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IMMUNOREGULATORY ACTIONS OF LIVE AND KILLED ENTEROCOCCI AND *E. COLI* PROBIOTICS

KURT ZIMMERMANN

Symbio Herborn Group, Herborn-Hörbach, Germany

SUMMARY

During the past two decades an aggressive marketing campaign for so-called „probiotics“ resulted in an increased consumer awareness for these „health-promoting bacteria“. Among them, certain lactic acid bacteria of human origin dominate. Effective market strategies in the past, associated with the consumption of these bacteria for attractive possibilities to improve well being and health, even for such medically relevant and serious disease entities like atopic allergy or inflammatory bowel disease. However, to effectively market these health-claims, probiotic bacteria need to have scientific credibility. The latter can only be achieved by well-controlled clinical studies together with pre-clinical well-designed experimental work using refined cell culture models preferably with human leukocytes. However, clinical data supporting the above claimed health benefits remained scant. So far, the best-documented clinical application of probiotics is the treatment of acute diarrhoea in adults and infants, as shown recently by well controlled small-scale studies.

Whereas the successful treatment of acute diarrhoea presumably needs the oral administration of viable bacteria, whose numbers could markedly differ between different fermented milk products, dependent on the manufacturing process and strain-specific properties, distinct immunomodulatory effects can be mediated by inactivated germs too. Hence, the central question to be answered by this presentation was, whether live or dead probiotic bacterial strains, in particular *E. coli* derived probiotics, *Enterococcus faecalis* and a mixture of both strains (Pro-Symbioflor®), may exert similar immunomodulatory effects, tentatively speculated to be useful for immune system related disorders.

To address this question may be the more important in view of the rising tendency of the food industry to use several state-of- the-art biotechnological tools to design „better“ probiotic strains aimed to achieve „optimal“ gut colonisation, better adhesion properties and metabolic profiles, anticancer properties, bacteriocin production and much more. Introducing such genetically modified bacteria into the human gastrointestinal tract could have a profound and still yet unknown impact on the physiology and complex interactions of the normal gut flora, so that probiotic bacteria with a suitable immunomodulatory profile, comparable to selected live strains, might be a more safe alternative, particular under conditions of long-term use.

The ability of mammalian host cells to distinguish harmful virulent pathogens from dead ones, point to the existence of so-called pathogen-associated molecular patterns (PAMS), recognised for example by the Toll-like receptor family of proteins. Other host defence strategies, represented by CD14 antigen, the acute phase proteins, the scavenger receptors, mannose binding lectin and much more emerged to be indispensable in promoting phagocytosis without promoting inflammation with subsequent more effective killing of Gram-positive as well as Gram-negative bacteria. Accordingly the presentation summarises recent published data with regard to those receptors involved in the recognition of Gram-positive and Gram-negative bacteria, which may be of importance in the recognition of *E. coli* and *Enterococcus faecalis*.

The results revealed similarities as well as differences in view of the molecular recognition of dead versus live bacteria and whole versus purified cell wall components illustrating, that the physical composition of their cell wall components have an impact for the subsequent activation of immune cells. Also, recent laboratory work providing evidence for powerful immunomodulatory actions of *Enterococcus faecalis* and *E. coli* were presented. Both strains, being the constituents of a „medical" probiotic preparation named Pro-Symbioflor®, possess a profound cytokine-modulating capacity suggesting that Th1-cells were the main target population. These distinct immunomodulatory properties of Pro-Symbioflor® may be of great importance for therapeutic interventions in Th2 dominated diseases.

PATHOGEN–ASSOCIATED MOLECULAR PATTERNS (PAMPs): KEY STRUCTURES NOT ONLY FOR ANTIMICROBIAL DEFENCE BUT ALSO FOR THE NON-PHLOGISTIC REMOVAL OF APOPTOTIC OR INJURED CELLS

For the survival of most multi-cellular organisms, the recognition of infecting microbes followed by the induction of an effective immune response is essential. Additionally, it is equally important, that the immune response is not induced upon the recognition of self antigens or non-infectious non-self antigens which seem to be of great importance, when injured or apoptotic cells must be removed without causing harm to the host. Thus, the central question is: How does the immune system decide which antigens to respond to and which to ignore? From an evolutionary point of view, microbes, such as bacteria, impose a general thread to the host organism, so that proper and effective recognition of microbial antigens is the first step to ensure the survival of mammalian cells.

But what are the responsible structures to interact with the immune system, as microbes normally possess a huge variety of potential harmful antigenic structures? These microbial structures should be expressed by as much as possible potentially harmful pathogens, enabling the host to broadly recognise and kill as much as possible pathogens with sufficient specificity. The problem comes to a solution as it has been suggested by *Medzhitov* et al. in 1997, that host cells infecting microbes are recognised by a limited number of highly conserved chemical

Table 1: Overview on PAMPs with their proposed ligands (for explanations see text)

PAMPs	Ligand(s)/functions
Soluble and membrane-bound CD14 (sCD14,mCD14) (*Sher* et al., 1991)	Interaction with LBP-bound monomers of LPS, sLPS > rLPS > diphosphoryl LipidA >Monophosphoryl Lipid A liporabinomannans of mycobacteria, soluble peptidoglycans, polymers of rhamnose and glucose of streptococci and whole bacterial cell walls of streptococci and staphylococci
LPS binding Protein (LBP) (*Fenton* and *Golenbock* , 1998)	Lipid A part of endotoxin
Scavenger receptor subtypes (SRs) (*Rigotti* et al., 1997)	SR-A and B subtypes interact with polyanionic ligands, such as structurally modified lipoproteins (oxidised LDLs, HDLs, etc.)
Mannose Binding Lectin (MBL) (*Stahl* and *Ezekowitz* , 1998)	A member of the collectin family, calcium-dependent binding of multiple lectin domains of a wide spectrum of oligosaccharides of several bacterial species
Serum amyloid P (SAP) (*Coker* et al., 2000)	A member of the pentraxin family of proteins, calcium dependent binding to FcG receptors like an opsonin
C-reactive protein (CRP) (*Fenton* and *Golenbock*, 1998; *Gregory* , 2000)	A member of the pentraxin family reacting with Phosphorylcholine domains in bacterial cell walls
Phosphatidyl Serine Receptor (PS-R) (*Gregory* , 2000)	Expressed as scavenger Receptor on Macrophages for non-phlogistic removal of apoptotic cells, cytokine inducible
Thrombospondin (TSP) (*Gregory* , 2000)	A matrix component enhanced on apoptotic cells, ligands reported to be CD36 or the Vitronectin receptor

structures produced only by microorganisms and not by multi-cellular hosts. Consequently, these structures are referred to as pathogen-associated molecular patterns (PAMPs) and comprise cell wall components of Gram-negative as well as Gram-positive bacteria as well. Their recognition by immune cells is followed by the induction of a more or less intense inflammatory response enabling the host to respond as fast as possible to the invading pathogens.

The concept of PAMPs, introduced by *Medzhitov* et al. (1997), was proposed to be mostly an element of the „primitive“ immune system. PAMPs can define ligands in the bacterial cell wall common for both Gram-positive and Gram-negative bacteria (such as peptidoglycans or lipoproteins/lipopeptides) or different ones, such as lipoteichoic acids (LTAs) or lipopolysaccharide (LPS). Of note, it turned out in the past years, that recognition of PAMPs operates quite efficiently in higher vertebrates as well. The different PAMPs together with their respective receptors (pattern recognition receptors, PRRs) compose therefore powerful recognition entities aimed to interact with manifold chemical entities highly conserved in microbes. Among PRRs are the serum amyloid P (SAP) (*Coker* et al., 2000), the newly described phosphatidylserine receptor (PS-R) (*Gregory*, 2000), the family of scavenger receptors (SRs) able to interact with modified lipoproteins (SR subtypes A and B) (*Rigotti*, 1997; *Zingg* et al., 2000; *Williams* et al., 1999; *Platt* and *Gordon*, 1998), the mannose binding lectin (MBL) (*Stahl* and *Ezekowitz*,

1998; *Turner*, 1998), the selectins (*Malhotra* and *Bird*, 1997), the C-reactive protein (CRP) and last not least the CD14 antigen (*Fenton* and *Golenbock*, 1998), as is summarised in Table 1.

