai, M., and Podolsky, D.K. (1998). *Am. J. Physiol.* **274**, 809–818.
36. Stahl, P.D. and Ezekowitz, R.A. (1998). *Curr. Opin. Immunol.* **10**:50–55.
37. Fraser, I.P., Koziel, H., and Ezekowitz, R.A. (1998). *Semin. Immunol.* **10**,363–372.
38. Park, S.H. and Bendelac, A. (2000). *Nature* **406**,788–792.
39. Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A. (1997). *Nature* **388**,394–397.
40. Medzhitov, R. and Janeway, C. (2000). *Trends Microbiol.* **8**,452-456.
41. Xu, Y., Tao, X., Shen, B., Horng, T., Medzhitov, R., Manley, J.L., et al. (2000). *Nature* **408**,111–115.
42. Yoshimura, A., Lien, E., Ingalls, R.R., Tuomanen, E., Dziarski, R., and Golenbock, D. (1999). *J. Immunol.* **163**,1–5.
43. Brightbill, H.D., Libraty, D.H., Krutzik, S.R., Yang, R.B., Belisle, J.T., Bleharski, J.R., et al. (1999). *Science* **285**,732–736.
44. Ingalls, R.R., Heine, H., Lien, E., Yoshimura, A., and Golenbock, D. (1999). *Infect. Dis. Clin. North Am.* **13**,341–353,vii.

45. Aderem, A. and Ulevitch, R.J. (2000). *Nature* **406**,782–787.
46. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., et al. (1999). *J. Exp. Med.* **189**,1777–1782.
47. Cario, E., Rosenberg, I.M., Brandwein, S.L., Beck, P.L., Reinecker, H.C., and Podolsky, D.K. (2000). *J. Immunol.* **164**,966–972.
48. Anderson, K.V. (2000). *Curr. Opin. Immunol.* **12**,13–19.
49. Miettinen, M., Matikainen, S., Vuopio-Varkila, J., Pirhonen, J., Varkila, K., Kurimoto, M., et al. (1998). *Infect. Immun.* **66**,6058–6062.
50. Kerneis, S., Bogdanova, A., Kraehenbuhl, J.P., and Pringault, E. (1997). *Science* **277**,949–952.
51. Lebman, D.A., Lee, F.D., and Coffman, R.L. (1990). *J. Immunol.* **144**,952–959.
52. Shockett, P. and Stavnezer, J. (1991). *J. Immunol.* **147**,4374–4383.
53. Coffman, R.L., Lebman, D.A., and Shrader, B. (1989). *J. Exp. Med.* **170**,1039–1044.
54. Kett, K., Baklien, K., Bakken, A., Kral, J. G., Fausa, O., and Brandtzaeg, P. (1995). *Gastroenterology* **109**,819–825.
55. Brandtzaeg, P., Baekkevold, E.S., Farstad, I.N., Jahnsen, F.L., Johansen, F.E., Nilsen, E.M., et al. (1999). *Immunol. Today* **20**,141–151.
56. Fujihashi, K., McGhee, J.R., Kweon, M.N., Cooper, M.D., Tonegawa, S., Takahashi, I., et al. (1996). *J. Exp. Med.* **183**,1929–1935.
57. Macpherson, A.J., Gatto, D., Sainsbury, E., Harriman, G.R., Hengartner, H., and Zinkernagel, R.M. (2000). *Science* **288**,2222–2226.
58. Kroese, F.G. and Bos, N.A. (1999). *Curr. Top. Microbiol. Immunol.* **246**,343–349.
59. Bos, N.A., Cebra, J.J., and Kroese, F.G. (2000). *Curr. Top. Microbiol. Immunol.* **252**,211-220.
60. Isolauri, E., Juntunen, M., Rautanen, T., Sillanaukee, P., and Koivula, T. (1991). *Pediatrics* **88**,90–97.
61. van der Waaij, L.A., Limburg, P.C., Mesander, G., and van der Waaij, D. (1996). *Gut* **38**,348–354.
