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THE AUTOVACCINE: LINK BETWEEN INNATE AND ADAPTIVE IMMUNITY

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SUMMARY

Autovaccines are prepared in a special well-standardised manufacturing procedure by the SymbioVaccin GmbH at Herborn from rough non-pathogenic autologous *E. coli*, which were derived from the patients own faecal flora. Autovaccines, being an integral component of the Microbiological Therapy are being used to treat chronic non-specific inflammatory disorders and are applied to patients mainly parenterally (i.c. or s.c.). Common molecular and cellular pathways by which bacterial cell wall components of different Gram-positive and Gram-negative pathogens interact with immunocompetent cell types are summarised, although defined „receptor" recognising structural elements of the autovaccine are unknown at present. However, the recently discovered family of the so called Toll-like receptor proteins and the elucidation of common signalling pathways shared by Toll-like receptors and important cytokine proteins makes it likely that the autovaccines use some of them to exert their immunoregulatory functions. Based on recent experimental work with a whole blood culture system, demonstrating precise immunomodulatory effects of the autovaccine, some cardinal functions of anti-inflammatory and pro-inflammatory cytokines like IL-4, IL-10 or interferon-γ were briefly outlined. These examples clearly underline the close connection of the cytokines affected by the autovaccine with the innate as well as the adaptive immune system, for the cells producing them constituted lymphoid cell types (T-cells, macrophages, B-lymphocytes) as well as non-lymphoid cells (mast cells, basophils, fibroblasts, Langerhans cells, dendritic cells, endothelial cells) orchestrating the immune response to viral and bacterial invaders.

The intriguing reconstitution production by auotovaccines of the interferon-γ in lymphocytes taken from an individual unable to respond to classical T-cell receptor mediated stimulation, is commented. Finally, an immunoregulatory model is proposed, by which the autovaccine, as a Lipid A analogue but avoid of any toxic properties associated with the Lipid A, may influence the innate and adaptive immune response in a remarkable way. This raises justified hope for the future to use autovaccines as a general anti-inflammatory and immunomodulating drug for the treatment of non-specific inflammation often accompanying dysregulated immune responses in humans with diseases like autoimmunity, cancer or IgE-mediated allergies.

THE TOLL LIKE RECEPTOR FAMILY: AN IMPORTANT LINK BETWEEN INNATE AND ADAPTIVE IMMUNITY

Toll-like receptors recently described in mammals are related to the *Drosophila* toll proteins and function to help the innate immune system recognising pathogen-associated molecular patterns (PAMS), that are expressed on infectious agents, but not on host cells. The search for homology of *Drosophila* Toll proteins with those in the mammalian system led to the discovery of the human Toll proteins by *Medhitov* (1997) and *Janeway* and *Medzhitov* (1999). Subsequently, different subtypes of TLRs were characterised in more detail, illustrated by Figure 1**.**

Based on the similarity in the cytoplasmic portions (designated the Toll-IL-1R or TIR domain), TLRs are related to the IL-1 receptor. However, the extracellular portions of the different TLR subtypes differ in length of their extracellular domains, typically composed of so-called leucine rich repeats (LRRs). In contrast, the IL-1R contains three immunoglobulin-like domains. The genes of these proteins are dispersed throughout the genome and despite their sometimes overlapping recognition of both gram-positive and gram-negative bacterial cell walls, the sequence homologies between different TLRs are not extensive: for example the extracellular domains of human TLR4 and human TLR2 are only 24% identical (*Akira* et al., 2001; *Beutler*, 2000; *Anderson*, 2000). Searching human and mouse databases revealed at present about 10 members of the TLR family, expressed in a cell type specific manner and presumably differentially regulated under conditions of inflammation by the environmental cytokine milieu (*Cario* and *Podolsky*, 2000; *Cario* et al., 2000).

As shown in Figure 1, TLRs are triggered by different agonistic stimuli which may induce after binding different signal transduction pathways (*Rabehi* et al., 2001; *Akira* et al., 2001).

For example agonists for TLR-4 include Gram-negative bacterial cell wall components, heat shock proteins, LPS, the plant product Taxol, viral proteins and flagella-derived proteins. In contrast, the subtype TLR2 was described to react predominantly with bacterial cell wall compounds specific for Gram-positive bacteria such as muramylpeptides or lipoteichic acids. Additionally, bacterial ligands present in both Gram-negative and Gram-positive bacterial cell walls such as lipoproteins and glycolipids bind to TLR2 as well as mannosylated phosphatidyl-Inositol and different lipoproteins from *Prophyromonas*, *Leptospira* and *Borrelia* species (*Akira* et al., 2001). Furthermore TLR2 recognises whole bacteria, yeast cell walls and peptidoglycans. The findings that TLR-2 could recognise some special LPS-subtypes expressed by *Leptospira* spp. and *Prophyromonas gingivalis* underline that subtle differences in the lipid-A structure might use different TLRs. Concerning the function of TLR6 (not shown in Figure 1) this subtype was reported to synergise with TLR2 in the recognition of Gram-positive bacteria, because cytoplasmic portions of the TLR2 functionally pairs with that of TLR1 or TLR6 stimulating cytokine production (*Akira* et al., 2001).