The latter glycoprotein is indispensable for the recognition of the Lipid A complex within the complex of lipid-binding-protein/LPS, together with a recently described new family of PRPs, the so called Toll-like receptor family (TLRs). Notably a new function of CD14 was recently described as a scavenger receptor for the clearance of apoptotic cells (*Gregory*, 2000). In particular, this newly described function of mCD14 could be of utmost importance for the organism to minimise or prevent overshooting inflammation associated with the removal of apoptotic and injured cells implicating that the receptor/ligand recognition process must generate signals for downregulation of inflammatory mediators. The report by *Gregory* et al. (2000) is therefore worthwhile to mention because the previously described functions of CD14 as a main LPS sensor generally were associated with „danger" for the immune system but turned out now to be involved in the limitation of inflammatory processes.

TOLL LIKE PROTEINS: RECEPTORS TO DISCRIMINATE BETWEEN GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

The first evidence for the involvement of Toll proteins in signalling against antibacterial defence came from analyses of *Drosophila* mutants, carrying loss-of-functions mutations in the various components of the Toll-pathway (*Lemaitre* et al., 1995; *Belvin* and *Anderson*, 1996). These mutants were unable to recognise fungal infections and produced among several other antibacterial peptides drosomycin, a major antifungal peptide. In *Drosophila*, the prototypic gene named Toll encodes a plasma membrane receptor, known to be involved in dorsal-ventral polarisation of the embryo. On the basis of using expressed sequence tags with homology to Toll, several members of the human Toll superfamily were recently cloned during the past years from human cDNA libraries including TLR4, originally designed human Toll (hToll) (*Medzhitov* et al., 1997; *Medzhitov*, 2000). Today's knowledge on members of the human Toll like receptor family and their putative ligands, the PAMPs of different bacterial species, are summarised in Figure 1.

Accordingly about 10 members of the TLR family are known at present. They represent type 1 transmembrane proteins, characterised extracellularily by so-called leucine rich repeats (LRRs) of different length. According to Figure 1, TLRs recognise PAMPs that often represent molecular signatures of a particular pathogen class: LPS is the main signature of Gram-negative bacterial cell walls and a huge amount of experimental data, obtained from gene knockout mice for different Toll proteins and transfection experiments with Toll receptors into cell lines, summarised by *Janeway* and *Medzhitov* (1999), *Beutler* (2000), *Anderson* (2000), *Heldwein* et al. (2001), *Brightbill* et al. (1999), and *Akira* et al. (2001), point to the prominent role of TLR4 as an important mammalian LPS sensor and receptor.

For example, a knockout mutation of TLR2 in mice has no effect on LPS signal transduction, as these animals were as competent in their response to LPS as their wildtype littermates. By contrast, a knockout in the TLR4 gene produced a phenotype completely unresponsive to

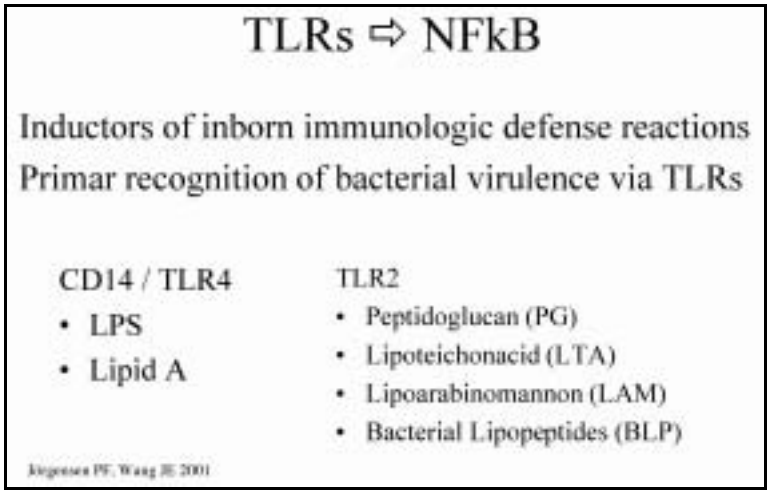

Figure 1: Subtypes of Toll-like receptors involved in the recognition of Gram-negative and Gram-positive bacterial cell walls

LPS stimulation. Furthermore, TLR4 mutations are also associated with hyporesponsiveness in humans. Most importantly, overexpression of TLR4 in embryonic human 293 kidney cells did not automatically conferred responsiveness to LPS suggesting that other additional molecule(s) are required for LPS signalling through TLR4. This molecule was subsequently identified as the secreted protein MD-2 (*Viriyakosol* et al., 2001). Transfection studies revealed that neither MD-2 nor TLR4 alone were able to confer responsiveness to LPS, but co-transfection did. MD-2 is physically associated with the extracellular domain of TLR4 on the cell surface (*Viriyakosol* et al., 2001).

According to Figure 1, the TLR2 recognises *lipoproteins* and *glycolipids* (reviewed by *Akira* et al., 2001; *Anderson* , 2000; and *Heldwein* et al., 2001), so that TLR2 is associated with the recognition of both Gram-negative and Gram-positive bacterial species. Moreover, other ligands included yeast cell walls, mycobacterial-derived lipoarabinomannans, whole mycobacteria, whole Gram-positive bacteria and Peptidoglycan. The TLR5 member was described to interact with flagellin, a 55 kD protein monomer obtained from bacterial flagellae. Flagellin is like the other bacterial cell wall components also a potent inducer of the inflammatory response accompanied by phagocytosis of bacteria, as it rapidly activates the NF-kappaB pathway (*Frendéus* et al., 2001; *Hedlund* et al., 2001) although it seemed to be partially independent of CD14. And last not least the TLR9 has been described to interact mainly with bacterial DNA, containing unmethylated CpG oligonucleotides (*Wagner*, 2001). Recently published data deal with CpG oligonucleotides as one of the most potent inducers of B-cell proliferation or dendritic cell maturation and activation, favouring the use of CpGs as potent adjuvans for vaccines. As TLR9 knockout mice were completely unresponsive to CpGs it seems likely that the TLR9 is essential in the signalling cascade of CpGs.

CD14 IN RECOGNITION OF PAMPs BY IMMUNOCOMPETENT CELLS INDUCED INHIBITORY AS WELL AS STIMULATORY SIGNALS

There is no doubt, that the major constituent of the outer membrane of Gram-negative bacteria plays a crucial role in mediating host response to Gram-negative bacterial infections by stimulating the release of inflammatory mediators, including cytokines from various target cells, such as endothelial cells, macrophages or polymorphonuclear cells. Beside that LPS activation of these mediators is thought to be responsible for the clinical manifestations of septic shock, these pathophysiological events may also play a role in chronic inflammatory disorders. Three cloned families of molecules on the surface of leukocytes are known to bind the Lipid A moiety responsible for the endotoxic activities of the LPS molecule.

These include the CD14, the macrophage scavenger receptors (SR-A family) and the β_2 or CD11/CD18 leukocyte integrins (*Fenton* and *Golenbock*, 1998). LPS binding to CD14 on the surface of immunocompetent cells is enhanced by serum factors, including the acute phase proteins and the LPS binding protein (LBP). LBP is known to catalyse the transfer of LPS monomers within the LPS/LBP-complex to the soluble or the membrane-bound form of CD14 (sCD14, mCD14). This serves to increase the sensitivity of cells towards LPS. These interactions of LBP with the Lipid A moiety of the LPS complex, intensely mediate and control bio-availability and the transport of LPS from biological fluids to responsive cell types and vice versa, opening sometimes under critical ill conditions, the life saving possibility for the organism to control neutralisation and stimulatory properties of this important molecule as best as possible. Details of these molecular interactions have been described extensively elsewhere (*Landmann* et al., 2000; *Malhotra* and *Bird*, 1997; *Mathison* et al., 1992; *Su* et al., 1995; *Tapping* et al., 1998; *Tobias* et al., 1999).

However, since the discovery of CD14 as a central LPS-receptor on mammalian cell types, a lot of subsequent work revealed two additional very remarkable unexpected findings: the first is that CD14 could interact not only with LPS of Gram-negative bacteria but with cell wall components of Gram-positive bacteria too, such as LTAs, lipoproteins, lipo-arabinomannans from *mycobacterium tuberculosis*, manuronic acid polymers from *Pseudomonas species*, soluble peptidoglycans from *S. aureus*, rhamnose-glucose polymers from *Streptococcus mutants* and insoluble cell wall components from several Gram-positive bacterial species (*Gupta* et al., 1996; 1999; *Heumann* et al., 1994).