62. Fehniger, T.A., Shah, M.H., Turner, M.J., VanDeusen, J.B., Whitman, S.P., Cooper, M.A., et al. (1999). *J. Immunol.* **162**,4511–4520.
63. Kagnoff, M.F. and Eckmann, L. (1997). *J. Clin. Invest.* **100**,6–10.
64. Panja, A., Goldberg, S., Eckmann, L., Krishen, P., and Mayer, L. (1998). *J. Immunol.* **161**,3675–3684.
65. Hershberg, R.M., Cho, D.H., Youakim, A., Bradley, M.B., Lee, J.S., Framson, P.E., et al. (1998). *J. Clin. Invest.* 102,792–803.
66. Blumberg, R.S., Terhorst, C., Bleicher, P., McDermott, F.V., Allan, C.H., Landau, S.B., et al. (1991). *J. Immunol.* **147**:2518–2524.
67. Reinecker, H.C. and Podolsky, D.K. (1995). *Proc. Natl. Acad. Sci. USA* **92**:8353–8357.
68. Raitano, A.B. and Korc, M. (1993). *Cancer Res.* **53**,636–640.
69. Eckmann, L., Stenson, W.F., Savidge, T.C., Lowe, D.C., Barrett, K.E., Fierer, J., et al. (1997). *J. Clin. Invest.* **100**,296–309.
70. Jung, H.C., Eckmann, L., Yang, S.K., Panja, A., Fierer, J., Morzycka-Wroblewska, E.,et al. (1995). *J. Clin. Invest.* **95**,55–65.
71. Schuerer-Maly, C.C., Eckmann, L., Kagnoff, M.F., Falco, M.T., and Maly, F.E. (1994). *Immunology* **81**,85–91.
72. Haller, D., Bode, C., Hammes, W.P., Pfeifer, A.M., Schiffrin, E.J., and Blum, S. (2000). *Gut* **47**,79–87.
73. Savkovic, S.D. (1997). *Am. J. Physiol.* **273**,1160–1167.
74. Muhldorfer, I., Blum, G., Donohue-Rolfe, A., Heier, H., Olschlager, T., Tschape, H., et al. (1996). *Res. Microbiol.* **147**,625–635.
75. Jung, H.C., Eckmann, L., Yang, S.-K., Panja, A., Fierer, J., Morzycka-Wroblewska, E., et al. (1995). *J. Clin. Invest.* **95**,55–65.

76. Madsen, K.L., Doyle, J.S., Jewell, L.D., Tavernini, M.M., and Fedorak, R.N. (1999). *Gastroenterology* **116**,1107–1114.
77. Dignass, A.U. and Podolsky, D.K. (1993). *Gastroenterology* **105**,1323–1332.
78. Planchon, S.M., Martins, C.A., Guerrant, R.L., and Roche, J.K. (1994). *J. Immunol.* **153**,5730–5739.
79. Miller, A., Lider, O., and Weiner, H.L. (1991). *J. Exp. Med.* **174**,791–798.
80. Rugtveit, J., Nilsen, E.M., Bakka, A., Carlsen, H., Brandtzaeg, P., and Scott, H. (1997) [published erratum appears in *Gastroenterology* 1997 Aug;**113(2)**,732]. *Gastroenterology* **112**,1493–1505.
81. Rogler, G., Hausmann, M., Vogl, D., Aschenbrenner, E., Andus, T., Falk, et al. (1998). *Clin. Exp. Immunol.* **112**,205–215.
82. Malizia, G., Calabrese, A., Cottone, M., Raimondo, M., Trejdosiewicz, L.K., Smart, C.J., et al. (1991). *Gastroenterology* **100**,150–159.
83. Allison, M.C. and Poulter, L.W. (1991). *Clin. Exp. Immunol.* **85**,504–509.
84. Grimm, M.C., Pullman, W.E., Bennett, G.M., Sullivan, P.J., Pavli, P., and Doe, W.F. (1995). *J. Gastroenterol. Hepatol.* **10**,387–395.