TLR5 recognises flagellin, a 55 kDa monomer protein derived from bacterial flagella. Thus, flagellin constitutes another important virulence factor of both Gram-positive and Gram-negative cell walls. Experimental evidence that TLR5 is involved in the recognition of flagellin came from expression of the flagellin gene into non-flagellated *E. coli* demon

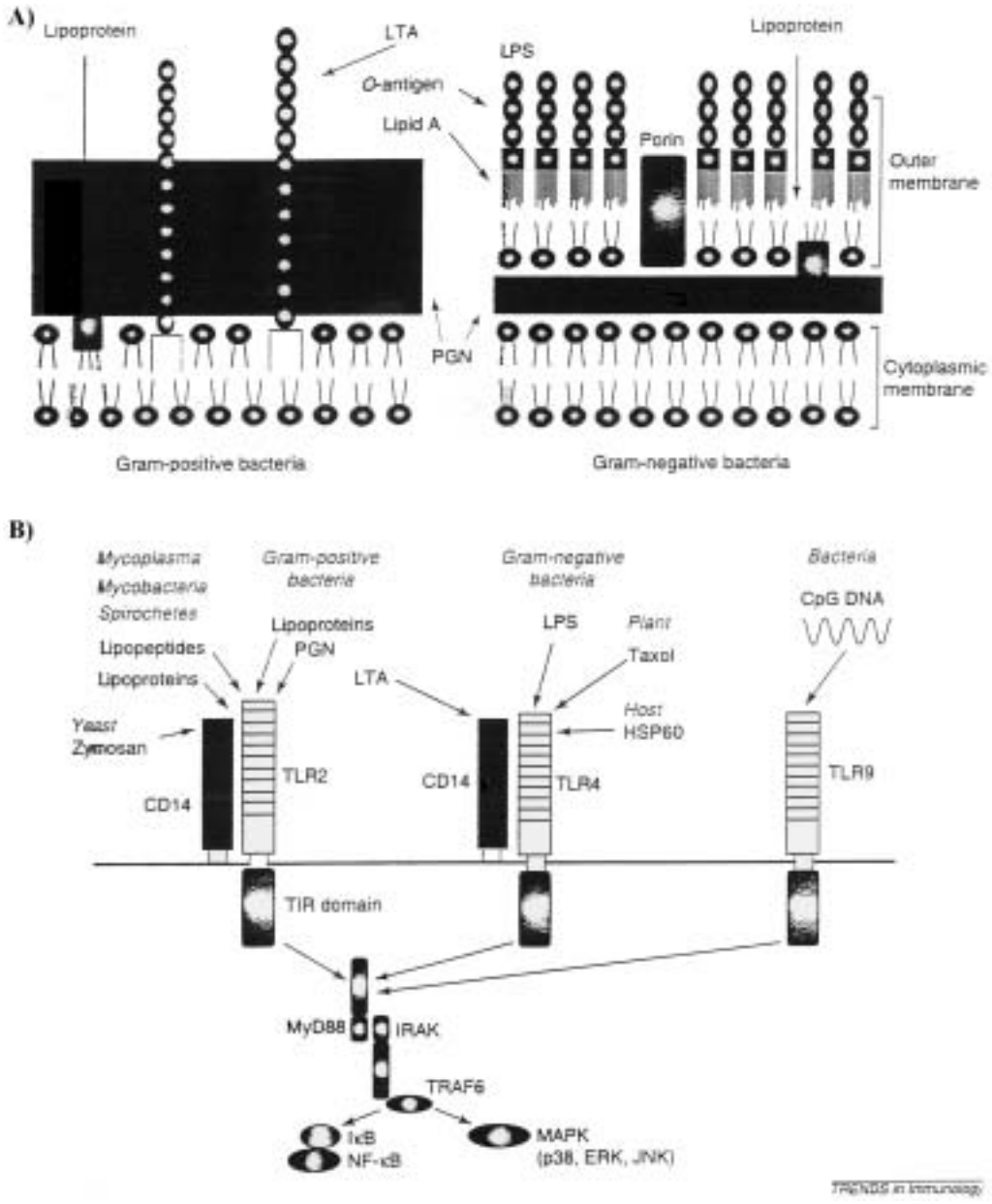

Figure 1: Subtypes of Toll like receptors.

strating that these bacteria were able to activate the TLR5, whereas deletion of the flagellin genes *from S. typhimurium* abrogated TLR- stimulating activity. At least TLR9 bind to bacterial-derived CpG oligonucleotides (*Wagner*, 2001). Again, this has been demonstrated by genetic engineered mice lacking the TLR9 gene, being completely unresponsive to stimulation by bacterial derived CpG oligonucleotides. In contrast, both null mutations for either TLR2 or TLR4 did not affect the ability of CpG oligonucleotides to enhance

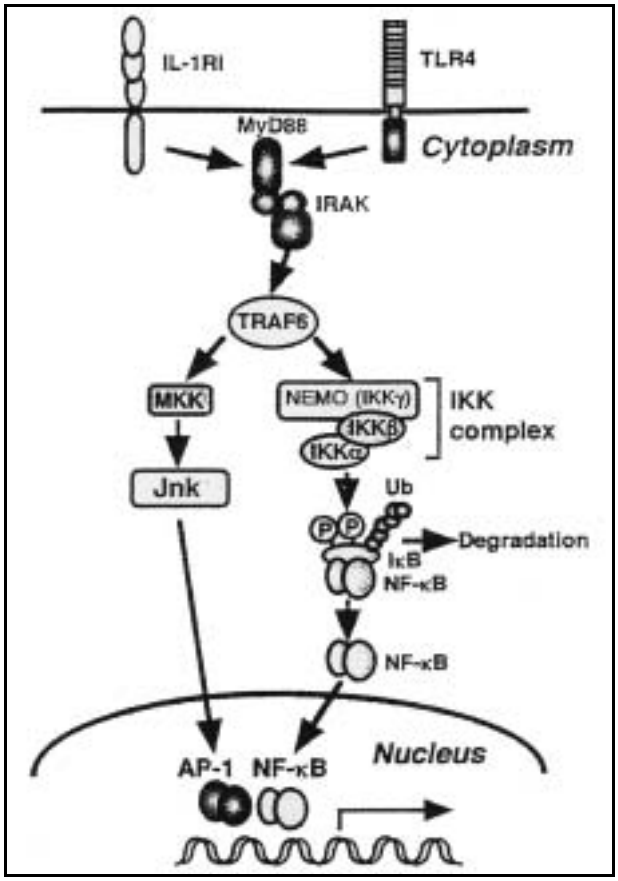

Figure 2: Signalling pathways mediated by Toll-like Receptor 4 in comparison to the IL-1R.

cytokine production by several cell types (B-cells, dendritic cells, monocytes) making it likely that this TLR subtype functions mainly as a sensor for bacterial DNA or DNA fragments (*Akira* et al., 2001).