These experimental findings open the possibility for the immune system to respond either in a more refined, but on the other hand, also in a broader manner to Gram-positive and Gram-negative bacteria as well. In the case of mixed bacterial infections, several different pathogens may invade the organism at the same time and the immune system has to decide how to handle them in the best way to avoid harm to the host. Nevertheless, signalling pathways induced by these molecular interactions may not be necessarily uniform but may include divergent as well as convergent signal transduction pathways, depending not only on the cell type, but also on the activation state of the cell in their natural environment.

In view of the particular composition of Pro-Symbioflor®, representing a

mixture of heat-inactivated Gram-positive and Gram-negative bacteria, together with their soluble and particulate cell wall components, synergistic actions of muramyldipeptides, Lipoteichoic acids (LTAs) together with LPS were shown by several publications, highlighting the dose-dependent effects of these bacterial stimulants concerning cytokine synthesis (*Rabehi* et al., 2001; *Liu* et al., 2001; *Sellati* et al., 1998; *Cauwels* et al., 1997; *Cleveland* et al., 1996; *Yang* et al., 2001).

In this context, the expression of certain cytokine genes, such as Interleukin-12 was reported to be synergistically activated by sequentially acting bacterial stimulants, for example LTAs and LPS, whereas the expression of other cytokine genes remained unaffected (*Cleveland* et al., 1996). Of note, the recent report by *Sugawara* et al. (1999) implicated clearly, that structural different LTAs from different bacterial species possess the capability to deliver to immune cells stimulating (agonistic) as well as inhibitory (antagonistic) signals, which may be of great importance, considering explanations for the immunomodulatory properties of such probiotic bacterial preparations composed of mixed bacterial strains such as Pro-Symbioflor® (described below).

The authors found, that depending on the level of expression of the CD14 antigen of different cell types (human monocytes, human gingival fibroblasts), LTAs were able to antagonise the effects of purified LPS and synthetic Lipid A, particularly, when high concentrations of LTAs were used to elicit cytokine induction (*Sugawara* et al., 1999).

The relevance of lipoproteins, being constituents of both Gram-negative and Gram-positive bacteria as important cytokine modulating PAMPs, was stressed by the report of *Giambartolomei* et al. (1999), showing the induction of pro- as well as anti-inflammatory cytokines (IL-6, IL-10) at the same time by the same pathogenic agent. The spirochete *Borrelia burgdorferi*, whose cell wall definitively lacks lipopolysaccharide but nevertheless proved to be a very strong immunomodulating agent in the cell wall of the heat-killed pathogen. Of utmost importance was the finding by the authors, that it was not the protein moiety itself found to play a role in cytokine induction, but the acylation of the peptide in form of tripalmitoyl-cysteine residues.

Of note, unlipidated outer surface proteins of *Borrelia burgdorferi* were unable to induce cytokines as IL-10 or IL-6 from human monocytes. It is reasonable to assume from these interesting experimental findings, that although LPS constitutes a biologically highly important immunomodulatory bacterial cell wall component, the acylation pattern of lipoproteins in Gram-negative and Gram-positive bacteria should be considered as strong immunomodulatory bacterial components too, which could promote the release by monocytes of a very differential cytokine profile.

Yet, considering the sometimes contrasting results with different bacterial species with regard to the activation of immune cells, it could be assumed, that these were highly influenced by the test system used, the leukocyte population under investigation, the sensitivity of immunocompetent cells, reflected by the density of receptors expressed, which correlates with the maturation state, the presence of serum in the culture and finally, the physicochemical composition of the bacterial cell wall-derived stimulants. All is being equally important for the net outcome of an immunological response. To underline these interesting findings in the literature, concerning the new important immunoregulatory role of the CD14 molecule as an important PAMP for interac-

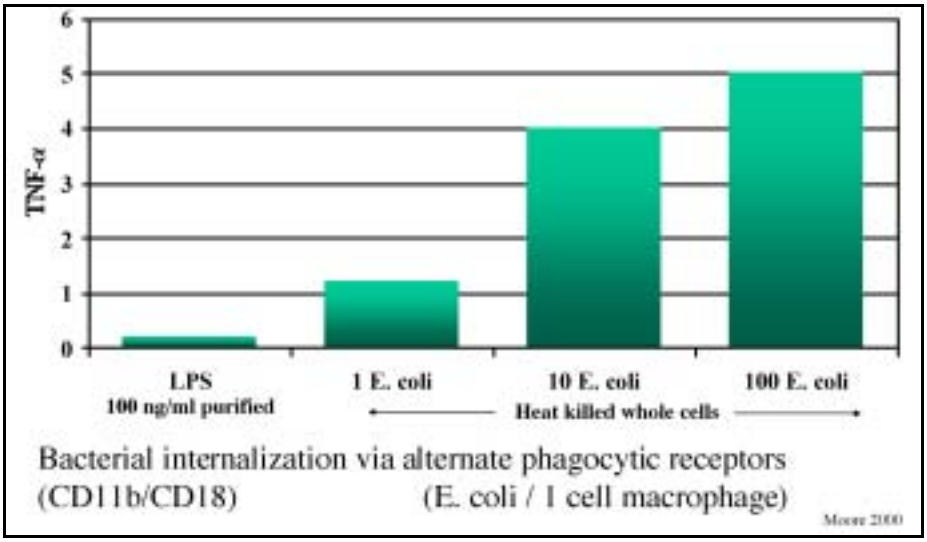

Figure 2: Stimulatory capacity of whole bacterial cells in CD14 knock out mice. It can be seen, that despite the failure of CD14 expression, whole bacterial cells of *E. coli* were able to induce TNF-α production in mouse macrophages derived from these animals, whereas even high concentrations of purified LPS remained ineffective, demonstrating that additional recognition structure in CD14 knock out animals operate together to sense bacteria.

tion with Gram-positive as well as Gram-negative bacteria, the Figures 2, 3, and 4 summarised some of the above mentioned data.

In Figure 2, the response of macrophages from CD14 knockout mice are shown with respect of the recognition of whole cells of *E. coli* compared to purified LPS (*Moore* et al., 2000). It can be seen that purified LPS were unable to induce TNF-α, chosen as a read out. However, despite the absence of CD14 on the cell membrane, murine macrophages produced dose-dependently remarkable amounts of TNF-α upon interaction with heat killed whole *E. coli* cells. This may be a clear hint for other molecules than CD14, to be involved in the recognition of whole bacteria, in comparison to purified LPS. The authors have suggested that the β_2 integrin CD11b/CD18 might have compensated for the loss of function of CD14. Moreover it is worthwhile to mention, that CD11/CD18 integrins do not have such a high affinity than the CD14 molecule for LPS in its monomeric form. Instead β_2 integrins preferentially were reported to interact with larger aggregates, including whole bacteria. The participation of β_2 Integrins in LPS recognition by CD14 knockout animals, was confirmed by the authors (*Moore* et al., 2000). Using inhibition experiments with neutrophil inhibitory factor, which blocked the integrin receptor, thereby diminishing the recognition of whole bacteria. About half of this CD14-independent response could be inhibited by integrin blockade abrogating TNF-α production.