85. Rugtveit, J., Brandtzaeg, P., Halstensen, T.S., Fausa, O., and Scott, H. (1994). *Gut* **35**,669–674.
86. Hessle, C., Hanson, L.A., and Wold, A.E. (1999). *Clin. Exp. Immunol.* **116**,276–282.
87. Bohn, E. and Autenrieth, I.B. (1996). *J. Immunol.* **156**,1458–1468.
88. Hall, S.S. (1995), IL-12 at the crossroads [news] [see comments]. *Science* **268**,1432–1434.
89. Cooper, A.M., Roberts, A.D., Rhoades, E.R., Callahan, J.E., Getzy, D.M., and Orme, I.M. (1995). *Immunology* **84**,423–432.
90. Pang, G., Buret, A., Batey, R.T., Chen, Q.Y., Couch, L., Cripps, A., et al. (1993). *Immunology* **79**,498–505.
91. Kiessling, R., Klein, E., and Wigzell, H. (1975). *Eur. J. Immunol.* **5**,112–117.
92. Mastroeni, P., Harrison, J.A., Chabalgoity, J.A., and Hormaeche, C.E. (1996). *Infect. Immun.* **64**,189–196.
93. Muller-Alouf, H., Alouf, J.E., Gerlach, D., Ozegowski, J.H., Fitting, C., and Cavaillon, J.M. (1994). *Infect. Immun.* **62**,4915–4921.
94. Gerard, C., Bruyns, C., Marchant, A., Abramowicz, D., Vandenabeele, P., Delvaux, A., et al. (1993). *J. Exp. Med.* **177**,547–550.
95. Lanier, L.L. (1998). *Annu. Rev. Immunol.* **16**,359–393.
96. Moretta, A., Bottino, C., Vitale, M., Pende, D., Biassoni, R., Mingari, M. C., et al. (1996). *Annu. Rev. Immunol.* **14**,619–648.
97. Galea-Lauri, J., Darling, D., Gan, S.U., Krivochtchapov, L., Kuiper, M., Gaken, J., et al. (1999). *J. Immunol.* **163**,62–70.
98. Trinchieri, G. (1995). *Annu. Rev. Immunol.* **13**,251–276.
99. Voss, S.D., Daley, J., Ritz, J., and Robertson, M.J.(1998). *J. Immunol.* **160**,1618–1626.
100. Fujiwara, S., Hashiba, H., Hirota, T., and Forstner, J.F. (1997). *Appl. Environ. Microbiol.* **63**,506–512.
101. Warren, H.S. and Kinnear, B.F. (1999). *J. Immunol.* **162**,735–742.
102. Muller, C., Szangolies, M., Kukel, S., Kiehl, M., Sorice, M., Griggi, T., et al. (1996). *Scand. J. Immunol.* **43**,583–592.
103. MacMicking, J., Xie, Q.W., and Nathan, C. (1997). *Annu. Rev. Immunol.* **15**,323–350.
104. Fort, M.M., Leach, M.W., and Rennick, D.M. (1998). *J. Immunol.* **161**,3256–3261.
105. Hogan, P.G., Hapel, A.J., and Doe, W.F. (1985). *J. Immunol.* **135**,1731–1738.
106. de Roos, N.M. and Katan, M.B. (2000). *Am. J. Clin. Nutr.* **71**,405–411.

107. Sanders, M.E. (1994), Lactic acid bacteria as promoters of human health, in *Functional Foods*, Goldberg, I., eds., Chapman & Hall, New York, pp. 294–322.
108. Mattila-Sandholm, T., Blum, S., Collins, J. K., Crittenden, R., de Vos, W., Dunne, C., et al. (1999). *Trends in Food Science & Technology* **10**,393–399.
109. Hooper, L.V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G., and Gordon, J.I. (2001). *Science* **291**,881–884.