INTRACELLULAR PATHWAYS INVOLVED IN SIGNALLING BY TLRs MAY BE DIVERGENT AS WELL AS CONVERGENT

Toll like receptors as type 1 transmembrane proteins are evolutionarily conserved between insects and humans, representing therefore the long searched „missing link" in the generation from an initially antigen non-specific immune response to the development of highly specific T-cell and B-cell mediated effector memory responses. The endogenous signalling pathways used by agonist-stimulated TLRs are partially unravelled (Figure 2). TLR4 signalling needs a small protein physically associated with the extracellular domain of TLR4 on the cell surface, that is the MD-2 protein. It was identified when TLR4 overexpression in a human fibroblast cell line called HEK293 did not automatically led to LPS responsiveness assuming that other molecules beside

TLR4 and CD14 may be essential for TLR4-mediated signalling. This was identified as the secreted protein MD-2 (*Viriyakosol* et al., 2001).

Considering the physical interactions of TLR4 with LPS it is not clear, whether TLR4 interact directly with the different bacterial cell wall components or whether CD14 transported LPS generates a signal able to initiate binding to TLR4, which resembles the pathways of the Toll protein activation cascade described for *Drosophila*. However, cross-linking experiments suggests that LPS is recognised in close proximity to CD14, MD-2 and TLR4. So far, the CD14 protein acts as a principal LPS binding protein so that it is reasonable to assume that these complexes interact with TLR4 in close association with the secreted MD-2, the latter increasing the affinity of LPS binding by stabilisation of the LPS/CD14/TLR4 complex. Indeed, this has recently been experimentally verified by the experimental work of Jiang et al. (2000) demonstrating, that LPS induced physical proximity between CD14 and the TLR4 prior to nuclear translocation of the NF-*k*B.

What may be of utmost importance concerning the partially overlapping signalling pathways of agonist stimulated TLRs and cytokine receptors is the observation, that the structural similarities between the IL-1R and TLRs in their cytoplasmic regions results in the use of common components for downstream signalling events, shown in Figure 2.

Binding of the respective ligands e.g. Interleukin-1β to IL-1R or LPS to the TLR4 was followed by recruitment of a cytoplasmic protein called myeloid differentiation antigen 88 (MyD88) through the TIR domain common for both receptors (*Akira* et al., 2001). The adaptor protein MyD88 links the TLR and the IL-R to the IL-1R associated kinase (IRAK) being a serine-threonine kinase linked to the pelle kinase of *Drosophila*. Upon phosphorylation of IRAK this kinase dissociated from the receptor complex and interacted with another adaptor protein also common for both pathways, the so-called TNF receptor-associated factor 6 (TRAF-6). Upon this step both pathways split into divergent signalling events distally: One activated the c-Jun NH_2 -terminal kinase (JNKs) or p38-mediated pathways whereas the other involved the degradation of the inhibitor of the NF-*k*B (=I*k*B) which binds normally to the cytoplasmic NF-kB family of transcription factors to hinder their import to the nucleus. Phosphorylation steps of the inhibitor of NF-*k*B is mediated by the I*k*B Kinase complex (=IKK) complex, containing several isoforms known as IKKα, β, and γ-isoforms which were shown to exert quite different functions (*Hatada* et al., 2000) in view of their extent of phosphorylative capacity. Meaning that the IKK-β is more potent that the IKK-α. Moreover IKK-β displayed a higher kinase activity towards the I*k*B-alpha subunit. The biological importance of this signaling pathway is illustrated by the fact that IKK-β homozygous deficient mice die as embryos and show massive liver degeneration due to hepatocyte apoptosis. Upon activation of the IKK-complex the inhibitor of NF-*k*B is sequestered to a ubiquitin-proteasome pathway to release the NF-kB p50-Rel dimers followed by their translocation to the nucleus and binding to their corresponding promotor regions of NF-*k*B inducible genes. So far the most intensively studied pathways of the IKK activation include those originating from the stimulation of the TNF-α IL-1β receptors (*Hatatda* et al., 2000).

Of note, MyD88 independent pathways has been reported recently with MyD88 homozygous deficient mice (*Akira* et al., 2001). These animals were completely unable to respond to

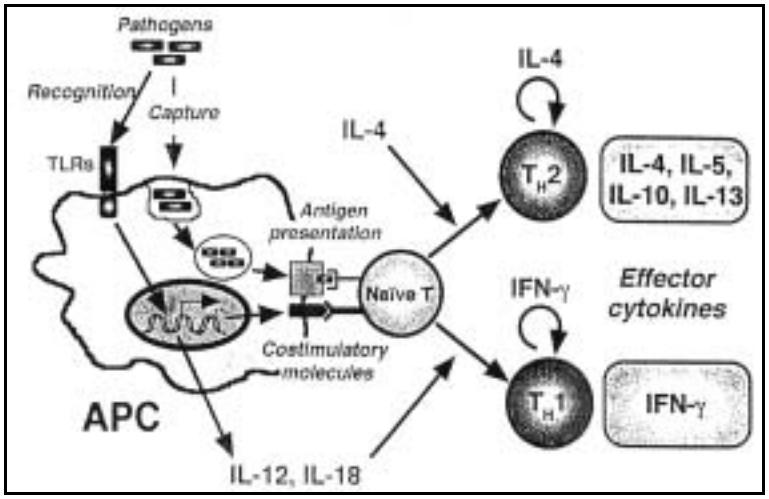

Figure 3: Impact of bacterial cell wall components on the polarisation of T-Helper cell sub-populations.