In Figure 3, the influence of different bacterial cell wall components on the level of expression of the CD14 molecule was shown. Interaction of human monocytes with purified bacterial cell wall components derived from Gram-positive bacteria resulted in a marked upregulation of CD14 expression. In contrast, stimulation by LPS of human monocytes downregulate CD14, underlining the complex role of the CD14 an-

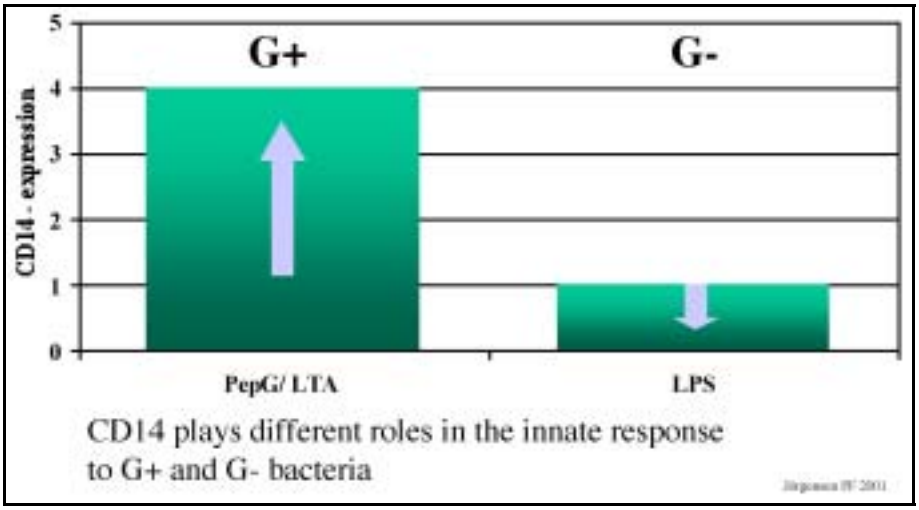

Figure 3: The important immunomodulatory roles of Gram-positive bacterial cell walls in comparison to Gram-negative bacterial cell walls in view of the level of CD14 expression monocytes It can be seen, that upon interaction of human monocytes with purified bacterial cell wall components derived from Gram-positive bacteria CD14 expression was highly increased. In contrast, stimulation by LPS of human monocytes down-regulate CD14, indicating the complex role of the PAMP CD14 in the recognition of different bacterial species.

tigen as an important PAMP operating in recognition of different bacterial species which could result in the generation of different signalling pathways. In Figure 4, the above mentioned synergistic effects of some distinct bacterial cell wall components on cytokine induction were summarised according to *Jörgensen* et al. (2001).

The importance of the experimental design for investigating bacterial immune cell interactions was outlined by *Cauwels* et al. (1997), reporting a dose-dependent cytokine production *ex vivo* with heparinised blood by LPS from *E. coli*, heat-inactivated whole pneumococci or purified cell walls which were all potent inducers of TNF-α, IL-1 and IL-6. The authors could further demonstrate in this system, that the whole blood assay is 1,000 fold more sensitive than the use of a human monocytic cell line THP-1 cells as it responded to as little as 1 ng LPS.

The cytokine response to 5 ng LPS corresponded according to *Cauwels* et al. (1997) to $5x10^5$ *E. coli* and proved to be similar to 1 µg cell wall (= 10^7) bacteria and 10^5 intact heat killed bacteria.

This means that isolated LPS was about 200 times more potent than the purified cell wall of pneumococci for induction of comparable levels of TNF-α, IL-1ß or IL-6, but roughly equipotent to whole pneumococci.

These experimental data underline not only the importance of the chemophysical composition of the bacterial cell wall components to interact with immune cells, but also the use of an appropriate sensitive test system to evaluate the binding specifities of different bacterial cell wall components from Gram-negative and Gram-positive bacteria. Moreover the authors suggested from their results the co-existence of CD14 dependent and CD14 independent stimulation pathways particularly by Gram-positive bacteria.

In addition, anti-CD14 antibodies could inhibit the response of whole

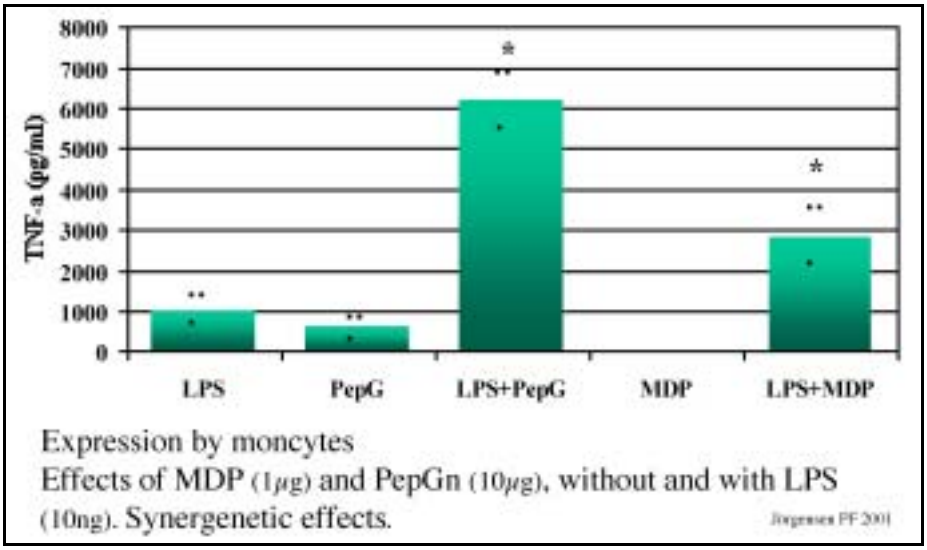

Figure 4: Synergistic effects of different bacterial cell wall compounds of either Gram-positive or Gram-negative bacteria on cytokine production (according to *Jörgensen* et al., 2001). Whereas purified MDP or peptidoglycans in the whole blood assay were only weakly effective for TNF-α production (shown in pg/ml), the simultaneous combination of both stimuli dramatically enhanced TNF-α production 3-fold to 6-fold.

blood cells to purified LPS, but not to stimulation with whole bacterial cells, which could not be abrogated by anti-CD14, indicating, that different mechanisms operating in whole blood for the recognition of whole bacteria compared to isolated cell wall components. This is shown in Figure 5.

DO DIFFERENCES EXIST BETWEEN LIVE VERSUS DEAD BACTERIA WITH REGARD TO THE CYTOKINE PROFILE THEY INDUCED?

The next question to be addressed in the context of the probiotic bacterial preparations manufactured by the SymbioPharm Herborn is to clarify, whether there will be differences between dead and live bacteria in terms of cytokine production.

The situation turned out to be equally complex as with the different PAMPs of Gram-negative and Gram-positive bacteria. Several conflicting reports support the conclusion, that, with regard to active immunisation procedures, living bacteria may be more potent than dead ones (*Cooper* et al., 1997; *Sher* et al., 1991; *Chambers* et al., 1997; *Zhan* and *Cheers*, 1998; *Cheers* and *Zhan*, 1996; *Sander* et al., 1995).

For example, *Cambers* et al. (1997) reported that protection of mice with virulent *Mycobacterium tuberculosis* was strongly associated with the inoculation of live but not dead BCG. Although live and dead bacteria induced comparable cellular responses during the first week after vaccination determined by immunohistochemical analyses of the draining lymph nodes, the typical migration of live parasites into local lymph nodes, which resulted in subsequent recruitment on mononuclear cells, was only seen with live bacteria.

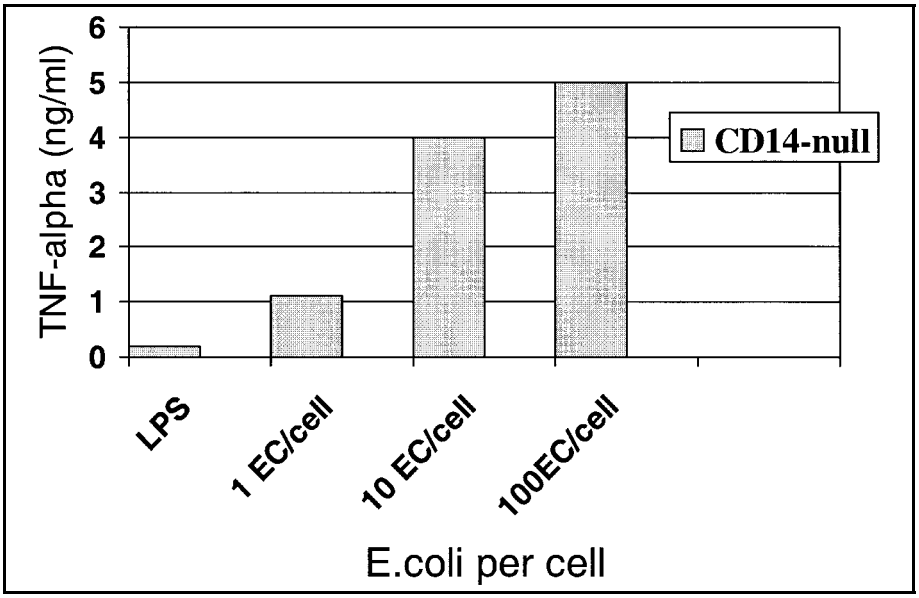

Figure 5: Different effects of anti-CD14 antibody aimed to inhibit signal transduction by bacterial cell wall components dependent of the test system and the physico-chemical composition of the bacterial components used. Whereas anti-CD14 inhibited the response of whole human blood leukocytes against purified LPS, no inhibition was observed with whole pneumococcal cell walls.

It is generally assumed, that the elimination of intracellular bacteria like mycobacteria, brucellae, listeriae or some salmonellae species required a strong Th1 biased immune response associated with a prominent release of IL-12 and interferon-gamma *Cheers* et al. (1996) as killed vaccines tend to be ineffective because they induced a strong Th2 driven response. A protective immune response, elicited by vaccination seemed to be strictly dependent on the generation of interleukin-12, which is a very important cytokine released after vaccination with attenuated but not dead BCG. However under in vitro conditions, IL-12 was reported to be released by live and dead bacteria (*Cheers* et al., 1996) so that the situation in view of IL-12 remains complex, because dead bacteria or their cell wall products could be either effective (*Mahon* et al., 1996) or not (*Zhan* and *Cheers*, 1995; 1998).

Further, not only species differences can play a prominent role in these observations, but also the structure of the bacterial cells too (*Mahon* et al., 1996; *Sander* et al., 1995): Using a murine respiratory infection model, the authors demonstrated, that infection with *Bordetella pertussis* (Gram-negative rod) or immunisation with a whole cell pertussis vaccine (e.g. the intact cell membrane with the natural configuration of lipopeptides, LPS and proteins) induced a strong antigen-specific Th1 dominated response. In contrast, immunisation with an a-cellular vaccine consisting of *B. pertussis* components or filamentous haemagglutinins generated a Th2 response, which was associated with delayed bacterial clearance from the lungs of the animals. However, addition of Interleukin-12 to the a-celluar vaccine strongly increased the Th1 response.

This may be a good example, how vaccine structure could influences the

Table 2: Characterisation of binding specifities of the AutoColiVaccine (ACV) compared to a commercial LPS preparation, a synthetic lipopeptide (PAM_3CSK_4), and a natural LPS (*S. friedenau*) using CHO-cells as test system

	CHO wildtype		CHO/CD14		CHO/CD14/TLR2	
TLR4	Natively expressed		Natively expressed		Natively expressed	
MD-2	Natively expressed		Natively expressed		Natively expressed	
CD14	-		Rec. Expressed		Rec. Expressed	
TLR2	-		-		Rec. Expressed	
Synthetic lipopeptide (PAM_3CSK_4)	-		-		>100 ng	
IL-1β control	↑		↑		↑	
AutoColiVaccine	-	-	+	>100 ng	+	>1000 ng
S. friedenau LPS	+	>100 ng	+	>1 ng	+	>10 ng
Commercial LPS (055:B5, *E. coli*)	+	>10 ng	+	>1 ng	+	>10 ng

Due to the failure to express TLR2, wild type CHO cells did not react with synthetic lipoproteins and only to high concentrations of LPS. When CD14 was additionally expressed, cells became responsive to the ACV at high concentration and, as could be expected the sensitivity to LPS increased due to CD14. Notably, CHO cell sensitivity to the ACV seemed to be increased in CD14 positive cells but this did not change after TLR2 expression. The result, that commercial LPS showed a rise in sensitivity after TLR2 expression could indicate for the presence of lipoproteins in the LPS, as the activity of *S. friedenau* LPS was comparable with that of commercial LPS regarding the activation of the CHO cells. Thus, the failure of the ACV to activate wild Type CHO cells might indicate a partially independent TLR4/MD-2 activation pathway triggered by the ACV.

outcome of an immune response. In the human system *Sander* et al. (1995) evaluated cytokine production of human leukocytes at the single cell level after stimulation with live attenuated *Mycobacteria bovis* BCG. It was shown, that although major cytokines like IL-12 and Interferon-γ were sequentially produced, later on a Th2 polarised lymphocyte response occurred, reflected by the appearance of IL-4, IL-5 and IL-10 intracellularly. This strongly indicates that in humans a mixed T-helper cell profile operating together for the successful elimination of *M. bovis*.

RECOGNITION OF A *E. COLI* PREPARATION IS PARTIALLY INDEPENDENT FROM THE CLASSICAL LPS RECEPTOR IN MAMMALS TLR-4/MD-2

Another important bacterial derived immunomodulator manufactured at Herborn is the AutoColiVaccine (ACV). This bacterial preparation derived from the stool flora of patients, resembled in its chemical composition in many parts the structure of Lipid A, but has different acylation patterns and a low endotoxic activity. Nevertheless it can be assumed, that commercially obtained LPS compared to the ACV will use possibly known receptors involved in the LPS-signalling pathway, such as the TLR-4-MD2/CD14 complex.

Together with an experimental group at Borstel it was investigated, which type of the known classical LPS receptors might be recognised by the ACV

Table 3: Characterisation of binding specifities of the AutoColiVaccine (ACV) compared to a commercial LPS preparation, a synthetic lipopeptide (PAM_3CSK_4) and a natural LPS (*S. friedenau*) using HEK293-fibroblasts as test system

	HEK293 wildtype		HEK293/TLR2		HEK 293/ TLR4/MD2		HEK 293/ CD14/TLR4/MD-2	
TLR4	-		-		Rec. Expressed		Rec. Expressed	
MD-2	-		-		Rec. Expressed		Rec. Expressed	
CD14	-		-		-		Rec. Expressed	
TLR2	-		Rec. Expressed		-		-	
Synthetic lipopeptide (PAM_3CSK_4)	-		>1000 ng		-		-	
TNF-_ control	↑		↑		↑		↑	
AutoColiVaccine	+	>1000 ng	+	>100 ng	+	>1000 ng	+	>10 ng
S. friedenau LPS	-	-	-	-	+	>100 ng	+	0,1 ng
Commercial LPS (055:B5, *E. coli*)	-	-	+	>100 ng	+	>10 ng	+	0,1 ng

Despite wild type HEK 293 fibroblasts did not express any of the classical LPS recognition structures, the ACV was able to stimulate the cells for increased IL-8 production. Most importantly, the expression of TLR2 by HEK 293 cells increased the sensitivity from 1000 to 100 ng and this was further enhanced after expression of CD14 (1000 to 10 ng). It is therefore reasonable to assume that the Lipid A analogue ACV contains additional molecules with specificity for TLR2 and CD14 but with a lower binding specificity for TLR4/MD2 compared to commercial LPS. This assumption may be also confirmed by the observation that HEK 293 wild type cells and HEK 293 TLR4/MD2 cells showed nearly the same reactivity against ACV with 1000 ng being necessary for cellular activation.

using binding studies with transfected cell lines. The latter represent a useful molecular tool to solve this question, because they harbour the cloned human TLR proteins together with their necessary adapter protein MD-2. Kirschning et al. (1998) and Yang et al. (1998) recently described the test system. The authors used the transfection of the human 293 embryonic kidney fibroblast cell line (HEK 293), with cloned human TLR-proteins and their necessary co-factors. As a parameter of HEK 293 activation upon challenge with the bacterial preparations, the release of IL-8 was determined after stimulation either with a commercial LPS preparation (*E. coli* 055:B5), a natural LPS from *S. friedenau* or the Lipid A analogue named ACV.

A second test system included Chinese hamster ovary cells (CHO-cells) co-transfected with a reporter gene to investigate the activation potential of LPS in comparison with ACV with respect to the expression of the CD25 surface marker. In this CHO cell line, the expression of CD25 was induced by a minimal structure of the NF-kB promotor from the selectin gene as described previously (*Yang* et al., 1998). The Tables 2 and 3 summarised the characteristics of the cell lines used together with the results.

The results from Tables 2 and 3 revealed some unexpected findings, which support evidence, that the receptors involved in the recognition of the ACV preparation seem to be partially independent from the classical TLR-4/MD-2 receptor system for the following reasons: In wild type (WT) CHO-cells containing the full repertoire of TLR-4/MD-2 the AutoColiVaccine

did not induce a marked CD25 expression compared to commercially obtained LPS.