IL-1, IL-18, LPS, lipopeptides and peptidogylcans indicating that MyD88 being indispensable for signalling by these bacterial cell wall compounds. However subtractive hybridisation cloning identified genes in mouse macrophages that were induced by LPS independently of MyD88. These genes include the so-called IFN-γ inducible genes among them the IP-10 (IFN-inducible protein 10), the glucocorticoid attenuated response gene 16 (GARG 16) and the IFN regulated gene 1 (IRG-1). The expression of these genes need TLR4 but downstream activation pathways may involve IFN regulatory factor 3 (IRF-3) and NK-*k*B according to *Akira* et al. (2001).

IMPACT OF BACTERIAL CELL WALL COMPOUNDS ON THE GENERATION OF A POLARISED T-HELPER CELL RESPONSE

Additional studies on TLR signalling revealed the participation of phosphatidyl-inositol 3 Kinase (PiP3 kinase) in TLR2 signalling which may also involved in T-cell receptor triggered activation of T-lymphocytes, generally being accompanied downstream by the activation of the calcineurin pathway (*Robey* and *Allison*, 1995; *Putney* et al., 1986; 1993; *Whitney* and *Sutherland*, 1972; *Pai* et al., 1994). TLR-2 signalling events were also reported to be mediated by G-protein sensitive pathways for example the small GTPase „ras“ (*Heldwein* et al., 2001), linking T-cell activation through the mitogen activated family of protein Kinases (MAP-Kinases). This latter very important family of signalling molecules found in cells of the innate and adaptive immune system include the extracellular related kinases (ERKs), the p38 MAP Kinases and the JNKs (*Rincon*, 2001). Thus it may be conceivable that intracellular signalling events mediated by TLRs upon binding of their respective bacterial ligands and for example the autovaccine may result in signalling cascades important for both the innate immune response (classical pro-in-

flammatory bacterial-induced cytokines like TNFα, IL-1β, or IL-8) and the adaptive immune response (Il-4, IL-5, INF-γ) (*Genot* and *Cantrell*, 2000).

Getting the puzzle together a very simplified scheme results shown in Figure 3: Here the ligation of TLRs and/or the presentation of bacterial antigens by professional phagocytes profoundly promote through monocyte activation the polarisation of the well known T-helper cell subpopulations into the Th1 or Th2 differentiation pathway, reflected by the long held paradigm that bacterial infections initiate a strongly polarised Th1 response according to *Romagnani* et al. (1994).

Hence, Figure 3 illustrate the impact of bacterial pathogens for the regulation of the T-helper lymphocyte development either by agonistic stimulation of the Toll-like receptors on antigen presenting cells (APCs) or their capture by phagocytosis resulting in the generation of cell mediated specific immunity. Accordingly, Figure 3 mainly focused on the instructive role some cytokines will have on the commitment and differentiation of the different T-helper cell subpopulations (*Romagnani*, 1996; *Dinarello*, 1999). By interaction of bacterial cell wall compounds or whole bacteria with TLRs, cytokines such as interleukin-12 or interleukin-18 are induced, which may skew the further development of naive CD4 T-cells into a predominant Th1 like differentiation pathway. Over the past several years the processes by which naive resting CD4 T-helper cells expand and differentiate into much larger populations of highly active effector T-cells and smaller populations of memory T-cells have been the subject of very intense research (*Jancovic* et al., 2000; *Swain*, 1999).

Triggering of the T-cell receptor by antigens presented by different APC subtypes profoundly affects the spatial and time-dependent interactions of the T-cell receptor/MHC-antigen complex, mediated by the stepwise upregulation of various co-stimulatory molecules subsequently to TCR ligation, leading to the generation of the „immunological synapse“ (*Lanzavecchia* and *Sallusto*, 2000). The model of the immunological synapse may be a useful one illustrating some structural similarities between neuronal and immunological signalling transduction events. It postulates that the sequential upregulation of co-stimulatory molecules and their binding to their respective ligands on APC subtype not only profoundly dictates T-cell proliferation, maturation and differentiation but also increased avidity of the TCR (*Margulies*, 2001; *Viola*, 1996).

In addition, the surrounding cytokine milieu at the time of antigenic contact like the presence of IL-4, synthesised by activated mast cells or basophils or the presence of IFN-γ, secreted by natural killer cells (NK-cells) through macrophage derived IL-12 respectively, may further direct the polarisation into either the Th2- or Th1-pathway respectively, whereupon activated effector cell populations continue to secrete their predominant cytokines as described by Romagnani et al. (1996). However, care should be taken to oversimplify this scheme for the following reasons: The polarisation of the immune response culminating in cell mediated, antiviral or humoral immune responses against diverse infectious agents should not be regarded as a stable phenomenon. This implies that the commitment of a polarised T-helper cell population may be also reversed dependent on the microenvironment in the tissue (*Aebischer* and *Stadler*, 1996).

Accordingly, it has been suggested that CD4 polarisation may represent distinct developmental stages in T-cell differentiation and maturation pathways rather than a lineage-dependent maturation pathway, which can further be

dramatically influenced by a variety of other parameters tissue (*Aebischer* and *Stadler*, 1996): Common hypotheses on the factors inducing and regulating T-helper cell commitment and activation include among several still unknown factors the duration and the strength of T-cell receptor activation (*Iezzi* et al., 1999), the class and number of co-stimulatory molecules upregulated (*Sperling* and *Bluestone*, 2001), the nature of the antigen-presenting cell (*O'Garra* and *Murphy*, 1996; *Liu* et al., 2001; *Banchereau* and *Steinman*, 1998). The characteristics of the antigen or pathogen itself (physical structure, high vs. low antigen doses, parenteral vs. mucosal penetration) do play a prominent role in the decision process of the immune system to ensure the most effective way for elimination of the pathogen. All these variables might have a profound impact on the outcome of an immune response in view of tolerance induction or specific immunity.