As WT CHO cells did not express the TLRs, they failed to react with the synthetic lipopeptide used as a control (PAM_3CSK_4), but were fully reactive to TNF-α. Additionally, despite HEK 293 WT cells were devoid of all classical LPS receptors, they responded to high concentrations of the ACV whereas the positive control LPS could not activate HEK 293 fibroblasts. And what may be of utmost importance is, that the introduction of the TLR-2 in CHO cells rendered them more responsive to the ACV.

The same was seen with the HEK 293 fibroblasts: Whereas WT HEK 293 cells could react to high concentrations of the ACV, but not to natural *S. friedenau* LPS or commercial LPS, the co-transfection of HEK 293 cells with TLR2 and CD14 seem to increase the sensitivity of the cells to ACV.

It may be concluded, that the ACV contained a compound with TLR2 specificity and these results do fit very well the above cited publications of *Giambartolomei* et al. (1999) and *Kreutz* et al. (1997) demonstrating the importance of the lipoproteins/lipopeptides in cytokine induction with the pattern of acylation of a synthetic PAM_3CSK_4 being more relevant for the capacity of a lipopeptide to modify cytokine synthesis.

Accordingly, the results by *Bainbridge* et al. (2001) implicated that although LPS (and ACV as an *E. coli* derived bacterial preparation) seem to be highly conserved in its core structure among different bacterial species, some subtypes of LPS even do not bind to the classical TLR4 receptor. Instead for example, the LPS from *Porphyromonas gingivalis* was reported to interact with TLR2 in stable transfected CHO cells. Furthermore *Porphyromonas gingivalis* LPS was able to submit stimulating signals to monocytes, while at the same time inhibiting endothelial cell activation by interfering with the p38MAP kinase pathway (*Bainbridge* et al., 2001).

THE PROBIOTIC PREPARATION PRO-SYMBIOFLOR®, COMPOSED OF HEAT-INACTIVATED *E. COLI* AND *ENTEROCOCCUS FAECALIS* INDUCED A CYTOKINE PROFILE CHARACTERISTIC FOR Th1 T-HELPER CELLS

The experimental results shown in Figures 6 to 9 contrasts some recent reports by *Hessle* et al., (2000) and *Haller* et al. (1999). Not only were differences between live and dead bacteria reported with respect to cytokine production, but growth-related differences and strain specific differences (*Hessle* et al., 2000) were also shown. For example *Hessle* et al. (2000) examined the synthesis of IL-10 and IL-12 by human blood monocytes after challenge with UV killed bacteria of 7 Gram-negative strains and 7 Gram-positive strains (commensals and pathogens). According to these experiments Gram-positive bacteria induced a predominant IL-12 production while Gram-negative bacteria enhanced preferentially the synthesis of IL-10. The lower capacity of Gram-negative bacteria to induce IL-12 was independent of IL-10 itself, as blocking IL-10 with antibodies did not resulted in an enhancement of IL-12 (*Haller* et al., 1999; *Hessle* et al., 2000).

In view of these findings, the series of experiments presented in Figures 6 to 9 argued more for a qualitative difference of some bacterial preparations rather than a species-origin determined

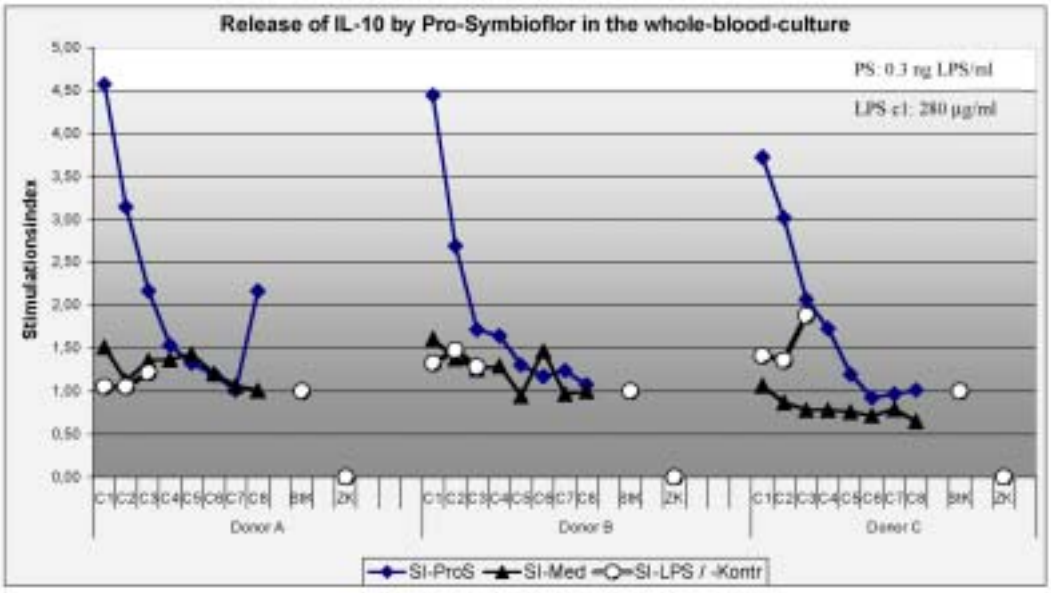

Figure 6: The effect of Pro-Symbioflor® on the LPS induced production of IL-10. Whole blood cell cultures were stimulated with sub-optimal concentration of opsonised zymosan together with eight different concentrations (C1–C8) of Pro-Symbioflor®. Medium-treated cells served to evaluate the background cytokine production and the positive control included LPS (*E. coli* 055:B5, Sigma). IL-10 was determined after 24h of incubation with ELISA. It can be seen that Pro-Symbioflor® dose-dependently stimulated the synthesis of IL-10 to a great extent, which was evident with leukocytes from all three donors tested.

difference. One probiotic preparation manufactured by SymbioPharm composed of live *Enterococcus faecalis* bacteria (Symbioflor 1®) was recently reported in a double-blind, randomised clinical study to significantly reduce number and severity of relapses in patients suffering from chronic recurrent bronchitis. An important medical disease entity thought to be associated with recurrent infections of the respiratory tract.

The latter may be a consequence of a suppressed immune system either due to inherited or to an adapted capacity of the immune system, to mount an appropriate antibacterial defence to effectively kill bacteria *Habermann* et al. (2001). To address the question, whether this clinically relevant effect could have been correlated with an immunomodulating effect of *Enterococcus faecalis* or *E. coli*, another probiotic bacterial preparation by SymbioPharm was investigated for immunomodulatory properties called Pro-Symbiofor®, using the whole blood cell culture system with blood from three healthy donors. The bacterial strains in Pro-Symbioflor® were of human origin and non-pathogenic, manufactured as a heat-inactivated preparation of cells and bacterial cell wall components with different concentrations of LPS and LTAs. The LPS content of Pro-Symbioflor® is in the range of about 0,3 ng/ml.

In these experiments, whole blood cultures were treated with eight different concentrations of Pro-Symbioflor® and sub-optimal concentrations of co-stimuli, able to activate specifically the synthesis of monokines (opsonises zymosan, IL-6, IL-10, IL-12) and lymphokines (anti CD3 plus anti-CD28, IL-4, IL-5 and IFN-γ) was used. The background control included unstimulated

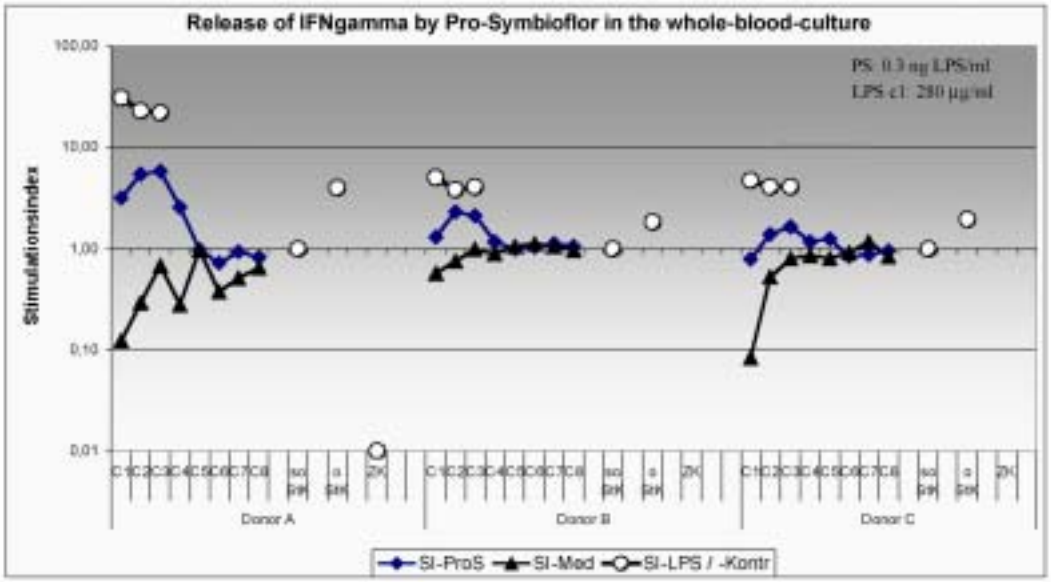

Figure 7: The effect of Pro-Symbioflor® on the LPS induced production of IFN-γ. As Co-stimulus a combination of anti-CD28 plus anti-CD3 antibodies was used. Pro-Symbioflor® dose-dependently induced great amounts of IFN-γ with leukocytes from all three donors tested.