From a biological point of view it is more reasonable to propose that in most instances the generation of an appropriate immune response against pathogens often depends on a mixed T-helper cell response (*Aebischer* and *Stadler*, 1996), so that only an over-activation of either T-cell population may be considered to have severe consequences for the host, particularly in view of the development of chronic inflammatory processes. Hence, a tight control of the immune response to infectious antigens by means of the regulation of cellular activity seems of utmost importance for the organism to avoid chronic inflammatory disorders or autoimmune diseases. Out of the numerous mechanisms underlying this control, the soluble mediators known as cytokines act as important immunoregulatory molecules. Their overproduction as well as their failure has been considered in the past to be associated with several important disease entities in humans, such as asthma or atopic diseases (*Brod*, 2000; *Barnes*, 2000; *Biedermann* et al., 2001; *Djukanovic*, 2000).

CYTOKINES INFLUENCED BY THE AUTOVACCINE IN WHOLE BLOOD CULTURES AND THEIR RELATION TO CHRONIC INFLAMMATORY DISEASE PROCESSES

The most popular functions of those cytokines whose synthesis were remarkably influenced by the autovaccine in the whole blood culture model is summarised in Table 1: It could be demonstrated that the autovaccine profoundly modulated the release of monokines as well as T-cell derived cytokines. Among them were IL- 4, IL-5, TNF-α, IL-10, IL-12 and IFN-γ. All cytokines, being profoundly modulated in their release by the autovaccine, are produced by lymphoid and non-lymphoid cell types (*Hunter* and *Reiner*, 2000). Thus their modulation by a bacterial immunomodulator reflects in an ideal way the important role of the autovaccine as a link between innate and adaptive immunity. IL-4 is considered to be the hallmark of those cytokines selectively produced in a Th2 type dominated immune response. It drives for example the differentiation of naive B-cells into IgE producing plasma cells. Therefore Th2 like T-lymphocytes are generally accepted to participate in type 1 allergic responses of the skin and the airways. However, CD8 T-cells were also reported to produce IL-4 (*Brown* and *Hural*, 1997). Originally, IL-4 has been described as a B-cell differentiation factor after antigenic stimulation.

Table 1: Short characteristic of immunoregulatory actions of cytokines whose synthesis was found to be modulated by the autovaccine in the whole blood culture system

Cytokine	Producers	Key functions in the immune response	References
IL-4	CD4-T-cells, mast-cells, basophils	Facilitates Th2 cell polarisation Activates Mast cells IgE-isotype switch in naive B-cells	Borish et al., 1996 Brown et al., 1997
IL-5	CD4-T-cells, eosinophils, Mast-cells	Eosinophil activator mast-cell activator	Borish et al., 1996 Teran et al., 1999
IL-10	Th1-cells, Th2-cells, T-reg-cells, Monocytes, macrophages, B-cells	Downregulates Th-1-cells and inhibits macrophage functions Growth factor for cytotoxic T-cells and mast cells	Borish et al., 1996
IFN-γ	NK-cells, Alveolar macrophages CD4-T-cells CD8-T-cells	Pro-inflammatory, anti-inflammatory and immunosuppressive actions dependent by the cellular activation state	Billau et al., 1990 Billau, 1996

IL-4 is mainly secreted by antigen-activated CD4 T-lymphocytes but mast cells, a subset of T-cells (the so called NKT-1 cells) and the basophils could release IL-4 (*Brown* and *Hural*, 1997). IL-4 stimulates the expression of co-stimulatory molecules like the B7-antigens, induced CD40 expression on B-cells and synergises with other B-cell activating cytokines like IL-2, IL-5 and IL-6 to increase antibody production. However, IL-4 possesses also anti-inflammatory properties as it inhibits monocyte production of IL-1, TNF-α and PGE_2.

Beside its action on B-cells and its pro-inflammatory effects during the initiation and maintenance of an allergic response, IL-4 controls the activation and differentiation of cytotoxic T-lymphocytes, which are essential for anti-tumour and antiviral immunity, so that IL-4 may be regarded as an important anti-tumour cytokine. Moreover, antibody-dependent cellular cytotoxicity and a downregulation of monocyte activation were general features attributes to IL-4 (*Borish*, 1996). During an allergic response, IL-4 enhances the recruitment of activated CD4 T lymphocytes into the airway epithelium by upregulating adhesion molecules on vascular endothelial cells.

In view of its potent pro-inflammatory role in allergy, IL-4 shares similarity with another mainly Th2-derived cytokine namely IL-5. IL-5 synthesis was found to be also markedly downregulated by the autovaccine in human leukocyte whole blood cultures. Beside its originally described growth promoting and differentiating effects on B-lymphocytes, IL-5 is a well known mediator of eosinophil activation (Borish, 1996; Teran, 1999). *In vitro* IL-5 was reported to induce chemo-attraction, trans-endothelial migration and superoxide production by human eosinophils and initiates their degranulation and prolonged their survival. The overexpression of the IL-5 gene in mice was accompanied by a fulminant eosinophilia, which can be blocked by a monoclonal antibody against IL-5.

In humans IL-5 mRNA was found to be upregulated in bronchial biopsies of asthmatic individuals and IL-5 protein was detected in the broncho-alveolar fluid of asthmatics (*Borish*, 1996; *Hamid* and *Minshall*, 2000). IL-5 is supposed to interfere with eosinophil survival by upregulation of the expression of anti-apoptotic molecules and the overexpression of the NF-*k*B transcription factors. IL-5 profoundly synergises with the cytokines IL-3 and GM-CSF in the recruitment of other non-lymphoid cell types profoundly involved in the generation and maintenance of an allergic response in the case of type 1 allergies. These are the mast cells and the basophils.

The overlapping and partially synergistic functions of IL-4 and IL-5 are good examples for the close interplay between different cytokines in disorders with an underlying dysregulation of the immune system and clearly underlines the clinical relevance and clinical importance of immunotherapeutic interventions. Targeting synthesis of different cytokines may result in a real immuno-modulating effect instead of a mere inhibition achieved by the suppression of only one cytokine (*Djukanovic* et al., 2000; *Biedermann* et al., 2001; *Creticos*, 2000). For the same reason, the modulation of the synthesis of interleukin-10 by the autovaccine constitutes an important means to mediate immunoregulating effects.