cells (ZK), and the stimulus control included *E. coli* serotype 055:B5 obtained from Sigma. The results of these experiments were illustrated in Figures 6 to 9.

The important anti-inflammatory cytokine IL-10 was dose-dependently increased in whole blood cultures of all three donors (Figure 6). The synthesis of IL-12 (Figure 7) showed more inter-individual variations and seemed to be increased within the concentration ranges C2 to C4. With whole blood cells from two donors, IL-12 was inhibited at the highest concentration of Pro-Symbioflor®. The important pro-inflammatory and immunoregulatory cytokine Interferon-γ proved to be consistently stimulated with whole blood cultures from all three donors (Figure 8) and the cytokines IL-5 (Figure 9) and IL-4 (data not shown) were profoundly suppressed over a wide concentration range. So far, although these are first results on immunomodulatory properties of Pro-Symbioflor®, these results were so consistent that they should justify the conclusion to suggest a profound influence of Pro-Symbioflor® on the activation of Th1-helper cells.

Thus, in the context of the previously described experimental findings reported by *Hessle* et al. (2000) or *Haller* et al. (1999), these results obviously do not encounter the immunopharmacological profile of Pro-Symbioflor® being a mixed heat-killed preparation of *Enterococcus faecalis* and *E. coli*. This „medical" probiotic was able to stimulate both cytokines at a high extent.

A reasonable explanation for these apparent differences may be the use of different test systems, the former involved separated cells, where the data reported herein were generated with the whole blood cell cultures. The latter resembles the *in vivo* conditions as close as possible, because the bacteria interact with immune cells in their natural environment allowing the full spectrum of receptor-ligand interactions as close as possible to the situation *in vivo*.

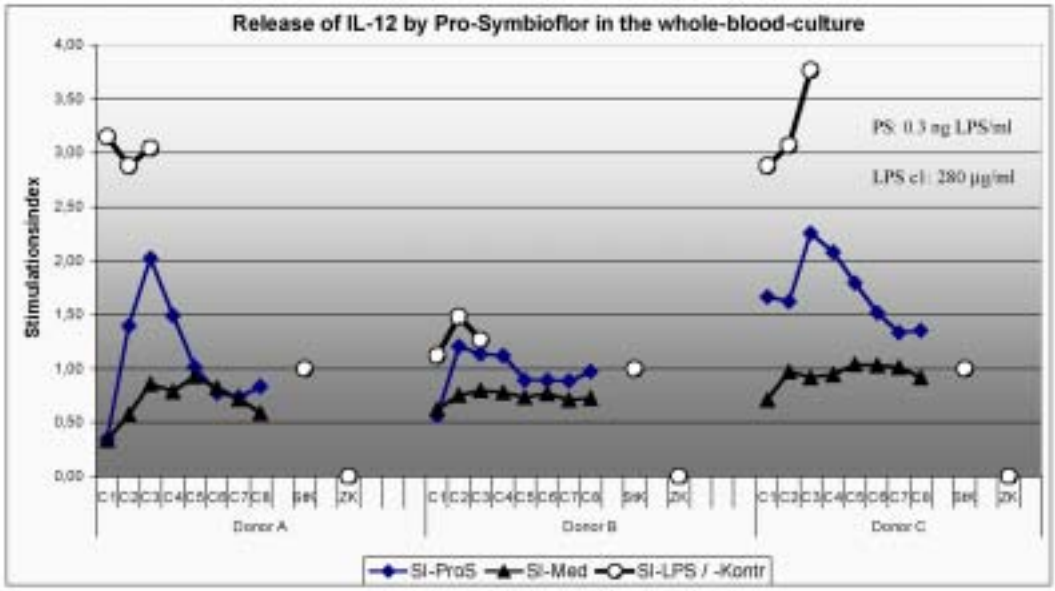

Figure 8: Pro-Symbioflor® profoundly affects the co-stimulated release of IL-12 (opsonised zymosan as co-activator, measurement after 24 h with ELISA) in whole blood cell cultures from three different donors. Although IL-12 production varied between donors in response to different concentrations of Pro-Symbioflor®, this important cytokine involved in the enhancement of cell-mediated immunity was profoundly modulated.

PRO-SYMBIOFLOR® FOR THE TREATMENT OF TYPE 1 ALLERGIC DISEASES?

Assumed, that in atopic allergy a predominant Th2 response dominate, it is tempting to speculate, that a bacterial preparation with such a remarkable immunomodulatory activity profile like Pro-Symbioflor® may be an ideal candidate for therapy of type 1 allergic diseases, characterised by an overproduction of IgE. Generally, bifidobacteria and lactobacilli as classical representatives of probiotic bacterial strains, were investigated extensively in the past in numerous experimental studies for their immunomodulatory properties and in some small controlled clinical trials for their „anti-allergic“ and anti-infective potential (reviewed by *Vaughan* et al., 1999; *Gill* and *Rutherfurs*, 2001; *German* et al., 1999; *de Roos* and *Katan*, 2000; *Gismondo* et al., 1999; *Sanders*, 2000; *McNaught* and *MacFie*, 2001; *Cross* and *Gill*, 2001; and *Cross* et al., 2001).

As an example, the list of probiotic bacteria-mediated effects include antimicrobial activity, colonisation resistance, antigen-non-specific immune system activation by cytokine induction and stimulation of phagocytosis of peripheral blood leukocytes, stimulation of secretory IgA production, anti-mutagenic effects, anti-genotoxic effects and influence on enzyme activity (*Sanders*, 2000). Unfortunately most of these health claims were until now not sufficiently supported by randomised, double-blind large controlled clinical studies.

The best documented clinical application for the use of probiotic bacteria seem to be preventing or shortening episodes of acute diarrhoea and gastro-

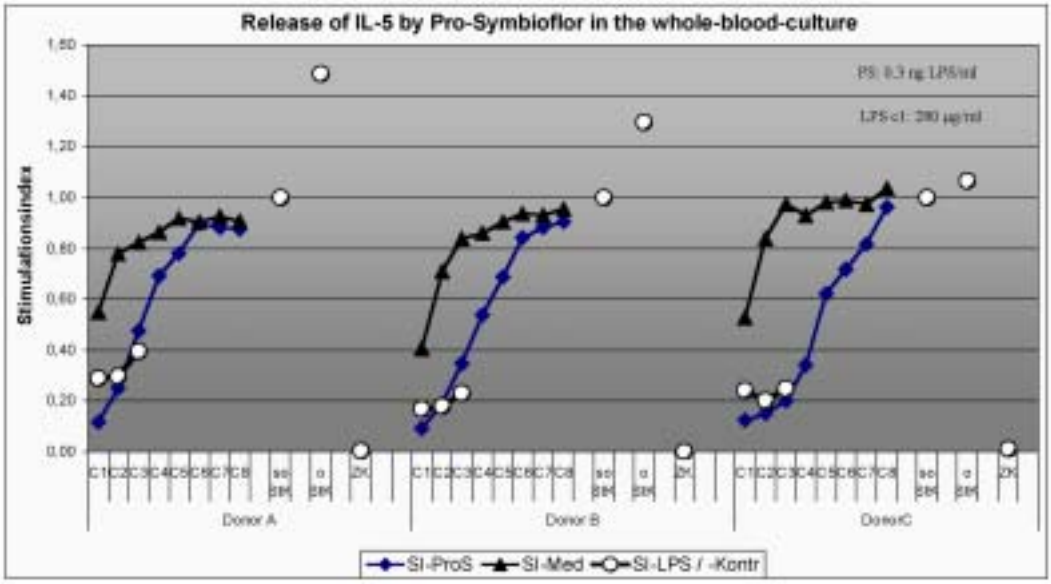

Figure 9: The effect of Pro-Symbioflor® on the co-stimulated production of IL-5. As Co-stimulus a combination of anti CD28 plus anti CD3 antibodies was used. Pro-Symbioflor® dose-dependently inhibited the release of IL-5 with leukocytes from all three donors tested over a wide concentration range. Data for IL-4 looked quite similar and were not presented here.

enteritis in infants and adults (*McNaught* et al., 2001; *Elmer* et al. 2001). Serious concerns may raise in view of the rising tendency of the food industry to genetically „design" food-related bacteria, specifically lactic acid bacteria, with new metabolic and functional properties not originally found in the parent strain (*Kuipers* et al., 2000; *Saarela* et al., 2000; *van der Werf* et al., 2001; *Dunne* et al., 2001) aimed to improve functional properties of the strains.

Presumably one reason for these extensive research activities may be the perhaps questionable assumption, that many of the claimed health promoting effects of probiotic bacteria could only be achieved with sufficient high numbers of bacteria (about 10^9 to 10^{10} per day), which should colonise the human gastrointestinal tract. Despite, that the survival of probiotic bacteria during passage through the human gut, when administered in fermented milk products, has been investigated intensely in recent years the survival rates have been estimated only to be about 20% - 40% for selected strains (*Bezkorovainy* et al., 2001).

Thus, although it is generally proposed that a probiotic bacteria mediated effect can be better achieved if the germs would adhere to the intestinal mucosa, reality has shown, that most of the bacteria passed into faeces without having attached to the mucosa or without having multiplied. Consequently, to get a continuous health effect by an exogenously introduced probiotic strain, the bacteria were recommended to be ingested by an individual continually.

However little is known, what defined molecular and functional properties an „ideal" probiotic bacterial strain must have to exert the above mentioned health effects. Simply, because under in vivo conditions the interactions of the introduced probiotic strain with the individual indigenous microflora of each

individual, might be extremely complex and influenced by several additional factors: Among them are the nutrition behaviour of an individual, the actual composition of the microbiota, external factors like stress and use of antibiotics or other pharmaceuticals. Therefore the important question arise, whether dead probiotic bacterial strains, if endowed with a suitable immunopharmacological profile, might be more safe for use in human in the context of immunomodulation, instead of ingesting large numbers of genetically modified strains, whose long term behaviour *in vivo* is presently largely unknown.

With regard to the immunopathogenesis of atopic allergy, there is compelling evidence from a vast amount of hundreds of published papers during the past 10 years, that established type 1 allergy is dominated by a preferential activation of Th2 cells, responsible for the initiation of atopic dermatitis or allergic asthma (*Rothe* and *Grant-Kels*, 1996; *Kapp*, 1995; *Vercelli*, 1995; *Masman* and *Sad*, 1996; *Romagnani*, 1997; *Leung*, 2000; 2001; *O'Garra* and *Murphy*, 1996). However, despite this intense research, the aetiology of e.g. atopic dermatitis, characterised as a chronic, highly purulent inflammatory skin disease with elevated levels of total and antigen-specific IgE and tissue eosinophilia, is largely unknown. Moreover, the cytokine profiles measured in biopsy specimens of atopic donors expressed high variability depending on the disease state (*Hamid* et al., 1994; 1996; *Thepen* et al., 1996; *Langeveld-Wildschut* et al., 2000; *de Vries* et al., 1997).

Together with other underlying factors, such as severe skin colonisation with super-antigenic exotoxin-producing *Staphylococcus aureus* (*Bunikowski* et al., 2000) or a chemokine triggered non-specific homing of specific lymphocytes into inflamed skin lesions (*Nickel* et al., 2000), contrasting results pointing to an IL-4 independent rise of IgE (*van der Pouw-Kraan* et al., 1994; *Virtanen* et al., 1995).

IL-4 is considered to be the main cytokine involved in the switch of antibody production by B-lymphocytes from IgG to IgE. All these reports strongly suggest that the immunopathogenesis of atopic eczema is not simply a mere shift towards a CD4-Th2 dominated immune response. This was recently confirmed by observations of a participation of CD8-positive T-cells in allergic responses (*Nakazawa* et al., 1997). Nevertheless, immunotherapeutic strategies used in the past in form of active desensitisation regimens using increasing doses of allergens implied, that an induction of the change in cytokine profiles which correlated with symptom improvement (*Hamid* et al., 1997; *Movérare* et al., 2000; *Secrist* et al., 1993) may be a reasonable strategy for the treatment of IgE mediated hypersensitivities.

A major breakthrough in the understanding of the etiopathogenesis of type 1 allergy which showed a dramatic rise during the past years, affecting approximately 30% of children during childhood (*Endres* et al., 2000), has come from the elucidation of the role of the gastrointestinal flora. The early manifestation of the disease soon after birth took place in a time span of about 6 months post partum, which decides whether atopic allergy will develop or not (*Bjorksten* et al., 2001).

A first clue came from the important observations of *Björksten* et al. (1999) about differing colonisations patterns of allergic Estonian and Swedish 2-year-old children. In the following years *He* et al. (2001) and *Ouwehand* et al. (1999) confirmed differences in the adhesion properties of the Gram-positive gut flora between healthy and allergic infants. Faecal *Bifidobacteriaceae* from

healthy infants expressed markedly higher adhesive properties than those of allergic infants. In addition, *Böttcher* et al. (2000) were able to demonstrate microflora-associated characteristics in faeces from allergic and non-allergic infants, by means of the measurement of the faecal concentrations of eight different short chain fatty acids.

They showed that allergic infants have lower levels of propionic, i-butyric, butyric, i-valeric and valeric acid and higher levels of i-caprioc acid. The latter short chain fatty acid has been associated with the presence of *Clostridium difficile*. Subsequently several experimental studies pointed to a possibly hopeful concept considering probiotic bacteria as a useful treatment of severe type 1 allergies such as atopic eczema (*Isolauri* et al., 1990; 2000; *Isolauri*, 1997; *Majamaa* and *Isolauri*, 1996; 1997; *Majamaa* et al., 1996; *Pessi* et al., 1998).

This intense research trend continued during in the following years and resulted in the appearance of first small clinical trials for the treatment of allergic infants by the oral application of Gram-positive probiotics (*Isolauri* et al., 2001; *Kalliomäki* et al., 2001; *Pelto* et al., 1998; *Pessi* et al., 2000; *Salminen* et al., 1995; 1996; *Simmering* and *Blaut*, 2001). In the light of these encouraging findings and the intriguing immunopathological profile of atopic diseases, it seems reasonable to assume, that a probiotic bacterial preparation such as Pro-Symbioflor® might help to improve symptoms associated with atopic eczema.

This the more, as Pro-Symbioflor® acts not by blocking only a distinct cytokine but instead activates those cytokine peptides responsible for the polarisation of T-helper lymphocytes into the Th1 direction while down-regulating those responsible for Th2 lymphocyte activation. This represents a real immunomodulatory capacity of Pro-Symbioflor® raising the question of the therapeutic value of such a „medical" probiotic for the treatment of type 1 allergy which will be the subject of further intense research in the field of probiotics in form of clinical trials.

To summarise the immunomodulatory properties of probiotic bacteria such as *E. coli* and *Enterococcus faecalis* and a mixture of them turned out to be highly complex, which may be the result of the initiation of different activatory and inhibitory pathways in immune cells. The outcome of such an interaction may not only be determined by the pathogenic class e.g. Gram-positive or Gram-negative bacteria, but much more by the sequential and spatial interactions of the PAMPs with their respective ligands, which is further modulated by the environmental cytokine milieu and the activation state of a given target cell population in a tissue.

These molecular interactions may induce, among other at present unknown effects of probiotics under in vivo situations, the elaboration of different cytokine profiles subsequently contributing to prominent changes in the activation of the innate as well as the adaptive immune system.

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