Interleukin-10 was originally described in the mouse system as a „cytokine synthesis inhibitor" predominantly acting on Th1 type T-lymphocytes and macrophages. However, there are important differences regarding the cellular source of IL-10 between different species: In the mouse system IL-10 is mainly produced by CD4 Th2 lymphocytes, whereas in the human system IL-10 can be synthesised by LPS activated monocytes/macrophages, by antigen activated Th1 and Th2 T-helper cells, by so called T-regulatory cells 1 (Tr1) and in an antigen non-specific way by mast cells and by B-lymphocytes.

IL-10 inhibits as an example interleukin-5 release by activated Th2 like cells thereby interfering with their growth and differentiation. Of note, IL-5 release was obviously suppressed by IL-10 in a different manner in response to different stimuli as demonstrated experimentally by Akdis et al. (2000), whereas in PMA/anti CD28 activated CD4 T-cells IL-5 release was abrogated, T-cells stimulated by the combination of ionomycin/PMA remained resistant to the inhibition by IL-5. This suggests that IL-10 suppression may target the activation signals generated by the costimulatory molecules B7/CD28**.**

In addition IL-10 downregulates the activation of macrophages, being one of the most important cell types linking innate and adaptive immunity. This inhibition was shown to be mediated either at the level of antigen-presentation by downregulation of co-stimulatory molecules or by the inhibition of the release of macrophage derived pro-inflammatory cytokines. Among them TNF-α, IL-1β, IL-6 or growth factors like GM-CSF or IL-3 were inhibited by IL-10. Likewise the activation of mast cells with their release of IL-3 and GM-CSF is negatively influenced by IL-10. Alle these actions are useful events to suppress the eosinophilic response generally observed in allergic asthma, leading in the past to the concept of IL-10 as an anti-allergic cytokine (*Pretolani* and *Goldman*, 1997). Most importantly IL-10 was found to interfere with the IFN-γ synthesis by activated CD4 Th1 helper lymphocytes participating in the activation of macrophages.

Particularly IFN-γ is considered to be one of most pleiotropic cytokines within the immune system, whose synthesis and expression in different

organs should be controlled very carefully, due to its highly pro-inflammatory effects on macrophages at one hand and its antiproliferative role on activated B-lymphocytes at the other hand.

By this two-sided effects of IFN-γ the complexity of activities of this cytokine become apparent. For example it can induce a striking induction of specific antibody production in activated B-cells but can also suppress antibody production. During infectious diseases, the proper activation of macrophages by IFN-γ helps the body to eliminate the infectious agent, but excess macrophage activation may cause severe acute or even chronic inflammation in target organs being sometimes fatal to the host (*Billau* and *Dijkmans*, 1990; *Billau*, 1996).

Accordingly, the cell type in the immune system being largely affected in its activation profile is obviously the monocyte/macrophage although other cell types like endothelial cells, dendritic cells or Langerhans cells may response to IFN-γ in some way. Thus important functions generally associated with immunoregulatory actions of IFN-γ are the dramatically enhancement of macrophage functions, for example intracellular killing of bacteria during cell mediated immune responses and lysis of tumour cells. Therefore a modulation of the IFN-γ response is considered to be ideally suited to affect innate as well as adaptive immunity. Considering the actions of IFN-γ on monocytes and macrophages, the activation state of the responding cell type dictates markedly the outcome of the response. In so far IFN-γ is often considered to function as a „priming signal". In view of the activation of Th2 T-lymphocytes during a type 1 allergic response, IFN-γ counteracts IgE response due to its antiproliferative effect on activated B-cells. Thus the balance between IL-4 and IFN-γ production may considered to be an important immunoregulatory pathway valuable for the downregulation of an overshooting Th2 dominated response (*Creticos*, 2000). Even in disease free intervals of allergic diseases such as allergic rhinitis, persistence of low level inflammation may cause over time a dangerous change in functions of the immune system so that a timely intervention in this process might be regarded as clinically very relevant to improve disease symptoms (*Ricca* et al., 2000).

SOME SPECIAL EFFCTS OF THE AUTOVACCINE ON LYMPHOCYTES ARE NOT SHARED BY OTHER CLASSICAL BACTERIAL DERIVED IMMUNOMODULATORS SUCH AS LPS

Clearly, some but not all cytokines modulated by the autovaccine, such as TNF-α, IL-10 or IL-6, can also be induced by the LPS molecule itself. So what are the real difference in the activation of the leukocytes by autovaccine or LPS? The complexity in the cytokine network increases as nearly every cell type in the body can respond to LPS with an upregulation of genes encoding a vast amount of growth factors, hormones or lipid mediators. Cellular responses upon LPS are dependent on the activation state and the surrounding cellular milieu, the type of the cell, number and affinity of receptors on the cell membrane and much more. So it is not surprising that Lipid A as the biological and toxic moiety of the LPS complex can beyond critical concentrations lying in the nanogram range induce fatal overactivation of the immune system resulting in the known sepsis syndrome and multi-organ failure. Not

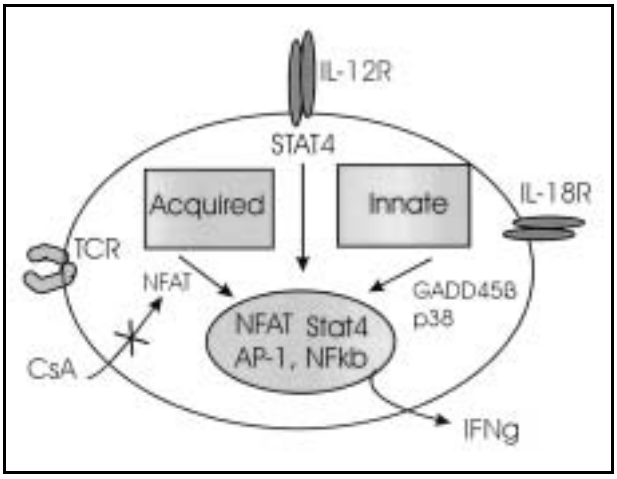

Figure 4: Innate and acquired pathways of Interferon-γ production.

so with the autovaccine: this molecule derived from rough *E. coli* has virtually any pyrogenic activity in rabbits and no apparent toxicity despite similarities in its structure with the Lipid A molecule.

So it may reasonably be assumed, that the responses of the immune system to autovaccine vs. Lipid A were only partially be comparable but even more complex with regard to the regulatory functions of autovaccine. Structurally, both autovaccine and Lipid A are partially related, but in view of the activation of the immune system, important differences emerged, demonstrated by the different dose-response curves in whole blood cultures of individual donors stimulated with autovaccine and the highly different inter-individual responses on the autologous bacteria compared to the homologous bacteria.

Generally it has been thought that IFN-γ may exclusively be produced by NK cells upon IL-12 released by macrophages or by TCR triggered T-lymphocytes in an antigen-dependent manner (*Billau* and *Dijkmans*, 1990). Considering the observed effects of the autovaccine being able to restore the Interferon-γ production by T-lymphocytes unable to respond to TCR ligation, some recent data may help to explain these findings.

For example this reconstitution of the IFN-γ production may agree with a recent report in the literature, that IFN-γ could also be induced in an antigen-independent manner by the combined actions of both IL-12 and IL-18 on T-lymphocytes, independent on the triggering of the T-cell receptor (*Yang* et al., 2001). Interleukin-18 being a member of the IL-1 family (*Akira*, 2000) signalling presumably through the same pathway like IL-1, shares intracellular similarities with the TLR-4 pathway, so that bacterial stimuli like autovaccine may also induce IL-18. However, an activation by autovaccine of IL-18 has not been tested yet.

The scheme illustrated in Figure 4 proposes therefore only a hypothetical pathway by which autovaccine could have restored the IFN-γ synthesis in T-cells from an individual, which could not activated by conventional T-cell agonists like anti-CD3 antibodies together with anti-CD28 antibodies. According to *Yang* et al. (2001) and *Nakanishi* et al. (2001), the combined action of IL-12 with IL-18 through their

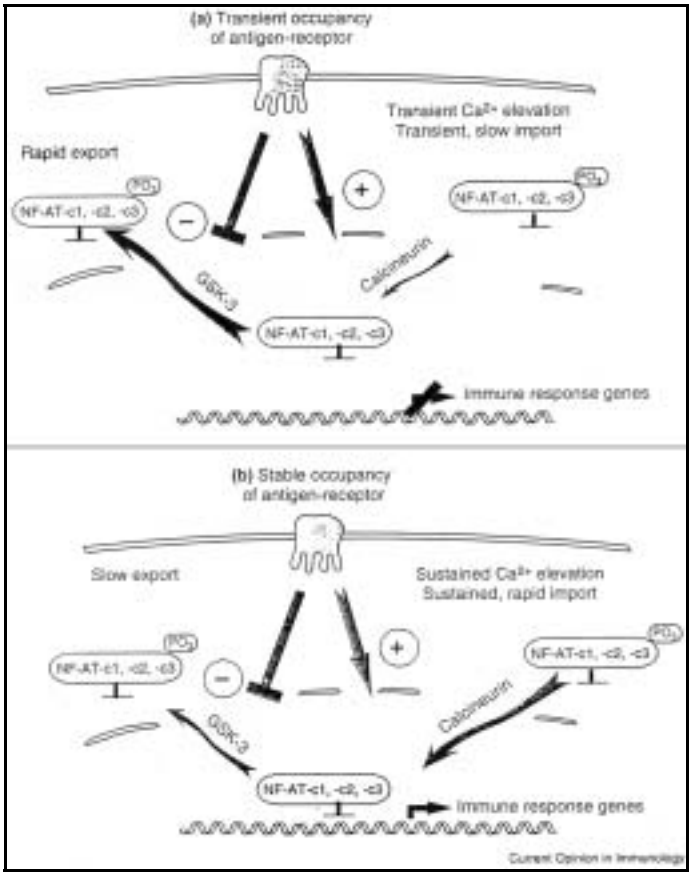

Figure 5: Hypothetical model how autovaccine Herborn might influence the calcium influx of T-lymphocytes.

corresponding receptors expressed on T-cells triggered the subsequent expression of a special cytoplasmic molecule of the MAP/ERK-kinase family e.g. GADD 45ß, which obviously bypasses the need for the classical pathway of a STAT-4 mediated IFN-γ production by macrophage derived IL-12.

The cytoplasmic expression of this molecule should enable CD4 positive T-helper cells to directly activate the NF-*k*B / AP transcriptional pathway for the activation of the IFN-γ gene in the case the cytokines IL-12 and IL-18 are *both* present.

As it has been demonstrated in the whole blood culture model that IL-12 was very efficiently modulated by the autovaccine and due to the fact that NK cells constitutively express both functionally active IL-12R and the IL-18R when freshly isolated, NK-cell derived IFN-γ could not be ruled out to explain the effects of autovaccine on IFN-γ production. However, the low number of NK-cells in whole blood argues against this possibility. In addition, the role of the macrophage as an IFN-γ-producing cell type is far from being clear: So far only one report by *Fenton* et al. (1997) described, that human alveolar macrophages were able to release IFN-γ after infection with *Mycobacterium tuberculosis* (*Fenton* et al., 1997).

Figure 5 should illustrate the role of autovaccine as a calcium-modulating

agent in the process of T-cell activation as a function of the duration of the subsequent activation of the calcium dependent phosphatase calcineurin. Assumed that autovaccine could restore defective TCR triggering by it s influence on the calcium influx into the cell, the figure based on the recent observations of *Neilson* et al. (2001).

In T-lymphocytes the transcription factors of the NF-ATc family are normally present in the cytoplasm in different phosphorylation states which hinders their nuclear import and binding to the corresponding promotor regions of respective cytokine genes on DNA, for example IL-2. The nuclear export of the transcription factors is mediated in this model by a member of the glycogen synthase Kinase 3 (GSK-3) which is constitutively active in T-cells. Under physiological conditions T-cells are often occasionally and transiently activated through their traffic from blood to lymph nodes and back during their search for potential harmful antigens. Occasionally the NF-ATc members may then enter the nucleus but are rapidly exported to hinder unfavourable activation of clonotypic T-cells in case of only low affinity binding of antigens.

Due to the observation that autovaccine binds strongly to calcium ions, the observed failure of the IFN-γ production upon ligation of the TCR by anti-CD3/anti-CD28 may presumably be substituted by the calcium-targeting effects of autovaccine, whereby too low intracellular calcium concentrations may be enhanced. Under low intracellular calcium concentrations the cytoplasmic phosphatase calcineurin is only marginally activated to dephosphorylate the NF-ATc transcription factors resulting in their enhanced export to the nucleus by the GSK-3. Assumed that the autovaccine could substitute for a prolonged calcium signal in an at present unknown way, the calcium concentration intracellularily may reach sufficient levels to allow an enhanced import of NF-ATc transcrption factors into the nucleus, simultaneously accompanied by a lower GSK-3 activity. This model was proposed by *Neilson* et al. (2001), according to observations by the authors that a mutation in human T-cells induced a failure to respond to specific TCR triggering or to ionomycin with a subsequent mobilisation of intracellular calcium stores. These special T-lymphocytes were supposed to be defective in the mobilisation of calcium ions. Interestingly, T-cell from these patients express all molecules required for T-cell stimulation at a normal level on the cell membrane but there seem to be a substantial abnormality in lymphocyte activation in the duration of the calcium signal. Thus defective calcium influx and/or inability of the T-cell to maintain sufficient prolonged intracellular calcium levels may have contributed to the observed defect in this special lymphocytes (*Pai* et al., 1994; *Ricca* et al., 2000; *Whitney* and *Sutherland*, 1972).

FUTURE WORK

How may these important features of the autovaccine be integrated into a hypothetical regulatory model of action?

It may be suggested (see Figure 6) that the autovaccine Herborn might interact similar to the classical LPS molecule with Toll-like receptor subtypes on a responsive cell type. However this interaction may be influenced by the aggregation state of the autovaccine (number and structure of „monomers“) and neighbouring receptors on immune cells potentially occupied by the autovaccine in a somewhat different manner such as

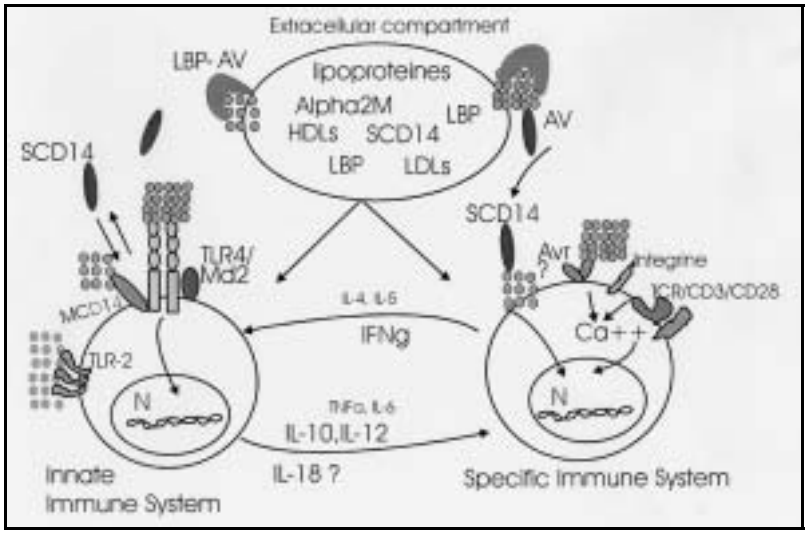

Figure 6: Model for the proposed immunoregulatory actions of autovaccine summarised in the context of the particular molecular structure of the autovaccine and its cytokine modulating capacities.

mCD14, integrins or lectin receptors like mannose binding protein. Additionally, lipoproteins in plasma, soluble CD14 and the Lipid A binding protein (LBP), generally known to regulate and control the traffic and biological activity of LPS in the blood stream (*Kitchens* and *Munford*, 1998; *Kitchens* et al., 1999; 2001; *Kitchens*, 2000) may also interfere with functions of the autovaccine on immunocompetent cells, resulting in the highly interindividual immunomodulating actions measured in the whole blood culture system. The downstream signalling events used by the autovaccine are largely unknown at present, so that in the context with this paper the proposed regulatory models of the actions of autovaccine illustrated in Figures 4, 5, and 6 are a mere speculatory one at present. Furthermore, surface-active properties of the autovaccine or electrostatic interactions of the autovaccine may also be taken into account and should and not be underestimated. Assuming different behaviour of aggregated and monomeric molecules, „monomeric“ molecules might profoundly interact for example with the so-called „lipid rafts“ in the eukaryotic cell membrane. This could have an impact on membrane fluidity thereby changing the conformation of raft-associated signalling molecules. To this end the autovaccine manufactured by the SymbioVaccin GmbH represents a remarkable class of bacterial-derived immunomodulators which to the present knowledge has not been described earlier in the literature with that individual specificity on lymphoid cells. From an immunological point of view it is highly interesting that a lipo-oligosaccharide preparation such as the autovaccine could initiate such a remarkable interindividual specific response on T-cells and on macrophages, without being strictly antigen-specific in the classical immunological sense. It will be of utmost importance for future work that this bacterial preparation, inducing probably a classical Th1 response in the whole blood culture system will be clinically evaluated for its effects in well designed, randomised clinical trials in very carefully selected indications.

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